SPECIFICATION

# TITLE OF THE INVENTION

# ANALYSIS OF AGONIST-ACTIVITY AND ANTAGONIST-ACTIVITY TO CYTOKININ RECEPTOR

## BACKGROUND OF THE INVENTION

### FIELD OF THE INVENTION

The present invention relates to a method for analyzing agonist-activity and antagonist-activity of an examinee to cytokinin receptor.

### DESCRIPTION OF THE RELATED ART

Cytokinins are plant hormones relevant to cell division and differentiation of higher plants and are well-known as very important physiologically active substances having functions of inducing division of cells of higher plants, differentiating the callus and pith to the stems and leaves, ethiolating and defoliating leaves, preventing the falling of fruit, breaking the dominance of the terminal bud and the like [Cytokinins: Chemistry, Activity, and Function, CRC press (1994)]. The substances having cytokinins-like activities are usable as plant growth regulators, for example, fruit-falling preventive agents for fruits such as apple, orange, and the like, plant-falling preventive agents for rice plants, barley, wheat and the like by regulating the height of the plants, and sweetness increasing agents for fruits after harvest.

As a method for finding substances having such cytokinins-like

activities, conventionally employed is a method for observing and evaluating the physiological changes by directly spraying examinee substances to plant.

The aforementioned method has problems that it requires to prepare the examinee substances in amounts sufficient to directly spray them to plant and also requires immensely long time to observe and evaluate the growth of the plant and the physiological changes of the plant after spraying of the examinee substances. Therefore, it has been desired that a variety of methods be developed for quickly finding substances having cytokinins-like activities with small amounts of examinee substances.

## SUMMARY OF THE INVENTION

Inventors of the present invention have intensively investigated in such situations and first found protein functioning as a cytokinin receptor and subsequently found it possible to analyze the agonist-activity and the antagonist-activity of examinee substances to a cytokinin receptor by using the cytokinin receptor ("the analysis method") and found it also possible to quickly search substances having cytokinins-like activities even in a small amount of examinee substances by employing the analysis method and consequently reached the present invention.

The present invention provides:

1: A method for analyzing antagonist-activity to a cytokinin receptor, which comprises (1) bringing an examinee substance and a substance having agonist-activity to the cytokinin receptor into contact with a transformed cell into which DNA coding the cytokinin receptor is introduced and

(2) measuring the existence or the quantity of intracellular signal transduction from the cytokinin receptor expressed in the transformed cell.

2: The method according to the above 1, wherein the transformed cell is a cell having a function of directly controlling the cell growth by intracellular signal transduction from the cytokinin receptor and the measurement of the existence or the quantity of the intracellular signal transduction is carried out using the quantity of the cell growth of the transformed cell as an indicator.

3: The method according to the above 1, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell so improved as to have histidine kinase activity lower than the intrinsic histidine kinase activity of the host cell.

4: The method according to the above 1, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell so improved as to have histidine kinase activity lower than the intrinsic histidine kinase activity of the host cell by deleting one or more of histidine kinase.

5: The method according to the above 1, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell having no cytokinin receptor.

6: The method according to the above 1, wherein the transformed cell is yeast.

7: The method according to the above 1, wherein the transformed cell is budding yeast.

8: The method according to the above 1, wherein the DNA coding the

cytokinin receptor is any one of DNA coding the cytokinin receptor selected from:

(a) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 6;

(b) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 2;

(c) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 4;

(d) a cytokinin receptor wherein said cytokinin receptor has at least one transmembrane region but less than that in its natural form.

(e) a cytokinin receptor having the amino acid sequence from amino acid number 196 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(f) a cytokinin receptor having the amino acid sequence from amino acid number 50 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(g) a cytokinin receptor having the amino acid sequence from amino acid number 32 to 1036 among the amino acid sequence represented by SEQ ID No: 4;

(h) a chimera-type cytokinin receptor comprising an extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, a histidine kinase region of the cytokinin receptor, and a receiver region of the histidine kinase, wherein said extracellular region, said transmembrane regions and said histidine kinase region are homogeneous to one another and the receiver region is heterogeneous thereto; and

(i) a cytokinin receptor having the amino acid sequence with deletion, substitution, or addition of one or a plurality of amino acids in the amino acid sequence of the cyctokinin receptor of (a), (b), (c), (e), (f), or (g).

9: A method for analyzing agonist-activity to a cytokinin receptor, which comprises (1) bringing an examinee substance into contact with a transformed cell into which DNA coding the cytokinin receptor is introduced and

(2) measuring the existence or the quantity of intracellular signal transduction from the cytokinin receptor expressed in the transformed cell, wherein

the DNA coding the cytokinin receptor is any one of DNA coding the cytokinin receptor selected from:

(b) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 2;

(c) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 4;

(d) a cytokinin receptor wherein said cytokinin receptor has at least one transmembrane region but less than that in its natural form.

(e) a cytokinin receptor having the amino acid sequence from amino acid number 196 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(f) a cytokinin receptor having the amino acid sequence from amino acid number 50 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(g) a cytokinin receptor having the amino acid sequence from amino acid

number 32 to 1036 among the amino acid sequence represented by SEQ ID No: 4;

(h) a chimera-type cytokinin receptor comprising an extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, a histidine kinase region of the cytokinin receptor, and a receiver region of the histidine kinase, wherein said extracellular region, said transmembrane regions and said histidine kinase region are homogeneous to one another and the receiver region is heterogeneous thereto; and

(i) a cytokinin receptor having the amino acid sequence with deletion, substitution, or addition of one or a plurality of amino acids in the amino acid sequence of the cyctokinin receptor of (b), (c), (e), (f), or (g).

10: The method according to the above 9, wherein the transformed cell is a cell having a function of directly controlling the cell growth by intracellular signal transduction from the cytokinin receptor and the measurement of the existence or the quantity of the intracellular signal transduction is carried out using the quantity of the cell growth of the transformed cell as an indicator.

11: The method according to the above 9, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell so improved as to have histidine kinase activity lower than the intrinsic histidine kinase activity of the host cell.

12: The method according to the above 9, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell so improved as to have histidine kinase activity lower than the intrinsic histidine kinase activity of the host cell by deleting

one or more of histidine kinase.

13: The method according to the above 9, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell having no cytokinin receptor.

14: The method according to the above 9, wherein the transformed cell is yeast.

15: The method according to the above 9, wherein the transformed cell is budding yeast.

16: A cytokinin receptor selected from:

(d) a cytokinin receptor wherein said cytokinin receptor has at least one transmembrane region but less than that in its natural form.

(e) a cytokinin receptor having the amino acid sequence from amino acid number 196 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(f) a cytokinin receptor having the amino acid sequence from amino acid number 50 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(g) a cytokinin receptor having the amino acid sequence from amino acid number 32 to 1036 among the amino acid sequence represented by SEQ ID No: 4;

(h) a chimera-type cytokinin receptor comprising an extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, a histidine kinase region of the cytokinin receptor, and a receiver region of the histidine kinase, wherein said extracellular region, said transmembrane regions and said histidine kinase region are homogeneous to one another

and the receiver region is heterogeneous thereto; and

(i) a cytokinin receptor having the amino acid sequence with deletion, substitution, or addition of one or a plurality of amino acids in the amino acid sequence of the cyctokinin receptor of (e), (f), or (g).

17: DNA coding the cytokinin receptor of the above 16.

18: A transformed cell into which DNA of the above 17 is introduced.

19: A method for detecting antagonist-activity to a cytokinin receptor, which comprises evaluating the antagonist-activity of two or more different examinee substances to the cytokinin receptor based on the difference obtained by comparison of the existence or the quantity of the intracellular signal transduction in a section where the examinee substances are independently used and measured by the analysis method of the above 1.

20: The method according to the above 19, wherein at least one substance among the two or more different examinee substances is a substance having no antagonist-activity to the cytokinin receptor.

21: A method for searching antagonist-active substance to a cytokinin receptor, which comprises selecting a substance having antagonist-activity to a cytokinin receptor based on the antagonist-activity to a cytokinin receptor evaluated by the detecting method of the above 19.

22: A plant growth regulator comprising the substances selected by the searching method of the above 21 as an active ingredient.

23: A method for detecting agonist-activity to a cytokinin receptor, which comprises evaluating the agonist-activity of two or more different examinee substances to the cytokinin receptor based on the difference

obtained by comparison of the existence or the quantity of the intracellular signal transduction in a section where the examinee substances are independently used and measured by the analysis method of the above 9.

24: The method according to the above 23, wherein at least one substance among the two or more different examinee substances is a substance having no agonist-activity to the cytokinin receptor.

25: A method for searching agonist-active substance to a cytokinin receptor, which comprises selecting a substance having agonist-activity to a cytokinin receptor based on the agonist-activity to a cytokinin receptor evaluated by the detecting method of the above 23.

26: A plant growth regulator comprising the substances selected by the searching method of the above 25 as an active ingredient.

Further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integer or step.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Hereinafter, the present invention will be described in detail.

A cytokinin receptor is protein having functions of controlling the propagation and differentiation of cells of higher plants based on the intracellular signal transduction mechanism so-called Two-Component regulatory system (or Histidine to Aspartic acidartic acid phosphorelay system) while being specifically bonded with cytokinins such as purine type cytokinins, e.g. kinetinee, zeatinee, and the like, and urea type cytokinins, e.g. N-phenyl-N'-(4-pyridyl)urea and the like. The cytokinin receptor to be used in the present invention belongs to the histidine kinase family and is protein composed of extracellular regions, transmembrane regions, histidine kinase regions (regions having histidine kinase activity in the cell and holding Histidine residue to be an active site), and receiver regions (regions having a reception part for phosphate group transfer and holding Aspartic acid residue to be an active site).

Practical examples of the cytokinin receptor are cytokinin receptors having the amino acid sequence represented by SEQ ID No: 2; cytokinin receptors having the amino acid sequence represented by SEQ ID No: 4; cytokinin receptors having the amino acid sequence represented by SEQ ID No: 6; cytokinin receptors having the amino acid sequence represented by SEQ ID No: 2 wherein one or a plurality of amino acids are deleted, substituted, or added; cytokinin receptors having the amino acid sequence represented by SEQ ID No: 4 wherein one or a plurality of amino acids are deleted, substituted, or added; cytokinin receptors having the amino acid

sequence represented by SEQ ID No: 6 wherein one or a plurality of amino acids are deleted, substituted, or added; cytokinin receptors having the amino acid sequence coded with DNA to be hybridized in stringent conditions with DNA having the nucleotide sequence coding the amino acid sequences represented by SEQ ID No: 2; cytokinin receptors having the amino acid sequence coded with DNA to be hybridized in stringent conditions with DNA having the nucleotide sequence coding the amino acid sequences represented by SEQ ID No: 4; cytokinin receptors having the amino acid sequence coded with DNA to be hybridized in stringent conditions with DNA having the nucleotide sequence coding the amino acid sequences represented by SEQ ID No: 6; cytokinin receptors of partially transmembrane region-deleted type which will be described later; chimera-type cytokinin receptors which will be described later; and the like. Incidentally, the phrase, "a plurality of amino acids", means more particularly about 2 to 20 amino acids and for example, 2 to 10 amino acids and 2 to 5 amino acids may be exemplified. Also, the phrase, "the amino acid sequences ... wherein one or a plurality of amino acids are deleted, substituted, or added", means as examples those having the amino acid sequences of which 80% or higher, preferably 90% or higher, and more preferably 95% or higher are identical with the sequences of the amino acids before the deletion, substitution or addition of amino acids (i.e. amino acid sequence identification of 80% or higher, particularly 90% or higher, and more preferably 95% or higher).

Those phrases, "deletion, substitution or addition of amino acids" or "which 80% or higher ... are identical" of course include the proteins

provided by the intracellular processing to which proteins having the amino acid sequences represented by SEQ ID Nos: 2, 4, and 6 are subjected, and the natural variations caused by differences in type of organisms from which the proteins are derived, differences in individual bodies, differences in tissues, and the like.

The phrase, "sequence identification", in the present invention means the identification and homology between two DNA sequences and between two protein sequences. The sequence identification may be determined by comparing two sequences aligned in the optimum states in a region of the sequences of the comparison objects. The DNA or proteins, the comparison objects, may have addition or deletion (e.g. gap and the like) in the optimum alignment of two sequences. Regarding such sequence identification, computation may be performed using, for example, Vector NTI by producing alignment by utilizing Clusta 1W algorithm [Nucleic Acid Res., 22 (22): 4673-4680 (1994)]. Incidentally, the sequence identification may be measured by a sequence analysis soft, practically Vector NTI, GENETYX-MAC and analysis tools provided in public database. The aforementioned public database may be, in general, accessible in, for example, home address; http://www.ddbj.nig.ac.jp.

Regarding the phrase, "be hybridized in stringent conditions", the term, hybridization, in this case may be performed, for example, according to a common method described in Molecular Cloning 2nd edition, written by Sambrook J., Frisch E. F., Maniatis T., issued by Cold Spring Harbor Laboratory press. Further, the phrase, "in stringent conditions", means, for example, that a hybrid is formed in a solution of 6 × SSC (a solution

containing 1.5 M NaCl and 0.15 M trisodium citrate is defined as 10 × SSC) at 65 ℃and then washed with 1 × SSC at a room temperature. The salt concentration in the washing step may be selected, for example, from the condition of 1 × SSC at a room temperature (a low stringent condition) to 0.1 × SSC at a room temperature (a high stringent condition). The temperature in the washing step may be selected, for example, from a room temperature (a low stringent condition) to 68℃ (a high stringent condition). Further, both of the salt concentration and the temperature may be changed.

(Production of a transformed cell into which DNA coding a cytokinin receptor is introduced)

A transformed cell into which DNA coding a cytokinin receptor is introduced may be obtained by introducing and expressing a DNA coding one of the above cytokinin receptors. For example, DNA having nucleotide sequence coding the amino acid sequence of the cytokinin receptor can be introduced into a host cell in the following manner. Hereinafter, one example of the method of the production of a transformed cell will be described.

(1) Preparation of cDNA

At first, the total RNA is prepared from plants such as higher plants according to the method described in Molecular Cloning 2nd edition written by J., Sambrook, E., F., Frishch, T., Maniastis.

Concretely, for example, after a part of tissues are sampled from a higher plant, the tissues are frozen in liquefied nitrogen and successively physically milled using a mortar and pestle or the like. The total RNA may

then be obtained by methods in which (a) the resulting milled product is mixed with a solution containing guanidine hydrochloride with phenol or SDS with phenol to obtain the total RNA or (b) the resulting milled product is mixed with a solution containing guanidine thiocyanate and further with CsCl and then subjected to centrifugal separation to obtain the total RNA. Examples of the higher plant include a monocotyledonous plant, e.g. rice, corn, barley, wheat and the like and a dicotyledonous plant, e.g. tobacco, soybean, Arabidopsis, and the like. For the aforementioned process, commercialized kits such as ISOGEN (produced by Nippon Gene Co.), RNeasy Total RNA Purification Kit (produced by QIAGEN Co.), and the like may be employed.

Next, mRNA is prepared from the total RNA. For example, the preparation may be carried out by a method utilizing the hybridization of oligo-dT chains bonded with cellulose or latex and poly-A chains of mRNA. For the operation, for example, commercialized kits such as mRNA Purification Kit (produced by Amersham Pharmacia Co.), Oligotex TM-dT30 (super) (produced by Takara Shuzo Co. Ltd.), and the like may be employed.

Further, cDNA is produced using the mRNA (mRNA having poly-A chains) may be prepared in the following manner. For example, oligo-dT chains or random primers are annealed with mRNA and then reacted with reverse transcriptase to produce cDNA. Further, the cDNA is reacted with, for example, RNaseH, DNA polymerase I to produce double chain cDNA. For the operation, for example, the following commercialized kits may be employed: SMARTTM PCR cDNA Synthesis Kit (produced by Clontech Co.), cDNA Synthesis Kit (produced by Takara Shuzo Co. Ltd.), cDNA Synthesis

Kit (produced by Amersham Pharmacia Co.), ZAP-cDNA Synthesis Kit (produced by Stratagene Co.) and the like.

(2) Cloning

The DNA coding the cytokinin receptor may be obtained by a polymerase chain reaction (hereinafter referred as to PCR) from the produced cDNA using, for example, DNA having partial nucleotide sequence of the nucleotide sequence of SEQ ID No: 1, 3 or 5 as a primer or by a hybridization method using DNA having partial nucleotide sequence of the nucleotide sequence of SEQ ID No: 1, 3 or 5 as a probe.

In the case of employing PCR, DNA usable as a primer set is those planned and synthesized based on the nucleotide sequences of about 20 bp to 40 bp, for example, the nucleotide sequences selected respectively from 5'-non-translation regions and 3'-non-translation regions of the nucleotide sequence represented by SEQ ID No: 1, 3, or 5. Examples of the primer set are sets of DNA of nucleotide sequence represented by SEQ ID No: 9 and DNA of nucleotide sequence represented by SEQ ID No: 10. The PCR solution to be used may be prepared by adding reaction solutions instructed by the kit to cDNA 250 ng. The conditions of the PCR may properly be changed depending on the primer set to be used and, for example, concrete conditions include as follows: keeping at 94℃ for 2 minutes, at about 8℃ for 3 minutes, and further repeating 40 cycles each of which comprises steps of keeping at 94℃ for 30 seconds, at 55 ℃ for 30 seconds, and at 72℃ for 4 minutes; and repeating 5 to 10 cycles each of which comprises steps of keeping at 94℃ for 5 seconds and at 72℃ for 4 minutes and further repeating about 20 to 40 cycles each of which comprises steps of keeping at

94℃ for 5 seconds and at 70℃ for 4 minutes. For the operation, the following commercialized kits may be, for example, employed: Takara Heraculase™ (produced by Takara Shuzo Co., Ltd.), DNA polymerase contained in Advantage cDNA PCR Kit (Clontech Co.), TAKARA Ex Taq (Takara Shuzo Co., Ltd.), PLATINUMTM PCR SUPER Mix (Lifetech Oriental Co.), and the like.

In the case of employing hybridization, cloning may be carried out according to a method described in, for example, "Cloning and Sequence", Experimental Manual of Plant Biotechnology (edited by Watanabe and Sugiura, Noson Bunka Publisher, 1989)

The probe to be used may be obtained by synthesizing DNA (with the chain length of about 200 nucleotides to 500 nucleotides) having partial nucleotide sequences of the nucleotide sequence represented by SEQ ID No: 1, 3 or 5 and labelling the DNA with radioisotope markers or fluorescent markers according to the known methods using, for example, Random Primed DNA Labelling Kit (Boehringer Co.), Random Primer DNA Labelling Kit Ver. 2 (Takara Shuzo Co., Ltd.), ECL Direct Nucleic Acid Labelling and Detection System (Amersham Pharmacia Co.), Megaprime DNA-labelling system (Amersham Pharmacia Co.) and the like.

Examples of the hybridization conditions include stringent conditions and the following conditions may be exemplified: keeping at 65℃ in the presence of 6 × SSC (0.9 M NaCl and 0.09 M trisodium citrate), 5 × Denhard's solution [0.1% (w/v) ficoll 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% BSA], 0.5% (w/v) SDS and 100 μg/ml degenerated salmon sperm DNA or in DIG EASY Hyb solution (Boeringer-Mannheim Co.) containing 100 μ

g/ml of degenerated salmon sperm DNA, successively keeping at a room temperature for 15 minutes two times in the presence of 1 × SSC (0.15 M NaCl and 0.015 M trisodium citrate) and 0.5% (w/v) SDS, and further keeping at 68°C for 30 minutes in the presence of 0.1 × SSC (0.015 M NaCl and 0.0015 M trisodium citrate) and 0.5% SDS.

In order to obtain DNA coding the cytokinin receptor of Arabidopsis, PCR can be carried out employing TAKARA LA taq™ (Takara Shuzo Co., Ltd.) and using a solution containing cDNA library phage of Arabidopsis (about 1,000,000 pfu) as a template and DNA having the nucleotide sequence represented by SEQ ID No: 11 and DNA having the nucleotide sequence represented by SEQ ID No: 12 as a primer set to amplify and obtain DNA to be a probe. The PCR solution to be used may be prepared by adding to 250 ng of cDNA library, the reaction solutions instructed by the kit.

The PCR conditions may be as follows: keeping at 94 °C for 2 minutes, at 8 °C for 3 minutes, and further repeating 40 cycles each of which comprising keeping at 94 °C for 30 seconds, at 55 °C for 30 seconds, and at 68 °C for 5 minutes.

Using the amplified and obtained DNA as a template, a $^{32}P$-labeled probe may be produced employing Megaprime DNA-labelling system kit (Amersham Pharmacia Co.) and using the reaction solutions instructed by the kit. Using the probe obtained in such a manner, colony hybridization is carried out by a conventional method, practically the hybridization is carried out keeping at 65 °C in the presence of 6 × SSC (0.9 M NaCl and 0.09 M trisodium citrate), 5 × Denhard's solution [0.1% (w/v) ficoll 400,

0.1% (w/v) polyvinylpyrrolidone, 0.1% BSA], 0.5% (w/v) SDS and 100 μg/ml degenerated salmon sperm DNA or in the DIG EASY Hyb solution (Boeringer-Mannheim Co.) containing 100 μg/ml of degenerated salmon sperm DNA, successively keeping at a room temperature for 15 minutes two times in the presence of 1 × SSC (0.15 M NaCl and 0.015 M trisodium citrate) and 0.5% (w/v) SDS, and further keeping at 68 ℃ for 30 minutes in the presence of 0.1 × SSC (0.015 M NaCl and 0.0015 M sodium citrate) and 0.5% SDS to obtain clone hybridized with the probe.

Further, DNA coding the cytokinin receptor may be prepared based on, for example, the nucleotide sequence represented by SEQ ID No: 1, 3 or 5 by chemical synthesis of polynucleotides according to a common method such as phosphite-triester method (Hunkapiller, M. et al., Nature, 310, 105, 1984).

The DNA coding the cytokinin receptor obtained in such a manner may be cloned in a vector by a common method described in, "Molecular Cloning: A Laboratory Manual 2nd edition" (1989), Cold Spring Harbor Laboratory Press; "Current Protocols In Molecular Biology" (1987), John Wiley & Sons, Inc. ISBNO-471-50338-X, and the like. The vector to be used may be, for example, pBlue Script II vector (produced by Stratagene Co.), pUC18/19 vector (produced by Takara Shuzo Co., Ltd.), TA cloning vector (produced by Invitrogen Co.), and the like

Incidentally, the nucleotide sequence of cloned DNA may be confirmed by, the Maxam Gilbert method (described in, for example, Maxam, A., M & W. Gilbert, Proc. Natl. Acad. Sci. USA, 74, 560, 1977, and the like), the Sanger method (described in, for example, Sanger, F. & A.R. Coulson, J.

Mol. Biol., 94, 441, 1975, Sanger, F. & Nicklen and A.R. Coulson., Proc. Natl. Acd. Sci. USA, 74, 5463, 1977, and the like). For the process, the following commercialized kits may be, for example, employed: Thermo Sequenase II dye terminator cycle sequencing kit (produced by Amersham Pharmacia Co.), Dye Terminator Cycle Sequencing FS Ready Reaction Kit (produced by PE Biosystems Japan Co.), and the like.

(3) Construction of expression vector

A expression vector of DNA coding the cytokinin receptor may be constructed according to a common method described in, for example, Molecular Cloning 2nd edition written by J., Sambrook, E., F., Frisch, & T., Maniastis, published by Cold Spring Harbor Laboratory Press.

Usable are vectors to be used in host cells to be transformed, for example, independently replicating vectors which contain genetic information possible to be duplicated in the host cells and further are possible to be isolated from the host cells and purified and may have detectable marker. More practically, in the case of using bacteria such as E. coli as the host cells, for example, Plasmid pUC 119 (produced by Takara Shuzo Co., Ltd.), Phagemid pBluescript II (Stratagene Co.) and the like may be used. In the case of using yeast as the host cells, for example, Plasmid pACT2 (Clontech Co.) and the like may be used. In the case of using plant cells as the host cells, for example, DNA coding the cytokinin receptor may be integrated with Plasmid pBI221 (Clontech Co.) to construct the vectors.

A expression vector possible to express DNA coding the cytokinin receptor in a host cell may be constructed by integrating a promoter with the aforementioned vectors in the upstream of the DNA coding the cytokinin

receptor in a binding manner of enabling to function in the host cell. In this case, the phrase, "in a binding manner of enabling to function", means that the promoter and the DNA coding the cytokinin receptor are bonded as to express the DNA coding the cytokinin receptor in the host cell under the control of the promoter. Usable as the promoter possible to function in the host cell in the case of using E. coli as the host cell are, for example, a promoter (*lacP*) of lactose operon of E. coli, a promoter (*trpP*) of triptophan operon, a promoter (*argP*) of arginine operon, a promoter (*galP*) of galactose operon, a tac-promoter, T7-promoter, T3-promoter, λ-phage promoter, (λ-pL, λ-pR) and the like. In the case of using yeast as the host cell, it may be prepared by a conventional genetic engineering method [described in Method in Enzymology 101 part (p.192-201) by Ammerer, et. al.] from *ADH1* promoter (the *ADH1* promoter is available from the yeast expression vector pAAH5 which contains the *ADH1* promoter and its terminator and which may be obtained from Washington Research Foundation). The *ADH1* promoter is included in US patent application number 299,733 of Washington Research Foundation and in the case that it is used for industrial and commercial purposes in USA., it is required to obtain permission from the patent holder. In the case of using a plant cell as the host cell, usable examples are a nopaline synthesis enzyme gene (*NOS*) promoter, an octopine synthesis enzyme gene (*OCT*) promoter, a cauliflower mosaic virus (CaMV)-derived 19S promoter, a CaMV-derived 35S promoter and the like.

Further, in the case of integrating DNA coding the cytokinin receptor into a vector already having a promoter possible to function in a

host cell, DNA coding the cytokinin receptor is inserted in the downstream of the promoter and the DNA coding the cytokinin receptor is inserted in a manner of enabling to function. For example, the aforementioned plasmid pACT 2 for yeast comprises the *ADH1* promoter and therefore, an expression vector possible to express the DNA coding the cytokinin receptor in yeast, such as CG1945 (Clontech Co.) may be constructed by inserting the DNA coding the cytokinin receptor in the downstream of the *ADH1* promoter of the plasmid pACT2.

(4) Production of transformed cell

A transformed cell to be used in the present invention may be produced by introducing the constructed expression vector into a host cell by a conventional method. As the host cell to be used for production of the transformed cell, examples are bacteria, yeast, plant cell and the like. As bacteria, examples are bacteria belonging to E. coli, Serratia, Bacillus, Brevibacterium, Corynebacterium, Microbacterium and the like. As yeast, examples are budding yeast and fission yeast. More particularly, examples are yeast belonging to Saccharomyces, Schizosaccharomyces and the like. As plant cell, examples are BY-2 strain, which is a cultured cell of tobacco, and BMS strain, which is a cultured cell of corn (Black Mexican Sweet), and the like.

As the method for introducing the expression vector into the aforementioned host cell, conventional introduction methods are employed according to the host cell to be transformed. For example, in the case of using bacteria as the host cell, the aforementioned expression vector may be introduced into a host cell by employing a conventional introduction method

such as a calcium chloride method and an electroporation method described in, "Molecular Cloning" (by J. Sambrook, et. al Cold Spring Harbor 1989). In the case of using yeast as a host cell, the aforementioned expression vector may be introduced into the host cell by employing Yeast transformation kit (produced by Clontech Co.) based on a lithium method. Further, in the case of using a plant cell as a host cell, the aforementioned expression vector may be introduced into the host cell by a conventional introduction method, for example, Agrobacterium infection method (Japanese Examined Patent Application No. 2 - 58917, Japanese Laid-Open Patent Application No. 60 - 70080), an electroporation method into a protoplast (Japanese Laid-Open Patent Application No. 60 - 251887, Japanese Laid-Open Patent Application No. 5 - 68575), a particle gun method (Japanese Laid-Open Patent Application No. 6 - 508316, Japanese Laid-Open Patent Application No. 63 - 258525).

(Transformed cell in which a cytokinin receptor of partially transmembrane region-deleted type, i.e. a cytokinin receptor of a transmembrane quantity variation type, is expressed)

A cytokinin receptor to be used in the present invention includes a cytokinin receptor wherein said cytokinin receptor has at least one transmembrane region but less than that in its natural form (commonly 2 to 4 transmembrane region) (incidentally, in the present invention, such cytokinin receptors are sometimes referred as to cytokinin receptors of partially transmembrane region-deleted type.) In this case, the phrase, "its natural form" means cytokinin receptors having an amino acid sequence most frequently existing among organisms having the similar nomenclature

and generally called also as wild-type cytokinin receptor.

Such cytokinin receptors of partially transmembrane regions-deleted type are cytokinin receptors whose transmembrane region structure may be determined by employing structure assumption software available in http://www.ch.embnet.org/software/TMPRED_form.html and whose transmembrane regions are partially deleted, for example, in 1 to 2 sites and are less in number than the number of the transmembrane regions of the natural type cytokinin receptors (i.e. natural form).

More particularly, examples of such cytokinin receptors include a cytokinin receptor having the amino acid sequence from amino acid number 196 to 1176 among the amino acid sequence represented by SEQ ID No: 2 (2 transmembrane regions); a cytokinin receptor having the amino acid sequence from amino acid number 50 to 1176 among the amino acid sequence represented by SEQ ID No: 2 (3 transmembrane regions); a cytokinin receptor having the amino acid sequence from amino acid number 32 to 1036 among the amino acid sequence represented by SEQ ID No: 4 (2 transmembrane regions); a cytokinin receptor having the amino acid sequence derived from the amino acid sequences of these cytokinin receptors wherein one or a plurality of amino acids are deleted, substituted or added, for example, cytokinin receptors having the amino acid sequence derived from the amino acid sequences in which one methionine is added to the amino-terminal and the like.

The DNA coding the cytokinin receptor may be constructed as to hold transmembrane regions in a less number than the number of the transmembrane regions of the natural type cytokinin receptors by partially

deleting the transmembrane regions by a conventional genetic engineering technique.

Production of the transformed cell in which the DNA coding the cytokinin receptor is introduced may be carried out according to the aforementioned method, "Production of transformed cell in which the DNA coding a cytokinin receptor is introduced".

(Transformed cell to express chimera-type cytokinin receptor)

A cytokinin receptor to be used in the present invention also includes a chimera-type cytokinin receptor comprising an extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, a histidine kinase region of the cytokinin receptor, and a receiver region for a histidine kinase. The chimera-type cytokinin receptor has the extracellular region, the transmembrane regions, and the histidine kinase region homogeneous to one another and the receiver region for the histidine kinase heterogeneous to these regions. For example, the chimera-type cytokinin receptor may have said extracellular region, transmembrane regions, and histidine kinase region derived from a cytokinin receptor selected from CRE1, AHK2, and AHK3, and have the receiver region derived from the osmosensor SLN1 in budding yeast.

In the above, histidine kinase regions of the cytokinin receptor may include the region present C-terminally downstream from the particular transmembrane region which is the most C-terminally downstream transmembrane region of the cytokinin receptor. Said histidine kinase region typically has five conservative motifs which are common to generic

histidine kinases as described in Annual Review of Genetics 23:311-336 (1989), Microbiological Reviews 53(4):450-490(1989), Science 262:539-544(1993), and the like. Examples of the region includes a region having the amino acid sequence from amino acid number 587 to 844 among the amino acid sequence represented by SEQ ID No: 2 in a case of AHK2, a region having the amino acid sequence from amino acid number 450 to 700 among the amino acid sequence represented by SEQ ID No: 4 in a case of AHK3 and a region having the amino acid sequence from amino acid number 449 to 714 among the amino acid sequence represented by SEQ ID No: 6 in a case of CRE1.

Receiver regions of the cytokinin receptor may include the region existing between the histidine kinase region and the C-terminal end of the cytokinin receptor. Said region having three conservative motifs which are common to generic histidine kinases as described in Annual Review of Genetics 23:311-336 (1989), Science 262:539-544(1993), and the like. Examples of the region includes a region having the amino acid sequence from amino acid number 891 to 1163 among the amino acid sequence represented by SEQ ID No: 2 in a case of AHK2, a region having the amino acid sequence from amino acid number 746 to 1018 among the amino acid sequence represented by SEQ ID No: 4 in a case of AHK3 and a region having the amino acid sequence from amino acid number 763 to 1038 among the amino acid sequence represented by SEQ ID No: 6 in a case of CRE1.

In addition, a sensor region for cytokinin means, for example, a region which is a part of any of the extracellular regions of the cytokinin receptor, wherein said sensor region is present between a transmembrane

region next to the histidine kinase region and a transmembrane region secondary close to the histidine kinase region. Said sensor region typically has 50% or more identification and homology between three cytokinin receptors of AHK2, AHK3 and CRE1 as described in Plant and Cell Physiology 42(2):231-235(2001) and the like. Examples of the region includes a region having the amino acid sequence from amino acid number 259 to 536 among the amino acid sequence represented by SEQ ID No: 2 in a case of AHK2, a region having the amino acid sequence from amino acid number 120 to 399 among the amino acid sequence represented by SEQ ID No: 4 in a case of AHK3 and a region having the amino acid sequence from amino acid number 132 to 398 among the amino acid sequence represented by SEQ ID No: 6 in a case of CRE1.

The histidine kinase protein has the following sequence in common in plants, e.g. higher plants, and microorganism. That is, histidine kinase protein is composed of an extracellular region, transmembrane region (generally about 2 to 4), histidine kinase region having histidine kinase activity and holding Histidine residue to be an active site, and receiver region having a reception part for a phosphoryl group transfer and holding Aspartic acid residue to be an active site. In the chimera-type cytokinin receptor, it is preferable that the extracellular region, the transmembrane regions, and the histidine kinase region are all derived from the identical type of cytokinin receptor, whereas the receiver regions are derived differently from type of the former cytokinin receptor.

It is sufficient for the receiver region of the chimera-type cytokinin receptor to have a function of receiving signals transmitted from the

histidine kinase region and transmitting them to the next step and any of receiver region may be usable as long as they can complement or improve the intrinsic functions of the receiver region of a histidine kinase protein comprising the homogeneously derived extracellular region, transmembrane regions, and histidine kinase region.

As such a receiver region, usable are, for example, a receiver region of histidine kinase proteins derived from microorganism (e.g. a receiver region of histidine kinase protein derived from a microorganism such as yeast, E. coli) and more particularly receiver region of histidine kinase protein coded in *SLN1* gene derived from budding yeast (e.g. the amino acid sequence represented by SEQ ID No: 7), receiver region of histidine kinase protein coded in Chey gene derived from Salmonella, receiver region of histidine kinase protein coded in *RcsC* gene, which is a hybrid sensor of E. coli [Maeda T, et al. Nature: 369 242-245, (1994): e.g. the amino acid sequence represented by SEQ ID No: 8], receiver region of histidine kinase protein coded in *Phks* gene relevant to cell cycle control of fission yeast [Shieh, JC, et al., Gene Dev. 11, 1008-1022(1997)].

The DNA coding the chimera-type cytokinin receptor may be constructed by respectively producing DNA for each of the extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, histidine kinase region of the cytokinin receptor, and receiver region for the histidine kinase protein, joining the DNA by a common genetic engineering technique so as to prevent appearance of any termination codon in the middle while using a proper linker so as to prevent frame shift. Incidentally, one DNA fragment may be utilized to provide the

DNA encoding the extracellular region of the cytokinin receptor and transmembrane regions of the cytokinin receptor or the DNA encoding the extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, and histidine kinase region of the cytokinin receptor.

The polynucleotides coding the aforementioned respective regions may respectively be produced by known methods. For example, in the case of production by PCR, at first, the oligonucleotides (5' side primers) having the nucleotide sequences of 5' terminal regions of respective regions to be amplified and oligonucleotides (3' side primers) having complementary nucleotide sequences to the nucleotide sequences of 3' terminal are designed and synthesized. The primers may be oligonucleotides of about 14 nucleotides to about 35 nucleotides in general and are preferable to contain in the 5' side of the primers, restriction enzyme recognition sequences usable at the time of ligating the polynucleotides amplified by the PCR to one another or these polynucleotides to vectors. Then, using the primers and the cDNA library as a template, amplification reactions may be carried out in the common reaction conditions employed for the PCR. As the template to be used in the case of producing the polynucleotides coding the extracellular region and the transmembrane regions of the cytokinin receptor or the histidine kinase region of the cytokinin receptor, usable is cDNA library derived from plants such as higher plants. Also as the template to be used in the case of producing the polynucleotides coding the receiver regions of the histidine kinase, usable is the total DNA or cDNA library derived from microorganism prepared by a common method.

The production of the transformed cell in which the DNA coding the

chimera-type cytokinin receptor is introduced may be carried out according to the aforementioned, "Production of the transformed cell in which the DNA coding a cytokinin receptor is introduced.".

(Intracellular signal transduction system relevant to cytokinin)

Measurement of the existence or the quantity of intracellular signal transduction from the cytokinin receptor expressed in the transformed cell produced by the above-described manner may be carried out by utilizing the intracellular signal transduction system originally present in the host cell used for production of the transformed cell. The existence or the quantity of intracellular signal transduction can be determined by employing the quantity of the cell growth of the transformed cell as an indicator. Alternatively, a regulator (and/or a mediator), which is a downstream signal transducer in the so-called Two-Component regulatory system, can be additionally introduced and expressed in the host cell and the expressed system may be used in the intracellular signal transduction system. Usable as the Two-Component regulatory system, for example, are Two-Component regulatory systems corresponding to 5 types of ethylene receptors; ETR1, ETR2, ERS1, ERS2, and EIN4; present in Arabidopsis [Chang et al., Science 262: 539-544 (1993), Hua et al., Science 269: 1712-1714 (1995), Sakai et al., Plant Cell Physiol 39: 1232-1239 (1998)] and AtHK1 having sensor functions to the osmotic pressure [Urao, Plant ell 11: 1743-1754 (1999)].

As the host cell to be used for production of such a transformed cell, usable are host cells improved as to have histidine kinase activity lower

than the intrinsic histidine kinase activity of the host cells. For example, it includes the host cells improved as to have histidine kinase activity lower than the intrinsic histidine kinase activity of the host cell by deleting one or more of histidine kinases. The phrase, "histidine kinase activity lower than" means that the quantity of a phosphoryl group transfer from Histidine residue (the active site of histidine kinase regions having histidine kinase activity) to Aspartic acid residue (the active site of receiver regions having a reception part) is decreased. Such improvements in the host cell may provide a change of the quantity of the cell growth, change of the morphology, change of the shape, change of the quantity of the biosynthesis of specific compound, change of the quantity of the metabolism of specific compound in the transformed cell. More particularly, the following strain [Maeda T et al., Nature 369: 242-245 (1994)] may be exemplified: a strain obtained by defecting the *SLN1* gene coding the protein having the osmotic pressure sensor function and derived from the budding yeast such as Saccharomyces cerevisiae and the like. Since the strain is defected in the histidine kinase protein existing in Saccharomyces cerevisiae, there can be more clearly detected the existence or the quantity of the intracellular signal transduction from the cytokinin receptor expressed in the transformed cell by using the quantity of the cell growth of the transformed cell as an indicator. Further, other preferable example includes a defective strain of the *RcsC* gene, which is a hybrid sensor derived from E. coli and a defective strain of *Phks* gene relevant to the cell cycle control of fission yeast.

(Method for analyzing agonist-activity and antagonist-activity to a cytokinin

receptor)

In the method for analyzing agonist-activity to a cytokinin receptor, examples of the first step of bringing an examinee substance into contact with a transformed cell into which DNA coding the cytokinin receptor is introduced include a method for culturing the transformed cell in a culture medium containing the examinee substance. In order to culture the transformed cell, the following cultures are usable: liquid-phase culture for culturing the transformed cell in a liquid culture medium and a solid-phase culture for culturing the transformed cell in a solid culture medium produced by adding agar or the like to the liquid culture medium. The concentration of an examinee substance in the culture medium is about 1 nM to about 1 mM and preferably about 10 nM to about 100 $\mu$ M. The culture time is, for example, 1 hour to 3 days and preferably 25 hours to 2 days. Incidentally, in the case of method for analyzing the agonist-activity to the cytokinin receptor, the culture containing no cytokinin may be used as the culture containing the examinee substance. When transforming the host cell to analyze the agonist-activity to a cytokinin receptor, it is preferable that the host cell is introduced with DNA encoding any one of the following cytokinin receptors: the cytokinin receptor having the amino acid sequence represented by SEQ ID No: 2; the cytokinin receptor having the amino acid sequence represented by SEQ ID No: 4; the cytokinin receptor wherein said cytokinin receptor has at least one transmembrane region but less than that in its natural form; the cytokinin receptor having the amino acid sequence from amino acid number 196 to 1176 among the amino acid sequence represented by SEQ ID No: 2; the cytokinin receptor having the

amino acid sequence from amino acid number 50 to 1176 among the amino acid sequence represented by SEQ ID No: 2; the cytokinin receptor having the amino acid sequence from amino acid number 32 to 1036 among the amino acid sequence represented by SEQ ID No: 4; the chimera-type cytokinin receptor comprising an extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, a histidine kinase region of the cytokinin receptor, and a receiver region of the histidine kinase, wherein said extracellular region, said transmembrane regions and said histidine kinase region are homogeneous to one another and the receiver region is heterogeneous thereto; and the cytokinin receptor having the amino acid sequence with deletion, substitution, or addition of one or a plurality of amino acids in the amino acid sequence of the above cyctokinin receptors.

In the method for analyzing the antagonist-activity to the cytokinin receptor, examples of the first step of bringing an examinee substance and a substance having the agonist-activity to the cytokinin receptor into contact with a transformed cell into which DNA coding the cytokinin receptor is introduced include a method for culturing the transformed cell in a culture medium containing the examinee substance and a substance having the agonist-activity to the cytokinin receptor. In order to culture the transformed cell, the following cultures are usable: liquid-phase culture for culturing the transformed cell in a liquid culture medium and a solid-phase culture for culturing the transformed cell in a solid culture medium produced by adding agar or the like to the liquid culture medium. The concentration of an examinee substance in the culture medium is about 1

nM to about 1 mM and preferably about 10 nM to about 100 $\mu$ M. The concentration of the substance (e.g. cytokinins such as trans-zeatin, cis-zeatin, benzyl adenine, thidiazuron and the like) having the agonist-activity to the cytokinin receptor is about 1 nM to about 1 mM and preferably about 10 nM to about 100 $\mu$ M. The culture time is, for example, 1 hour to 3 days and preferably 25 hours to 2 days. Incidentally, in the case of method for analyzing the antagonist-activity to the cytokinin receptor, the culture containing a cytokinin may be used as the culture medium containing the examinee substance and the substance having the agonist-activity to the cytokinin receptor. When transforming the host cell to analyze the antagonist-activity to a cytokinin receptor, it is preferable that the host cell is introduced with DNA encoding any one of the following cytokinin recpetors: the cytokinin receptor having the amino acid sequence represented by SEQ ID No: 2; the cytokinin receptor having the amino acid sequence represented by SEQ ID No: 4; the cytokinin receptor having the amino acid sequence represented by SEQ ID No: 6; the cytokinin receptor wherein said cytokinin receptor has at least one transmembrane region but less than that in its natural form; the cytokinin receptor having the amino acid sequence from amino acid number 196 to 1176 among the amino acid sequence represented by SEQ ID No: 2; the cytokinin receptor having the amino acid sequence from amino acid number 50 to 1176 among the amino acid sequence represented by SEQ ID No: 2; the cytokinin receptor having the amino acid sequence from amino acid number 32 to 1036 among the amino acid sequence represented by SEQ ID No: 4; the chimera-type cytokinin receptor comprising an extracellular region of the cytokinin

receptor, transmembrane regions of the cytokinin receptor, a histidine kinase region of the cytokinin receptor, and a receiver region of the histidine kinase, wherein said extracellular region, said transmembrane regions and said histidine kinase region are homogeneous to one another and the receiver region is heterogeneous thereto; and the cytokinin receptor having the amino acid sequence with deletion, substitution, or addition of one or a plurality of amino acids in the amino acid sequence of the above cyctokinin receptors.

The agonist activity or the antagonist activity of the examinee to the cytokinin receptor can be detected by evaluating the agonist-activity or antagonist-activity of two or more examinee substances. The agonist-activity or the antagonist activity can be provided for the evaluation by conducting the aforementioned method for analyzing the agonist-activity or the antagonist-activity to the cytokinin receptor. For example, the evaluation of the agonist-activity or antagonist activity can be based on the difference between the two or more examinee substances, which are obtained by comparison of the existence or the quantity of the intracellular signal transduction. In utilizing the two or more different examinee substances, the examinee substances are typically utilized independently in different systems. It is preferable that at least one of the two or more examinee substances has no agonist-activity or antagonist-activity to the cytokinin receptor.

More particularly, for example, in the case of using a transformed cell (that is, a transformed cell having a function of directly controlling the cell growth by intracellular signal transduction from the cytokinin receptor)

produced using TM182 (*sln1* Δ) [Maeda T et al, Nature 369: 242-245 (1994)], a *SLN1* genetically defected strain in which, for example, *PTP2* Tyrosine phosphatase gene [Ota et al. Proc. N. A. Sci., USA, 89, 2355-2359 (1992)] is introduced as a host cell, the agonist-activity to the cytokinin receptor may be measured using, as an indicator, the quantity of the cell growth of the transformed cell in a culture medium (an agar culture medium or a liquid culture medium) containing glucose as a carbon source, for example, a DOLU + Glu culture medium. In the case of the DOLU + Glu culture medium to which the examinee substance is added, the examinee substance found capable of growing the transformed cell can be evaluated as a substance having the agonist-activity to the cytokinin receptor. On the other hand, in the case of the DOLU+Glu culture medium to which the examinee substance and a substance having the agonist-activity to the cytokinin receptor are added, the examinee substance found capable of suppressing or inhibiting the growth of the transformed cell can be evaluated as a substance having the antagonist-activity to the cytokinin receptor.

Incidentally, as a blank, the growth of the transformed cell in a culture medium using galactose as the carbon source in place of glucose, for example, a DOLU + Gal culture medium, can be investigated independently of the existence of the examinee substance.

Further, in the case of using a transformed cell (that is, a transformed cell having a function of directly controlling the cell growth by the intracellular signal transduction from the cytokinin receptor) produced by employing fission yeast as a host cell, such as a Phks genetically defected

strain, the fission pattern of the fission yeast may be observed with a microscope. In this case, when the examinee substance is added to the culture medium containing no substance having the agonist-activity to the cytokinin receptor, the examinee substance found capable of normal fission and propagation of the transformed cell can be evaluated as a substance having the agonist-activity to the cytokinin receptor. On the other hand, if the culture medium contains an examinee substance and a substance having the agonist-activity to the cytokinin receptor, the examinee substance found capable of suppressing or inhibiting the normal fission and propagation of the transformed cell can be evaluated as a substance having the antagonist-activity to the cytokinin receptor.

Furthermore, in the case of using a transformed cell (that is, a transformed cell having a function of directly controlling reporter gene expression by the intracellular signal transduction from the cytokinin receptor) produced by employing as the host cell an E. coli defective in RcsC gene into which cps-LacZ is introduced, the X-Gal coloring may be observed in an agar culture medium or a liquid culture medium [Suzuki et al. Plant Cell Physiol 42: 107-113 (2001)]. In this case, if the culture medium contains the examinee substance and no substance having the agonist-activity to the cytokinin receptor, the examinee substance found capable of coloring the transformed cell to be blue can be evaluated as a substance having the agonist-activity to the cytokinin receptor. On the other hand, if the culture medium contains the examinee substance and a substance having the agonist-activity to the cytokinin receptor, the examinee substance found capable of disappearing coloring the blue of the

transformed cell can be evaluated as a substance having the antagonist-activity to the cytokinin receptor.

Moreover, an agonist-active substance or an antagonist-active substance to the cytokinin receptor may be searched by selecting substances having the agonist-active substance or the antagonist-active substance to the cytokinin receptor based on the agonist-activity or the antagonist-activity to the cytokinin receptor evaluated by the aforementioned detection methods.

Further, a substance selected by the above described detection methods may be utilized as an active ingredient of a plant growth regulator.

The plants to be the objects to be treated with the aforementioned plant growth regulator are, for example, decorative plants such as flowering plants and ornamental foliage plants; cultivating plants such as crop, vegetable, fruit and the like; fibrous plants; trees; lawn and the like.

The growth regulator is generally mixed with a solid carrier, a liquid carrier, and the like and further, if needed, mixed with a surfactant and other auxiliary agents for the formulation of an agricultural and horticultural agent and formulated in an emulsion agent, a hydrating agent, a suspension agent, a solution agent and the like. In these agricultural and horticultural agents, an agonist-active substance or an antagonist-active substance to the cytokinin receptor may be contained generally in 0.5 to 90 % by weight and preferably in 1 to 80 % by weight.

Usable as the solid carrier to be used for the formulation of an agricultural and horticultural agent are, for example, clays (kaolinite, kieselguhr, synthesized hydrated silicon oxide, intercalated clays, bentonite,

acidic white clay, and the like), talc, other inorganic minerals (sericite, quartz powder, sulfur powder, activated carbon, calcium carbonate, and the like), chemical fertilizers (ammonium sulfate, ammonium phosphate, ammonium nitrate, ammonium chloride, urea, and the like) in finely powdered state or in granular state and usable as the liquid carrier are, for example, water, alcohols (methanol, ethanol and the like), ketones (acetone, methyl ethyl ketone, cylohexanone and the like), aromatic hydrocarbons (toluene, xylene, ethylbenzene, methylnaphthalene and the like), non-aromatic hydrocarbons (hexane, cyclohexane, kerosene and the like), esters (ethyl acetate, butyl acetate and the like), nitriles (actonitrile, isobutylnitrile and the like), ethers (dioxane, diisopropyl ether and the like), acid amides (dimethylformamide, dimethylacetamide and the like), halohydrocarbons (dichloroethane, trichloroethane and the like), etc.

As the surfactant, usable are, for example, alkylsulfuric acid esters, alkylsulfonic acid salts, alkylarylsulfonic acid salts, alkylaryl ethers and their polyoxyethylene compounds, polyethylene glycols, polyhydric alcohol esters, sugaralcohols and the like.

As other auxiliary agents for the formulation of agricultural and horticultural agents, usable are solidification agents and dispersanst such as casein, gelatin, polysaccharides (starch, acacia, cellulose derivatives, alginic acid and the like), lignin derivatives, bentonite, synthesized water-soluble polymer [poly(vinyl alcohol), poly(vinyl pyrolidone), poly(acrylic acid) and the like] and the like, and stabilizers such as PAP (acidic isopropyl phosphate), BHT (2,6-tert-butyl-4-methylphenol), BHA (2-/3-tert-butyl-4-methoxyphenol), plant oils, mineral oils, aliphatic acids,

aliphatic acid esters and the like.

The agonist-active substance or the antagonist-active substance to the cytokinin receptor made to be agricultural and horticultural agents is used as it is or diluted with water to carry out treatment for the stem and leave parts, branch and leave parts, and flower and fruit parts of plants by spraying, for fruits by immersion, and for fruits by application. The plant growth regulator is used for the object plants to carry out the treatment once or a plurality of times.

In the case of using the plant growth regulator for the purpose of suppressing the dropping of fruits, the plant growth regulator is diluted with water and the resulting diluted agent is sprayed to the fruit parts and branch and leave parts before harvest.

In the case of using the plant growth regulator for the purpose of suppressing the ball dropping of cotton, the plant growth regulator is diluted with water and the resulting diluted agent is sprayed to the balls and stem and leave parts of cotton before harvest.

The plant growth regulator may be used for the treatment of growing plants or of plants after harvest.

The appilcation amount of the agonist-active substance or the antagonist-active substance to the cytokinin receptor in an agricultural and horticultural agent is generally 1 to 8000 g per 1 hectare, although it is changed depending on the state of the agricultural and horticultural agent, the timing for the treatment, the method for the treatment, the site for the treatment, and the object plant to be treated. Also in the case of using the plant growth regulator while diluting the agent with water, the

concentration of the agent is generally 0.0001 to 1000 mM and preferably 0.001 to 10 mM, although it is changed depending on the state of the agricultural and horticultural agent, the timing for the treatment, the method for the treatment, the site for the treatment, and the object plant to be treated.

Next, hereinafter given are formulation examples of an agricultural and horticultural agent produced from an agonist-active substance or an antagonist-active substance to the cytokinin receptor and used as a plant growth regulator. The parts in the following description of the examples denotes the parts by weight.

Formulation example 1

A hydrating agent was obtained by sufficiently pulverizing and mixing 50 parts of an agonist-active substance or an antagonist-active substance to the cytokinin receptor, 3 parts of calcium ligninsulfonate, 2 parts of sodium laurylsulfate, and 45 parts of synthesized hydrated silicon oxide.

Formulation example 2

A hydrating agent was obtained by sufficiently pulverizing and mixing 70 parts of an agonist-active substance or an antagonist-active substance to the cytokinin receptor, 3 parts of calcium ligninsulfonate, 2 parts of sodium laurylsulfate, and 25 parts of synthesized hydrated silicon oxide.

Formulation example 3

An emulsion agent was obtained by mixing 40 parts of an agonist-active substance or an antagonist-active substance to the cytokinin

receptor, 3 parts of polyoxyethylene sorbitane monoolate, 2 parts of CMC (carboxymethyl cellulose), and 52 parts of water and wet-pulverizing the resultant mixture to be 5 $\mu$ m or smaller in the particle size.

The present invention makes it possible to analyze the agonist-activity and the antagonist-activity to the cytokinin receptor and also makes it possible to quickly search the substances having the agonist-activity and the antagonist-activity to the cytokinin receptor even in a small amount of examinee substances by employing the analysis method.

EXAMPLES

Hereinafter, although the present invention will be described in details with the reference to examples, the present invention is not at all restricted to these examples.

(Example 1) Production of Arabidopsis cDNA phage library for CRE1 cloning

Seeds of Arabidopsis thaliana ecotype Wassilewskija were sterilized with 70% of ethyl alcohol for 1 minute and further sterilized with 1.5% of sodium hypochlorite for 10 minutes. The resulting seeds were well washed with sterilized water and then cultured for 2 weeks in GM culture medium [4.3 g Murashige and Skoog's basal salt mixture, 1% sucrose, 10 ml of 5% MES-KOH (pH 5.7), 0.3% Phytagel$^{TM}$ (SIGMA)] to obtain 5g of the plant. After the plant was frozen in liquefied nitrogen and physically milled with a mortar and a pestle. The resulting milled product was mixed with a mixed solution of 10 ml of an extraction buffer [200 mM Tris-HCl (pH 8.5), 100 mM NaCl, 10 mM EDTA, 0.5% SDS, 14 mM $\beta$ -mercaptoethanol] and 10 g of phenol. After being mixed by a Vortex mixer, the resulting mixture was

mixed further with 10 ml of chloroform and vigorously stirred and subjected to centrifugal separation at 10,000 rotation for 20 minutes. The recovered aqueous layer was mixed with LiCl in the concentration to be 2M of the final concentration, left still at -80°C for 3 hours, thawed and subjected to centrifugal separation at 10,000 rotation for 20 minutes to recover a precipitate. The recovered precipitate was dissolved in 2 ml of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and then further mixed with 0.2 ml of 3 M sodium acetate (pH 5.2) and 5 ml ethanol and subjected to centrifugal separation to recover RNA as a precipitate. Further, the precipitate (RNA) was subjected to treatment with Oligotex™ dT30super (Nippon Rosch Co.) to extract RNA integrated with polyA.

The production of phage cDNA library from the extracted RNA integrated with polyA was carried out employing ZAP-cDNAR Synthesis Kit (Stratagene Co.) according to the instruction. The potency of the produced phage cDNA library was 500,000 PFU.

(Example 2) Production of DNA probe of CRE1

The PCR was carried out employing TAKARA LA Taq™ (Takara Shuzo Co., Ltd.) and using a phage solution (about 1,000,000 PFU) of the phage cDNA library produced in the example 1 as a template and DNA having the nucleotide sequence represented by SEQ ID No: 11 and DNA having the nucleotide sequence represented by SEQ ID No: 12 as the primers to amplify DNA. The procedure will be described in details below.

A PCR solution was prepared by adding a reaction composition containing dNTP and the like to the phage 1,000,000 pft and respective primer DNA each in 0.2 $\mu$ M according to the instruction of the kit and the

desired DNA fragment was amplified in PCR conditions: keeping at 94℃ for 2 minutes, and further repeating 40 cycles each of which comprised keeping at 94℃ for 30 seconds, at 55℃ for 30 seconds, and at 68℃ for 5 minute. Next, using the amplified DNA fragment as a template, a probe labelled with $^{32}P$ was prepared employing Megaprime DNA-labelling system kit (Amersham Pharmacia Co.). Incidentally, reaction solution (25 μl) was prepared by adding 2.0 MBq of $^{32}P$ dCTP to 25 ng of amplified DNA fragment and adding a reaction composition instructed by the kit. The labelling reaction was carried out at 37℃ for 10 minutes.

(Example 3) Production of phage cDNA clone holding CRE1

The cloning of desired CRE1 gene was carried out by plaque hybridization using the probe prepared by the example 2. Detailed description will be given below.

Using the phage cDNA library produced in the example 1 and according to the instruction of ZAP·cDNAR Synthesis Kit, plaque was produced. DNA was adsorbed on a nitrocellulose filter from the produced plaque and then treated with UV rays to be fixed on the filter. Using the filter prepared in such a manner, hybridized phage cDNA clone was obtained by keeping at 65°C in the presence of 6 × SSC (0.9 M NaCl and 0.09 M trisodium citrate), 5 × Denhard's solution [0.1% (w/v) ficoll 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% BSA], 0.5% (w/v) SDS and 100 μg/ml degenerated salmon sperm DNA or in DIG EASY Hyb solution (Boeringer-Mannheim Co.) containing 100 μg/ml of degenerated salmon sperm DNA, successively keeping at a room temperature for 15 minutes two times in the presence of 1 × SSC (0.15 M NaCl and 0.015 M trisodium

citrate) and 0.5% (w/v) SDS, and further keeping at 68℃ for 30 minutes in the presence of 0.1 × SSC (0.015 M NaCl and 0.0015 M trisodium citrate) and 0.5% SDS.

(Example 4) Cloning of CRE1 cDNA

Using the cDNA of the phage cDNA clone obtained by the example 3 as a template and DNA having the nucleotide sequence represented by SEQ ID No: 13 and DNA represented by SEQ ID No: 14 as primers, DNA having the nucleotide sequence represented by SEQ ID No: 5 was amplified by PCR. Detailed description will be given below.

The PCR was carried out employing Herculase Enhanced DNA Polymerase (TOYOBO Co., Ltd.) in the reaction conditions of keeping at 94℃ for 1 minute, and further repeating 25 cycles each of which comprised keeping at 94℃ for 30 seconds, at 55℃ for 30 seconds, and at 72℃ for 4 minute. Incidentally, the PCR solution (50 μl) was prepared by adding a reaction composition containing dNTP and the like to 500 ng of cDNA of the phage cDNA clone and respective primer DNA each in 100 ng according to the instruction of the kit.

The desired DNA fragment was amplified in such a manner.

(Example 5) Construction of CRE1 expression vector

After p415CYC1, a yeast expression vector, [Munberg et al. Gene: 156 119-122 (1995), available from ATCC library (No. 873821)] was digested with a restriction enzyme Sma I and then using T4 DNA ligase, DNA having the nucleotide sequence represented by SEQ ID No: 5 and obtained by the example 4 was ligated downstream of the CYC 1 promoter sequence of the expression vector p415CYC1 as to be integrated to express the desired

protein in yeast. The constructed DNA was confirmed to be in the right direction and its nucleotide sequence was confirmed to be the nucleotide sequence represented by SEQ ID No: 5 by an automatic DNA sequencer and then the expression plasmid p415CYC·CRE 1 was obtained.

(Example 6) Cloning of AHK3 (AAF99730) cDNA

Seeds of Arabidopsis thaliana ecotype Wassilewskija were sterilized with 70% of ethyl alcohol for 1 minute and further sterilized with 1.5% of sodium hypochlorite for 10 minutes. The resulting seeds were well washed with sterilized water and then cultured for 2 weeks in GM culture medium [4.3 g Murashige and Skoog's basal salt mixture, 1% sucrose, 10 ml of 5% MES·KOH (pH 5.7), 0.3% Phytagel™ (SIGMA)] to obtain 5g of the plant. After the plant was frozen in liquefied nitrogen and physically milled with a mortar and a pestle. The resulting milled product was mixed with a mixed solution of 10 ml of an extraction buffer [200 mM Tris·HCl (pH 8.5), 100 mM NaCl, 10 mM EDTA, 0.5% SDS, 14 mM $\beta$ ·mercaptoethanol] and 10 g of phenol. After being mixed by a Voltex mixer, the resulting mixture was mixed further with 10 ml of chloroform and vigorously stirred and subjected to centrifugal separation at 10,000 rotation for 20 minutes. The recovered aqueous layer was mixed with LiCl in the concentration to be 2M of the final concentration, left still at ·80℃ for 3 hours, thawed and subjected to centrifugal separation at 10,000 rotation for 20 minutes to recover a precipitate. The recovered precipitate was dissolved in 2 ml of TE [10 mM Tris·HCl (pH 8.0), 1 mM EDTA] and then further mixed with 0.2 ml of 3 M sodium acetate (pH 5.2) and 5 ml ethanol and subjected to centrifugal separation to recover RNA as a precipitate. Further, 40 $\mu$ g of the

precipitate (RNA) was mixed with 30 unit of FPLC pure™ DnaseI (Amersham-Pharmacia) and 60 unit of Superace (Ambion) to remove mixed genome DNA and the resulting RNA was subjected to the phenol/chloroform treatment and ethanol treatment to purify the RNA. Next, using the purified RNA as a template and oligo (dT) 12-18 (Amersham-Pharmacia) as a primer, RT-PCR was carried out. The RT-PCR was carried out employing Superscript II (GIBCO BRL Co.) at 42℃ for 40 minutes. Incidentally the PT-PCR solution was prepared according to the method described in instruction of the Superscript II.

The desired cDNA was amplified in such a manner.

Using the amplified cDNA as a template and DNA having the nucleotide sequence represented by SEQ ID No: 16 and DNA having the nucleotide sequence represented by SEQ ID No: 17 as primers, DNA having the nucleotide sequence represented by SEQ ID No: 3 was amplified by PCR. The PCR was carried out using Herculase Enhanced DNA Polymerase (TOYOBO Co., Ltd.) in the reaction conditions of keeping at 94℃ for 1 minute, and further repeating 41 cycles each of which comprised keeping at 94℃ for 30 seconds, at 55℃ for 30 seconds, and at 72℃ for 4 minute. Incidentally, the PCR solution (50 μl) was prepared by adding a reaction composition containing dNTP and the like to 500 ng of the template DNA and respective primer DNA each in 100 ng according to the instruction of the kit.

The desired DNA fragment was amplified in such a manner.

(Example 7) Construction of pHM-1

After p415CYC1 [Munberg et al. Gene: 156 119-122 (1995); available

from ATCC library (No. 87382)] was digested with the restriction enzyme Spe I and BamH I, synthesized DNA fragments (linkers) having the nucleotide sequence represented by SEQ ID Nos: 18 and 15 were inserted to the expression vector p415CYC1 using T4 DNA ligaseas to newly add the restriction enzyme sites Sac II, Apa I, Nhe I to the plasmid p415CYC1 and construct pHM-1.

(Example 8) Construction of AHK3 expression vector

After the pHM-1 was digested with the restriction enzyme Sal I and Sac II, and then using T4 DNA ligase, DNA having the nucleotide sequence represented by SEQ ID No: 3 was introduced downstream of the CYC1 promoter sequence of the expression vector pHM-1 as to be integrated to express the desired protein in yeast. The constructed DNA was confirmed to be right in the direction and its nucleotide sequence was confirmed to be right in the sequence by an automatic DNA sequencer and thus the expression plasmid p415CYC-AHK3 was obtained.

(Example 9) Cloning of AHK2(BAB09274)cDNA

Using the cDNA prepared in the example 6 (the cDNA prepared by reverse transcription of the total RNA) as a template and DNA having the nucleotide sequence represented by SEQ ID No: 19 and DNA having the nucleotide sequence represented by SEQ ID No: 20 as primers, DNA having the nucleotide sequence represented by SEQ ID No: 1 was amplified by PCR. The PCR was carried out employing TAKARA Pfu Turbo denature (Takara Shuzo Co., Ltd.) in the reaction conditions of keeping at 94℃ for 1 minute, and further repeating 30 cycles each of which comprised keeping at 94℃ for 30 seconds, at 55℃ for 30 seconds, and at 72℃ for 4 minute. Incidentally,

the PCR solution (50 μl) was prepared by adding a reaction composition containing dNTP and the like to 500 ng of the template DNA and respective primer DNA each in 50 ng according to the instruction of the kit.

The desired DNA fragment was amplified in such a manner.

(Example 10) Cloning of AHK2(BAB09274)Δ cDNA

Using the cDNA prepared in example 6 (the cDNA prepared by reverse transcription of the total RNA) as a template and DNA having the nucleotide sequence represented by SEQ ID No: 21 and DNA having the nucleotide sequence represented by SEQ ID No: 22 as primers, PCR was carried out to amplify DNA having the nucleotide sequence represented by SEQ ID No: 1 in which ATG was added to the 5' terminal sites of the nucleotide sequence from the nucleotide numbers 586 to 3531. The PCR was carried out employing TAKARA Pfu Turbo denature (Takara Shuzo Co., Ltd.) in the reaction conditions of keeping at 94℃ for 1 minute, and further repeating 30 cycles each of which comprised keeping at 94℃ for 30 seconds, at 55℃ for 30 seconds, and at 72℃ for 4 minute. Incidentally, the PCR solution (50 μl) was prepared by adding a reaction composition containing dNTP and the like to 500 ng of the template DNA and respective primer DNA each in 50 ng according to the instruction of the kit.

The desired DNA fragment was amplified in such a manner.

(Example 11) Construction of AHK2 and AHK2 Δ expression vectors

After pHM-1 was digested with a restriction enzyme Sac II and Nhe I and then using T4 DNA ligase, DNA fragments obtained by the example 9 and the example 10 were respectively ligated downstream to the CYC 1 promoter sequence of the expression vector pHM-1 as to be integrated to

express the desired protein in yeast. The introduced DNA was confirmed to be right in the direction and its nucleotide sequence was confirmed to be right in the sequence by an automatic DNA sequencer and thus the expression plasmid p415CYC-AHK 2 and p415CYC-AHK2Δ were obtained.

(Example 12) Production of transformed cells TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3

Transformation of TM182 (*sln1*Δ ), *SLN1* genetically detected strain, [Maeda T et al. Nature: 369 242-245 (1994)] was carried out using the obtained expression plasmid p415CYC-CRE1 (the example 5), p415CYC-AHK2 (the example 11), p415CYC-AHK2Δ (the example 11) and p415CYC-AHK3 (the example 8). The transformation was carried out by employing Polyethylene glycol/lithium acetate (PEG/LiAc)-mediated transformation method according to the description of VII. Library Transformation & Screening Protocols disclosed in MATCHAMAKER Two-Hybrid System 3 User Manual p. 22 from CLONTECH Co. Transformed cells TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3 were produced by selecting in the DOLU + Gal culture medium the transformed cells having a disappearance of leucine nutrient requirement.

(Example 13) Response of transformed cells TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3 to cytokinin (Part 1)

A culture solution 10 μ l (about 800 clones of yeast) of the transformed cells TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3 produced by the example 12 was spotted on DOLU + Glu agar media containing 10 μ M of trans-zeatin and cultured at 30°Cfor 30 hours.

After incubation, the growth state of the transformed cells was observed and photographed by a digital camera.

As a result, each of the transformed cells TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3 showed high growth in the case of using the DOLU + Glu agar culture media containing trans-zeatin as compared with that in the case of using the DOLU + Glu agar culture media containing no trans-zeatin. The results showed that the transformed cells responded to cytokinin and that they were possible to be grown in the DOLU + Glu agar culture media. Further the transformed cells were found possible to be grown in the DOLU + Gal agar culture media independently of the existence trans-zeatin.

(Example 14) Response of transformed cells TM182-CRE1, TM182-AHK2Δ and TM182-AHK2 to cytokinin (Part 2)

Culture solutions of the transformed cells TM182-CRE1, TM182-AHK2Δ and TM182-AHK2 produced by the example 12 were spotted on DOLU + Glu agar media containing cytokinin in a variety of concentrations (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM) and cultured at 30°C for 30 hours. After incubation, the growth state of the transformed cells was observed and photographed by a digital camera. The lowest observed supply concentrations of trans-zeatin and cis-zeatin in which the respective transformed cells could be grown were shown in Table 1.

Table 1

| cytokinin | TM182-CRE1 | TM182-AHK2 | TM182-AHK2Δ |
|---|---|---|---|
| trans-zeatin | 10μ M | 1μ M | 100nM |
| cis-zeatin | no growth | 10μ M | 1μ M |

(Example 15) Method for searching substance having agonist-activity to cytokinin receptor (Part 1)

The transformed cells TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3 produced in the example 12 were inoculated in 200 ml of DOLU + Gal culture media and pre-cultured at 30℃ for 36 hours. The pre-cultured solutions were diluted with DOLU + Glu media as to become 0.100 as the optical density ($OD_{600}$) and further the resultants were diluted with DOLU + Glu media at a dilution rate of 1/200 to obtain diluted pre-cultured solutions.

An assay plate was prepared by filling respective wells of a 96-well plate with 20 μ l of solutions which were prepared by diluting DMSO solution (10mM) of each examinee substance with DOLU + Glu medium at a dilution rate of 1/100, said solutions containing each examinee substance to be 100 μ M at the final concentration. Simultaneously, an assay plate only filled with 20 μ l of solutions which were prepared by diluting DMSO solution with DOLU + Glu medium, said solutions containing no examinee substance, was prepared as the blank sections.

The diluted pre-cultured solutions were added in 200 μ l each into the respective wells of both assay plates and cultured at 30℃ for 24 hours and then the optical density ($OD_{620}$) of each well was measured by a plate

reader. The agonist-activity of the examinee substances to the cytokinin receptor was detected by comparing the optical density measured in the testing sections to which the examinee substances were added with the optical density measured in the blank sections. The optical density of the culture solutions of the transformed cells in the testing sections to which the examinee substances were added were shown in Table 2. Compound B showing the higher optical density measured in the testing sections to which the examinee substances were added than that in the blank sections were selected as agonist-active substance to the cytokinin receptor.

Table 2

| Examinee substance | TM182-AHK 2 | TM182-AHK 2Δ | TM182-AHK 3 |
|---|---|---|---|
| DMSO | 0.01 | 0 | 0.51 |
| Compound A*1 | 0.01 | 0 | 0.54 |
| Compound B*2 | 0.45 | 0.89 | 0.88 |

*1 Abscisic acid (=negative control)

*2 6-Benzyl aminopurine (=positive control)

(Example 16) Method for searching substance having antagonist-activity to cytokinin receptor (Part 2)

The transformed cells TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3 produced in the example 12 are inoculated in 200 ml of DOLU + Gal culture media and cultured at 30℃ for 30 hours to obtain

pre-cultured solutions.

An assay plate is prepared by filling respective wells of a 96-well plate with 200 μl of each DOLU + Glu medium mixed with 1 μM of trans-zeatin (cytokinin), and into the assay plate is put each examinee substance to be 1 μM at the final concentration. Simultaneously, an assay plate only filled with 200 μl of the DOLU + Gal medium mixed with 1 μM of trans-zeatin is prepared as the blank sections

The pre-cultured solutions are added in 20 μl each into the respective wells of both assay plates and cultured at 30℃ for 30 hours and then the optical density of each well is measured by a plate reader. The antagonist-activity of the examinee substances to the cytokinin receptor is detected by comparing the optical density measured in the testing section to which the examinee substances are added with the optical density measured in the blank sections. The examinee substances showing the lower optical density measured in the testing sections to which the examinee substances are added than that in the blank sections are selected as antagonist-active substance to the cytokinin receptor.

(Example 17) Method for searching substance having agonist-activity to cytokinin receptor (Part 3)

The transformed cells TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3 produced in the example 12 are inoculated in 200 ml of DOLU + Gal culture media and cultured at 30℃ for 30 hours to obtain pre-cultured solutions.

The pre-cultured transformed cells (TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3) are spot-added in 10 μl to the

respective DOLU + Glu agar culture media to which agonist-active substances to the cytokinin receptor selected by the example 15 are added while their concentration being changed from 10 nM to 100 $\mu$ M and then are cultured at 30°C for 30 hours. After incubation, the intensity of the agonist-activity of the examinee substances to the cytokinin receptor is detected and confirmed based on the lowest concentration at which transformed cells (TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3) are observed to grow.

(Example 18) Method for searching substance having antagonist-activity to cytokinin receptor (Part 4)

The transformed cells TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3 produced in the example 12 are inoculated in 200 ml of DOLU + Gal culture media and cultured at 30°C for 30 hours to obtain pre-cultured solutions.

The pre-cultured transformed cells (TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3) are spot-added in 10 $\mu$ l to the respective DOLU + Gul agar culture media to which antagonist-active substances to the cytokinin receptor selected by the example 16 are added while their concentration being changed from 10 nM to 100 $\mu$ M and also 10 $\mu$ M of trans-zeatin (cytokinin) is added and then is cultured at 30°C for 30 hours. After incubation, the intensity of the antagonist-activity of the examinee substances to the cytokinin receptor is detected and confirmed based on the lowest concentration at which transformed cells (TM182-CRE1, TM182-AHK2, TM182-AHK2Δ and TM182-AHK3) are not observed to grow.

Hereinafter, the medium compositions to be used in the present invention will be described:

(a) DOLU + Glu culture medium

| | |
|---|---|
| Bacto-yeast nitrogen base without amino acids | 6.7g |
| Glucose | 20g |
| Drop-out mix (x) | 2.0g |
| Distilled water | 1000ml |

(b)DOLU + GAL culture medium

| | |
|---|---|
| Bacto-yeast nitrogen base without amino acids | 6.7g |
| Glucose | 20g |
| Drop-out mix (x) | 2.0g |
| Distilled water | 1000ml |

(x) Drop-out mix: Drop-out mix is a combination of the following ingredients.

| | | | |
|---|---|---|---|
| Adenine | 0.5g | Lysine | 2.0g |
| Alanine | 2.0g | Methionine | 2.0g |
| Arginine | 2.0g | para-Aminobenzoic acid | 0.2g |
| Asparagine | 2.0g | Phenylalanine | 2.0g |
| Aspartic acid | 2.0g | Proline | 2.0g |
| Cysteine | 2.0g | Serine | 2.0g |
| Glutamine | 2.0g | Threonine | 2.0g |
| Glutamic acid | 2.0g | Trytophan | 2.0g |
| Glycine | 2.0g | Tyrosine | 2.0g |
| Histidine | 2.0g | Valine | 2.0g |
| Inositol | 2.0g | Isoleucine | 2.0g |

(c) DOLU + Glu agar culture medium

A solid culture medium prepared by adding 2% (W/V) of agar into the culture medium (a)

(d) DOLU + GAL agar culture medium

A solid culture medium prepared by adding 2% (W/V) of agar into the culture medium (c)

" Sequence table free text"

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Oligonucleotide primer designed for PCR

SEQ ID No: 10

Oligonucleotide primer designed for PCR

SEQ ID No: 11

Oligonucleotide primer designed for PCR

SEQ ID No: 12

Oligonucleotide primer designed for PCR

SEQ ID No: 13

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Oligonucleotide primer designed for PCR

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Oligonucleotide primer designed for PCR

SEQ ID No: 18

Oligonucleotide primer designed for PCR

SEQ ID No: 19

Oligonucleotide primer designed for PCR

SEQ ID No: 20

Oligonucleotide primer designed for PCR

SEQ ID No: 21

Oligonucleotide primer designed for PCR

SEQ ID No: 22

Oligonucleotide primer designed for PCR

WHAT IS CLAIMED IS:

Claim 1: A method for analyzing antagonist-activity to a cytokinin receptor, which comprises (1) bringing an examinee substance and a substance having agonist-activity to the cytokinin receptor into contact with a transformed cell into which DNA coding the cytokinin receptor is introduced and (2) measuring the existence or the quantity of intracellular signal transduction from the cytokinin receptor expressed in the transformed cell.

Claim 2: The method according to claim 1, wherein growth of said transformed cell is controlled by intracellular signal transduction from said cytokinin,receptor, and wherein said existence or quantity of intracellular signal transduction is determined by measuring growth of said transformed cell.

Claim 3: The method according to claim 1, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell so improved as to have histidine kinase activity lower than the intrinsic histidine kinase activity of the host cell.

Claim 4: The method according to claim 1, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell so improved as to have histidine kinase activity lower than the intrinsic histidine kinase activity of the host cell by deleting one or more of histidine kinase.

Claim 5: The method according to claim 1, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell having no cytokinin receptor.

Claim 6: The method according to claim 1, wherein the transformed

cell is yeast.

Claim 7: The method according to claim 1, wherein the transformed cell is budding yeast.

Claim 8: The method according to claim 1, wherein the DNA coding the cytokinin receptor is any one of DNA coding the cytokinin receptor selected from:

(a) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 6;

(b) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 2;

(c) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 4;

(d) a cytokinin receptor wherein said cytokinin receptor has at least one transmembrane region but less than that in its natural form;

(e) a cytokinin receptor having the amino acid sequence from amino acid number 196 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(f) a cytokinin receptor having the amino acid sequence from amino acid number 50 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(g) a cytokinin receptor having the amino acid sequence from amino acid number 32 to 1036 among the amino acid sequence represented by SEQ ID No: 4;

(h) a chimera-type cytokinin receptor comprising an extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, a

histidine kinase region of the cytokinin receptor, and a receiver region of the histidine kinase, wherein said extracellular region, said transmembrane regions and said histidine kinase region are homogeneous to one another and the receiver region is heterogeneous thereto; and

(1) a cytokinin receptor having the amino acid sequence with deletion, substitution, or addition of one or a plurality of amino acids in the amino acid sequence of the cyctokinin receptor of (a), (b), (c), (e), (f), or (g), wherein said cytokinin receptor is encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide represented by the nucleotide sequence selected from the group consisting of SEQ ID Nos: 1, 3, and 5, and wherein said stringent conditions comprise hybridization at 6 X SSC at 65°C and washing in the presence of 0.1 X SSC and 0.5% SDS at 68°C for 30 minutes.

Claim 9: A method for analyzing agonist-activity to a cytokinin receptor, which comprises (1) bringing an examinee substance into contact with a transformed cell into which DNA coding the cytokinin receptor is introduced and (2) measuring the existence or the quantity of intracellular signal transduction from the cytokinin receptor expressed in the transformed cell, wherein

the DNA coding the cytokinin receptor is any one of DNA coding the cytokinin receptor selected from:

(b) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 2;

(c) a cytokinin receptor having the amino acid sequence represented by SEQ ID No: 4;

(d) a cytokinin receptor wherein said cytokinin receptor has at least one transmembrane region but less than that in its natural form;

(e) a cytokinin receptor having the amino acid sequence from amino acid number 196 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(f) a cytokinin receptor having the amino acid sequence from amino acid number 50 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(g) a cytokinin receptor having the amino acid sequence from amino acid number 32 to 1036 among the amino acid sequence represented by SEQ ID No: 4;

(h) a chimera-type cytokinin receptor comprising an extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, a histidine kinase region of the cytokinin receptor, and a receiver region of the histidine kinase, wherein said extracellular region, said transmembrane regions and said histidine kinase region are homogeneous to one another and the receiver region is heterogeneous thereto; and

(i) a cytokinin receptor having the amino acid sequence with deletion, substitution, or addition of one or a plurality of amino acids in the amino acid sequence of the cyctokinin receptor of (b), (c), (e), (f), or (g), wherein said cytokinin receptor is encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide represented by the nucleotide sequence selected from the group consisting of SEQ ID Nos: 1, 3, and 5, and wherein said stringent conditions comprise hybridization at 6 X SSC at 65°C and washing in the presence of 0.1 X SSC and 0.5% SDS at 68°C for 30 minutes.

Claim 10: The method according to claim 9, wherein growth of said transformed cell is controlled by intracellular signal transduction from said

cytokinin receptor, and wherein said existence or quantity of intracellular signal transduction is determined by measuring growth of said transformed cell.

Claim 11: The method according to claim 9, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell so improved as to have histidine kinase activity lower than the intrinsic histidine kinase activity of the host cell.

Claim 12: The method according to claim 9, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell so improved as to have histidine kinase activity lower than the intrinsic histidine kinase activity of the host cell by deleting one or more of histidine kinase.

Claim 13: The method according to claim 9, wherein the transformed cell is a transformed cell generated by introducing DNA coding the cytokinin receptor into a host cell having no cytokinin receptor.

Claim 14: The method according to claim 9, wherein the transformed cell is yeast.

Claim 15: The method according to claim 9, wherein the transformed cell is budding yeast.

Claim 16: A cytokinin receptor selected from:

(d) a cytokinin receptor wherein said cytokinin receptor has at least one transmembrane region but less than that in its natural form;

(e) a cytokinin receptor having the amino acid sequence from amino acid number 196 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(f) a cytokinin receptor having the amino acid sequence from amino acid number 50 to 1176 among the amino acid sequence represented by SEQ ID No: 2;

(g) a cytokinin receptor having the amino acid sequence from amino acid number 32 to 1036 among the amino acid sequence represented by SEQ ID No: 4;

(h) a chimera-type cytokinin receptor comprising an extracellular region of the cytokinin receptor, transmembrane regions of the cytokinin receptor, a histidine kinase region of the cytokinin receptor, and a receiver region of the histidine kinase, wherein said extracellular region, said transmembrane regions and said histidine kinase region are homogeneous to one another and the receiver region is heterogeneous thereto; and

(i) a cytokinin receptor having the amino acid sequence with deletion, substitution, or addition of one or a plurality of amino acids in the amino acid sequence of the cyctokinin receptor of (e), (f), or (g), wherein said cytokinin receptor is encoded by a polynucleotide that hybridizes under stringent conditions to a polynucleotide represented by the nucleotide sequence selected from the group consisting of SEQ ID Nos: 1, 3, and 5, and wherein said stringent conditions comprise hybridization at 6 X SSC at 65°C and washing in the presence of 0.1 X SSC and 0.5% SDS at 68°C for 30 minutes.

Claim 17: DNA coding the cytokinin receptor of claim 16.

Claim 18: A transformed cell into which DNA of claim 17 is introduced.

Claim 19: A method for analysing antagonist-activity to a cytokinin receptor, comprising:

(1) bringing an examinee substance and a substance having agonist-activity to the cytokinin receptor into contact with a transformed cell into which DNA coding the cytokinin receptor is introduced;

(2) measuring an existence or quantity of intracellular signal transduction from the cytokinin receptor expressed in the transformed cell; and

(3) comparing the existence or quantity measured in (2) with a second existence or quantity of intracellular signal transduction from said cytokinin receptor measured in the absence of said examinee substance but in presence of another examinee substance.

Claim 20: The method according to claim 19, wherein another examinee substance is a substance having no antagonist-activity to said cytokinin receptor.

Claim 21: A method for searching antagonist-active substance to a cytokinin receptor, which comprises selecting a substance having antagonist-activity to a cytokinin receptor based on the antagonist-activity to a cytokinin receptor evaluated by the detecting method of claim 19.

Claim 22: Use of a composition to regulate plant growth, wherein the composition comprises a substance selected by the searching method of claim 21 as an active ingredient and a carrier.

Claim 23: A method for detecting agonist-activity to a cytokinin receptor, which comprises evaluating the agonist-activity of two or more different examinee substances to the cytokinin receptor based on the difference obtained by comparison of the existence or the quantity of the intracellular signal transduction in a section where the examinee substances are independently used and measured by the analysis method of claim 9.

Claim 24: The method according to claim 23, wherein at least one substance among the two or more different examinee substances is a substance having no agonist-activity to the cytokinin receptor.

Claim 25: A method for searching agonist-active substance to a cytokinin receptor, which comprises selecting a substance having agonist-activity to a cytokinin receptor based on the agonist-activity to a cytokinin receptor evaluated by the detecting method of claim 23.

Claim 26: Use of a composition to regulate plant growth, wherein the composition comprises a substance selected by the searching method of claim 25 as an active ingredient and a carrier.

SEQUENCE LISTING

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<120> ANALYSIS OF AGONIST-ACTIVITY AND ANTAGONIST-ACTIVITY TO CYTOKININ RECEPTOR

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<170> PatentIn Ver. 2.1

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370 375 380

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Gln Glu Thr Val Ser His Ile Val Ser Val Asp Met Met Ser Gly Glu
385 390 395 400

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Glu Asp Arg Glu Asn Ile Leu Arg Ala Arg Ala Ser Gly Lys Gly Val
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Ser Leu Asp Phe Gly Asp Pro Ser Arg Asn His Glu Met His Cys Arg
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755 760 765

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770 775 780

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995 1000 1005

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Ile Glu Asp Lys Ser Gly Leu Leu Val Gly Ser Val Gly Asp Leu Glu
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Lys Thr Lys Met Thr Thr Leu Lys Lys Lys Asn Lys Met Trp Phe Trp
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Asn Lys Ile Ser Ser Ser Gly Leu Lys Ile Pro Ser Phe Ser Tyr Gln
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Val Val Trp Val Val Phe Trp Val Leu Val Ser Ile Trp Thr Phe Trp
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Tyr Phe Ser Ser Gln Ala Met Glu Lys Arg Lys Glu Thr Leu Ala Ser
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130 135 140

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Gly Lys Ile Pro Ser Ala Ile Asp Gln Arg Thr Phe Ser Glu Tyr Thr
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Asp Arg Thr Ser Phe Glu Arg Pro Leu Thr Ser Gly Val Ala Tyr Ala
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Arg Ser Ser Gly Lys Gly Val Leu Thr Ala Pro Phe Pro Leu Ile Lys
275 280 285

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340 345 350

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Asn Ser Ile Lys Phe Thr Glu Lys Gly His Ile Phe Val Thr Val His
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ttg gtg gat gag cta ttt gaa tct atc gat gga gag aca gca tca tct 1824

Leu Val Asp Glu Leu Phe Glu Ser Ile Asp Gly Glu Thr Ala Ser Ser
595 600 605

ccg gaa agt aca ctg agt ggg ctt cca gtt gca gac cgg cag agg agc 1872

Pro Glu Ser Thr Leu Ser Gly Leu Pro Val Ala Asp Arg Gln Arg Ser

610 615 620

tgg gaa aac ttt aaa gct ttc agc tcc aac ggg cat cgg agc ttt gaa 1920

Trp Glu Asn Phe Lys Ala Phe Ser Ser Asn Gly His Arg Ser Phe Glu
625 630 635 640

cca tct ccc cct gat ata aac cta atc gtc tca gtt gag gat act ggc 1968

Pro Ser Pro Pro Asp Ile Asn Leu Ile Val Ser Val Glu Asp Thr Gly
645 650 655

gta ggg atc cct gta gaa gcg cag tcc cgt att ttt acg cct ttc atg 2016

Val Gly Ile Pro Val Glu Ala Gln Ser Arg Ile Phe Thr Pro Phe Met
660 665 670

caa gtc gga cca tcc ata tcc agg acg cat gga ggc aca gga att gga 2064

Gln Val Gly Pro Ser Ile Ser Arg Thr His Gly Gly Thr Gly Ile Gly
675 680 685

ctt agc ata agc aaa tgt cta gtt gga ctg atg aag gga gaa att gga 2112

Leu Ser Ile Ser Lys Cys Leu Val Gly Leu Met Lys Gly Glu Ile Gly
690 695 700

ttc tcg agt act ccc aag gtt ggg tcc aca ttc aca ttt act gct gta 2160

Phe Ser Ser Thr Pro Lys Val Gly Ser Thr Phe Thr Phe Thr Ala Val
705 710 715 720

ttt tcc aat ggg atg caa cca gct gaa aga aag aat gac aac aac cag 2208

Phe Ser Asn Gly Met Gln Pro Ala Glu Arg Lys Asn Asp Asn Asn Gln
725 730 735

ccc ata ttc tcg gaa ttc cgg ggc atg aaa gct gtg gtt gtg gac cat 2256

Pro Ile Phe Ser Glu Phe Arg Gly Met Lys Ala Val Val Val Asp His
740 745 750

agg cct gca agg gca aaa gtc tcg tgg tac cat ttt cag cgt ctt gga 2304

Arg Pro Ala Arg Ala Lys Val Ser Trp Tyr His Phe Gln Arg Leu Gly
755 760 765

att cga gtc gaa gta gtt cca cgt gtt gaa cag gct cta cat tat ctg 2352

Ile Arg Val Glu Val Val Pro Arg Val Glu Gln Ala Leu His Tyr Leu
770 775 780

aag att ggt act acc act gtg aat atg ata ctc ata gag caa gaa ata 2400

Lys Ile Gly Thr Thr Thr Val Asn Met Ile Leu Ile Glu Gln Glu Ile
785 790 795 800

tgg aat agg gaa gca gat gat ttc att aaa aag cta cag aaa gac cct 2448

Trp Asn Arg Glu Ala Asp Asp Phe Ile Lys Lys Leu Gln Lys Asp Pro
805 810 815

ctt ttc ctt tct cct aag ttg att ttg tta gca aac tca gta gaa tcg 2496

Leu Phe Leu Ser Pro Lys Leu Ile Leu Leu Ala Asn Ser Val Glu Ser
820 825 830

tca ata tca gag gct tta tgc acc ggt ata gat cct cca ata gtg ata 2544

Ser Ile Ser Glu Ala Leu Cys Thr Gly Ile Asp Pro Pro Ile Val Ile
835 840 845

gtg aaa cca ttg agg gcg agt atg cta gca gca act ttg cag agg gga 2592

Val Lys Pro Leu Arg Ala Ser Met Leu Ala Ala Thr Leu Gln Arg Gly
850 855 860

ttg ggt att gga atc aga gaa cca cct caa cac aag gga cct cct gct 2640

Leu Gly Ile Gly Ile Arg Glu Pro Pro Gln His Lys Gly Pro Pro Ala
865 870 875 880

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885 890 895

gat aac aac gta aac ctc aga gtg gca gcg gga gct ctg aaa aag tac 2736

Asp Asn Asn Val Asn Leu Arg Val Ala Ala Gly Ala Leu Lys Lys Tyr
900 905 910

gga gct gat gtg gtc tgc gct gag agt ggg ata aag gca atc tca ttg 2784

Gly Ala Asp Val Val Cys Ala Glu Ser Gly Ile Lys Ala Ile Ser Leu
915 920 925

ctt aag cca cct cac gag ttt gat gct tgc ttc atg gac att cag atg 2832

Leu Lys Pro Pro His Glu Phe Asp Ala Cys Phe Met Asp Ile Gln Met
930 935 940

cca gaa atg gat gga ttt gaa gct aca agg aga ata cga gat atg gaa 2880

Pro Glu Met Asp Gly Phe Glu Ala Thr Arg Arg Ile Arg Asp Met Glu
945 950 955 960

gag gag atg aac aag aga ata aag aat ggg gag gct ttg ata gta gag 2928

Glu Glu Met Asn Lys Arg Ile Lys Asn Gly Glu Ala Leu Ile Val Glu
965 970 975

aac ggt aac aaa aca agc tgg cat ctt ccg gta tta gca atg acg gca 2976

Asn Gly Asn Lys Thr Ser Trp His Leu Pro Val Leu Ala Met Thr Ala
980 985 990

gat gtg atc caa gca acg cat gag gaa tgt ctg aag tgt gga atg gat 3024

Asp Val Ile Gln Ala Thr His Glu Glu Cys Leu Lys Cys Gly Met Asp
995 1000 1005

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Gly Tyr Val Ser Lys Pro Phe Glu Ala Glu Gln Leu Tyr Arg Glu Val
1010 1015 1020

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Ile Glu Asp Lys Ser Gly Leu Leu Val Gly Ser Val Gly Asp Leu Glu
35 40 45

Lys Thr Lys Met Thr Thr Leu Lys Lys Lys Asn Lys Met Trp Phe Trp
50 55 60

Asn Lys Ile Ser Ser Ser Gly Leu Lys Ile Pro Ser Phe Ser Tyr Gln
65 70 75 80

Phe Leu Gly Ser Val Lys Phe Asn Lys Ala Trp Trp Arg Lys Leu Val
85 90 95

Val Val Trp Val Val Phe Trp Val Leu Val Ser Ile Trp Thr Phe Trp
100 105 110

Tyr Phe Ser Ser Gln Ala Met Glu Lys Arg Lys Glu Thr Leu Ala Ser
115 120 125

Met Cys Asp Glu Arg Ala Arg Met Leu Gln Asp Gln Phe Asn Val Ser

130 135 140

Met Asn His Val Gln Ala Met Ser Ile Leu Ile Ser Thr Phe His His

145 150 155 160

Gly Lys Ile Pro Ser Ala Ile Asp Gln Arg Thr Phe Ser Glu Tyr Thr

165 170 175

Asp Arg Thr Ser Phe Glu Arg Pro Leu Thr Ser Gly Val Ala Tyr Ala

180 185 190

Met Arg Val Leu His Ser Glu Arg Glu Glu Phe Glu Arg Gln Gln Gly

195 200 205

Trp Thr Ile Arg Lys Met Tyr Ser Leu Glu Gln Asn Pro Val His Lys

210 215 220

Asp Asp Tyr Asp Leu Glu Ala Leu Glu Pro Ser Pro Val Gln Glu Glu

225 230 235 240

Tyr Ala Pro Val Ile Phe Ala Gln Asp Thr Val Ser His Val Val Ser

245 250 255

Leu Asp Met Leu Ser Gly Lys Glu Asp Arg Glu Asn Val Leu Arg Ala

260 265 270

Arg Ser Ser Gly Lys Gly Val Leu Thr Ala Pro Phe Pro Leu Ile Lys

275 280 285

Thr Asn Arg Leu Gly Val Ile Leu Thr Phe Ala Val Tyr Lys Arg Asp

290 295 300

Leu Pro Ser Asn Ala Thr Pro Lys Glu Arg Ile Glu Ala Thr Asn Gly

305 310 315 320

Tyr Leu Gly Gly Val Phe Asp Ile Glu Ser Leu Val Glu Asn Leu Leu

325 330 335

Gln Gln Leu Ala Ser Lys Gln Thr Ile Leu Val Asn Val Tyr Asp Ile

340 345 350

Thr Asn His Ser Gln Pro Ile Ser Met Tyr Gly Thr Asn Val Ser Ala

355 360 365

Asp Gly Leu Glu Arg Val Ser Pro Leu Ile Phe Gly Asp Pro Leu Arg

370 375 380

Lys His Glu Met Arg Cys Arg Phe Lys Gln Lys Pro Pro Trp Pro Val

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Leu Ser Met Val Thr Ser Phe Gly Ile Leu Val Ile Ala Leu Leu Val

405 410 415

Ala His Ile Ile His Ala Thr Val Ser Arg Ile His Lys Val Glu Glu

420 425 430

Asp Cys Asp Lys Met Lys Gln Leu Lys Lys Lys Ala Glu Ala Ala Asp

435 440 445

Val Ala Lys Ser Gln Phe Leu Ala Thr Val Ser His Glu Ile Arg Thr

450 455 460

Pro Met Asn Gly Val Leu Gly Met Leu His Met Leu Met Asp Thr Glu

465 470 475 480

Leu Asp Val Thr Gln Gln Asp Tyr Val Arg Thr Ala Gln Ala Ser Gly

485 490 495

Lys Ala Leu Val Ser Leu Ile Asn Glu Val Leu Asp Gln Ala Lys Ile

500 505 510

Glu Ser Gly Lys Leu Glu Leu Glu Glu Val Arg Phe Asp Leu Arg Gly

515 520 525

Ile Leu Asp Asp Val Leu Ser Leu Phe Ser Ser Lys Ser Gln Gln Lys

530 535 540

Gly Val Glu Leu Ala Val Tyr Ile Ser Asp Arg Val Pro Asp Met Leu

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Ile Gly Asp Pro Gly Arg Phe Arg Gln Ile Leu Thr Asn Leu Met Gly

565 570 575

Asn Ser Ile Lys Phe Thr Glu Lys Gly His Ile Phe Val Thr Val His

580 585 590

Leu Val Asp Glu Leu Phe Glu Ser Ile Asp Gly Glu Thr Ala Ser Ser

595 600 605

Pro Glu Ser Thr Leu Ser Gly Leu Pro Val Ala Asp Arg Gln Arg Ser

610 615 620

Trp Glu Asn Phe Lys Ala Phe Ser Ser Asn Gly His Arg Ser Phe Glu

625 630 635 640

Pro Ser Pro Pro Asp Ile Asn Leu Ile Val Ser Val Glu Asp Thr Gly

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Val Gly Ile Pro Val Glu Ala Gln Ser Arg Ile Phe Thr Pro Phe Met

660 665 670

Gln Val Gly Pro Ser Ile Ser Arg Thr His Gly Gly Thr Gly Ile Gly

675 680 685

Leu Ser Ile Ser Lys Cys Leu Val Gly Leu Met Lys Gly Glu Ile Gly

690 695 700

Phe Ser Ser Thr Pro Lys Val Gly Ser Thr Phe Thr Phe Thr Ala Val

705 710 715 720

Phe Ser Asn Gly Met Gln Pro Ala Glu Arg Lys Asn Asp Asn Asn Gln

725 730 735

Pro Ile Phe Ser Glu Phe Arg Gly Met Lys Ala Val Val Val Asp His

740 745 750

Arg Pro Ala Arg Ala Lys Val Ser Trp Tyr His Phe Gln Arg Leu Gly

755 760 765

Ile Arg Val Glu Val Val Pro Arg Val Glu Gln Ala Leu His Tyr Leu

770 775 780

Lys Ile Gly Thr Thr Thr Val Asn Met Ile Leu Ile Glu Gln Glu Ile

785 790 795 800

Trp Asn Arg Glu Ala Asp Asp Phe Ile Lys Lys Leu Gln Lys Asp Pro

805 810 815

Leu Phe Leu Ser Pro Lys Leu Ile Leu Leu Ala Asn Ser Val Glu Ser

820 825 830

Ser Ile Ser Glu Ala Leu Cys Thr Gly Ile Asp Pro Pro Ile Val Ile

835 840 845

Val Lys Pro Leu Arg Ala Ser Met Leu Ala Ala Thr Leu Gln Arg Gly

850 855 860

Leu Gly Ile Gly Ile Arg Glu Pro Pro Gln His Lys Gly Pro Pro Ala

865 870 875 880

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885 890 895

Asp Asn Asn Val Asn Leu Arg Val Ala Ala Gly Ala Leu Lys Lys Tyr

900 905 910

Gly Ala Asp Val Val Cys Ala Glu Ser Gly Ile Lys Ala Ile Ser Leu

915 920 925

Leu Lys Pro Pro His Glu Phe Asp Ala Cys Phe Met Asp Ile Gln Met

930 935 940

Pro Glu Met Asp Gly Phe Glu Ala Thr Arg Arg Ile Arg Asp Met Glu

945 950 955 960

Glu Glu Met Asn Lys Arg Ile Lys Asn Gly Glu Ala Leu Ile Val Glu

965 970 975

Asn Gly Asn Lys Thr Ser Trp His Leu Pro Val Leu Ala Met Thr Ala

980 985 990

Asp Val Ile Gln Ala Thr His Glu Glu Cys Leu Lys Cys Gly Met Asp

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Arg Ile Glu Ile Ser Asp Ser Glu Ser Leu Glu Asn Leu Lys Ser Ser
20 25 30

gat ttt tat caa ctg ggt ggt ggt ggt gct ctg aat tcg tca gaa aag 144

Asp Phe Tyr Gln Leu Gly Gly Gly Gly Ala Leu Asn Ser Ser Glu Lys
35 40 45

ccg aga aag atc gat ttt tgg cgt tcg ggg ttg atg ggt ttt gcg aag 192

Pro Arg Lys Ile Asp Phe Trp Arg Ser Gly Leu Met Gly Phe Ala Lys
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atg cag cag cag caa cag ctt cag cat tca gtg gcg gtg aag atg aac 240

Met Gln Gln Gln Gln Gln Leu Gln His Ser Val Ala Val Lys Met Asn
65 70 75 80

aat aat aat aat aac gat cta atg ggt aat aaa aaa ggg tca act ttc 288

Asn Asn Asn Asn Asn Asp Leu Met Gly Asn Lys Lys Gly Ser Thr Phe
85 90 95

ata caa gaa cat cga gca ttg tta cca aaa gct ttg att ctg tgg atc 336

Ile Gln Glu His Arg Ala Leu Leu Pro Lys Ala Leu Ile Leu Trp Ile
100 105 110

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Ile Ile Val Gly Phe Ile Ser Ser Gly Ile Tyr Gln Trp Met Asp Asp
115 120 125

gct aat aag att aga agg gaa gag gtt ttg gtc agc atg tgt gat caa 432

Ala Asn Lys Ile Arg Arg Glu Glu Val Leu Val Ser Met Cys Asp Gln
130 135 140

aga gct aga atg ttg cag gat caa ttt agt gtt agt gtt aat cat gtt 480

Arg Ala Arg Met Leu Gln Asp Gln Phe Ser Val Ser Val Asn His Val
145 150 155 160

cat gct ttg gct att ctc gtc tcc act ttt cat tac cac aag aac cct 528

His Ala Leu Ala Ile Leu Val Ser Thr Phe His Tyr His Lys Asn Pro
165 170 175

tct gca att gat cag gag aca ttt gcg gag tac acg gca aga aca gca 576

Ser Ala Ile Asp Gln Glu Thr Phe Ala Glu Tyr Thr Ala Arg Thr Ala
180 185 190

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Phe Glu Arg Pro Leu Leu Ser Gly Val Ala Tyr Ala Glu Lys Val Val
195 200 205

aat ttt gag agg gag atg ttt gag cgg cag cac aat tgg gtt ata aag 672

Asn Phe Glu Arg Glu Met Phe Glu Arg Gln His Asn Trp Val Ile Lys
210 215 220

aca atg gat aga gga gag cct tca ccg gtt agg gat gag tat gct cct 720

Thr Met Asp Arg Gly Glu Pro Ser Pro Val Arg Asp Glu Tyr Ala Pro
225 230 235 240

gtt ata ttc tct caa gat agt gtc tct tac ctt gag tca ctc gat atg 768

Val Ile Phe Ser Gln Asp Ser Val Ser Tyr Leu Glu Ser Leu Asp Met
245 250 255

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Met Ser Gly Glu Glu Asp Arg Glu Asn Ile Leu Arg Ala Arg Glu Thr
260 265 270

gga aaa gct gtc ttg act agc cct ttt agg ttg ttg gaa act cac cat 864

Gly Lys Ala Val Leu Thr Ser Pro Phe Arg Leu Leu Glu Thr His His
275 280 285

ctc gga gtt gtg ttg aca ttc cct gtc tac aag tct tct ctt cct gaa 912

Leu Gly Val Val Leu Thr Phe Pro Val Tyr Lys Ser Ser Leu Pro Glu
290 295 300

aat ccg act gtc gaa gag cgt att gca gcc act gca ggg tac ctt ggt 960

Asn Pro Thr Val Glu Glu Arg Ile Ala Ala Thr Ala Gly Tyr Leu Gly
305 310 315 320

ggt gcg ttt gat gtg gag tct cta gtc gag aat tta ctt ggt cag ctt 1008

Gly Ala Phe Asp Val Glu Ser Leu Val Glu Asn Leu Leu Gly Gln Leu
325 330 335

gct ggt aac caa gca ata gtt gtg cat gtg tat gat atc acc aat gca 1056

Ala Gly Asn Gln Ala Ile Val Val His Val Tyr Asp Ile Thr Asn Ala

340 345 350

tca gat cca ctt gtc atg tat ggt aat caa gat gaa gaa gcc gac aga 1104

Ser Asp Pro Leu Val Met Tyr Gly Asn Gln Asp Glu Glu Ala Asp Arg
355 360 365

tct ctc tct cat gag agc aag ctc gat ttt gga gac ccc ttc agg aaa 1152

Ser Leu Ser His Glu Ser Lys Leu Asp Phe Gly Asp Pro Phe Arg Lys
370 375 380

cat aag atg ata tgc agg tac cac caa aag gca cca ata cca ttg aat 1200

His Lys Met Ile Cys Arg Tyr His Gln Lys Ala Pro Ile Pro Leu Asn
385 390 395 400

gtg ctc aca act gtg cca ttg ttc ttt gcg att ggt ttc ttg gtg ggt 1248

Val Leu Thr Thr Val Pro Leu Phe Phe Ala Ile Gly Phe Leu Val Gly
405 410 415

tat ata ctg tat ggt gca gct atg cac ata gta aaa gtc gaa gat gat 1296

Tyr Ile Leu Tyr Gly Ala Ala Met His Ile Val Lys Val Glu Asp Asp
420 425 430

ttc cat gaa atg caa gag ctt aaa gtg cga gca gaa gct gct gat gtc 1344

Phe His Glu Met Gln Glu Leu Lys Val Arg Ala Glu Ala Ala Asp Val
435 440 445

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Ser Ser Thr Gln Arg Asp Tyr Ala Gln Thr Ala Gln Val Cys Gly Lys
485 490 495

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Ala Leu Ile Ala Leu Ile Asn Glu Val Leu Asp Arg Ala Lys Ile Glu
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530 535 540

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Ile Glu Leu Ala Val Phe Val Ser Asp Lys Val Pro Glu Ile Val Lys
545 550 555 560

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565 570 575

tcg gtt aaa ttc aca gag aaa gga cat atc ttt gtt aaa gtc cat ctt 1776

Ser Val Lys Phe Thr Glu Lys Gly His Ile Phe Val Lys Val His Leu
580 585 590

gcg gaa caa tca aaa gat gaa tct gaa ccg aaa aat gca ttg aat ggt 1824

Ala Glu Gln Ser Lys Asp Glu Ser Glu Pro Lys Asn Ala Leu Asn Gly
595 600 605

gga gtg tct gaa gaa atg atc gtt gtt tcc aaa cag tca agt tac aac 1872

Gly Val Ser Glu Glu Met Ile Val Val Ser Lys Gln Ser Ser Tyr Asn
610 615 620

aca ttg agc ggt tac gaa gct gct gat ggt cgg aat agc tgg gat tca 1920

Thr Leu Ser Gly Tyr Glu Ala Ala Asp Gly Arg Asn Ser Trp Asp Ser
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Phe Lys His Leu Val Ser Glu Glu Gln Ser Leu Ser Glu Phe Asp Ile
645 650 655

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Ser Ser Asn Val Arg Leu Met Val Ser Ile Glu Asp Thr Gly Ile Gly
660 665 670

atc cct tta gtt gca caa ggc cgt gtg ttt atg ccg ttt atg caa gca 2064

Ile Pro Leu Val Ala Gln Gly Arg Val Phe Met Pro Phe Met Gln Ala
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gat agc tcg act tca aga aac tat gga ggt act ggt att ggt ttg agt 2112

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690 695 700

ata agc aag tgt ctt gtt gaa ctt atg cgt ggt cag ata aat ttc ata 2160

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agc cgg cct cat att gga agc acg ttc tgg ttc acg gct gtt tta gag 2208

Ser Arg Pro His Ile Gly Ser Thr Phe Trp Phe Thr Ala Val Leu Glu
725 730 735

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Lys Cys Asp Lys Cys Ser Ala Ile Asn His Met Lys Lys Pro Asn Val
740 745 750

gaa cac ttg cct tct act ttt aaa gga atg aaa gct ata gtt gtt gat 2304

Glu His Leu Pro Ser Thr Phe Lys Gly Met Lys Ala Ile Val Val Asp

755 760 765

gct aag cct gtt aga gct gct gtg act aga tac cat atg aaa aga ctc 2352

Ala Lys Pro Val Arg Ala Ala Val Thr Arg Tyr His Met Lys Arg Leu
770 775 780

gga atc aat gtt gat gtc gtg aca agt ctc aaa acc gct gtt gtt gca 2400

Gly Ile Asn Val Asp Val Val Thr Ser Leu Lys Thr Ala Val Val Ala
785 790 795 800

gct gct gcg ttt gaa aga aac ggt tct cct ctc cca aca aaa ccg caa 2448

Ala Ala Ala Phe Glu Arg Asn Gly Ser Pro Leu Pro Thr Lys Pro Gln
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820 825 830

aat gac tca gag att cgt tta ttg aat tca aga acc aac gga aac gtt 2544

Asn Asp Ser Glu Ile Arg Leu Leu Asn Ser Arg Thr Asn Gly Asn Val
835 840 845

cat cac aag tct ccg aaa cta gct cta ttc gca aca aac atc aca aat 2592

His His Lys Ser Pro Lys Leu Ala Leu Phe Ala Thr Asn Ile Thr Asn
850 855 860

tcg gag ttc gac aga gct aaa tcc gca gga ttt gca gat acg gta ata 2640

Ser Glu Phe Asp Arg Ala Lys Ser Ala Gly Phe Ala Asp Thr Val Ile
865 870 875 880

atg aaa ccg tta aga gca agc atg att ggg gcg tgt ctg caa caa gtt 2688

Met Lys Pro Leu Arg Ala Ser Met Ile Gly Ala Cys Leu Gln Gln Val
885 890 895

ctc gag ctg aga aaa aca aga caa caa cat cca gaa gga tca tca ccc 2736

Leu Glu Leu Arg Lys Thr Arg Gln Gln His Pro Glu Gly Ser Ser Pro
900 905 910

gca act ctc aag agc ttg ctt aca ggg aag aag att ctt gtg gtt gat 2784

Ala Thr Leu Lys Ser Leu Leu Thr Gly Lys Lys Ile Leu Val Val Asp
915 920 925

gat aat ata gtt aac agg aga gta gct gca gga gct ctc aag aaa ttt 2832

Asp Asn Ile Val Asn Arg Arg Val Ala Ala Gly Ala Leu Lys Lys Phe
930 935 940

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Gly Ala Glu Val Val Cys Ala Glu Ser Gly Gln Val Ala Leu Gly Leu
945 950 955 960

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965 970 975

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Pro Gln Met Asp Gly Phe Glu Ala Thr Arg Gln Ile Arg Met Met Glu
980 985 990

aag gaa gct aaa gag aag acg aat ctc gaa tgg cat tta ccg att cta 3024

Lys Glu Ala Lys Glu Lys Thr Asn Leu Glu Trp His Leu Pro Ile Leu
995 1000 1005

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Pro Arg Lys Ile Asp Phe Trp Arg Ser Gly Leu Met Gly Phe Ala Lys

50 55 60

Met Gln Gln Gln Gln Gln Leu Gln His Ser Val Ala Val Lys Met Asn

65 70 75 80

Asn Asn Asn Asn Asn Asp Leu Met Gly Asn Lys Lys Gly Ser Thr Phe

85 90 95

Ile Gln Glu His Arg Ala Leu Leu Pro Lys Ala Leu Ile Leu Trp Ile

100 105 110

Ile Ile Val Gly Phe Ile Ser Ser Gly Ile Tyr Gln Trp Met Asp Asp

115 120 125

Ala Asn Lys Ile Arg Arg Glu Glu Val Leu Val Ser Met Cys Asp Gln

130 135 140

Arg Ala Arg Met Leu Gln Asp Gln Phe Ser Val Ser Val Asn His Val

145 150 155 160

His Ala Leu Ala Ile Leu Val Ser Thr Phe His Tyr His Lys Asn Pro

165 170 175

Ser Ala Ile Asp Gln Glu Thr Phe Ala Glu Tyr Thr Ala Arg Thr Ala

180 185 190

Phe Glu Arg Pro Leu Leu Ser Gly Val Ala Tyr Ala Glu Lys Val Val

195 200 205

Asn Phe Glu Arg Glu Met Phe Glu Arg Gln His Asn Trp Val Ile Lys

210 215 220

Thr Met Asp Arg Gly Glu Pro Ser Pro Val Arg Asp Glu Tyr Ala Pro

225 230 235 240

Val Ile Phe Ser Gln Asp Ser Val Ser Tyr Leu Glu Ser Leu Asp Met

245 250 255

Met Ser Gly Glu Glu Asp Arg Glu Asn Ile Leu Arg Ala Arg Glu Thr

260 265 270

Gly Lys Ala Val Leu Thr Ser Pro Phe Arg Leu Leu Glu Thr His His

275 280 285

Leu Gly Val Val Leu Thr Phe Pro Val Tyr Lys Ser Ser Leu Pro Glu

290 295 300

Asn Pro Thr Val Glu Glu Arg Ile Ala Ala Thr Ala Gly Tyr Leu Gly

305 310 315 320

Gly Ala Phe Asp Val Glu Ser Leu Val Glu Asn Leu Leu Gly Gln Leu

325 330 335

Ala Gly Asn Gln Ala Ile Val Val His Val Tyr Asp Ile Thr Asn Ala

340 345 350

Ser Asp Pro Leu Val Met Tyr Gly Asn Gln Asp Glu Glu Ala Asp Arg

355 360 365

Ser Leu Ser His Glu Ser Lys Leu Asp Phe Gly Asp Pro Phe Arg Lys

370 375 380

His Lys Met Ile Cys Arg Tyr His Gln Lys Ala Pro Ile Pro Leu Asn

385 390 395 400

Val Leu Thr Thr Val Pro Leu Phe Phe Ala Ile Gly Phe Leu Val Gly

405 410 415

Tyr Ile Leu Tyr Gly Ala Ala Met His Ile Val Lys Val Glu Asp Asp

420 425 430

Phe His Glu Met Gln Glu Leu Lys Val Arg Ala Glu Ala Ala Asp Val

435 440 445

Ala Lys Ser Gln Phe Leu Ala Thr Val Ser His Glu Ile Arg Thr Pro

450 455 460

Met Asn Gly Ile Leu Gly Met Leu Ala Met Leu Leu Asp Thr Glu Leu

465 470 475 480

Ser Ser Thr Gln Arg Asp Tyr Ala Gln Thr Ala Gln Val Cys Gly Lys

485 490 495

Ala Leu Ile Ala Leu Ile Asn Glu Val Leu Asp Arg Ala Lys Ile Glu

500 505 510

Ala Gly Lys Leu Glu Leu Glu Ser Val Pro Phe Asp Ile Arg Ser Ile

515 520 525

Leu Asp Asp Val Leu Ser Leu Phe Ser Glu Glu Ser Arg Asn Lys Gly

530 535 540

Ile Glu Leu Ala Val Phe Val Ser Asp Lys Val Pro Glu Ile Val Lys

545 550 555 560

Gly Asp Ser Gly Arg Phe Arg Gln Ile Ile Ile Asn Leu Val Gly Asn

565 570 575

Ser Val Lys Phe Thr Glu Lys Gly His Ile Phe Val Lys Val His Leu

580 585 590

Ala Glu Gln Ser Lys Asp Glu Ser Glu Pro Lys Asn Ala Leu Asn Gly

595 600 605

Gly Val Ser Glu Glu Met Ile Val Val Ser Lys Gln Ser Ser Tyr Asn

610 615 620

Thr Leu Ser Gly Tyr Glu Ala Ala Asp Gly Arg Asn Ser Trp Asp Ser

625 630 635 640

Phe Lys His Leu Val Ser Glu Glu Gln Ser Leu Ser Glu Phe Asp Ile

645 650 655

Ser Ser Asn Val Arg Leu Met Val Ser Ile Glu Asp Thr Gly Ile Gly

660 665 670

Ile Pro Leu Val Ala Gln Gly Arg Val Phe Met Pro Phe Met Gln Ala

675 680 685

Asp Ser Ser Thr Ser Arg Asn Tyr Gly Gly Thr Gly Ile Gly Leu Ser

690 695 700

Ile Ser Lys Cys Leu Val Glu Leu Met Arg Gly Gln Ile Asn Phe Ile

705 710 715 720

Ser Arg Pro His Ile Gly Ser Thr Phe Trp Phe Thr Ala Val Leu Glu

725 730 735

Lys Cys Asp Lys Cys Ser Ala Ile Asn His Met Lys Lys Pro Asn Val

740 745 750

Glu His Leu Pro Ser Thr Phe Lys Gly Met Lys Ala Ile Val Val Asp

755 760 765

Ala Lys Pro Val Arg Ala Ala Val Thr Arg Tyr His Met Lys Arg Leu

770 775 780

Gly Ile Asn Val Asp Val Val Thr Ser Leu Lys Thr Ala Val Val Ala

785 790 795 800

Ala Ala Ala Phe Glu Arg Asn Gly Ser Pro Leu Pro Thr Lys Pro Gln

805 810 815

Leu Asp Met Ile Leu Val Glu Lys Asp Ser Trp Ile Ser Thr Glu Asp

820 825 830

Asn Asp Ser Glu Ile Arg Leu Leu Asn Ser Arg Thr Asn Gly Asn Val

835 840 845

His His Lys Ser Pro Lys Leu Ala Leu Phe Ala Thr Asn Ile Thr Asn

850 855 860

Ser Glu Phe Asp Arg Ala Lys Ser Ala Gly Phe Ala Asp Thr Val Ile

865 870 875 880

Met Lys Pro Leu Arg Ala Ser Met Ile Gly Ala Cys Leu Gln Gln Val

885 890 895

Leu Glu Leu Arg Lys Thr Arg Gln Gln His Pro Glu Gly Ser Ser Pro

900 905 910

Ala Thr Leu Lys Ser Leu Leu Thr Gly Lys Lys Ile Leu Val Val Asp

915 920 925

Asp Asn Ile Val Asn Arg Arg Val Ala Ala Gly Ala Leu Lys Lys Phe

930 935 940

Gly Ala Glu Val Val Cys Ala Glu Ser Gly Gln Val Ala Leu Gly Leu

945 950 955 960

Leu Gln Ile Pro His Thr Phe Asp Ala Cys Phe Met Asp Ile Gln Met

965 970 975

Pro Gln Met Asp Gly Phe Glu Ala Thr Arg Gln Ile Arg Met Met Glu

980 985 990

Lys Glu Ala Lys Glu Lys Thr Asn Leu Glu Trp His Leu Pro Ile Leu

995 1000 1005

Ala Met Thr Ala Asp Val Ile His Ala Thr Tyr Glu Glu Cys Leu Lys

1010 1015 1020

Ser Gly Met Asp Gly Tyr Val Ser Lys Pro Phe Glu Glu Glu Asn Leu

1025 1030 1035 1040

Tyr Lys Ser Val Ala Lys Ser Phe Lys Pro Asn Pro Ile Ser Pro Ser

1045 1050 1055

Ser

<210> 7

<211> 125

<212> PRT

<213> Saccharomyces cerevisiae

<400> 7

Glu Thr Ser Val Lys Ile Leu Val Val Glu Asp Asn His Val Asn Gln
1 5 10 15

Glu Val Ile Lys Arg Met Leu Asn Leu Glu Gly Ile Glu Asn Ile Glu
20 25 30

Leu Ala Cys Asp Gly Gln Glu Ala Phe Asp Lys Val Lys Glu Leu Thr
35 40 45

Ser Lys Gly Glu Asn Tyr Asn Met Ile Phe Met Asp Val Gln Met Pro
50 55 60

Lys Val Asp Gly Leu Leu Ser Thr Lys Met Ile Arg Arg Asp Leu Gly
65 70 75 80

Tyr Thr Ser Pro Ile Val Ala Leu Thr Ala Phe Ala Asp Asp Ser Asn
85 90 95

Ile Lys Glu Cys Leu Glu Ser Gly Met Asn Gly Phe Leu Ser Lys Pro
100 105 110

Ile Lys Arg Pro Lys Leu Lys Thr Ile Leu Thr Glu Phe
115 120 125

<210> 8

<211> 118

<212> PRT

<213> Escherichia coli

<400> 1

Asn Asp Asp Met Met Ile Leu Val Val Asp Asp His Pro Ile Asn Arg
1 5 10 15

Arg Leu Leu Ala Asp Gln Leu Gly Ser Leu Gly Tyr Gln Cys Lys Thr
20 25 30

Ala Asn Asp Gly Val Asp Ala Leu Asn Val Leu Ser Lys Asn His Ile
35 40 45

Asp Ile Val Leu Ser Asp Val Asn Met Pro Asn Met Asp Gly Tyr Arg

50 55 60

Leu Thr Gln Arg Ile Arg Gln Leu Gly Leu Thr Leu Pro Val Ile Gly
65 70 75 80

Val Thr Ala Asn Ala Leu Ala Glu Glu Lys Gln Arg Cys Leu Glu Ser
85 90 95

Gly Met Asp Ser Cys Leu Ser Lys Pro Val Thr Leu Asp Val Ile Lys
100 105 110

Gln Ser Leu Thr Leu Tyr
115

<210> 9

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

<400> 9

tccccgcgga aaatgttctt acggttaggt ag 32

<210> 10

<211> 33

<212> DNA

<213> Artificial Sequence

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tcggtcgact tatgattctg tatctgaagg cga 33

<210> 11

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tcagatatga actgggcact caac 24

<210> 12

<211> 24

<212> DNA

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<223> Description of Artificial Sequence:Designed
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ctcaatgctt ttgttccttg actc 24

<210> 13

<211> 31

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:Designed

oligonucleotide primer for PCR

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accatgaact gggcactcaa caatcatcaa g 31

<210> 14

<211> 30

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:Designed
oligonucleotide primer for PCR

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ggattacgac gaaggtgaga taggattagg 30

<210> 15

<211> 32

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence:Designed
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gatcccagct agctagggcc ctaccgcggg ga 32

<210> 16

<211> 32

<212> DNA

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tccccgcgga aaatgttctt acggttaggt ag 32

<210> 17

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<212> DNA

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<210> 18

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ctagtccccg cggtagggcc ctagctagct gg 32

<210> 19

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tccccgcgga aaatgtctat aacttgtgag c 31

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<210> 21

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<210> 22

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