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(54) **A gene expression vector using the gene expression regulating region of the ADP ribosylation factor**

Expressionsvektor mit Regulationsregion vom ADP-Ribosylierungsfaktor

Vecteur d'expression utilisant la région régulatrice du facteur de ribosylation de l'ADP

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(73) Proprietor: **SAPPORO BREWERIES LTD.**
Tokyo 150-8686 (JP)

(72) Inventors:
- **Kuroda, Hisao, c/o Sapporo Breweries Ltd.**
  Nitta-cho, Nitta-gun, Gunma-ken (JP)
- **Hirota, Naohiko, c/o Sapporo Breweries Ltd.**
  Nitta-cho, Nitta-gun, Gunma-ken (JP)
- **Ito, Kazutoshi, c/o Sapporo Breweries Ltd.**
  Nitta-cho, Nitta-gun, Gunma-ken (JP)

(74) Representative: **VOSSIUS & PARTNER**
Postfach 86 07 67
81634 München (DE)

(56) References cited:
- **FEBS LETTERS, vol. 316, no. 2, 1993 pages 133-136, F. REGAD ET AL. 'cDNA cloning and expression of an Arabidopsis GTP-binding protein of the ARF family'**
- **JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 7, 1993 pages 4863-4872, I.M. SERVENTI ET AL. 'Characterization of the gene for ADP-ribosylation Factor (ARF) 2'**

Remarks:
The file contains technical information submitted after the application was filed and not included in this specification

EP 0 681 028 B9

Description

[0001] This invention relates to the field of genetic engineering of plants, and more specifically, to regulation of gene expression using a gene expression regulating region of the ADP ribosylation factor gene in plants.

[0002] Due to recent progress in genetic engineering, research on the plant breeding at the genetic level (so-called molecular plant breeding) is now being carried out intensively. In order to achieve a successful plant breeding, using genetic engineering techniques, the useful genes in the plants must be expressed and made to function to achieve a specific purpose efficiently. Generally, therefore, to regulate gene expression and achieve the desired purpose, a gene expression regulating region in the plant is isolated for use.

[0003] Typical gene expression regulating regions that have so far been used in this way are, for example, the 35S promoter of the tobacco mosaic virus (Guilley, H., et al (1982), Cell 30, 763-773), and the actin gene promoter derived from rice plants (McElroy, D. et al, (1990), The Plant Cell, 2, 163-171).

[0004] However, the number of common multi purpose promoters in plants is still limited, and considering that time, place and degree of expression are also important in any plant that is to be modified, there are very few actual cases where the gene expression regulating region of a plant can actually be utilized. This is due to the fact that the technology, whereby useful genes that have been separated or manufactured for the plant breeding can be freely expressed and made to function in the plant, is not yet very advanced.

[0005] The establishment and accumulation of techniques for regulating gene expression in plants with a view to making useful genes function efficiently to serve a specific purpose, has therefore been long awaited.

[0006] This invention was conceived in order to resolve the above problems, and aims to construct a genetic expression vector that can express genes in a tissue-specific, efficient manner. More specifically, it aims to provide a gene expression vector that, using genetic engineering techniques, can express useful genes efficiently to serve a specific purpose in the plant breeding or the production of substances using plant tissues or cells.

[0007] In order to achieve the above aims, this invention features the use of the promoter derived from an ADP ribosylation factor gene as a plant promoter.

[0008] Within the meaning of the present invention the term "derived from an ADP ribosylation factor gene" refers to promoter sequences or sequences of a gene expression regulating region of the ADP ribosylation factor gene comprising nucleotide substitutions, deletions or additions with respect to the wild type sequences without altering the functional activity of the elements as determined in the expression analyses.

[0009] Still more specifically, this invention comprises a DNA fragment having a base pairs 1 to 3027 in SEQ ID NO: 1:, or a DNA fragment comprising part of this fragment having tissue specific promoter activity.

[0010] This invention further comprises an expression vector comprising any of the aforesaid DNA fragment.

[0011] Still further, this invention comprises a transformant comprising any of the aforesaid expression vector.

[0012] By using the gene expression regulating region of this ADP ribosylation factor (referred to hereinafter as ARF) gene for the gene expression, a desired gene can be efficiently expressed in any plant of interest, and utilized for plant breeding and the production of useful substances using plant culture cells.

[0013] ARF was first discovered as an enhancing factor for cholera toxin. In mammals, it is present in particularly large amounts in the brain, accounting for 1% of total protein (Kahn, R., and Gilman, A. G. (1984), The Journal of Biological Chemistry, 259, 6228-6234). According to recent research, ARF is a protein with a molecular weight of approximately 20 kD that commonly exists in eukaryotes, and is known to be a type of low molecular weight G protein that contributes to intracellular transport (Serafini, T., et al (1991) Cell, 67, 239-253). Its gene structure has been reported for yeast, mammals, and dicotyledons (Sewell, J.L., Proc. Natl. Acad. Sci. USA (1988) 85, 4620-4624).

[0014] The research leading to the present invention aimed at constructing tissue-specific gene expression systems in barley. The inventors discovered that the ARF gene is characteristic in that it is actively expressed in barley seeds, roots and callus, but strongly suppressed or almost not expressed at all in the leaves.

[0015] The Inventors' research was the first of its kind to investigate ARF from the viewpoint of gene expression rather than that of function, and was also the first to show that the expression region of the ARF gene could be used as a vector of industrial utility. It is hoped that the expression vector constructed from the gene expression region of ARF according to this invention, will find wide application as a characteristic gene expression system for the plant breeding and production of substances using plant tissues or cells with the use of genetic engineering techniques.

Isolation of cDNA of ARF

[0016] The Inventors, using differential screening techniques, succeeded in cloning the cDNA of the ARF gene that is expressed tissue-specifically and at a high level, and isolating a full length cDNA. Next, by cloning the upstream region of the ARF gene, they verified its promoter activity, and created a gene expression vector using the gene expression regulating region of this gene.

Acquisition of Expression Vector

[0017] The Inventors screened the gene library of barley, isolated a DNA fragment in the upstream region of this gene, and combined this fragment with a reporter gene so as to construct an expression vector.

[0018] More specifically, a gene expression vector using the promoter of the ARF gene according to this invention may be obtained by the following methods.

Differential Screening

[0019] In general, expression-specific genes may be cloned from cells and tissues having different genetic expressions according to the method of Takahashi et al (Takahashi, Y., Kuroda, H. et al (1989), Proc. Natl. Acad. Sci. USA 86, 9279-9283). For example, after extracting polyA$^+$ RNA from roots, cDNA is synthesized, and the cDNA fragments are cloned into a lambda phage vector or plasmid vector to produce a cDNA library. Part of the library is subjected to plating, transferred to a nylon filter, and hybridized with a radioactive probe of leaves and roots. The clone which is expressed specifically in the roots is then screened. The sequence of bases in the clone may be determined by the usual methods.

Cloning of Promoter

[0020] General methods for cloning fragments in the upstream region of genes are given in detail in Sambrook, J., Frisch, E.F., Maniatis, T., Molecular Cloning, A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press. According to this invention for example, as the base sequence of cDNA is well known, suitable restriction enzymes may be used to cleave chromosomal DNA and the genome Southern method used to determine the size of the fragments obtained in the upstream region. Next, barley chromosomal DNA is separated, cleaved with these enzymes, and this DNA used to construct a chromosomal library. If a radioactive cDNA probe is synthesized and this library is screened, a desired upstream region may be cloned.

Verification of Function of Gene Expression Vector

[0021] To determine whether the upstream region so obtained functions as a promoter, it is usual to ligate the upstream region to a reporter gene, introduce it into a suitable cell or tissue, and verify the expression of the reporter gene by enzyme activity. The detailed method is described in, for example, Draper, J., et al, Plant Genetic Transformation and Gene Expression, A Laboratory Manual (1988), Blackwell Scientific Publications.

[0022] The function of the vector according to this invention was verified by introducing it into a barley culture cell protoplast, and analyzing the resulting gene expression.

[0023] According to this invention, an upstream region DNA fragment that had been separated, was ligated to a β-glucuronidase gene, and introduced into barley culture cell protoplast. After several days of culturing, promoter function was verified by extracting total protein, and measuring enzyme activity in total protein using 4-methyl umbelliferyl glucuronide (4-MUG) as substrate. As a result, it was found that this DNA fragment may be used as a gene expression vector.

Isolating Methods for DNA Fragments Which Code for an ARF Promoter

[0024] A DNA fragment having a base SEQ ID NO: 1: could be obtained by a method described below. First, synthetic PCR primers having the sequence corresponding to base sequence 1 to 50 and synthetic PCR primers having the sequence complementary to 2976 to 3027 are synthesized by DNA synthesizers, for example 391 DNA Synthesizer (ABI inc.) or Gene Assembler (Pharmacia inc.). A barley genomic DNA is purified by CTAB methods or SDS-phenol methods (fully described in Plant Genetic Transformation and Gene expression, Draper et al). Finally, DNA fragments having the base sequence of ARF promoter are synthesized by the Polymerase Chain Reaction with use of, for example, LA PCR kit (Takara inc.) or XL-Wax100 (Perkin Elmer inc.).

[0025] Alternatively, DNA fragments having the base sequence of an ARF promoter are synthesized directly. For example, synthetic DNA having the base sequence 1 to 110 of SEQ ID NO: 1: and synthetic DNA having the base sequence complementary to 1 to 16 are synthesized.

[0026] Double strand DNA having the base sequence 1 to 110 of SEQ ID NO: 1: is obtained by Klenow filling reaction with these primers. With the same method, a DNA fragment having the base sequence of 100 to 190 is obtained. Next, by a general molecular cloning method, these two DNA fragments are ligated by use of the restriction enzyme ApaI which recognition site is located in the sequence 104. Finally, a full length DNA fragment having the base sequence 1 to 3027 of SEQ ID NO: 1: is obtained by repeating the procedure described above.

Brief Description of the Drawings

[0027]

Fig. 1 is a drawing showing the results, at the amino acid level, of a computer search of the homology between the cDNA clone R151 and the ARF gene in man, bovine and yeast. In the figure, - indicates common amino acid sequences, while the shading shows amino acids that are thought to be GTP binding sites.
Fig. 2 is a diagram of a restriction enzyme map of the clone λR15.
Fig. 3 is a drawing showing a process for constructing a gene expression vector.
Fig. 4 is a drawing showing the results of a study of the activity of the promoter (R15 promoter (pSBG418)) according to this invention.

[0028] As described hereintofore, according to this invention, full length cDNA was isolated by differential screening. The gene library of barley was screened so as to isolate a DNA fragment in the upstream region of this gene, and this fragment was combined with a reporter gene so as to construct a gene expression vector. This vector was then introduced into the protoplast of barley culture cells, and its function was verified by analyzing genetic expression. The invention will now be described in further detail with reference to specific examples, however it is understood that the invention is not to be construed as being limited to them in any way.

Example 1

Construction of the cDNA library expressed in roots

[0029] The roots(10 g) of young barley plants (cultivar Haruna Nijo) whereof the first and second leaves had opened was separated, frozen in liquid nitrogen and crushed to a powder in a blender in liquid nitrogen. After allowing the liquid nitrogen to evaporate, 100 ml of 4 M guanidine isocyanate in 0.1 M Tris-HCl (pH 7.0) and 100 ml of a phenol:chloroform: isoamyl alcohol mixture (25:24:1) were added, and the resulting mixture blended in the blender for several minutes. The suspension was transferred to a centrifuge tube, centrifuged at 4000 x g for 20 min, and the aqueous layer recovered. To this an equal volume of the phenol:chloroform:isoamyl alcohol (25:24:1) mixture was added, the resulting mixture centrifuged, and the aqueous layer recovered. This procedure was repeated until the intermediate layer between the phenol layer and the water layer had disappeared, then 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol were added so to the aqueous layer as to precipitate total nucleic acids. After dissolving the precipitate in 10 ml 0.5 M KCl/10 mM Tris-HCl (pH 8), polyA$^+$ RNA was purified using an oligo dT cellulose (Boehringer Inc.) column.
[0030] cDNA was synthesized from 2 μg of this polyA$^+$ RNA using a cDNA synthesis kit (Amersham Inc., cDNA synthesis system plus). A λgt10 library was then constructed using this cDNA and cDNA cloning system λgt10 (Amersham Inc.).

Example 2

Differential screening

[0031] PolyA$^+$ RNA was purified from leaves of young barley plants whereof the first and second leaves had opened, using the method of Example 1. After the λgt10 library constructed in Example 1 was subjected to plating to give 10^3 pfu/plate, it was transferred to a high bond N$^+$ filter (Amersham Inc.) and fixed with alkali. This was repeated twice so as to prepare two filters. The filters were hybridized with a radioactive cDNA probe (10^5 cpm/μg polyA$^+$ RNA), synthesized using 2 μg of polyA$^+$ RNA from roots and leaves, respectively, as a template, in 20 ml of hybridization solution (6xSSC, 1% SDS) at 65°C for 24 hrs, and washed four times with a wash solution (2xSSC, 1% SDS) at 65°C. After drying the filters, an autoradiograph was performed, and the signal strength of each phage clone was compared. Clones that showed a strong signal in roots were recovered from the original plate.

Example 3

Structural analysis of cDNA clones

[0032] Various phage clones were grown, and the DNA recovered. The cDNA insert was separated from this DNA, and cloned with pUC118 or pUC119 (Takarashuzo Inc.). The base sequence was determined by the di-deoxy method using an ABI Inc. 373 ADNA sequencer.

Example 4

Analysis of cDNA clone R151

[0033] Several cDNA base sequences were determined, and a computer search was performed. As a result, approximately 90% homology was found at the amino acid level between one of these, cDNA clone R151, and the ARF gene in man, bovine and yeast (Fig. 1). When this cDNA clone was used as a probe to perform a Northern blot analysis, it was found that this gene was very strongly expressed in barley seeds, roots and culture cells, but hardly at all in leaves.

Example 5

Cloning of the upstream DNA fragment

[0034] Genomic Southern was performed on barley chromosomal DNA cleaved by the restriction enzyme HindIII, using an upstream DNA fragment containing an ARF protein N terminal region obtained by HindIII cleavage of this cDNA clone R151 as a probe. As a result, a 5 kb band was detected which could be cloned, and which is thought to contain a promoter region. Barley chromosomal DNA was therefore cleaved by HindIII, DNA fragments near 5 kb separated by electrophoresis, and ligated to the HindIII site of Lambda Blue MidTM (Clontech Inc.) in order to construct a library. This library was screened by the above probe to obtain the clone λR15. In other words, according to this embodiment, a genomic library was screened using the HindIII upstream probe R151 so as to isolate λDNA.

Example 6

Structural analysis of upstream DNA fragments

[0035] After cloning the above fragments at the HindIII site of pUC118, a restriction enzyme map was constructed (Fig. 2). Enzymes that do not cleave λR15 are EcoRI, ClaI, SalI, KpnI, NaeI, SacII, SmaI. As a result, from the restriction map of λR15, the N terminus of ARF protein is probably located effectively in the center, and its orientation is probably as shown in Fig. 2. To determine the precise position of the N terminus, a polymerase chain reaction (PCR) was performed using the synthetic primer 5'-ACGAATTCATGGGG CTCACGTTTACCAAGCTG-3' and M13 primer (Takarashuzo Inc.) that recognize base sequences encoding the N terminus shown in Sequence Table 2, and the sizes of DNA fragments after the reaction were determined. It was found that the N terminus is situated at approximately 2.3 kbp from downstream. The HindIII (1) - BamHI (3070) fragment corresponding to the upstream part containing a region near the N terminus was therefore cloned to pUC118, and the base sequences were determined by the method shown in the examples so as to obtain the results shown in Sequence Table 1.

Example 7

Construction of a gene expression vector, and expression analysis (Construction of a gene expression vector)

[0036] The β-glucuronidase gene, which is a reporter gene, was ligated to the ARF promoter fragment, and promoter activity was measured. pBI101 shown in Fig. 3(a) (Toyobo Inc.) was cleaved by the restriction enzymes HindIII and EcoRI, a 2280 bps fragment was recovered by electrophoresis, then cloned into pUC118 at the HindIII and EcoRI sites to construct pSBG419 (Fig. 3(b)). Next, pSBG419 was cleaved by the restriction enzyme PstI, and self ligated after blunting using a Takarashuzo DNA blunting kit to construct pSBG420 (Fig. 3(c)). Using the same method, the upstream DNA fragment shown in Example 5 was cloned at the HindIII site of pSBG420 to construct pSBG421 (Fig. 3(d)). pSBG421 was then inserted into E. coli DH5 (Toyobo Inc.) by the usual method. pSBG421 was cleaved by the restriction enzymes XhoI and SphI, and cloned with the fragment XhoI (2700) - NiaIII (3010) shown in the Sequence Table I to construct pSBG418 (Fig. 3(e)). pSBG418 has a structure wherein a β-glucuronidase fused gene, comprising the codons for the amino acids methionine, proline, valine, asparagine, serine, arginine, glycine, serine, proline, glycine, glycine, glutamine, serine and leucine which are attached to the N terminus downstream of the ARF promoter, is linked. Transcription of the β-glucuronidase fused gene is induced by the action of the ARF promoter.

Expression analysis

[0037] Next, a transient expression test was performed using the protoplast of a suspension culture B53 derived from an immature embryo of wild barley (from Hordeum bulbosum), and promoter activity was examined (Fig. 4). Using enzyme solutions, approximately 10^8 protoplasts were isolated from the cell suspension at the 5th or 7th day of sub-

culture. To the resuspended protoplasts (2 x 10^6) 2 nmol pSBG418, pBI221 (Toyobo Inc.) and calf thymus DNA as carrier DNA were added so that the total was 60 μg, and electroporation was performed using a Biorad gene pulser at 900 V/cm and 125 μF. After recovering the protoplasts, they were suspended in 15 ml of MSD4, 0.6 M mannitol, and cultured in a 9-cm Petri dish at 25°C for 3 days. After culture, the cells were recovered, solubilized in Gus extract buffer (50 mM phosphate buffer (pH 7.0), 10 mM EDTA (pH 7.0), 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, 10 mM mercaptoethanol), and a GUS assay was performed on 100 μg of soluble protein using substrate 4MUG.

[0038] The samples showed specific activities of 8.9 nmol 4-MU$mg^{-1}hr^{-1}$ soluble protein and 0.3 nmol 4-MU$mg^{-1}hr^{-1}$ soluble protein, respectively. It is therefore clear that the promoter of this invention (R15 promoter (pSBG418)) shows a higher activity, for both the 5th and 7th day subcultures, than the 35S promoter (pBI221) that has been used conventionally. In particular, the 7th day subculture shows approximately 30 times the activity of the 35S promoter.

[0039] If it is desired to express other useful genes, these useful genes may be inserted instead of the β-glucuronidase fusion gene. This can easily be accomplished using known, common techniques by those skilled in the art.

[0040] As described hereintofore, the gene expression vector constructed using the ARF gene expression region according to this invention, has wide application as a characteristic gene expression system in the plant breeding and in the production of substances using plant tissues or cells with the use of genetic engineering techniques. The gene expression vector employing the gene expression regulating region in the AFR gene of this invention, therefore, makes a significant contribution to efficiently expressing a desired gene in a plant for a specific purpose, improving plant species, and producing useful substances using plant culture cells.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Sapporo Breweries Limited
(B) STREET: 20-1, Ebisu 4-chome, Shibuya-ku
(C) CITY: Tokyo
(E) COUNTRY: Japan
(F) POSTAL CODE (ZIP): 150

(ii) TITLE OF INVENTION: A GENE EXPRESSION VECTOR USING THE GENE EXPRESSION REGULATING REGION OF THE ADP RIBOSYLATION FACTOR

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP Hei 6-71048
(B) FILING DATE: 08-APR-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3088 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AAGCTTCTAG ATCACCGAAG ATGGGCCAGA GGGGGGCCAG GGGCTGCCCA GGCGCCCTGC 60

TGGCGTGGCC AGGCCCTAGG CCGCGCCAGG AGGCCGCCTG GGTGGGCCCC ACCTCCTCCG 120

GTGCCCTCCT TTGGCCTATA TTTAGAGTCC CGAGAGGAAA CCCTTCCACA ACTTCCAGAA 180

TCGCGAATTT CTCCATCGTT CCGTTGCCGC AGCGCTTCCG AGATCGGGAG CGTCAGGAGA 240

CCTCTTCCCG ATACCCTGCC GGAGGGAGGA TTGACCTTCG GGAGCTTCTC CACCGCAATC 300

AACGCTTCCC GGACGTGCCG TGAGTAGTCC TCCTTGGACC ATGGGTCCAT GACCAGTAAC 360

TATGTGATGT CTTCTCTCCA ATCTTGTGCT TCATTGGTTA GTCCTTGTGA GCTGCCCTAC 420

ATGATCAAGG CATCTATGTA ATTCTCTTGC TATTGCTATG CTCGGTTTGT TGGGATCCGA 480

TGAATTATGA GATTATGTTC AGATTGTTAT GAGTTATATA TTTGATTATC CTTTTATATA 540

ACGTTTCTTA GTGATTCATG CATGTTCTCT ATTGCTATTT ATTGCTTTGG TCGAGTAGTA 600

GATTGTAACT CCAAGAGGGA GCGTTATGCA TGATTATGGG TTCATGCCCC TCGATGTCTT 660

GCTTGAGTGA CATGAACATG AGACTAGGGG ATGTGCTGTT GCCACCAGGG AGAAAAAAAT 720

GATGCTTGTG ACCTCAGTTG CAAGGATTAT TTACCTCACA CATAGTTCGT TAATGAAGTT 780

GTCCGTTACT TTGAGTTTAC ACTTTGGATG GGGCTCGCAA CTTAATACCG GAGAGATGTT 840

CTGGATAGAT ATCTCAAGGT GGATGATTAG TAAGTAGATG CTGATGAATA AACGGTCTAC 900

TTGTCTTGAC GTACTGCCCA TTACTATTGA GCCTATAACT ATCAAGTAGC ATAATTAGCA 960

TTGTGGTGCG TTCATAATTC TGTCAATTGC CCAACTGTGA TTTGTTTATC ATAGCATAGT 1020

TGTTTATCGT CTTTTGGAAG AGATACATCA CTAGTGAACA TCATGTGACA CCGGTCAATA 1080

TCACCACCAT TGTTTACACA TCCATCATTT ACCGCTTTCA TTTACTTTTC CGTTGCAATC 1140

ACTATTACCT TCCCGCTTGT GTTTTGATCC TTTGCAAACT ACAAGGTCGG AGAGATTGGC 1200

AACCTCTCTG TACTCGTTGG GAGCAAAGTT ATTTGTTGTG TGTGCAGGTC CACGTCTTCT 1260

ACTGACCAGA ACAGGAGACA CCTACTTGTT GGTCCTACGA GTCCTCTTGG TTTGTTAAAC 1320

CTTACAGTTC CCGTGTGAGG AAAAACTTGT TGCTGATTAC ATCTCAACAT TTCACTTTGG 1380

GTAATCAACT AGGTGCGAGA AATATATATA CATCTCTCGT CATCAAAAGG CGAAAGTTAT 1440

TTTATTATCT CATTAAAAGT TTCAAAAAAT TTAAATTCCA CTCAATTCCA ATCAATCACT 1500

CAAGCAAGCA AAACATATAA TTGATTATTA TAACATTTCA TTTTTTGGGA TGTTACATGA 1560

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TTTATAGTAC AGTGTCATAA TGTTCGATCG CAACCAAACA AAGAGCAATT TCATAGTTTT 1680

TACATGCGCC TCATTATTCA TAGAAGTATA GTGGACATAA GATCCTCGTC ATTTTTGCCC 1740

TGTTAGAACG GATATCGAAG CCTCCATGCT CACTCTTTCG TCGGCTTCTC TTTATCATCC 1800

ACCTCCTTCT TCACCTTTCC CAACTTGACC GTCAAGCACT TCAAGATCCT AACACGGACA 1860

GTTTCTACGA TTATTTTTGC ATCGTCATCC AACACATTCA TGTTCAAGGT CAACATCTTT 1920

ATATCTTCCG AAACGGTCGC GAGCTCAACT TGCTCATAGT TTTTTTAGGC AGCAGAAGTT 1980

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TAAAAAATAT TCCTATTTGA TTTACTATGC TTATTATTAA AAATCCGACA TTATATTTTT 2100

TTTTCTGTTC ATGAGTCACA ACTATTCCCT TTTTTTCAGA CGGAAATCGC AGTTATTGCC 2160

GACATACTAC TAATGTCGAT GTACACGGTC CACATTTACC GCCCAAGTTT AATGTGACCG 2220

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CACCTAAAGA AAAGAAAAAG TTAATGTCAC CGCACACAGA CGGACCCACC TAACAGCTGG    2280
CCCACGCTGC CTTCTATGCG CCTTCCACGA AGAAATCCGG CCCCACCTGC CGGTGACCCT    2340
CCAAGCACGT CCCTATATAG ACTTCCTCGT GTGCGGCCAT CTCGGTCTCT CTCTCTCTCC    2400
TCCATTCCTC TCTCCTCCTC CTGCCACCGA TCCCAAACCA GGGAGGAACC CGGCGCGAGG    2460
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CCTGTGATCC GATCGGTGGT TTGGTCGCTC GATTCGTCAT ACTATTTTTC GTTGTTGTGA    2640
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TAGATTCGAT CCGTTTCTCG AGGGTGCGGC TCGAGGAATC TGCCGCGTTT GACCGGCTTC    2760
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AATCTGGTCC GGTTTAGCGC TGTTCGTGCG CCGTGGCGTG GATCTGGCGC TGCTGCCCTC    2880
AGATCTTGTA GATCTAGTTG GGTGGCTTCA ATTCTGGAGT ATTTTTTTAT TGTTGTTATT    2940
ATAGAACGAG CCTGTGTCTG TGTTGATCTA TGGGTCTGAC GCTTCGGCCT TGAATTTGTT    3000
TCGTTTGATT TCAGCGCAGG AGCGGACATG GGGCTCACGT TCACTAAGCT GTTCAGTCGG    3060
CTCTTCGCCA AGAAGGAGAT GAGGATCC                                       3088
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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ACGAATTCAT GGGGCTCACG TTTACCAAGC TG                                  32
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Claims

1. An expression vector comprising a tissue specific gene expression regulating region which is derived from an ADP ribosylation factor gene of barley whereby the term "derived" refers to nucleotide substitutions, deletions or additions with respect to the wild type sequences without attening the functional activity of the elements as determined in the expression analyses.

2. The expression vector of claim 1, wherein the gene expression regulating region is a DNA fragment comprising the base sequence 1 to 3027 of SEQ ID NO:1, or a DNA fragment comprising part of said fragment having still tissue specific promoter activity.

3. The expression vector of claim 1 or 2 further comprising a reporter gene.

4. The expression vector of claim 3, wherein the reporter gene is the β-glucuronidase gene.

5. The expression vector of claim 4, which is pSBG418 with a structure as shown in Figure 3.

6. A gene expression regulating region of an ADP ribosylation factor gene of barley which is a DNA fragment comprising the base sequence 1 to 3027 of SEQ ID NO:1, or a DNA fragment comprising part of said fragment having still promoter activity.

7. A method for expression of a foreign gene in a plant cell, plant tissue or plant comprising introducing a desired foreign gene into an expression vector according to any one of claims 1 to 5 and specifically expressing the gene in the plant cell, plant tissue or plant.

8. A plant cell, plant tissue or plant obtained by the method of claim 7.

9. A plant cell, plant tissue or plant comprising the expression vector of or any one of claims 1 to 5.

Patentansprüche

1. Expressionsvektor, umfassend eine eine gewebespezifische Genexpression regulierende Region, die von einem ADP-Ribosylierungsfaktor-Gen aus Gerste abgeleitet ist, wobei sich der Begriff "abgeleitet" auf Nucleotidsubstitutionen, -Deletionen oder -Additionen in Hinblick auf die Wildtyp-Sequenzen bezieht, ohne die in den Expressionsanalysen bestimmte funktionelle Aktivität der Elemente zu ändern.

2. Expressionsvektor nach Anspruch 1, wobei die die Genexpression regulierende Region ein DNA-Fragment ist, das die Basensequenz 1 bis 3027 von SEQ ID NR. 1 umfasst, oder ein DNA-Fragment ist, das einen Teil des Fragments umfasst und noch gewebespezifische Promotoraktivität aufweist.

3. Expressionsvektor nach Anspruch 1 oder 2, der weiterhin ein Reportergen umfasst.

4. Expressionsvektor nach Anspruch 3, wobei das Reportergen das β-Glucuronidasegen ist.

5. Expressionsvektor nach Anspruch 4, der pSBG418 mit einer in Figur 3 gezeigten Struktur ist.

6. Genexpression regulierende Region eines ADP Ribosylierungsfaktor-Gens aus Gerste, das ein DNA-Fragment ist, das die Basensequenz 1 bis 3027 von SEQ ID NR. 1 umfasst, oder ein DNA-Fragment ist, das einen Teil des Fragments umfasst und noch Promotoraktivität aufweist.

7. Verfahren zur Expression eines Fremdgens in einer Pflanzenzelle, einem Pflanzengewebe oder einer Pflanze, das das Einführen eines erwünschten Fremdgens in einen Expressionsvektor nach einem der Ansprüche 1 bis 5 und die spezifische Expression des Gens in der Pflanzenzelle, dem Pflanzengewebe oder der Pflanze umfasst.

8. Pflanzenzelle, Pflanzengewebe oder Pflanze, die/das/die durch das Verfahren nach Anspruch 7 erhalten wird.

9. Pflanzenzelle, Pflanzengewebe oder Pflanze, die/das/die den Expressionsvektor nach einem der Ansprüche 1 bis 5 umfasst.

Revendications

1. Vecteur d'expression comprenant une région de régulation de l'expression d'un gène spécifique d'un tissu qui est dérivée du gène du facteur de ribosylation de l'ADP de l'orge, dérivée se référant à des substitutions, délétions ou additions de nucléotide par rapport aux séquences de type sauvage sans altération de l'activité fonctionnelle des éléments, comme déterminé par les analyses de l'expression.

2. Vecteur d'expression selon la revendication 1, dans lequel la région de régulation de l'expression du gène est un fragment d'ADN comprenant la séquence de bases 1 à 3027 de SEQ ID NO:1, ou un fragment d'ADN comprenant une partie dudit fragment ayant conservé l'activité de promoteur spécifique de tissu.

3. Vecteur d'expression selon la revendication 1 ou 2, comprenant en outre un gène rapporteur.

4. Vecteur d'expression selon la revendication 3, dans lequel le gène rapporteur est le gène de la β-glucuronidase.

5. Vecteur d'expression selon la revendication 4, qui est pSBG418 avec une structure telle que représentée à la figure 3.

6. Région de régulation de l'expression de gène issue d'un gène du facteur de ribosylation de l'ADP de l'orge, qui est un fragment d'ADN comprenant la séquence de bases 1 à 3027 de SEQ ID NO:1, ou un fragment d'ADN comprenant une partie dudit fragment ayant conservé l'activité de promoteur.

7. Méthode pour l'expression d'un gène étranger dans une cellule végétale, tissu végétal ou plante comprenant l'introduction d'un gène étranger désiré dans un vecteur d'expression selon l'une quelconque des revendications 1 à 5 et l'expression spécifique du gène dans la cellule végétale, le tissu végétal ou la plante.

8. Cellule végétale, tissu végétal ou plante obtenus par la méthode selon la revendication 7.

9. Cellule végétale, tissu végétal ou plante comprenant le vecteur d'expression selon l'une quelconque des revendications 1 à 5.

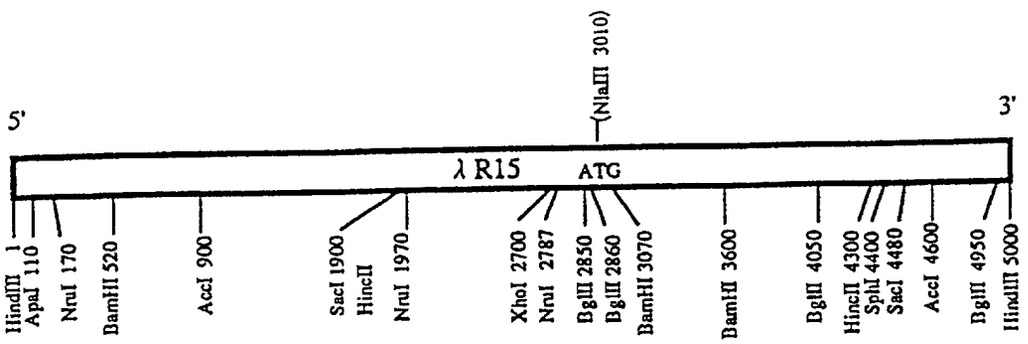
```
                      10         20         30         40         50         60
Barley ARF    MGLTFTKLFS RLFAKKEMRI LMVGLDAAGK TTILYKLKLG EIVTTIPTIG FNVETVEYKN
Human ARF3    --NI-GN-LK S-IG------ ---------- ---------- ---------- ----------
Human ARF1    --NI-AN--K G--G------ ---------- ---------- ---------- ----------
Bovine ARF1   --NI-AN--K G--G------ ---------- ---------- ---------- ----------
Bovine ARF2   --NV-E---K S--G------ ---------- ---------- ---------- ----------
Yeast ARF     ---FA----S N--GN----- ------G--- --V------- -VI------- ------Q---

                      70         80         90        100        110        120
Barley ARF    ISFTVWDVGG QDKIRPLWRH YFQNTQGLIF VVDSNDRDRV VEARDELHRM LNEDELRDAV
Human ARF3    ---------- ---------- ---------- -------E-- N---E--M-- -A--------
Human ARF1    ---------- ---------- ---------- -------E-- N---E--M-- -A--------
Bovine ARF1   ---------- ---------- ---------- -------E-- N---E--M-- -A--------
Bovine ARF2   ---------- ---------- ---------- -------E-- N---E--T-- -A--------
Yeast ARF     ---------- --R--S---- -YR--E-V-- -------S-I G---EVMQ-- -------N-A

                     130        140        150        160        170        181
Barley ARF    LLVFANKQDL PNAMNAAEIT DKLGLHSLRQ RHWYIQSTCA TTGEGLYEGL DWLSSNIANKS
Human ARF3    ---------- ---------- ---------H -N----A--- -S-D------ ---ANQLK--K
Human ARF1    ---------- ---------- ---------H -N----A--- -S-D------ ----NQLR-QK
Bovine ARF1   ---------- ---------- ---------H -N----A--- -S-D------ ----NQLR-QK
Bovine ARF2   ----V----- ---------- ---------- -N----A--- -S-D------ ----NQLK-QK
Yeast ARF     W--------- -E--S----- E------I-N -P-F--A--- -S-------- E---NSLK-ST
```

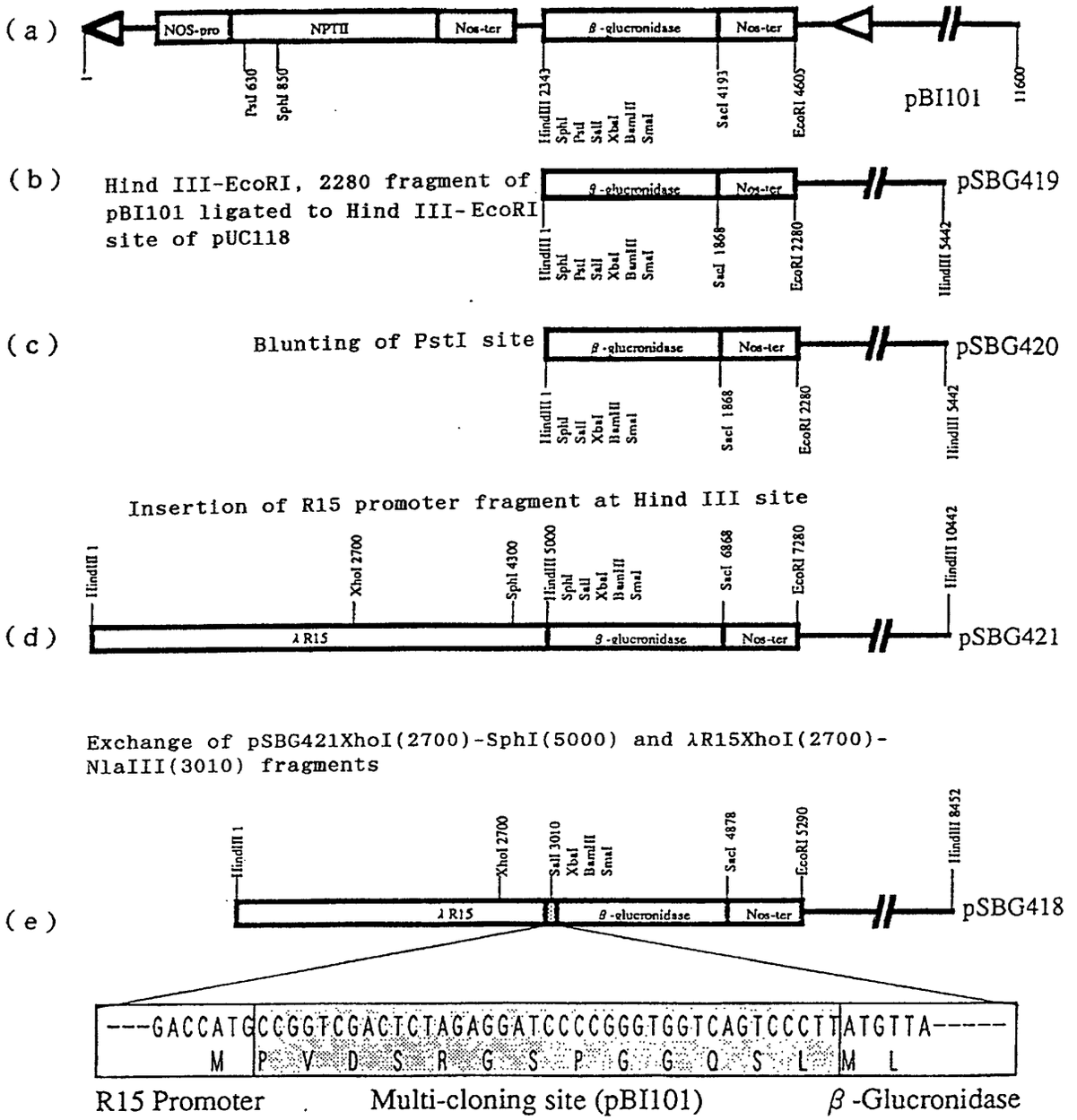
Homology of barley ARF(R151) with human, bovine and yeast ARF

Fig. 1



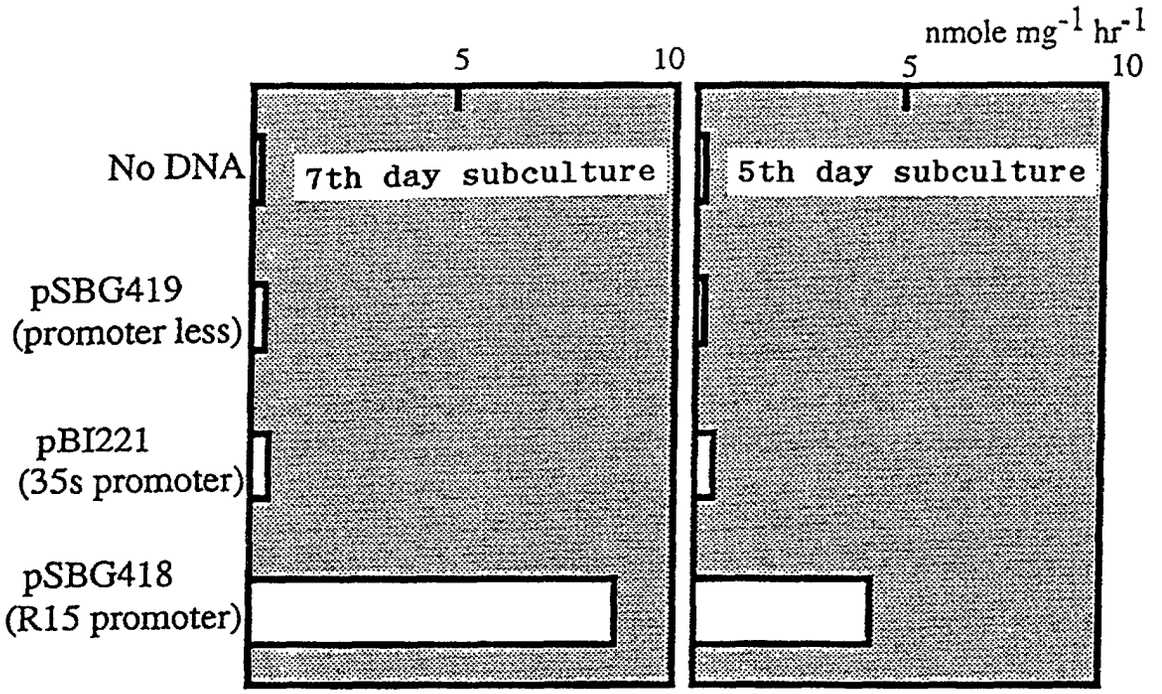
Restriction enzyme map of λR15

Fig. 2



Construction of pSBG418

Fig. 3



Analysis of R15 promoter activity

Fig. 4