

METHODS FOR PRODUCING SECRETED PROTEINS

5

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods for producing proteins secreted out of plant cells.

10 Description of the Related Art

When a heterologous protein gene is introduced into a plant host cell and is highly expressed, secreting the expressed heterologous protein into the extracellular apoplast to accumulate therein may be envisioned as a measure to reduce
15 a load on the host cell.

Methods for secreting a heterologous protein into an extracellular apoplast may include a method of expressing the heterologous protein in the form of a fusion protein in which a signal sequence that directs endoplasmic reticulum (ER)
20 transport is linked to the amino terminus of the heterologous protein. The signal sequence allows the linked protein to penetrate into the endoplasmic reticulum lumen, and the signal sequence is cleaved from the heterologous protein by a signal peptidase in the ER membrane to release the heterologous
25 protein into the ER lumen. The heterologous protein is folded in the ER lumen, and then secreted into the extracellular apoplast by the intracellular transport system.

Glycinin is a soybean seed storage protein, and after it

is translated in the form of a proglycinin precursor having a signal sequence for ER transport, the signal sequence is cleaved from the precursor in the ER membrane to release a proglycinin. Proglycinin forms a trimer in the ER and is transported into a protein storage vacuole, and then is processed into a mature glycinin to form a hexamer. Maruyama et al. (The Plant Cell, 2006, vol. 18, p. 1253-1273) reported that in an experiment of transiently expressing a green fluorescent protein (hereinafter, sometimes referred to as GFP) in a soybean immature seed, only when a glycinin signal sequence for ER transport that was flanked on its carboxy terminus by 9-amino acids sequence derived from a proglycinin amino-terminal region was fused to the amino terminus of a GFP to express, the GFP was secreted into the extracellular apoplast. Kawagoe et al. (The Plant Cell, 2005, vol. 17, p. 1141-1153) reported that in an experiment of expressing a GFP in a recombinant rice immature seed, when a glycinin signal sequence for ER transport that was flanked on its carboxy terminus by 2-amino acids sequence derived from a proglycinin amino-terminal region and a few other amino acids was fused to the amino terminus of a GFP to express, the GFP was not secreted into the extracellular apoplast.

In production of useful proteins, to maintain original useful properties such as enzyme activities or physiological functions of an intended heterologous protein, it is preferred that heterologous proteins to be produced have no additional heterologous sequences or short ones, if any.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide improved methods for secreting an intended heterologous protein into an extracellular apoplast of a plant cell using a glycinin signal sequence for ER transport, in production of
5 the heterologous protein in plant cells.

The present invention provides:

1. a method for producing a heterologous protein secreted out of a plant cell comprising:
10 introducing into a plant cell genome a DNA encoding an amino acid sequence that comprises a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein the signal sequence is directly fused to the amino acid sequence of the heterologous
15 protein or one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein; and
expressing the DNA;
2. the method according to the item 1, wherein one amino
20 acid is inserted between the signal sequence and the amino acid sequence of the heterologous protein;
3. the method according to the item 2, wherein the one amino acid is selected from among serine, alanine and methionine;
- 25 4. the method according to any one of the items 1 to 3, wherein the glycinin signal sequence for endoplasmic reticulum transport is the amino acid sequence of SEQ ID NO:1;
5. a method for producing a recombinant plant cell

secreting a heterologous protein comprising:

introducing into a plant cell genome a DNA encoding an amino acid sequence that comprises a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein the signal sequence is directly fused to the amino acid sequence of the heterologous protein or one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein;

6. a plant expression plasmid comprising a DNA encoding an amino acid sequence that comprises a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein the signal sequence is directly fused to the amino acid sequence of the heterologous protein or one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein;

7. the plasmid according to the item 6, wherein one amino acid is inserted between the signal sequence and the amino acid sequence of the heterologous protein;

8. a DNA encoding an amino acid sequence that comprises a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein;

9. a plant cell with a genome into which a DNA encoding an amino acid sequence is introduced, the amino acid sequence

comprising a glycinin signal sequence for endoplasmic
reticulum transport and an amino acid sequence of a
heterologous protein, wherein the signal sequence is directly
fused to the amino acid sequence of the heterologous protein
5 or one or two amino acids are inserted between the signal
sequence and the amino acid sequence of the heterologous
protein; and

10. a plant with a genome into which a DNA encoding an
amino acid sequence is introduced, the amino acid sequence
10 comprising a glycinin signal sequence for endoplasmic
reticulum transport and an amino acid sequence of a
heterologous protein, wherein the signal sequence is directly
fused to the amino acid sequence of the heterologous protein
or one or two amino acids are inserted between the signal
15 sequence and the amino acid sequence of the heterologous
protein, or a seed of the plant; and the like.

According to the present invention, it is possible to
secrete an intended heterologous protein in a functional form
into an extracellular apoplast of a plant cell without fusing
20 a relatively long peptide to the heterologous protein, in
production of the heterologous protein in plant cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a structural schematic diagram of the plasmid
25 pSUM-35S-GY1SP S-GFP.

Fig. 2 is a structural schematic diagram of the plasmid
pSUM-35 S-GY1SP-GFP.

Fig. 3 is a structural schematic diagram of the plasmid

pSUM-35S-GY1SP M-GFP.

Fig. 4 is a structural schematic diagram of the plasmid pSUM-35S-GY1SP A-GFP.

Fig. 5 is a structural schematic diagram of the plasmid
5 pSUM-35S-GY1SP S-Lip1soy.

Fig. 6 is a structural schematic diagram of the plasmid pSUM-35 S-Lip1soy.

Fig. 7 is a structural schematic diagram of the plasmid pBI-35S-GY1SP S-Lip1soy.

10 Fig. 8 is a structural schematic diagram of the plasmid pBI-35 S-Lip1soy.

Fig. 9 is a histogram showing transcription level of the lipase gene.

15 Fig. 10 is an image showing results of detecting lipase protein by Western blot analysis.

Fig. 11 is an image showing results of detecting lipase protein by Western blot analysis from fractionation of a tobacco in which the plasmid pBI-35S-GY1SP S-Lip1soy has been introduced.

20 Fig. 12 is a structural schematic diagram of the plasmid pSUM-GY1-GY1SP S-Lip1soy.

Fig. 13 is an image showing results of detecting lipase protein by Western blot analysis.

25 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the present invention, the term "out of plant cells" means the outside of the plant cell membrane.

The term "extracellular apoplast" means a region outside

of plant cell membrane, specifically includes a vessel or xylem, a cell wall, an intercellular space, and the like, and includes culture medium used in cell culture.

The term "secretion" in the present invention refers to transporting proteins into the extracellular apoplast.

In general, as the extracellular apoplast is low in protein content, mainly consists of water and ions, it may accumulate heterologous proteins in high concentration.

In the present specification, "an amino acid sequence that comprises a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein the signal sequence is directly fused to the amino acid sequence of the heterologous protein or one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein" may be referred to as "an amino acid sequence used in the present invention".

The DNA encoding "an amino acid used in the present invention" may be referred to as a "DNA used in the present invention".

The "DNA used in the present invention" may be single-stranded DNA or double-stranded DNA.

"An amino acid sequence used in the present invention" is an amino acid sequence of a fusion protein containing an amino acid represented by the following (1) or (2).

In the present specification, an amino acid sequence is listed from the amino terminal to the carboxyl terminal.

(1) an amino acid sequence in which a glycinin signal sequence for endoplasmic reticulum transport is directly fused to an amino acid sequence of a heterologous protein.

(2) an amino acid sequence in which one or two amino acids are inserted between a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein.

A glycinin signal sequence for endoplasmic reticulum transport used in the present invention is a signal sequence for transport into the endoplasmic reticulum (ER) located in the amino terminal of a proglycinin precursor. The signal sequence has a function to allow the protein linked to its carboxyl terminus to penetrate into the endoplasmic reticulum lumen and is cleaved off by a signal peptidase in the ER membrane. Five (5) subunits (G1 to G5) are present in glycinin, and a signal sequence for ER transport of each subunit is known (Nielsen et al., The Plant Cell (1989) Vol. 1, pp 313-328) (SEQ ID NOS: 1 to 5). A glycinin signal sequence for endoplasmic reticulum transport used in the present invention may specifically include the amino acid sequence of any one of SEQ ID NOS: 1 to 5, and preferably the amino acid sequence of SEQ ID NO: 1, but it is not limited to as far as it has a function as a glycinin signal sequence for endoplasmic reticulum transport. For example, glycinin signal sequence for endoplasmic reticulum transport to be found in the future and variant of glycinin signal sequence for endoplasmic reticulum transport may be used.

Signal sequence for ER transport of subunit G1:

MAKLVFSLCFLLFSGCCFA (SEQ ID NO:1)

Signal sequence for ER transport of subunit G2:

MAKLVLSLCFLLFSGCFA (SEQ ID NO:2)

Signal sequence for ER transport of subunit G3:

5 MAKLVLSLCFLLFSGCCFA (SEQ ID NO:3)

Signal sequence for ER transport of subunit G4:

MGKPFITLSLSSLCLLLSSACFA (SEQ ID NO:4)

Signal sequence for ER transport of subunit G5:

MGKPFITLSLSSLCLLLSSACFA (SEQ ID NO:5)

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A "heterologous protein" in the present invention is a protein intended to be produced by introducing its structural gene into a host plant cell genome, and a protein that is not native to the amino acid sequence linked to its amino terminus.

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The "heterologous protein" includes not only a protein which is not produced naturally in the host plant cell, but also a protein naturally produced in the host plant cell. When the "heterologous protein" is directly linked to a glycinin signal sequence for endoplasmic reticulum transport, it is a protein

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other than glycinin.

A "heterologous protein" in the present invention is preferably a mature protein. A "heterologous protein" in the present invention is preferably a protein suitable to be expressed in a plant cell. The present invention is suitable for producing proteins which are not operably folded when
25 expressed in a prokaryotic cell such as E. coli and which are operably folded when expressed in plant cells. A "heterologous protein" may include industrially useful

proteins such as industrial enzymes, vaccines, physiologically active materials, and antibodies. Specifically, a "heterologous protein" may include lipase (for example, refer to Longhi et al., 1992, Biochimica et Biophysica Acta (1992) Vol. 1131 (2), pp227-232), amylase, pectinase, β -glucanase, phytase, lactase, and the like as an industrial enzyme; chicken Newcastle disease vaccine, vaccine against porcine edema disease, vaccine against porcine protozoal disease, hepatitis B vaccine, and the like as a vaccine; hematopoietic factor, hormone, interferons, and the like as a physiologically active material; and immunoglobulin and the like as an antibody.

A "heterologous protein" in the present invention is preferably a protein which does not have a sequence to reduce the extracellular secretion efficiency. Examples of the sequence to reduce the extracellular secretion efficiency may include an endoplasmic reticulum retention signal sequence and the amino acid sequence of any one of SEQ ID NOs: 1 to 3 described in U.S. Patent No. 7,368,628.

A "heterologous protein" in the present invention is preferably a protein other than soybean glycinin.

Amino acid sequences of these proteins may be obtained by using protein engineering techniques and genetic engineering techniques known in the art, or may be obtained from a variety of databases available in the art. Nucleotide sequences encoding amino acid sequences of these proteins may be obtained by using conventional genetic engineering

techniques, or may be obtained from a variety of databases available in the art.

In the present invention, a glycinin signal sequence for
5 endoplasmic reticulum transport is directly linked to an amino
acid sequence of a heterologous protein or one or two amino
acids are inserted between the signal sequence and an amino
acid sequence of a heterologous protein. Depending on the
property of a desired heterologous protein, but it may be
10 preferable that one or two amino acids are inserted between
the signal sequence and the amino acid sequence of the
heterologous protein for a good yield of the heterologous
protein to be obtained without degradation, and may be more
preferable that one amino acid is inserted between the signal
15 sequence and the amino acid sequence of the heterologous
protein. The "one amino acid" may include one amino acid
selected among serine, alanine, and methionine. The sequence
of the "two amino acids" is preferably an amino acid sequence
other than FS (phenylalanine-serine).

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A DNA encoding "an amino acid sequence that comprises a
glycinin signal sequence for endoplasmic reticulum transport
and an amino acid sequence of a heterologous protein, wherein
the signal sequence is directly fused to the amino acid
25 sequence of the heterologous protein or one or two amino acids
are inserted between the signal sequence and the amino acid
sequence of the heterologous protein" may be prepared by
conventional genetic engineering techniques. For example,

based on nucleotide sequences, "a DNA used in the present invention" is chemically synthesized. A DNA encoding a heterologous protein may be linked in frame to a DNA comprising (a) a nucleotide sequence encoding a glycinin
5 signal sequence for endoplasmic reticulum transport or (b) a nucleotide sequence in which a nucleotide sequence encoding one or two amino acids is added to the 3' end of the nucleotide sequence of (a) by using conventional genetic engineering techniques. More specifically, it may be prepared
10 based on the methods described in Examples below.

In the production method of the present invention, the "DNA used in the present invention" described above is introduced into a plant cell genome to be expressed.

15 A method of introducing DNA may include genetic engineering methods known in the art. For example, a plant expression plasmid containing "a DNA used in the present invention" is introduced by using a genetic cell engineering technique suitable for a host plant cell. These techniques
20 may include an Agrobacterium method, particle gun method, electroporation method, and calcium phosphate method.

In response to a gene transfer method employed, a plant expression plasmid containing "a DNA used in the present
25 invention" may be constructed by inserting the "DNA used in the present invention" into, for example, a Ti plasmid-derived vector such as pBI101 and pBI121, or a Ri plasmid-derived vector, or a conventional pUC-based E. coli vector such as

pBI221 using conventional genetic engineering methods.

The plant expression plasmid contains a promoter and a terminator to express a fusion protein of the present invention.

5 The corresponding promoter is not specifically limited to as far as it can function in a plant cell, and include a constitutive promoter such as cauliflower mosaic virus 35S promoter (International Patent Publication WO84/02913), ubiquitin promoter (International Patent Publication
10 WO01/094394) and actin promoter (International Patent Publication WO00/070067), a tissue-specific promoter such as soybean seed glycinin promoter (European Patent Publication EP0571741), carrot CR16G1 promoter (US Patent No.5959176) and common bean phaseolin promoter (International Patent
15 Publication WO91/013993), and a chemical inducible promoter such as corticosteroid responsive promoter (Aoyama T & Chua NH, 1997, Plant J. 11,605-612; U.S. Patent No. 6,063,985) and ethanol responsive promoter (Caddick MX et al., 1998, Nature Biotech. 16, 177-180; International Patent Publication
20 WO93/21334).

 The terminator is not specifically limited to as far as it can function in a plant cell, and may include, NOS terminator (International Patent Publication WO84/02913), soybean seed glycinin terminator (European Patent Publication
25 EP0571741) and carrot CR16 terminator (US Patent No.7202083).

Also, the plant expression plasmid may contain a selective marker such as auxotrophic marker or drug resistant marker.

A cell with a genome into which "a DNA used in the present invention" has been introduced may be selected and purified by characteristics of the selective marker, for example, resistance to kanamycin, introduced along with the DNA.

Since the plant expression plasmid has been incorporated into the genome of a plant cell, a sequence of "a DNA used in the present invention" is transferred to the genome of a daughter cell after cell division and as a result, it is possible to stably express the DNA even in the progeny.

A plant cell with a genome into which "a DNA used in the present invention" will be introduced, that is, a host plant cell is preferably a cell of a plant for which gene transfer method, tissue culture method, technique of redifferentiation from a cell or a callus, and the cultivation technique are established. Also, the host plant cell is preferably a cell of a plant species important for industry such as agriculture or useful in studies such as genome analyses. These plants may include, soybean, common bean, green pea, peanut, castor bean, rice, corn, cotton, rapeseed, wheat, barley, cucumber, eggplant, carrot, potato, taro, sweet potato, pumpkin, garlic, onion, Japanese cedar, pine, willow, poplar, eucalyptus, tobacco, birdsfoot trefoil, alfalfa, clover, thale cress, sorghum, sesame, sunflower, Jerusalem artichoke, switchgrass, pampas grass, Jatropha curcas, oil palm, coconut palm, olive, sugarcane, sugar beet, cassava, and kenaf.

A cell with a genome into which "a DNA used in the

present invention" has been introduced may be cultured under
suitable conditions by using MS culture medium and the like.
From the cell into which "a DNA used in the present invention"
has been introduced, a plant (body) into which "a DNA used in
5 the present invention" has been introduced may be obtained by
the conventional redifferentiation methods. Also, from the
plant, its seed may be obtained.

A heterologous protein of interest may be produced by
10 expressing and secreting by a plant cell of the present
invention, or a plant of the present invention.

The resulting produced protein can be recovered
appropriately by a combination of ordinary isolation and
purification methods. For example, culture of the plant cell
15 of the present invention or homogenized fraction of the plant
or plant cell of the present invention can be recovered and
used as a fraction containing the "heterologous protein" of
interest. A fraction containing the "heterologous protein" of
interest can also be obtained by a centrifugation of the
20 culture after completion of the culture and subsequent
recovering of the supernatant. A further purified
"heterologous protein" of interest can be recovered by
subjecting the supernatant fraction described above to various
chromatographic procedures such as ion exchange chromatography,
25 hydrophobic interaction chromatography, gel filtration
chromatography, affinity chromatography and the like.

A method of recovering an extracellular apoplast

solution in which "heterologous proteins" are accumulated may include methods described in experimental protocols (for example, 'Experimental protocol for proteins of a plant, The functions and structures of proteins approached from genes and organisms', Kenzo Nakamura et al., 1998, SHUJUN Co., Ltd., ISBN 4-87962-180-3) or literatures (for example, Boernke et al., Planta (2002) Vol. 214, pp356-364). For example, a buffer having a high ionic strength such as 1 M sodium chloride or 1 M potassium chloride may be sucked into a plant cell under low pressures and then the cell may be centrifuged at low speeds to recover an extracellular apoplast solution.

Examples

The present invention will be described in detail with reference to examples, but the present invention is not limited to the examples.

Example 1

Construction of a green fluorescent protein expression plasmid

In order to express Aequorea Victoria-derived green fluorescent protein (hereinafter, it may be referred to as GFP) in a plant cell, an expression plasmid was constructed, the plasmid containing a DNA in which a DNA encoding a glycinin signal sequence for endoplasmic reticulum transport (hereinafter, it may referred to as GY1SP) and a DNA encoding a green fluorescent protein has been linked without causing a codon frame shift (that is, in-frame).

(1) Construction of a GFP secretory expression plasmid

An oligonucleotide consisting of the nucleotide sequence

of SEQ ID NO: 6 and encoding a green fluorescent protein was synthesized. A PCR was performed by using the synthesized oligonucleotide as a template, and an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 7 and an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 8 as primers to amplify a DNA fragment encoding GFP and having a NheI site and a SacI site respectively at each of the 5'- and 3'-end of the fragment. For the PCR, a DNA polymerase (trade name: KOD-Plus, manufactured by TOYOBO Co., Ltd.) was used and conditions of the reactions were as followed: incubation at 94°C for 2 minutes, followed by 30 cycles each for 94°C for 30 seconds, 50°C for 30 seconds, and 68°C for 60 seconds; and followed by incubation at 68°C for 3 minutes. The amplified DNA fragment was purified from the reaction solution by using a DNA fragment purification kit (trade name: MagExtractor-PCR & Gel Clean Up, manufactured by TOYOBO Co., Ltd.), and the purified DNA fragment was ligated by using a gene cloning kit (trade name: TaKaRa BKL Kit, manufactured by TaKaRa Co., Ltd.) to the SmaI site of the plasmid pUC118. The ligation product was introduced into competent cells of E. coli DH5 α strains (manufactured by TOYOBO Co., Ltd.) and ampicillin resistant strains were selected. Nucleotide sequences of plasmids from the selected resistant strains were analyzed by using a reaction kit for nucleotide sequence analysis (trade name: BigDye Terminator v3.1 Cycle Sequencing Kit, manufactured by Applied Biosystems Japan. Ltd ABI) and a nucleotide sequence analyzer (trade name: ABI Prism 3100 Genetic Analyzer, manufactured by Applied Biosystems Japan.

Ltd ABI). A plasmid having an intended nucleotide sequence was digested with NheI and SacI and was obtained a 0.7 kbp DNA fragment encoding a polypeptide in which a serine residue has been fused to the amino terminus of GFP.

5 SEQ ID NO:6: 5'-

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGG
CGACGTGAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCA
AGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGGCCCCACCCTCGTG
ACCACCTTCACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGA
10 CTTCTTCAAGTCCGCCATGCCCCAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACG
ACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATC
GAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAA
CTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACT
TCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAAC
15 ACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCCGACAACCACTACCTGAGCACCCAGTCCGC
CCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCG
CCGGGATCACTCACGGCATGGACGAGCTGTACAAGTAA-3'

SEQ ID NO:7: 5'-gctagcgtgagcaagggcgaggagctgttcacc-3'

SEQ ID NO:8: 5'-gagctcttacttgtacagctcgtccatgccgtg-3'

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Terminally-phosphorylated synthetic oligonucleotides with the nucleotide sequences of SEQ ID NOs:9 and 10 respectively were mixed at an equivalent mole ratio, heated at 65°C for 5 minutes, and then cooled slowly to room temperature
25 to prepare a linker encoding a glycinin signal sequence for ER transport (SEQ ID NO: 1). The prepared linker and the 0.7 kbp DNA fragment encoding the polypeptide in which a serine residue has been fused to the amino terminus of GFP were

ligated to the plasmid pBI221 (GenBank Accession Number AF502128) digested with BamHI and SacI and transformed into E. coli in a similar manner. Nucleotide sequences of selected resistant strains were analyzed and was obtained the plasmid
5 pSUM-35S-GY1SP S-GFP (Fig. 1) containing the nucleotide sequence of SEQ ID NO: 11 and expressing a fusion protein in which a serine residue has been inserted between GY1SP and GFP.

SEQ ID NO:9:

10 5'-gatccatggccaagctagttttttccctttgttttctgcttttcagtggctgctgcttcg-3'

SEQ ID NO:10:

5'-ctagcgaagcagcagccactgaaaagcagaaaacaaagggaaaaaactagcttggccatg-3'

SEQ ID NO:11: 5'-

GGATCCATGGCCAAGCTAGTTTTTCCCTTTGTTTTCTGCTTTTCAGTGGCTGCTGCTTCGC
15 TAGCGTGAGCAAGGGCGAGGAGCTGTTACCCGGGGTGGTGCCCATCCTGGTCGAGCTGGACG
GCGACGTGAACGGCCACAAGTTCAGCGTGTCGGGCGAGGGCGAGGGCGATGCCACCTACGGC
AAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCCGTGCCCTGGCCCCACCCTCGT
GACCACCTTCACCTACGGCGTGCACTGCTTCAGCCGCTACCCCGACCATGAAGCAGCACG
ACTTCTTCAAGTCCGCCATGCCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGAC
20 GACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCAT
CGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACA
ACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAAC
TTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAA
CACCCCCATCGGGCGACGGCCCCGTGCTGCTGCCCCGACAACCACTACCTGAGCACCCAGTCCG
25 CCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCC
GCCGGGATCACTCACGGCATGGACGAGCTGTACAAGTAAGAGCTC-3'

A first-round PCR was performed by using the plasmid

pSUM-35S-GY1SP S-GFP as a template, an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 12 as a forward primer, and an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 13, 14, or 15 as a reverse primer for modification, respectively. Second-round PCRs were performed by using a portion of each reaction solution of the first-round PCR as a template, the oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 12 as a forward primer, and an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 16 as a reverse primer, to amplify a DNA fragment having a BamHI site and a SacI site respectively at each of the 5'- and 3'-end of the fragment and encoding a fusion protein in which an alternative amino acid residue is inserted between GY1SP and GFP. For the first-round PCR, a DNA polymerase (trade name: PrimeSTAR HS DNA Polymerase, manufactured by TaKaRa Co., Ltd.) was used and conditions of the reactions were 30 cycles each for 98°C for 10 seconds, 55°C for 5 seconds, and 72°C for 30 seconds. The second-round PCRs were performed in the same manner as the first-round PCR except that the incubation time at 72°C was modified to 60 seconds.

Each of the amplified DNA fragments was purified, digested with BamHI and SacI, and inserted between the BamHI site and the SacI site of the plasmid pSUM-35S-GY1SP S-GFP by replacing the DNA fragment of pSUM-35S-GY1SP S-GFP encoding a fusion protein in which a serine residue has been inserted between GY1SP and GFP with the BamHI and SacI-digested amplified DNA fragment to obtain the following plasmids.

By using the oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 13 as a reverse primer for modification in the first-round PCR, was obtained a plasmid pSUM-35S-GY1SP-GFP (Fig. 2) expressing a fusion protein in which GY1SP and GFP are directly linked with no additional amino acid residue insertion.

By using the oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 14 as a reverse primer for modification in the first-round PCR, was obtained a plasmid pSUM-35S-GY1SP M-GFP (Fig. 3) expressing a fusion protein in which a methionine residue has been inserted between GY1SP and GFP.

By using the oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 15 as a reverse primer for modification in the first-round PCR, was obtained a plasmid pSUM-35S-GY1SP A-GFP (Fig. 4) expressing a fusion protein in which an alanine residue has been inserted between GY1SP and GFP.

SEQ ID NO:12: 5'-gagtcaggatccatggccaagctagttttttcc-3'

SEQ ID NO:13: 5'-cttgctcacagcgaagcagcagcc-3'

SEQ ID NO:14: 5'-cttgctcaccatagcgaagcagcagcc-3'

SEQ ID NO:15: 5'-cttgctcacagcagcgaagcagca-3'

SEQ ID NO:16: 5'-gttcgagagcttcttacttgtacagctcgtccat-3'

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 17 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a glycine residue has been inserted

between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 18 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a leucine residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 19 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which an isoleucine residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 20 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a valine residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 21 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a proline residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 22 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a threonine residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 23 as a reverse primer for modification

in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a histidine residue has been inserted between GY1SP and GFP.

5 By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 24 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a glutamine residue has been inserted between GY1SP and GFP.

10 By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 25 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a glutamic acid residue has been inserted between GY1SP and GFP.

15 By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 26 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which an asparagine residue has been inserted between GY1SP and GFP.

20 By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 27 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which an aspartic acid residue has been inserted between GY1SP and GFP.

25 By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 28 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a lysin residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 29 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a cysteine residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 30 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which an arginine residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 31 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a tyrosine residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 32 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a tryptophan residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 33 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a phenylalanine residue has been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 34 as a reverse primer for modification in the first-round PCR, is obtained a plasmid expressing a

fusion protein in which a serine residue and a glycine residue have been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 35 as a reverse primer for modification
5 in the first-round PCR, is obtained a plasmid expressing a fusion protein in which a serine residue and an alanine residue have been inserted between GY1SP and GFP.

By using an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 36 as a reverse primer for modification
10 in the first-round PCR, is obtained a plasmid expressing a fusion protein in which an alanine residue and a glycine residue have been inserted between GY1SP and GFP.

SEQ ID NO:17: 5'-cttgctcacgccagcgaagcagca-3'
SEQ ID NO:18: 5'-cttgctcacaagagcgaagcagcagcc-3'
15 SEQ ID NO:19: 5'-cttgctcacgatagcgaagcagcagcc-3'
SEQ ID NO:20: 5'-cttgctcacaacagcgaagcagcagcc-3'
SEQ ID NO:21: 5'-cttgctcacgggagcgaagcagca-3'
SEQ ID NO:22: 5'-cttgctcacagtagcgaagcagcagcc-3'
SEQ ID NO:23: 5'-cttgctcacgtgagcgaagcagca-3'
20 SEQ ID NO:24: 5'-cttgctcacttgagcgaagcagcagcc-3'
SEQ ID NO:25: 5'-cttgctcacttcagcgaagcagcagcc-3'
SEQ ID NO:26: 5'-cttgctcacgtagcgaagcagcagcc-3'
SEQ ID NO:27: 5'-cttgctcacatcagcgaagcagcagcc-3'
SEQ ID NO:28: 5'-cttgctcaccttagcgaagcagcagcc-3'
25 SEQ ID NO:29: 5'-cttgctcacgcaagcgaagcagca-3'
SEQ ID NO:30: 5'-cttgctcactctagcgaagcagcagcc-3'
SEQ ID NO:31: 5'-cttgctcacgtaagcgaagcagcagcc-3'
SEQ ID NO:32: 5'-cttgctcacccaagcgaagcagca-3'

SEQ ID NO:33: 5'-cttgctcacgaaagcgaagcagcagcc-3'

SEQ ID NO:34: 5'-cttgctcacgccgctagcgaagca-3'

SEQ ID NO:35: 5'-cttgctcacagcgctagcgaagcagca-3'

SEQ ID NO:36: 5'-cttgctcacgccagcagcgaagca-3'

5

(2) Construction of a GFP-expressing binary vector for infection introduction

Each of the plasmids described in Example 1(1) is digested with HindIII and EcoRI to obtain a 1.9-kbp DNA fragment. Each of the obtained DNA fragments is ligated to the plasmid pBI121 (GenBank Accession Number AF485783) previously-digested with BamHI and SacI to obtain a binary vector plasmid expressing a fusion protein in which GY1SP and GFP are directly linked or one or two amino acids are inserted between GY1SP and GFP.

Example 2

Analysis of GFP expression in recombinant tobacco cultured cells

(1) Introduction of GFP expression plasmid into tobacco cultured cells and selection of recombinant cultured cells

An expression plasmid prepared in Example 1(1) was introduced into suspension cultured tobacco BY-2 cells by using a gene transfer system (trade name: PDS-1000/He System, manufactured by Bio-Rad Co., Ltd.). The preparation of cultured cells for gene transfer and gene transfer manipulation were conducted according to a method by IIDA et al. (Experimental Study Gene Transfer Experimental Method 1 -

Particle gun method-, Plant Cell Engineering, 1992, Vol. 4, No. 1, pp 43-48).

Three (3) to 5 days after the gene transfer manipulation, cultured cells were transferred onto a modified MS medium (MS
5 inorganic salts (Murashige and Skoog, Physiol. Plant. (1962) Vol. 15, pp473-493), 3% sucrose, 1 μ M 2,4-D, 1 mg/L thiamin-HCl, 100 mg/L myo-inositol, 200 mg/L KH_2PO_4) containing 30 mg/L of kanamycin and solidified with 0.8% agar and cultured in the dark at 23 to 25°C for 1 month to select cell masses resistant
10 to kanamycin. The selected cell masses were cultured on a modified MS agar medium containing 200 mg/L of kanamycin for another 3 weeks, grown cell masses were transferred to a fresh medium with the same composition, and then another culture for 3 to 4 weeks was further performed twice to establish
15 recombinant cell strains. After 2 weeks of rotary culture of the obtained cell strains was performed in a modified MS liquid medium containing 200 mg/L of kanamycin in the dark at 23 to 25°C at 140 rpm, another 2 weeks of culture was performed in a fresh medium under the same conditions to
20 purify the recombinant cell strains and stabilize the growth of each cell strain.

Subsequently, a rotary culture of the recombinant cell strains was performed in a modified MS liquid medium without kanamycin under the same conditions, and 0.3 to 0.4 g of cells
25 were transferred onto a fresh medium just before the cell growth would reach a saturated state to subculture.

(2) Analysis of GFP secretion amount in recombinant tobacco

cultured cells

GFP secreted from the recombinant cultured cell obtained as described above into a culture medium and GFP accumulating in the cells were quantitatively measured by using a Western blotting method and an immunostaining method to calculate the secretion amount of the GFP.

When the growth rate was increasing exponentially as a result of a rotary culture of the recombinant cultured cells, 20 mL of the culture was recovered and centrifuged at 8,000 rpm for 10 minutes at room temperature and obtained a culture supernatant and cultured cells. The culture supernatant was concentrated by using a centrifugal filter unit (trade name: Centriprep YM-10, manufactured by Millipore Co. Ltd.) until the volume of the supernatant was reduced to about 1/200 of the original volume, and the concentrate obtained was used as a medium fraction. The cultured cells of 0.4 g were frozen with liquid nitrogen, glass beads (diameter 0.25 to 0.5 mm) of the same weight as the cell weight and 300 μ L of PBS buffer were added, and then the cells were ground by a grinding apparatus (trade name: Mixermill MM-300, manufactured by QIAGEN Co., Ltd.). After the obtained ground product was centrifuged at 15,000 rpm, at 4°C for 5 minutes, the supernatant was recovered and centrifuged at 15,000 rpm, at 4°C for 5 minutes. The supernatant was recovered and used as a whole cell fraction. An aliquot of each fraction was collected and was subjected to a measurement of the protein concentration by using a protein coloration reagent (trade name: Bio-Rad Protein Assay Dye Reagent Concentrate,

manufactured by Bio-Rad Co., Ltd.) and a bovine serum albumin preparation (manufacture by Sigma Co., Ltd.) as a standard.

The aliquot of each fraction and equal volume of the reagent buffer (2-fold concentrate, containing 2-mercapto ethanol, manufacture by Nacalai Tesque, Inc.) were mixed, and the mixture was subjected to a heat treatment at 100°C for 3 minutes, and the resulting mixture was stored in ice. The mixture was applied to a SDS-PAGE gel (trade name: multi gel II Mini, manufactured by Cosmo Bio Co., Ltd.) and was electrophoresed under current of 30 mA per gel in the SDS-PAGE electrophoresis buffer (0.1% SDS, 25 mM tris, 192 mM glycine) for 1 hour. A recombinant GFP manufactured by Cosmo Bio Co., Ltd was electrophoresed as a standard for protein content determination. After the electrophoresis, the gel was slowly shook in a transfer buffer (0.0375% SDS, 48 mM tris, 39 mM glycine, 20% methanol) at room temperature for 15 minutes, and then was subjected to electroblotting by using a Trans blotting SD cell manufactured by Bio-Rad Co., Ltd. at 10 V for 1 hour to transfer proteins from the gel to a PVDF membrane (trade name: Immobilon-P, manufactured by Millipore Co., Ltd.). After the membrane was washed with a washing buffer (TBS buffer containing 0.1% Tween) for 10 minutes, the membrane was slowly shook in a washing buffer containing 2% of ECL Advance Blocking Agent (manufactured by GE HealthCare Co., Ltd.) for 1 hour at room temperature to perform a blocking. Subsequently, the membrane was washed twice with a washing buffer for 5 minutes, and then it was slowly shook in a 4,000-fold dilution of GFP Epitope Tag manufactured by Affinity BioReagents Co.,

Ltd. with Can Get Signal Immunoreaction Enhancer Solution manufactured by TOYOBO Co., Ltd. at room temperature for 40 minutes as a primary antibody reaction. After the membrane was washed with a washing buffer once for 2 minutes, twice for 5 minutes, and once for 15 minutes, the membrane was slowly shook in 5,000-fold dilutions of ECL Anti-rabbit IgG, Horseradish Peroxidase-Liked Species-Specific Whole Antibody from donkey, manufactured by GE HealthCare Co., Ltd. and S-Protein-HRP conjugate, manufactured by GE Healthcare, with Can Get Signal Immunoreaction Enhancer Solution, manufactured by TOYOBO Co., Ltd. at room temperature for 1 hour as a secondary antibody reaction. Subsequently, after the membrane was washed three times with a washing buffer for 5 minutes, The membrane was subjected to GFP signal detection by using ECL Advanced Western Blotting Detection Kit manufactured by GE Healthcare Co., Ltd., a chemiluminescence detection device (trade name: ChemiDoc XRS, manufactured by Bio-Rad Co., Ltd.) and an image analysis software (trade name: Quantity One, manufactured by Bio-Rad Co., Ltd.) and the signal intensity was measured.

In the medium fraction of the each recombinant tobacco cultured cells to which the plasmid pSUM-35S-GY1SP-GFP, pSUM-35S-GY1SP S-GFP, pSUM-35S-GY1SP M-GFP, or pSUM-35S-GY1SP A-GFP has been introduced, a signal of GFP having an appropriate molecular weight was detected. Each of the secretion amounts of GFP per milliliter of culture was calculated as 2.4 ng for pSUM-35S-GY1SP-GFP introduced cells, 20.7 ng for pSUM-35S-GY1SP S-GFP introduced cells, 10.1 ng for pSUM-35S-GY1SP M-GFP

introduced cells, and 1.8 ng for pSUM-35S-GY1SP A-GFP introduced cells, and from these results, it was apparent that heterologous proteins were secreted out of the plant cells. Also, each of the ratios of GFP not degraded to GFP secreted was calculated as 17% for pSUM-35S-GY1SP-GFP introduced cells, 61% for pSUM-35S-GY1SP S-GFP introduced cells, 49% for pSUM-35S-GY1SP M-GFP introduced cells, and 38% for pSUM-35S-GY1SP A-GFP introduced cells. From these results, it could be seen that an intended heterologous protein was extracellularly secreted when GY1SP is linked to the heterologous protein, and also it has a greater tendency to have high yields of the heterologous protein without any degradation when one amino acid is inserted between GY1SP and the heterologous protein, compared to the case where GY1SP is directly linked to the heterologous protein.

Example 3

Construction of a secretory lipase expression plasmid

To express *Candida rugosa*-derived lipase in a plant cell, an expression plasmid was constructed, the plasmid containing a DNA in which a DNA encoding a glycinin signal sequence for endoplasmic reticulum transport and a DNA encoding a lipase has been linked without causing a codon frame shift.

(1) Construction of a secretory lipase expression plasmid

The nucleotide sequence of SEQ ID NO: 37 was designed based on the amino acid sequence (GenBank Accession Number P20261) of *Candida rugosa*-derived lipase by selecting codons to be matched with plant codon usage, and DNA having the

nucleotide sequence was synthesized. The synthesized DNA was inserted between the BamHI site and the HindIII site of the plasmid pTV118N (manufactured Takara Bio Inc.) to obtain plasmid SYN224-78 containing a nucleotide sequence encoding the lipase with modified codons.

A PCR was performed by using the plasmid SYN224-78 as a template, and an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 38 and an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 39 as primers to amplify a DNA fragment having a NheI site and a SacI site respectively at each of the 5'- and 3'-end of the fragment. For the PCR, a DNA polymerase (trade name: KOD-Plus, manufactured by TOYOBO Co., Ltd.) was used and conditions of the reactions were as followed: incubation at 94°C for 2 minutes, followed by 30 cycles each for 94°C for 30 seconds, 50°C for 30 seconds, and 68°C for 90 seconds; and followed by incubation at 68°C for 3 minutes. The amplified DNA fragment was purified from the reaction solution by using a DNA fragment purification kit (trade name: MagExtractor-PCR & Gel Clean Up, manufactured by TOYOBO Co., Ltd.), and the purified DNA fragment was ligated by using a gene cloning kit (trade name: TaKaRa BKL Kit, manufactured by TaKaRa Co., Ltd.) to the SmaI site of the plasmid pUC118. The ligation product was introduced into competent cells of E. coli DH5 α strains (manufactured by TOYOBO Co., Ltd.) and ampicillin resistant strains were selected. Nucleotide sequences of plasmids from the selected resistant strains were analyzed by using a reaction kit for nucleotide sequence analysis (trade name: BigDye Terminator

v3.1 Cycle Sequencing Kit, manufactured by Applied Biosystems Japan. Ltd ABI) and a nucleotide sequence analyzer (trade name: ABI Prism 3100 Genetic Analyzer, manufactured by Applied Biosystems Japan. Ltd ABI). A plasmid having an intended
5 nucleotide sequence was digested with NheI and SacI and was obtained a 1.6 kbp DNA fragment encoding a polypeptide in which a serine residue has been fused to the amino terminus of lipase. The lipase-encoding DNA fragment was inserted between the NheI site and the SacI site of the plasmid pSUM-35S-GY1SP
10 S-GFP by replacing the DNA fragment of pSUM-35S-GY1SP S-GFP encoding a fusion protein in which a serine residue has been inserted between GY1SP and GFP with the lipase-encoding DNA fragment to obtain the plasmid pSUM-35S-GY1SP S-Lip1soy (Fig. 5) containing the nucleotide sequence of SEQ ID NO: 40 and
15 expressing a fusion protein in which a serine residue has been inserted between GY1SP and lipase.

SEQ ID NO:37: 5'-

GGATCCATGGCTCCAACTGCAACTCTTGCTAACGGTGATACCATTACTGGACTTAACGCTAT
TATCAATGAGGCATTCTCGGTATTCTTTTGCTGAGCCACCTGTTGGTAACCTTAGATTCA
20 AGGACCCAGTTCCTTACTCCGGATCACTTGATGGTCAGAAGTTTACTTCTTACGGACCATCC
TGCATGCAACAGAATCCAGAAGGTACCTATGAAGAGAACCTCCCAAAGGCTGCACTTGATCT
TGTGATGCAGTCCAAAGTTTTCGAGGCTGTGTCTCCTTCATCCGAGGACTGTCTCACTATTA
ATGTTGTGAGGCCACCTGGAACCAAGGCTGGTGCAAACCTTCCAGTTATGCTTTGGATCTTT
GGTGGAGGTTTCGAGGTTGGTGGAACCTTCAACTTTTCTCTCCAGCTCAAATGATCACTAAGTC
25 TATTGCTATGGGTAAACCAATCATTCATGTTTCAGTGAATTACCGTGTGTCTTCATGGGGAT
TCCTCGCAGGTGATGAGATTAAGGCTGAAGGTTTCAGCTAACGCTGGACTTAAAGACCAGAGA
CTTGGTATGCAATGGGTTGCAGATAATATTGCTGCTTTTGGAGGTGACCCTACCAAGGTGAC
TATCTTCGGAGAGTCCGCAGGTTCTATGTCTGTTATGTGTACATTCTTTGGAACGATGGAG

ACAATACTTATAAGGGTAAACCACTCTTCAGAGCTGGAATTATGCAATCTGGTGCTATGGTG
 CCTTCAGACGCAGTTGATGGAATCTACGGTAACGAGATTTTGGATCTTCTTGCTTCCAATGC
 TGGATGTGGTTCTGCATCCGATAAGCTCGCTTGCCTTAGGGGTGTGTCTCAGACACACTTG
 AAGATGCTACTAACAATACCCAGGATTCCTCGCATATTCATCTCTTAGACTTTCATACCTT
 5 CCTAGGCCAGACGGTGTTAACATTACAGATGACATGTATGCTCTTGTGAGAGAGGGTAAATA
 TGCTAATATCCCTGTTATTATTGGAGATCAGAACGACGAAGGTACTTCTTCGGAACATCCT
 CACTCAATGTGACTACCGACGCTCAGGCAAGAGAGTACTTTAAGCAATCTTTCGTTTCATGCT
 TCAGATGCAGAAATTGACACTCTTATGACTGCTTATCCAGGTGATATTACTCAAGGCTCCCC
 TTTTGACACAGGCATTCTTAACGCTCTCACTCCACAATTCAAGAGGATTTTCAGCAGTTCTTG
 10 GCGATCTTGGTTTTACCCTCGCTAGACGTTATTTCCCTTAATCATTACACTGGTGGAACAAAG
 TATTCTTTCCTTTCAAACAGCTTTCGGTCTCCAGTGCTTGGAACCTTTCCTCAACGA
 CATCGTTTTCCAGGATTATCTTCTCGGTTCCGGTCTCTTATTTATAATAACGCTTTCATTG
 CATTCGCTACCGACCTTGATCCAAATACTGCTGGACTCCTTGTTAAGTGGCCTGAATACACA
 TCCTCTTCCCAAAGTGGTAACAACCTTATGATGATCAATGCTCTCGGCTTTTATACTGGTAA
 15 AGACAACCTTCAGAACCGCAGGATACGATGCTCTTTTCTCTAATCCTCCATCATTCTTCGTGT
 GAGAGCTC-3'
 SEQ ID NO:38: 5'-GCTAGCGCTCCAAGTGAACCTCTTGCTAAC-3'
 SEQ ID NO:39: 5'-GAGCTCTCACACGAAGAATGATGGAGGATTAGAG-3'
 SEQ ID NO:40: 5'-
 20 GGATCCATGGCCAAGCTAGTTTTTCCCTTTGTTTTCTGCTTTTCAGTGGCTGCTGCTTCGC
 TAGCGCTCCAAGTGAACCTCTTGCTAACGGTGATACCTTACTGGACTTAACGCTATTATCA
 ATGAGGCATTCCTCGGTATTCCTTTTGCTGAGCCACCTGTTGGTAACCTTAGATTCAAGGAC
 CCAGTTCCTTACTCCGGATCACTTGATGGTCAGAAAGTTACTTCTTACGGACCATCCTGCAT
 GCAACAGAATCCAGAAGGTACCTATGAAGAGAACCTCCCAAAGGCTGCACTTGATCTTGTGA
 25 TGCAGTCCAAAGTTTTCGAGGCTGTGTCTCTTCATCCGAGGACTGTCTCACTATTAATGTT
 GTGAGGCCACCTGGAACCAAGGCTGGTGCAAACCTTCCAGTTATGCTTTGGATCTTTGGTGG
 AGGTTTCGAGGTTGGTGGAACTTCAACTTTTCCCTCCAGCTCAAATGATCACTAAGTCTATTG
 CTATGGGTAAACCAATCATTTCATGTTTCAGTGAATTACCGTGTGTCTTCATGGGGATTCCCTC

GCAGGTGATGAGATTAAGGCTGAAGGTTTCAGCTAACGCTGGACTTAAAGACCAGAGACTTGG
 TATGCAATGGGTTGCAGATAATATTGCTGCTTTTGGAGGTGACCCTACCAAGGTGACTATCT
 TCGGAGAGTCCGCAGGTTCTATGTCTGTTATGTGTACATTCTTTGGAACGATGGAGACAAT
 ACTTATAAGGGTAAACCACTCTTCAGAGCTGGAATTATGCAATCTGGTGCTATGGTGCCTTC
 5 AGACGCAGTTGATGGAATCTACGGTAACGAGATTTTTGATCTTCTTGCTTCCAATGCTGGAT
 GTGGTTCTGCATCCGATAAGCTCGCTTGCCTTAGGGGTGTGTCCTCAGACACACTTGAAGAT
 GCTACTAACAATACCCAGGATTCTCGCATATTCATCTTTAGACTTTCATACCTTCCTAG
 GCCAGACGGTGTTAACATTACAGATGACATGTATGCTCTTGTGAGAGAGGGTAAATATGCTA
 ATATCCCTGTTATTATTGGAGATCAGAACGACGAAGGTACTTTCTTCGGAACATCCTCACTC
 10 AATGTGACTACCGACGCTCAGGCAAGAGAGTACTTTAAGCAATCTTTCGTTTCATGCTTCAGA
 TGCAGAAATTGACACTCTTATGACTGCTTATCCAGGTGATATTACTCAAGGCTCCCCTTTTG
 ACACAGGCATTCTTAACGCTCTCACTCCACAATTCAAGAGGATTCAGCAGTTCTTGGCGAT
 CTTGGTTTTACCCTCGCTAGACGTTATTTCTTAATCATTACACTGGTGGAACAAAGTATTC
 TTTCTTTTCAAAACAGCTTTCCGGTCTCCCAGTGCTTGGAACTTTCCACTCAAACGACATCG
 15 TTTTCCAGGATTATCTTCTCGGTTCCGGTCTCTTATTTATAATAACGCTTTCATTGCATTC
 GCTACCGACCTTGATCCAAATACTGCTGGACTCCTTGTTAAGTGGCCTGAATACACATCCTC
 TTCCCAAAGTGGTAAACAACCTTATGATGATCAATGCTCTCGGTCTTTATACTGGTAAAGACA
 ACTTCAGAACCGCAGGATACGATGCTCTTTTCTCTAATCCTCCATCATTCTTCGTGTGAGAG
 CTC-3'

20

(2) Construction of a cytosolic lipase expression plasmid

Plasmid SYN224-78 was digested with BamHI and SacI and a
 DNA fragment comprising the nucleotide sequence of SEQ ID NO:
 37 was purified. The obtained DNA fragment was ligated to
 25 plasmid pBI221 digested with BamHI and SacI to obtain plasmid
 pSUM-35S-Lip1soy (Fig. 6) expressing the lipase to which GY1SP
 was not linked to.

(3) Construction of a lipase expression binary vector for transfection

Each of the plasmids obtained in examples 3(1) and (2) was digested with BamHI and SacI to obtain a DNA fragment containing a lipase coding region. Each of the DNA fragments
5 was ligated to the plasmid pBI121 previously-digested with BamHI and SacI, to obtain binary vector plasmid pBI-35S-GY1SP S-LipIsoy (Fig. 7) expressing a fusion protein in which a serine residue has been inserted between GY1SP and lipase, and
10 binary vector plasmid pBI-35S-LipIsoy (Fig. 8) expressing lipase to which GY1SP was not linked to.

(4) Preparation of anti-rabbit lipase antiserum

Plasmid SYN224-78 was digested with BamHI and SacI to
15 obtain 1.6kbp-DNA fragment encoding the lipase. The obtained DNA fragment was inserted between BamHI site and HindIII site of plasmid pQE-80L (manufactured by QIAGEN Inc.) to obtain plasmid expressing a lipase to which histidine-tag peptide was linked at the amino-terminal. The expression plasmid was
20 introduced into the E. coli JM109 strain and the lipase protein to which histidine-tag peptide has been linked was purified from the obtained transformed E. coli strain. A rabbit was immunized by using the purified protein as an antigen and an antiserum (AGC TECHNO GLASS CO., LTD)
25 containing anti-lipase antibody was obtained.

Example 4

Analysis of expression of lipase in recombinant tobacco

(1) Introduction of a lipase expression vector into tobacco and selection of recombinant tobacco

Each of the lipase expression binary vectors prepared in Example 3 (3) was introduced into tobacco through the Agrobacterium infection method. The transfection was performed according to the method by Uchimiya (Plant gene manipulation manual, Method for producing transgenic plant, Kodansha Scientific Ltd., 1990, ISBN4-06-153513-7).

Each of lipase expression binary vectors was introduced into Agrobacterium tumefaciens LBA4404 (manufactured by Clontech) and the resulting transfected Agrobacterium cells were cultured overnight in an LB liquid medium containing 300mg/L streptomycin, 100mg/L rifampicin, and 25 mg/L kanamycin. Leaf discs taken from aseptically-cultured tobacco (Nicotiana tabacum strain SR-1) were dipped in the overnight culture, planted on MS agar medium (MS inorganic salts, MS vitamins, 3% sucrose, 0.8% agar) with 0.1 mg/L naphthalene acetic acid (NAA) and 1.0 mg/L benzyladenine (BA) added, and then cultured under fluorescent light at 23°C to 25°C for 2 days. These cultured leaf discs were washed with sterilized water, and cultured on MS agar medium with 0.1 mg/L NAA, 1.0 mg/L BA, and 500 mg/L cefotaxime added for 7 days to eliminate Agrobacterium. Then, the cultured leaf disks were transferred to MS agar medium with 0.1 mg/L NAA, 1.0 mg/L BA, 500 mg/L cefotaxime, and 100 mg/L kanamycin added. The culture of the leaf disks was performed for 2 months by transferring them to fresh medium having the above composition every 2 weeks to induce adventitious budding.

Adventitious buds were transferred to and rooted on MS agar medium with 100 mg/L kanamycin added, to raise them to young plants. The regenerated plants were transferred to potting soil, acclimated to the external environment in a growth chamber, then grown in a greenhouse. After growing the plants in a greenhouse, seeds were harvested.

(2) Lipase expression assay of recombinant tobacco by western blotting method

Lipase expressed in the recombinant tobacco obtained as described above was detected through western blotting and immunostaining method.

A recombinant tobacco leaf disc of about a 2-cm square was placed in a sampling tube with one zirconia bead (5mmφ, YTZ ball, manufactured by Nikkato Corp.), rapidly frozen under liquid nitrogen, and ground using a grinding apparatus (trade name: Mixermill MM-300, manufactured by Qiagen). After mixing the ground products with 300μL of PBS buffer, the mixture was centrifuged at 15,000 rpm for 5 minutes at 4°C, the supernatant was collected and centrifuged at 15,000 rpm for 5 minutes at 4°C, and the supernatant was collected. The protein concentration was measured by the method described in Example 2 (2), and lipase protein was detected through SDS-PAGE, western blotting, and immunostaining. As the SDS-PAGE gel (trade name: PAG mini 'Daiichi' manufactured by Daiichi Pure Chemicals Co., Ltd.) was used. In the antibody reaction of a membrane after the protein transfer, the rabbit antiserum described in Example 3 (4) was used as a primary antibody, and

the goat anti-rabbit IgG-AP manufactured by Santa Cruz Biotechnology was used as a secondary antibody. The membrane, after reacting with antibody, was subjected to color-development using the AP kit (manufactured by Bio-Rad) to
5 detect the lipase protein.

As a result of detection through the western blotting, a relatively high level of lipase expression was observed in 15 individuals among 22 individuals of recombinant tobaccos to which pBI-35S-GY1SP S-Lip1soy was introduced, and in 16
10 individuals among 98 individuals of recombinant tobaccos where pBI-35S-Lip1soy was introduced. T1 seeds obtained from the individuals were seeded on modified MS agar medium containing 50 mg/L kanamycin under aseptic conditions. Then, individuals showing resistance to kanamycin were selected and the selected
15 individuals were grown to obtain T2 seeds. The T2 seeds were seeded again on kanamycin-added media under aseptic conditions, and lines without kanamycin-sensitive individuals were selected as a lipase expression fixed line.

20 (3) Analysis of transcription level of lipase gene through real-time PCR

Seeds of selected T2 fixed line were seeded, and a leaf disc of about 2 cm square was sampled after 12 days of acclimation. Total RNA was extracted from the leaf disc by
25 using a plant RNA extraction kit (trade name: RNeasy Plant Mini Kit, manufactured by Qiagen), and cDNA was synthesized from the obtained total RNA by using a cDNA synthesis kit (trade name: ReverTra Ace, manufactured by Toyobo). The

quantification of lipase mRNA was performed using the synthesized cDNA as a template, and an oligonucleotide consisting of the nucleotide sequence of SEQ ID No: 41 and an oligonucleotide consisting of the nucleotide sequence of SEQ ID No: 42 as primers, and using a real-time PCR apparatus (trade name: 7500 Fast Real-Time PCR System, manufactured by Applied Biosystems). For an internal standard, the quantity of mRNA of the tobacco ubiquitin gene (GenBank Accession Number U66264) was measured using oligonucleotides consisting of the nucleotide sequences of SEQ ID NOs: 43 and 44 respectively as primers.

As a result, individuals showing the comparable lipase gene transcription level were obtained in the selected T2 fixed lines to which pBI-35S-GY1SP S-Lip1soy or pBI-35S-Lip1soy has been introduced (Fig. 9).

SEQ ID No:41: 5'-TTGATCTTCTTGCTTCCAATGC-3'

SEQ ID No:42: 5'-GTCTGAGGACACACCCCTAAGG-3'

SEQ ID No:43: 5'-GAAGCAGCTCGAGGATGGAA-3'

SEQ ID No:44: 5'-GACGGGTTGACTCTTTCTGGAT-3'

20

(4) Analysis of expression level of lipase protein in recombinant tobacco

From a leaf disc of the recombinant tobacco individuals having the comparable transcription level of lipase gene, whole cell fraction was fractionated, and extracellular apoplast fraction and cell debris fraction were fractionated according to the method by Boernke et al. (2002, Planta 214, pp. 356-364). Then, the amount of lipase protein was measured

in each of the fractions through western blotting and immunostaining method to calculate the amount of secreted lipase protein.

Leaf disks of 0.1 to 0.2 g were taken from each individual after 17 days of acclimation, and proteins were extracted according to the method described in Example 4(2), which was used as a whole cell fraction.

In a similar manner, 5 to 9 g of leafs were taken from each individual after 17 days of acclimation, midribs and major veins were removed from the leafs, and the leafs were cut into 1 to 2 cm square leaf discs. The leaf discs were infiltrated with 100 mL of 1 M KCl solution under vacuum for 5 minutes. The resulting leaf discs were centrifuged at 1,000 g for 3 minutes at 4°C and the supernatant was collected, which was used as an extracellular apoplast solution. The solution was further concentrated until the liquid volume was reduced to about 1/20 of the original volume by using centrifugal filter units (trade name: Centriprep YM-10, manufactured by Millipore) and Microcon YM-10, which was used as an extracellular apoplast fraction.

In addition, after the collection of the extracellular apoplast solution, a portion of the precipitate was collected, and then protein was extracted therefrom according to the method described in Example 4(2), which was used as a cell debris fraction. Measurement of protein concentration, SDS-PAGE, and western blotting were performed according to the method described in Example 2(2) for these fractions. ECL Plus Western Blotting Detection Reagent manufactured by GE

Health Care was used as a detection reagent for immunostaining method, and a wash buffer solution in which 5% of skim milk was dissolved was used as a blocking solution. In a primary antibody reaction, a solution of the rabbit anti-lipase antiserum described in Example 3(5) diluted 10,000 times with Can Get Signal Immunoreaction Enhancer Solution (manufactured by Toyobo) was used. In a secondary antibody reaction, solutions of ECL Anti-rabbit IgG, Horseradish Peroxidase-Linked Species-Specific Whole Antibody (donkey-derived) manufactured by GE Health Care, and S-protein-HRP conjugate manufactured by GE Health Care diluted 10,000 times and 30,000 times, respectively, with Can Get Signal Immunoreaction Enhancer Solution manufactured by TOYOBO were used. As a standard, Lipase OF (manufactured by Meito Transportation Co., Ltd.) was used.

As a result, signal of lipase protein was detected in the recombinant tobacco in which plasmid pBI-35S-GY1SP S-Lip1soy was introduced (Fig. 10). As a result of the fractionation, lipase signal was detected in the extracellular apoplast fraction, indicating the secretion of the lipase protein (Fig. 11). As a result of calculation of the amount of lipase production and the amount of lipase secretion based on the electrophoresis level and the signal intensity of the standard, it was found that 28.9 μg of lipase protein was accumulated for each 1 g wet weight of the leaf, and particularly, 1.76 μg of the lipase was secreted into the extracellular apoplast.

(5) Measurement of enzyme activity of lipase in recombinant tobacco cell fraction

Activity measurement was performed on the fraction, in which lipase protein was detected, by using an enzyme activity measurement reagent (trade name: Lipase Kit S, manufactured by 5 Dainippon Sumitomo Pharma Co., Ltd.). As a standard, Lipase OF (manufactured by Meito Transportation Co., Ltd.) was used.

As a result, it was found that, in the recombinant tobacco in which plasmid pBI-35S-GY1SP S-Lip1soy was 10 introduced, the activities of the whole cell fraction and the extracellular apoplast fraction were 5.7 IU and 37.3 IU, respectively, for each 1 g of total protein. According to the above described results, it was found that the recombinant tobacco in which the plasmid pBI-35S-GY1SP S-Lip1soy was 15 introduced produced enzymatically-active lipase and secreted the enzymatically-active lipase to the extracellular apoplast region.

Furthermore, it is supposed that the lipase secreted to the extracellular apoplast region has high activity and 20 folding and processing is performed accurately.

Example 5

Construction of a lipase secretory expression plasmid containing glycinin gene promoter and terminator

25 To express *Candida rugosa*-derived lipase in a plant seed, was constructed seed-specific expression plasmid in which glycinin gene promoter and terminator were operably linked to DNA encoding an intended protein.

A PCR was performed by using the plasmid pSUM-GY1 disclosed in European Patent application 0571741 as a template, an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 45 and an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 46 as primers to amplify a glycinin gene promoter fragment having a BamHI site at 3'-end of the fragment. For the PCR, a DNA polymerase (trade name: KOD-Plus, manufactured by TOYOBO Co., Ltd.) was used and conditions of the reactions were as followed: incubation at 94°C for 2 minutes, followed by 25 cycles each for 94°C for 30 seconds, 40°C for 30 seconds, and 68°C for 2 minutes; and followed by incubation at 68°C for 3 minutes. The amplified DNA fragment obtained was ligated to PCR product insertion site of the plasmid pCR2.1-TOPO (trade name, manufactured by Invitrogen Inc.).

In a similar manner, PCR was performed with an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 47 and an oligonucleotide consisting of the nucleotide sequence of SEQ ID NO: 48 as primers to amplify a glycinin gene terminator fragment having a SacI site and a HindIII site respectively at each of the 5'- and 3'-end of the fragment. The amplified DNA fragment was ligated between the SacI site and the HindIII site of the plasmid into which the glycinin gene promoter fragment has been inserted.

Terminally-phosphorylated synthetic oligonucleotides with the nucleotide sequences of SEQ ID NOs:49 and 50 respectively were mixed at an equivalent mole ratio, heated at 65°C for 5 minutes, and then cooled slowly to prepare a linker.

The prepared linker was inserted between the BamHI site and the SacI site of the plasmid into which the glycinin gene promoter fragment and the glycinin gene terminator fragment have been inserted. The resulting plasmid was digested with
5 EcoRI and HindIII and was inserted into plasmid pUC19 (Takara bio Inc.).

The ligation product was introduced into competent cells (Toyobo Inc.) of the E. coli DH5 α strain ampicillin resistant strains were selected. Nucleotide sequence of plasmid of the
10 selected ampicillin resistant strain was analyzed by using the nucleotide sequence analysis reaction kit, BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem Inc.) and the nucleotide sequence analyzer, ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc.) to obtain plasmid having glycinin
15 gene promoter and terminator.

The linker having the glycinin signal sequence for ER transport prepared in Example 1 (1), and 1.6kpb DNA fragment encoding a polypeptide in which a serine residue has been fused to the amino terminus of lipase prepared in Example 3(1)
20 were inserted between the BamHI site and the SacI site of the plasmid having glycinin gene promoter and terminator. Thus, was obtained plasmid pSUM-GY1-GY1SP S-Lip1soy (Fig. 12) containing the nucleotide sequence of SEQ ID NO: 51, containing glycinin gene promoter and terminator, and
25 expressing a fusion protein in which a serine residue has been inserted between GY1SP and lipase.

SEQ ID NO:45: gttttccagtcacgac

SEQ ID NO:46: ggatccGACTGATGAGTGTTTAAGGACCAATG

SEQ ID NO:47: gagctcAACCAATAAATAATAATAATAATG
 SEQ ID NO:48: aagcttCAAGTCATGAAGAACCTGATAAGACGTC
 SEQ ID NO:49: gatecgcggccgcgagct
 SEQ ID NO:50: cgcggccgcg
 5 SEQ ID NO:51: 5'--
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GTTCTTCATGACTTG-3'

Example 6

Analysis of lipase expression in recombinant soybean seed

- 5 (1) Introduction of a lipase expression vector into a soybean
and selection of recombinant soybean

Lipase expression plasmids prepared in Example 3 (1) and
Example 5 respectively were introduced into soybean spherical
somatic embryo by the particle gun method,, and the
10 introduction of the lipase genes into the selected spherical
somatic embryo was identified through PCR.

A hypocotyl segment of an immature embryo extracted from
sterilized soybean immature seed was cut into two immature
cotyledons. Then, the immature cotyledons were placed in
15 induction medium (MS inorganic salts, B5 media vitamins
(Gamborg et al., Exp. Cell Res. (1968) Vol. 50, pp. 151-158),
180 μ M 2,4-D, 3% sucrose, 0.2% gel-lyte, pH 7.0), and cultured
at a temperature 23°C to 25°C for approximately 1 month (at 23
hours light and 1 hour dark conditions everyday). The thus-
20 formed spherical somatic embryos were transplanted to
proliferation medium (MS inorganic salts, B5 media vitamins,
90 μ M 2,4-D, 3% sucrose, 0.2% gel-lyte, pH 5.8), and then
transplanted again to a fresh medium having the same
composition every 2 or 3 weeks to perform culture 5 to 8 times
25 under the same conditions.

Each of lipase expression plasmids prepared in Example 3
(1) and Example 5 was mixed with the same amount of a plasmid
in which DNA (GenBank Accession number: V01499) encoding E.

coli-derived APH4 has been linked downstream to the promoter of plasmid pG8CRG1-2 described in US Patent No.6218598, and was introduced into the spherical somatic embryos cultured for 3 to 4 days in a fresh proliferation medium by using a gene transfer apparatus (trade name: PDS-1000/He system, manufactured by Bio-Rad). Three (3) to 4 days after the introduction treatment, the embryos were transferred to selection medium (MS inorganic salts, B5 media vitamins, 30 μ M hygromycin, 90 μ M 2,4-D, 3% sucrose, pH 5.8) solidified with 0.2% gel-lyte or liquid selection medium, and cultured at a temperature 23°C to 25°C at 23 hours light and 1 hour dark conditions. The embryos were transferred to a fresh medium having the same composition every 2 or 3 weeks to perform culturing 5 to 8 times under the same condition. Further, in the case of the liquid selection medium, rotary culture was performed at 100 rpm.

The selected spherical somatic embryos were sampled, 40 μ L of a nucleic acid extraction reagent (trade name: PrepMan Ultra Reagent, manufactured by Applied Biosystems) was added to the embryos, and heated to 100°C for 10 minutes. After cooling, centrifugation was performed at 15,000 rpm and 22°C for 1 minute and the supernatant was collected, which was used as a genomic DNA solution. PCR was performed using the extracted genomic DNA solution as a template, and an oligonucleotide consisting of the nucleotide sequence of SEQ ID No: 52 and an oligonucleotide consisting of the nucleotide sequence of SEQ ID No: 53 as primers, and spherical somatic embryos in which lipase genes have been introduced were

selected. For the PCR, a DNA polymerase (trade name: TaKaRa Ex Taq Hot Start Version, manufactured by Takara Bio Inc.) was used and conditions of the reactions were as followed:

incubation at 94°C for 2 minutes, followed by 30 cycles each
5 for 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for minutes; and followed by incubation at 72°C for 2 minutes.

SEQ ID NO:52: ctggtgcaaacccttcagtt

SEQ ID NO:53: ttatcggatgcagaaccaca

10 The selected recombinant spherical somatic embryos were transplanted to emersion medium (MS inorganic salts, B5 media vitamins, 3% sucrose, 0.2% gel-lyte, pH 5.8), cultured for 6 to 8 weeks, and then dried for 1 to 2 weeks. The somatic embryos which became white to yellow cotyledon-shaped embryos
15 were transferred to germination medium (MS inorganic salts, B5 media vitamins, 3% sucrose, 0.2% gel-lyte, pH 5.8) and cultured for 2 weeks. The regenerated individuals rooted and developed true leaves were transferred to potting soil and acclimated in a growth chamber. After growing the individuals
20 in a greenhouse, seeds were harvested.

(2) Analysis of expression amount of lipase protein in recombinant soybean seed by Western blotting method

Proteins were extracted from recombinant soybean seed
25 and was quantified by Western blotting method and the immunostaining method to calculate the production amount of lipase protein.

The recombinant soybean seed which was allowed to absorb

water overnight was divided into four pieces, and each piece was put in a tube containing one zirconia bead (5 mmφ, YTZ ball) (manufactured by Nikkato Corp.), and was rapidly frozen under liquid nitrogen. The frozen seeds were ground by using
5 a grinding apparatus (trade name: Mixermill MM-300, manufactured by QIAGEN gmbh.), mixed with PBS buffer with being added by 300 μL by using the mixer mill, and centrifuged at 15,000 rpm, 4°C for 5 minutes to collect supernatant. The collected supernatant was centrifuged at 15,000 rpm for 5
10 minutes at 4°C, and the supernatant was collected. The concentration of protein was measured by the method described in Example 2(2), and then SDS-PAGE, Western blot and immunostaining were performed. In the primary antibody reaction, solution obtained by diluting 50,000 times the
15 rabbit anti-lipase antiserum described in Example 3(5) with Can Get Signal Immunoreaction Enhancer Solution (manufactured by Toyobo Inc.) was used, and in the secondary antibody reaction, solution obtained by diluting ECL Anti-rabbit IgG, Horseradish Peroxidase-Linked Species-Specific Whole Antibody
20 (donkey-derived) (manufactured by GE health care Inc.) and S-protein-HRP conjugate (manufactured by GE health care Inc.) 50,000 times with Can Get Signal Immunoreaction Enhancer Solution (manufactured by Toyobo Inc.) was used. The lipase OF (manufactured by Meito Industry Inc.) was used as a
25 standard.

As a result, a signal of lipase protein was detected from the recombinant soybean seed to which the plasmid pSUM-GY1-GY1SP S-Lip1soy has been introduced(Fig. 13). The

production amount of lipase was calculated based on the mobility on electrophoresis and a signal intensity of the standard, and it was found that lipase protein corresponding to about 0.5% of soluble protein was produced.

5

(3) Analysis of amino-terminal amino acid sequence of lipase in recombinant soybean seed

Ammonium sulphate was added by 30% to protein extract of the recombinant soybean seed from which the signal of lipase protein was detected, mixed, and centrifuged at 15,000 rpm, 4°C for 20 minutes. PBS buffer was added to the obtained precipitates and mixed to measure the protein concentration and perform the Western blot analysis by the method disclosed in Example 2(2). A membrane onto which protein was transferred was shaken for 10 minutes by using staining solution (0.1% CBB, 50% methanol, 10% acetic acid), was destained twice by using destaining solution (50% methanol, 10% acetic acid), and was washed twice by using washing solution. A band detected at a position of about 60 kDa was cut and provided for the analysis of amino-terminal amino acid sequence.

As a result, the amino acid sequence of SEQ ID NO: 54, which is a serine residue followed by an amino acid sequence of lipase, was detected, and it was found that the expressed fusion protein was processed immediately downstream of the glycinin signal sequence for ER transport.

SEQ ID NO:54: SAPTA

(4) Measurement of enzyme activity of lipase in protein extract of a recombinant soybean seed

A measurement of enzyme activity was performed on protein extract of a recombinant soybean seed in which lipase proteins were detected. Substrate 4-Nitrophenyl decanoate manufactured by SIGMA Co. Ltd. was dissolved in DMSO and the mixture was added into 50 mM potassium phosphate buffer (pH 6.50) containing 0.5% Triton X-100 manufactured by Nacalai Tesque, Inc. to prepare a 2.5 mM dilution as a substrate solution. 30 μ L of protein extract of a recombinant soybean seed in which a plasmid pSUM-GY1-GY1SP S-Lip1soy was introduced was mixed with 370 μ L of 2.5 mM substrate solution to perform a reaction at 30°C for 10 minutes. Acetone of 800 μ L was added and mixed as a reaction stop solution. The resulting reaction solution was centrifuged at 15,000 rpm for 1 minute at 22°C, and the optical density of 1 mL supernatant was measured at 410 nm. 4-nitrophenol manufactured by Fluka Co. Ltd. was used as a standard. In addition, 1U is defined as the amount of an enzyme to produce 1 μ mol of 4-nitrophenol as a hydrolysate of the substrate per minute.

As a result, it could be seen that a recombinant soybean seed in which the plasmid pSUM-GY1-GY1SP S-Lip1soy has been introduced had 17.8 U of lipase activity per gram of dry seed weight. From these results, it was apparent that a recombinant soybean seed in which the plasmid pSUM-GY1-GY1SP S-Lip1soy has been introduced accumulated lipase having enzyme activity.

WHAT IS CLAIMED IS:

1. A method for producing a heterologous protein secreted out of a plant cell comprising:

5 introducing into a plant cell genome a DNA encoding an amino acid sequence that comprises a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein the signal sequence is directly fused to the amino acid sequence of the heterologous protein
10 or one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein; and expressing the DNA.

2. The method according to claim 1, wherein one amino
15 acid is inserted between the signal sequence and the amino acid sequence of the heterologous protein.

3. The method according to claim 2, wherein the one amino acid is selected from among serine, alanine and methionine.
20

4. The method according to any one of claims 1 to 3, wherein the glycinin signal sequence for endoplasmic reticulum transport is the amino acid sequence of SEQ ID NO:1.

25 5. A method for producing a transgenic plant cell secreting a heterologous protein comprising:

introducing into a plant cell genome a DNA encoding an amino acid sequence that comprises a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of
30 a heterologous protein, wherein the signal sequence is directly fused to the amino acid sequence of the heterologous protein or one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein.

6. A plant expression plasmid comprising a DNA encoding an amino acid sequence that comprises a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein.

7. The plasmid according to claim 6, wherein one amino acid is inserted between the signal sequence and the amino acid sequence of the heterologous protein.

8. A DNA encoding an amino acid sequence that comprises a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein.

9. A plant cell with a genome into which a DNA encoding an amino acid sequence is introduced, the amino acid sequence comprising a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein the signal sequence is directly fused to the amino acid sequence of the heterologous protein or one or two amino acids are inserted between the signal sequence and the amino acid sequence of the heterologous protein.

10. A plant with a genome into which a DNA encoding an amino acid sequence is introduced, the amino acid sequence comprising a glycinin signal sequence for endoplasmic reticulum transport and an amino acid sequence of a heterologous protein, wherein the signal sequence is directly fused to the amino acid sequence of the heterologous protein or one or two amino acids are inserted between the signal sequence and the amino acid

sequence of the heterologous protein; or

a seed of the plant, the seed inheriting said DNA from the plant.

5 11. The method according to claim 5, wherein one amino acid is inserted between the signal sequence and the amino acid sequence of the heterologous protein.

12. The method according to claim 11, wherein the one
10 amino acid is selected from among serine, alanine and methionine.

13. The plasmid according to claim 7, wherein the one amino acid is selected from among serine, alanine and methionine.

15 14. The DNA according to claim 8, wherein one amino acid is inserted between the signal sequence and the amino acid sequence of the heterologous protein.

15. The DNA according to claim 14, wherein the one amino
20 acid is selected from among serine, alanine and methionine.

16. The plant cell according to claim 9, wherein one amino acid is inserted between the signal sequence and the amino acid sequence of the heterologous protein.

25

17. The plant cell according to claim 16, wherein the one amino acid is selected from among serine, alanine and methionine.

30 18. The plant or the seed according to claim 10, wherein one amino acid is inserted between the signal sequence and the amino acid sequence of the heterologous protein.

19. The plant or the seed according to claim 18, wherein the one amino acid is selected from among serine, alanine and methionine.

SEQUENCE LISTING

<110> Sumitomo Chemical Co., Ltd.

<120> METHODS FOR PRODUCING SECRETED PROTEINS

<130> S21222

<150> JP2008-184479

<151> 2008-07-16

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