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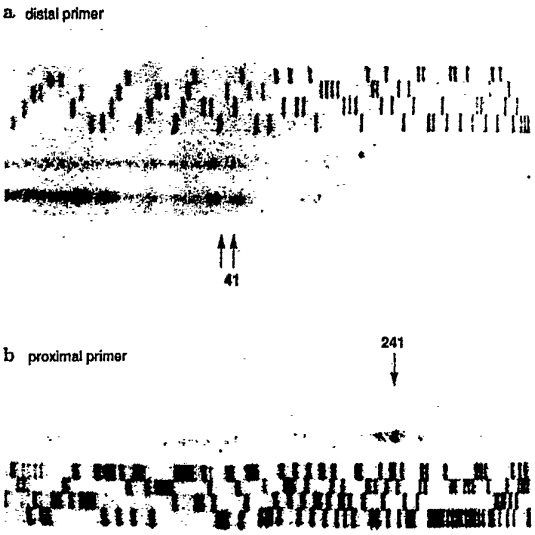

(57) Abstract

There is disclosed a gene encoding barley (1→3, 1→4)-β-glucanase isoenzyme EI; nucleotide sequences encoding for promoter and enhancer regions of said gene; procedures for the isolation of said gene; synthetic nucleotide sequences characterizing said gene, or promoter regions of said gene, or enhancer region of said gene, or the gene encoding barley (1→3, 1→4)-β-glucanase isoenzyme II. Applications/uses for the isolated gene or isolated or synthetic nucleotide sequences according to the invention, principally relate to the malting and brewing industries, for instance, transferring the isolated gene or isolated or synthetic nucleotide sequences into barley so to generate improved varieties, or preparing large quantities of enzymes with barley (1→3, 1→4)-β-glucanase activity for use as an additive in the malting and brewing industries.

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+ DESIGNATIONS OF "SU"

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"GENE ENCODING BARLEY ENZYME"

BACKGROUND OF THE INVENTION

This invention provides a gene encoding barley (1→3,1→4)-ß-glucanase isoenzyme I (EI); nucleotide sequences encoding for promoter and enhancer regions of said gene; procedures for the isolation of said gene; synthetic nucleotide sequences characterizing said gene, or promoter regions of said gene, or enhancer regions of said gene, or the gene encoding barley (1→3,1→4)-ß-glucanase isoenzyme II (cDNA for EII); applications/uses for said isolated gene and said synthetic nucleotide sequences, including molecular cloning of the gene encoding said isoenzyme I or the said synthetic nucleotide sequences, and preparation of "synthetic polypeptides" having said isoenzyme I or II activity, which may be modified with respect to thermal stability or other properties through protein engineering.

Endosperm cell wall degradation is important in the production of malt for the brewing industry, where barley is germinated under conditions designed to maximise endosperm modification while limiting seedling growth. A commonly-used indicator of malt quality is the amount of material which can subsequently be extracted from the malt with hot water (malt extract). High levels of (1→3,1→4)-ß-glucan adversely affect the recovery of malt extract probably because the extent of cell wall degradation, and hence storage polymer mobilisation, is diminished. Conversely (1→3,1→4)-ß-glucanase activity is positively correlated with endosperm modification and malt extract. Therefore the ability of a barley variety to rapidly produce high

levels of (1→3,1→4)-ß-glucanases is an important quality determinant.

Furthermore, if significant levels of high molecular weight (1→3,1→4)-ß-glucan are present in the mash/malt extract, whether this be caused by a high initial (1→3,1→4)-ß-glucan content in the barley, by an inability of the grain to generate sufficient (1→3,1→4)-ß-glucanase for its degradation during malting and mashing, or by the use of unmalted barley as a mash adjunct, the polysaccharide can cause serious filtration difficulties in the brewing process and can contribute to haze formation in the final product. The high viscosity of aqueous solutions of barley (1→3,1→4)-ß-glucan has also been implicated in problems encountered in the stockfeed industry.

Two (1→3,1→4)-ß-glucan endohydrolase (EC 3.2.1.73) isoenzymes are synthesized in germinating barley grain, where they function to depolymerize (1→3,1→4)-ß-glucans in cell walls of the starchy endosperm (Fincher, 1989). These polysaccharides constitute up to 70-75% by weight of the walls. The purified (1→3,1→4)-ß-glucanases differ in their apparent molecular weight, isoelectric point, carbohydrate content and thermal stability (Woodward & Fincher, 1982a,b). Partial amino acid sequence analyses indicate that the enzymes exhibit a high degree of positional identity and represent the products of two separate genes which probably arose by duplication of a common ancestral gene (Woodward et al., 1982).

More recent evidence based on Southern blot analyses confirms the presence of two (1→3,1→4)-ß-glucanase genes in the barley genome and indicates that the genes are located on different chromosomes (Loi

et al., 1988). The genetics of barley (1→3,1→4)-ß-glucanases are significant because the ability of the grain to rapidly produce high levels of the enzymes is closely correlated with malting performance (Stuart et al., 1988) and is therefore an important selection criterion in breeding programs, as dealt with more fully below under the heading of "Discussion".

A cDNA clone encoding barley (1→3,1→4)-ß-glucanase isoenzyme EII (Fincher et al., 1986) has been used in hybridization histochemical studies to define the expression sites and developmental regulation of the (1→3,1→4)-ß-glucanase genes in intact, germinating barley grain (McFadden et al., 1988). Expression of the (1→3,1→4)-ß-glucanase genes, as measured by mRNA accumulation, is first detected in the epithelial layer of the scutellum. Levels of (1→3, 1→4)-ß-glucanase mRNA then decrease in the scuteller epithelium but increase in the aleurone, where induction of gene expression progresses from the proximal to distal end of the grain (McFadden et al., 1988).

Although the probe used to monitor (1→3,1→4)-ß-glucanase gene expression did not discriminate between the two individual genes, earlier in vitro investigations with isolated barley aleurone layers and scutella indicated that isoenzyme EI is synthesized predominantly in the scutellum, while isoenzyme EII is synthesized exclusively in the aleurone (Stuart et al., 1986). The phytohormone gibberellic acid enhances the rate of (1→3,1→4)-ß-glucanase secretion from aleurone layers and from isolated scutella (Stuart et al., 1986).

It appears therefore that expression of the two (1→3,1→4)-ß-glucanase genes in germinating barley represents a relatively uncomplicated system for the

examination of both hormonal and tissue-specific regulation of plant genes. We have isolated and sequenced a gene encoding barley (1→3,1→4)-ß-glucanase isoenzyme EI, and identified potential regulatory elements in the promoter region, and used euplasmic wheat-barley addition lines to identify the chromosomes that carry the two genes.

STATEMENT OF INVENTION

In accordance with the present invention, there is provided an isolated DNA sequence which encodes for barley (1→3,1→4)-ß-glucanase isoenzyme I, said isolated DNA sequence having a nucleotide sequence in accordance with Fig. 1 of the accompanying drawings, said nucleotide sequence having been submitted to the GenBank/EMBL Data Bank on 5th September 1990 and accorded accession number X56260.

In accordance with the present invention, there is also provided isolated or synthetic DNA sequences consisting of portions of the nucleotide sequence according to Fig. 1, such portions including:

(i) DNA sequence which encodes for barley (1→3,1→4)-ß-glucanase isoenzyme I, said DNA sequence having from nucleotides 3144 to 4061 of the nucleotide sequence according to Fig. 1 of the accompanying drawings, in particular, said nucleotides 3144 to 4061 modified at nucleotides 3711 to 3719, especially the substitution of cytosine with adenine at nucleotide 3712, so as to improve the heat stability of the barley (1→3,1→4)-ß-glucanase isoenzyme I translated therefrom; or

(ii) DNA sequence which encodes for a promotor region of barley (1→3, 1→4)-ß-glucanase isoenzyme I,

said promoter region being the sequence from nucleotides 1 to 3000 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings, in particular, said nucleotides 1 to 620, preferably 1 to 480, more preferably 1 to 474, of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings; or

(iii) DNA sequence which encodes for a promoter region of barley (1→3,1→4)-ß-glucanase isoenzyme I, said promoter region being the sequence from nucleotides 620 to 2840, preferably 1800 to 2840, more preferably 1800 to 2836, of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings; or

(iv) DNA sequence which encodes for the intron region of barley (1→3,1→4)-ß-gluacanase isoenzyme I, said intron region being the sequence from nucleotides 619 to 3132 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings; or

(v) DNA sequence which encodes for an enhancer region of barley (1→3, 1→4)-ß-glucanase isoenzyme I, said enhancer region being the sequence from nucleotides 4060 to 4643 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings,

the indicated promoter sequences having particular importance in transformation of barley with modified genetic material coding for barley (1→3, 1→4,)-ß-glucanase isoenzyme I or II, especially barley (1→3, 1→4)-ß-glucanase isoenzyme I with improved thermal stability.

In accordance with the present invention, there is also provided a synthetic or copy DNA (c-DNA) sequence which encodes for barley (1→3, 1→4)-ß-

glucanase isoenzyme I, said DNA sequence having a nucleotide sequence conforming with Fig. 9 of the accompanying drawings, as well as a synthetic or copy DNA (c-DNA) sequence which encodes for barley (1→3,1→4)-ß-glucanase isoenzyme II, said DNA sequence having a nucleotide sequence conforming with Fig. 10 of the accompanying drawings.

In accordance with the present invention, there is also provided plasmids or phages or other vectors, or micro-organisms, in particular, yeasts, embodying such an isolated or synthetic DNA sequence.

In accordance with the present invention, there is also provided a method for the preparation of such a plasmid or phage or other vector, which comprises isolating the specified DNA sequence from barley plant material by digesting an appropriate DNA sample of the barley plant material with a restriction endonuclease; locating genomic DNA fragments carrying (1→3,1→4)-ß-glucanase sequences using fragments of a (1→3,1→4)-ß-glucanase copy DNA (cDNA); then excising and inserting the appropriate DNA sequence into the plasmid or phage or other vector.

In accordance with the present invention, there is also provided synthetic polypeptides having barley (1→3,1→4)-ß-glucanase activity, obtained by protein engineering or recombinant DNA expression, employing such an isolate or synthetic DNA sequence, or such a plasmid or phage or other vector, or such a microorganism, in particular, yeast.

In accordance with the present invention, there is also provided synthetic polypeptides having barley (1→3,1→4)-ß-glucanase isoenzyme I or II activity,

said polypeptides having an amino acid sequence in general conformity with any one of Figs. 1, 9 and 10 of the accompanying drawings but in a which a portion(s) of the respective amino acid sequence has been substituted with: (i) one or more amino acids of those in the amino acid sequences shown for barley (1→3,1→4)-ß-glucanase isoenzyme I or II, which provides barley (1→3,1→4)-ß-glucanase isoenzyme I- or II-like activity with improved thermal stability and is readily reproducible via recombinant DNA (rDNA) in bacterial or other expression systems; or (ii) one or more larger peptides having an amino acid sequence in general conformity with any one of Figs. 1, 9 and 10 of the accompanying drawings but in which a portion(s) of the respective amino acid sequence optionally has a deletion and/or substitution variant thereof and/or one or more additional amino acids directly attached to its amino terminus or carboxy terminus, or optionally is modified by glycosylation or phosphorylation or acetylation such that the resultant peptide can be used with or without further processing to provide a biological activity of barley (1→3,1→4)-ß-glucanase isoenzyme I or II in substantial measure.

In accordance with the present invention, there is also provided a process for producing a synthetic polypeptide exhibiting barley (1→3,1→4)-ß-glucanase activity, comprising the steps of:

a) providing the indicated vector comprising the isolated or synthetic nucleotide sequence wherein the nucleotide sequence is capable of being expressed by a host containing the vector;

b) incorporating the vector into the host; and

c) maintaining the host containing the vector under conditions suitable for transcription and translation of the nucleotide sequence into said synthetic polypeptide.

In accordance with the present invention, there is also provided use in the malting or brewing industries, of:

(i) such an isolated or synthetic DNA sequence in the production of barley grains having enhanced barley (1→3,1→4)-ß-glucan degradation quality; or

(ii) such a plasmid or phage or other vector in the production of barley grains having having enhanced barley (1→3,1→4)-ß-glucan degradation quality; or

(iii) such a microorganism, in particular, yeast, in the production of polypeptides that degrade barley (1→3,1→4)-ß-glucan; or

(iv) such a synthetic polypeptide that degrades barley (1→3,1→4)-ß-glucan.

In accordance with the present invention, there is also provided malting processes or brewing processes involving the use of:

(i) barley grains embodying such an isolated or synthetic DNA sequence so as to enhance the degradation of barley (1→3,1→4)-ß-glucan; or

(ii) barley grains embodying such an isolated or synthetic DNA sequence introduced via such a plasmid or phage or other vector so as to enhance the degradation of barley (1→3,1→4)-ß-glucan; or

(iii) such a synthetic polypeptide so as to enhance the degradation of barley (1→3,1→4)-ß-glucan.

With regard to the nucleotide sequences set out in Figs. 1, 9 and 10 of the accompanying drawings, it will be understood that the indicated sequences also embrace the sequence set out in the respective figure of drawings except for the omission or addition or substitution or rearrangement of any functionally non-critical (a) nucleotide constituents thereof, or (b) fragments thereof, or (c) multiple fragments thereof, which omission or addition or substitution or rearrangement does not significantly affect the (1→3,1→4)-ß-glucanase isoenzyme I or II activity of proteins encoded by the nucleotide sequence of in the respective figure of drawings, or which do not significantly affect the promotor region(s) of the DNA sequence, or which do not significantly affect the enhancer region of the DNA sequence.

DETAILED DESCRIPTION

Discussing the background to the present invention, two (1→3,1→4)-ß-glucan 4-glucanohydrolase (EC 3.2.1.73) isoenzymes are expressed in germinated barley grain, where they function to depolymerize the (1→3,1→4)-ß-glucans that constitute 70-75% by weight of cell walls of the starchy endosperm (Fincher 1989). The purified (1→3,1→4)-ß-glucanases differ in their apparent molecular weight, isoelectric point, carbohydrate content and thermal stability, but exhibit identical substrate specificity and share similar kinetic properties (Woodward and Fincher 1982a,b). Partial amino acid sequence analyses indicate that the enzymes are similar and represent the products of two separate genes (Woodward et al. 1982).

A cDNA clone encoding barley (1→3,1→4)-ß-glucanase isoenzyme EII has been used in hybridization histochemical studies to define the expression sites and developmental regulation of the genes in intact, germinated barley grain (McFadden et al. 1988). Gene expression, as measured by mRNA accumulation, is first detected in the epithelial layer of the scutellum. Levels of (1→3,1→4)-ß-glucanase mRNA then decrease in the scutellar epithelium but increase in the aleurone, where induction of gene expression progresses from the proximal to distal end of the grain. However, the probe used to monitor transcription of (1→3,1→4)-ß-glucanase genes in these experiments did not discriminate between the two individual genes (McFadden et al. 1988).

In the present invention we have isolated a gene encoding barley (1→3,1→4)-ß-glucanase isoenzyme EI and near full length c-DNAs for the EI and EII isoenzymes. This has enabled the complete amino acid sequence of isoenzyme EI to be determined and allowed a comparison of protein and mRNA sequences for the EI and EII isoenzymes of barley (1→3,1→4)-ß-glucanase. Based on this comparison, EI- or EII-specific oligonucleotide probes have been prepared and used to demonstrate differential expression of the two genes in the scutellum and aleurone of germinated grain and in young leaves.

Thus, in accordance with the present invention we have isolated a gene for isoenzyme EI from a barley genomic library and determined the nucleotide sequence of a 4643 bp fragment. The gene is located on barley chromosome 5 while the gene for (1→3,1→4)-ß-glucanase isoenzyme EII is carried on chromosome 1. The isoenzyme EI gene contains a single 2514 bp intron that is

inserted in codon 25 of a sequence encoding a signal peptide of 28 amino acids. The coding region of the mature enzyme is characterized by a high (G+C) content, which results from an extreme bias towards the use of these nucleotides in the wobble base position of codons. Determination of the nucleotide sequence of the gene has enabled the complete primary structure of the enzyme to be deduced; isoenzyme EI shows 92% positional identity with the primary sequence of (1→3,1→4)-ß-glucanase isoenzyme EII at both the nucleotide and amino acid level. However, the nucleotide sequences of the two genes diverge markedly in their 3' untranslated regions. Oligonucleotide probes specific for these 3' untranslated regions were used to define the expression sites of the two genes by Northern analysis and by amplifying specific cDNAs through the polymerase chain reaction. In the tissues examined, transcription of the isoenzyme EII gene is restricted to the aleurone layer of germinated grain. In contrast, the gene for isoenzyme EI is transcribed at relatively high levels in young leaves, but also in the scutellum and aleurone of germinated grain.

The DNA sequences according to the invention, encoding for barley (1→3,1→4)-ß-glucanase isoenzyme I, may be isolated from barley plant material by methods known in principle to persons skilled in the art, for instance, by a method as set out below, in which DNA samples of the barley plant material are digested with a restriction endonuclease; genomic DNA fragments carrying (1→3,1→4)-ß-glucanase sequences are located using fragments of a (1→3,1→4)- ß-glucanase copy DNA (cDNA) or synthetic oligonucleotides with sequences corresponding to portions of the cDNA or gene sequence; then the appropriate DNA sequences can be excised for insertion into a plasmid vector for storage.

The isolated or synthetic DNA sequences according to the invention may be employed in conventional genetic engineering techniques, for example, in transferring the isolated or synthetic nucleotide sequences into yeast to remove residual ß-glucan in beer production, as a result of which the invention further provides a plasmid or phage or other vector comprising the isolated or synthetic DNA sequences of the invention, as well as microorganisms containing the isolated or synthetic DNA sequences.

Thus, applications/uses for the isolated or synthetic nucleotide sequences according to the invention principally relate to the malting and brewing industries, for instance transferring the isolated or synthetic nucleotide sequences into barley so as to generate improved varieties; or transferring the isolated or synthetic nucleotide sequences into yeast to remove residual ß-glucan in beer production; or so as to generate thermostable said isoenzymes EI and EII by site-directed or general mutagenesis; or so as to enhance levels of expression by genetic engineering of the promoter sequence elements, said isoenzymes EI and EII functioning to remove cell walls in the starchy endosperm of germinating barley grains, essentially for successful germination of grain, the importance of which lies in the direct correlation of (1→3,1→4)-ß-glucanase levels with malting quality of barleys, i.e. the greater the levels of the enzyme, the better the malting quality.

In particular, the gene encoding for barley (1→3,1→4)-ß-glucanase isoenzyme I modified at nucleotides 3711 to 3719, especially the substitution of cytosine with adenine at nucleotide position 3712 of Fig. 1 of the accompanying drawings, so as to improve the heat

stability of the barley (1→3, 1→4)-ß-glucanse isoenzyme I translated therefrom, may be expressed: (a) in barley by transforming barley with the modified gene so as to produce improved varieties; or (b) in cultured cells to produce large quantities of the enzyme with improved heat stability, which may be used as an additive in the malting or brewing industries.

Thus, in accordance with the present invention, there is provided "synthetic polypeptides" having barley (1→3,1→4)-ß-glucanase isoenzyme I activity. The "synthetic polypeptides" of the present invention include barley (1→3,1→4)-ß-glucanase isoenzyme I with the amino acid sequence recited herein but with: (i) the substitution of one or more amino acids of those in the amino acid sequences shown herein for barley (1→3, 1→4)-ß-glucanase isoenzyme I, which is expected to have barley (1→3,1→4)-ß-glucanase isoenzyme I-like activity with improved thermal stability and to be readily producible via recombinant DNA (rDNA) in bacterial or other expression systems; or (ii) various larger peptides containing the recited sequence (or such a deletion and/or substitution variant thereof) together with one or more additional amino acids directly attached to its amino and/or carboxy terminus, as well as such peptides otherwise modified at their termini or elsewhere, e.g. by glycosylation, phosphorylation, acetylation or the like, to the extent such peptides can be used (with or without further processing) to provide a biological activity of barley (1→3,1→4)-ß-glucanase isoenzyme I in substantial measure.

As used herein, the term "synthetic polypeptides" means peptides produced by a technique (e.g. protein engineering or recombinant DNA expression) other than its natural production in a living plant.

Accordingly, the "synthetic" polypeptides of this invention are to be distinguished from peptides produced in living plants via expression of DNA occurring naturally in those plants. As produced, such "synthetic polypeptides" are normally free from peptides of barley (and usually other plant) origin.

In accordance with the present invention, there is further provided a process for producing a polypeptide exhibiting barley (1→3,1→4)-ß-glucanase isoenzyme I activity, comprising the steps of:

a) providing a vector comprising the isolated or synthetic nucleotide sequence according to the invention, wherein the nucleotide sequence is capable of being expressed by a host containing the vector;

b) incorporating the vector into the host; and

c) maintaining the host containing the vector under conditions suitable for transcription and translation of the nucleotide sequence into said polypeptide,

although the preparation of proteins from the isolated or synthetic DNA sequence of the present invention can be carried out by other methods known in principle to persons skilled in the art.

A preferred method for producing the synthetic barley (1→3,1→4)-ß-glucanase isoenzyme I peptides of the present invention is by rDNA technology utilizing host cells such as bacteria (e.g. E. coli) or eucaryotic cells such as yeast or cultured insect cells. Modifications of the DNA sequences herein can be made to

affect their efficiency of peptide production in a desired host cell. Such modifications include, but are not limited to: host-preferred codon substitution; construction of DNA coding for fusion proteins including barley (1→3,1→4)-ß-glucanase isoenzyme I; substitution of codons to eliminate or enhance mRNA structural features affecting their translation; and other modifications that improve production of barley (1→3,1→4)-ß-glucanase isoenzyme I in the selected host cell.

The isolated or synthetic DNA sequences of the present invention, as well as plasmids or phages or other vectors or microorganisms embodying such isolated or synthetic DNA sequences, beside synthetic polypeptides obtained by protein engineering or recombinant DNA expression, employing such vectors or microorganism, have particular use in the malting or brewing industries.

Barley is usually germanated under controlled conditions for about 4 days, the conditions being designed to maximize the levels of hydrolytic enzymes, e.g. (1→3,1→4)-ß-glucanases, α-amylases, proteases, etc., in the grain to minimize the amount of seedling growth (i.e. root and shoot growth), so as to limit the incorporation into the vegetative tissues of the seedling, of grain nutrients that are ultimately required by the brewing yeast. When malting is complete, the grain is generally dried in a kiln at temperatures of up to 85°C and transported from the malthouse to the brewery.

Brewers crush or mill the malted barley and

extract soluble sugars, oligosaccharides, peptides, amino acids etc. into hot water, which might initially be 45-65°C, in a process called mashing, which results in the loss of much of the (1→3,1→4)-ß-glucanase that survives the kilning. Other enzymes, such as α-amylases, can degrade their substrates during the extraction period to produce a higher proportion of low molecular weight molecules that can subsequently be used by the yeast to support their growth and to provide the yeast with fermentable sugars and disaccharides for ethanol production in the beer.

The synthetic polypeptide of the present invention can be added at the mashing stage so as to enhance the degradation of (1→3, 1→4,)-ß-glucans. The "mash" may stand or be mixed for a period of time to effect extraction, and the soluble extract may then be separated from the spent grains by a variety of methods. If the separation of soluble extract from the spent grains involves filtration or drainage of the extract through the bed of spent grains, run off times can be dramatically increased by highly viscous, residual (1→3, 1→4,)-ß-glucans. Thereafter, there are a number of steps in which the extract is boiled to precipitate proteins and to denature the enzymes. Hops may be added at this stage to the extract to give the bitter flavour, and then the extract is cooled prior to fermentation. At each step, precipitates may be removed by filtration or centrifugation, and residual (1→3, 1→4,)-ß-glucans can slow down these steps.

The cooled extract is then usually "spiked" with a yeast culture, which grows over a period of several days, at cool temperatures, on the nutrients of the malt extract, and in the near-anaerobic conditions, the sugars and disaccharides are fermented to ethanol.

At the end of the fermentation, the yeast are removed, together with any material that precipitated during the fermentation, and this again is usually achieved by centrifugation or filtration, residual (1→3, 1→4)-ß-glucans also slowing down this step.

The transformation of barley with isolated or synthetic DNA of the present invention may be carried out by known procedures. Thus, isolated or synthetic DNA of the present invention may be introduced/reintroduced into barley by:

(i) excising the modified gene (consisting of the original or modified promoter, the intron, the original or modified protein coding region with attached signal peptide region, and the 3'-untranslated region extending beyond the putative polyadenylation site) from the plasmid or phage or yeast vector used in the manipulation processes;

(ii) splicing the excised modified gene into a plasmid vector containing an antibiotic-resistant gene (such as the neomycin phosphoryl transferase gene, which vectors are commercially available) that can be used to select transgenic cells in which the introduced DNA has been stably incorporated into the chromosome of the barley cell, the selection being based on the acquired resistance of the barley cells to the antibiotic;

(iii introducing the new vector into barley cells by conventional techniques such as making protoplasts (naked cells from which the cell walls have been enzymatically removed) and treating them with polyethylene glycol (PEG) in the presence of the

DNA, or using a microparticle gun to incorporate DNA coated onto micro-tungsten particles, into the chromosome;

(iv) allowing the barley cells to recover from the treatment, generate new cell walls and start dividing again, and then selecting for recombinant transgenic cell lines by applying the neomycin antibiotic, and then growing the resistant cells into small colonies;

(v) changing the medium on which the cells are growing to induce formation of roots and shoots and when the plantlet is well established, transfering the plant to soil so as to allow it to mature;

(vi) testing that the mature plant is fertile, by its ability to set fertile seed, and testing the (1→3,1→4)-ß-glucanase activity of the transgenic plant.

The transgenic barley so produced could be re-introduced into breeding programs and the new character could be cross-bred into agronomically acceptable barleys and seed distributed to farmers, as such manipulations would probably result in some changes in the original barley and different barley varieties are bred to suit the conditions of particular local environments.

The following description illustrates specific embodiments of the invention. They are not to be taken as limiting the scope of the invention. Various modifications will be apparent to those skilled in the art, with or without the other disclosures herein.

MATERIALS AND METHODS

Plant Material

Barley (Hordeum vulgare, cv Clipper) was obtained from the Victorian Crops Research Institute, Horsham, Victoria, Australia and from the cereal collection of the John Innes Centre for Plant Science Research, Norwich, U.K. Other barley cultivars, hexaploid wheats (Triticum aestivum) and various aneuploid wheat stocks (Sears 1966; Sears and Sears 1978) were as described by Sharp et al. (1988). For germination experiments, barley grains were surface sterilized with 2.5% (w/v) sodium hypochlorite for 20 min at room temperature, washed thoroughly with 10mM HCI and water, and soaked in sterile water containing 100 µg/ml neomycin, 100µg/ml chloramphenicol and 100 units/ml nystatin for 16 h at room temperature. Grain was germinated on filter paper in sterile petri dishes at 22°C. Tissues were excised for RNA isolation at selected times after the initiation of germination, as follows: scutella, 1 day; aleurone layers, 3 days; coleoptiles, 6 days; young roots and root hairs, 2 days; young leaves, approximately 10 days (that is, the first leaves to emerge from the coleoptile). Mature leaves, stems and roots obtained from glasshouse-grown barley plants approximately 4 weeks after planting were washed thoroughly and surface sterilized in sodium hypochlorite before RNA isolation.

RNA Isolation and Northern Analysis

Total RNA was extracted from tissue ground to a fine powder under liquid N_2 in a guanidinium thiocyanate buffer and purified by equilibrium centrifugation in caesium trifluoroacetate (Okayama et al. 1987) or, alternatively, by extraction into 50mM Tris-HCl, pH9.0

(containing 10mM EDTA, 2% w/v SDS, 10mM ß-mercaptoethanol, 100mM NaCl and 0.1% w/v proteinase K), exhaustive removal of protein with phenol-chloroform and recovery of RNA by ethanol precipitation (Baulcombe and Buffard 1983). The RNA samples (5-10µg) were separated on 1% (w/v) agarose gels in 2.2M formaldehyde (Sambrook et al. 1989) and transferred to nitrocellulose filters (Hybond-C Extra, Amersham Corp.). To ensure approximately equal loadings, control gels were stained with ethidium bromide for comparison of the relative intensities of ribosomal RNA bands. Filters were prehybridized at 60°C for 4 h in 6x standard saline citrate (SSC 150mM NaCl/15mM sodium citrate, pH7.0) containing 1% (w/v) SDS, 1x Denhardt's solution (Denhardt 1966), and 0.1 mg/ml herring sperm DNA. Oligonucloetide probes (30-mers) were synthesized using solid phase phosphoramidite chemistry on an Applied Biosystems Model 380A DNA synthesizer and end-labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Sambrook et al. 1989). Filters were hybridized with oligonucleotide probes at 60°C for 16 hr in prehybridization buffer containing 10% (w/v) dextran sulphate. Filters were washed exhaustively in 3 x SSC/0.1% SDS at 60°C. DNA probes were labelled to a specific activity of more than 10^9µdpm/µg with [α-^{32}P]dCTP using random sequence hexanucleotides (Feinberg and Vogelstein 1983). Prehybridization of filters for DNA probes was effected in 2x SSC containing 1% (w/v) SDS, 1 x Denhardt's solution and 0.1µg/ml herring sperm DNA for 4 hr at 68°C and hybridization at 68°C for 16 hr in the same buffer containing 10% (w/v) dextran sulphate. Filters were washed to 0.2x SSC/0.1% SDS at 68°C prior to autoradiography on Kodak XAR X-ray film.

DNA Isolation and Southern Analysis

Isolation of genomic DNA, restriction enzyme digestion, agarose gel electrophoresis, alkaline Southern blotting to Amersham Hybond-N Plus membranes and hybridization procedures were as described by Sharp et al (1988). Near full-length (1→3,1→4)-ß-glucanase cDNAs were used as probes.

Amplification of Specific cDNAs

First strand cDNA was prepared from 5-10µg total RNA primed with appropriate oligonucleotides by reverse transcriptase (Sambrook et al. 1989) in a total volume of 50 µl. After 1 hr at 42°C, 2.5µl of the first strand cDNA reaction was mixed in a total volume of 50µl with flanking oligonucleotides and Taq polymerase, and incubated under mineral oil in a Perkin-Elmer Cetus (Norwalk, CT, USA) DNA Thermal Cycler as follows; 94°C/2 min; 94°C/30 sec, 40°C/20 sec, 72°C/2 min, repeated 30x; 72°C/10 min; 4°C. The polymerase chain reaction (PCR) products were examined by agarose or polyacrylamide gel electrophoresis.

Screening the Barley Genomic Library

The barley genomic library was obtained from Clontec Laboratories Inc. (Palo Alto, CA, USA). The library was prepared from partially digested DNA from 7-day old seedlings of Hordeum vulgare L. (var. NK 1558) in the cloning vector EMBL3. The library was plated out on a lawn of E. coli NM 538 cells and screened by hybridization of nitrocellulose filter plaque replicas (Sambrook et al. 1989) using as a probe the 734 bp HinfI fragment of a (1→3,1→4)-ß-glucanse cDNA (Fincher et al. 1986). The probe was labelled and prehybridization, hybridization and washing procedures were essentially as described by Loi et al. (1988). Following plaque purification of positive clones, phage DNA was isolated

and the genomic DNA insert analysed with restriction endonucleases. A 3.8 kb HindIII fragment, a 4.2 kb SalI fragment, and 1.5 kb and 1.8 kb BamHI fragments were sub-cloned into the corresponding sites of plasmid pUC19 for sequence analysis.

Isolation of cDNA clones

For the generation of cDNA libraries, poly A(+) RNA was extracted from scutella excised from the grain and incubated for 2 days at 25°C in 10mM $CaCl_2$ and 5μM gibberellic acid (GA), and from aleurone layers after 1.5 days incubation. Procedures for the synthesis of cDNA, the preparation of cDNA libraries in λgt11 and screening were as described elsewhere (Doan and Fincher 1988; Hoj et al. 1989). Both libraries were screened with a cDNA encoding barley (1→3,1→4)-ß-glucanase isoenzyme EII (Fincher et al. 1986).

Nucleotide Sequence Analysis

The dideoxynucleotide chain termination procedure of Sanger et al. (1977) was used to sequence the (1→3,1→4)-ß- glucanase genomic clone. Appropriate restriction endonuclease fragments were recovered from agarose gels with Geneclean (BIO101) and subcloned into pUC19 or into M13mp18 or mp19 for sequencing. Computer analysis were performed with the Pustell Sequence Analysis Programs (International Biotechnologies Inc., New Haven, CT, USA), the Wisconsin Genetics Computer Group package (University of Wisconsin, Madison, WI, USA; Devereux et al. 1984) and with the Staden programs (MRC Laboratory of Molecular Biology, Cambridge, UK; Staden 1986).

Primer Extension

Total RNA preparations (5-50μg) from young leaves and scutella were annealed with 10ng end-labelled oligonucleotide specific for the 5' untranslated regions of the isoenzyme EI gene in deionized 50% (v/v) formamide at 34°C for 16 h after heating the incubation mixture at 85°C for 10 min (Sambrook et al. 1989). After recovery of the RNA by ethanol precipitation, reverse transcription and polyacrylamide gel electrophoresis of the extended primers were as described by Sambrook et al. (1989).

RESULTS

Isolation of a genomic clone

From approximately 5 x 10^5 plaques, two genomic clones were isolated using cDNA for barley (1→3,1→4)-ß-glucanase isoenzyme EII as a probe. The two clones had identical restriction maps with EcoRI, BamHI and HindIII (not shown). A 4643 bp segment of one genomic clone was sequenced in both directions (Fig. 1). The gene can be identified as barley (1→3,1→4)-ß-glucanase isoenzyme EI from the presence of sequence encoding the first 40 amino acids of the mature isoenzyme EI (Fig. 1 cf. Woodward et al. 1982) and because sequences further 3' in the same open-reading frame exactly match those obtained from tryptic peptides of the purified protein (unpublished data). The deduced amino acid composition in that open reading frame also corresponds closely to that determined directly from the protein, suggesting that the amino acid sequence was deduced from the correct reading frame (Table 1).

Isolation of cDNA clones

The isoenzyme EII cDNA clone (Fincher et al. 1986) used

to screen the library was less than full-length and did not include non-coding sequence. Additional cDNA clones were therefore isolated to obtain further sequence information of isoenzyme EII mRNAs and also for comparison of isoenzyme EI mRNA with the genomic sequence. A 1431 bp cDNA encoding isoenzyme EI, identified from the deduced protein-coding sequence, was isolated from the scutellum cDNA library and exhibited more than 98% positional identity with the genomic clone where the sequences overlap although, as discussed below, the genomic sequence is interrupted by a single large intron. This isoenzyme EI cDNA includes the complete coding sequence of the mRNA and non-translated 5' and 3' flanking sequences. A 1229 bp isoenzyme EII cDNA obtained from the aleurone cDNA library was also identified from the deduced amino acid sequence. The isoenzyme EII cDNA had associated 5' and 3' non-translated sequences, including a short poly(A) tail. The sequence of the 3' region of the isoenzyme EI cDNA showed 98% positional identity with a 286 bp cDNA isolated by Jackson et al. (1986). The c-DNAs encoding for isoenzymes EI and EII are shown in Fig. 9 and Fig. 10, respectively.

Analysis of the genomic sequence

A comparison of the sequence of the isoenzyme EI cDNA clone with the genomic DNA sequence showed that the gene is interrupted by a single large intron close to the codon for the NH_2-terminal isoleucine residue of the mature enzyme in the region encoding the putative signal peptide (Fig. 1). It was thought that the cDNA clone is almost intact, as its 5' end is between the TATA box of a putative promoter that we have designated the distal promoter (Table 2), and an initiating methionine codon in a context that reasonably matches the plant consensus

sequence (Table 2). The peptide encoded by the 5' exon resembles a signal peptide, having a relatively hydrophilic NH_2-terminal region and a hydrophobic core which becomes more hydrophilic towards its COOH-terminus, where there is a helix-breaking proline residue and a large polar amino acid (glutamine) (Fig. 1, Watson 1984). The absence of a charged amino acid close to the NH_2-terminus (Fig. 1) is unusual but not unique in eukaryotic signal peptides (Watson 1984).

The intron is typical of plant introns. Both the 5' and 3' boundary sequences at positions 619 and 3132 (Table 2) are similar to the consensus sequence for plant introns and there are stop codons in all reading frames within the 3' region of the intron (Fig. 1). The high (A+T) content near the intron boundaries is a characteristic of plant introns required for effective splicing of adjacent exons (Goodall and Fillipowicz 1989). The ACTAAC motif beginning 26 bp from the 3' end of the intron corresponds in both position and sequence to the consensus sequence for the branch point of type II introns of plant genes (Goodall and Fillipowicz 1989). Although this intron is much longer than the average length of 249 bp reported by Hawkins (1988) in a survey of plant intron lengths, there is at least one example of a larger plant intron (Yanofsky et al. 1990). Analysis of the nucleotide sequence of the genomic DNA suggested that there is the potential for an additional exon that is not shown in the cDNA sequences. This additional exon would be contained within the large intron; the 3' boundary of the exon would be at nucleotide 3077 of the gene (Fig. 1) and would have many features of the extreme 5' exon of a plant gene. These features include an adjacent promoter, referred to as the proximal promoter, comprising a CAAT sequence that begins 30 bp 5' to a possible TATA box that is located,

in turn, approximately 220 nucleotide pairs on the 5' side (Fig. 1; Table 2) of an ATG codon initiating the open frame of the putative exon. The nucleotide sequence surrounding the ATG translation start point of this putative exon is very similar to the optimal sequence for initiation by eukaryotic ribosomes (CCACCATGG; Kozak 1986) and also is in reasonable agreement with consensus sequences derived from plant genes (Table 2). Although the initiation codon is not the first after the putative transcription start point (Fig. 1), the others are found in sub-optimal contexts (cf. Kozak 1986). The open reading frame following this initiating codon would encode a peptide with the three distinct domains commonly found in eukaryotic signal peptide sequences (Watson 1984) and would be in frame with the open reading frame of the 3' exon in a spliced mRNA. At the NH_2-terminal end of the deduced polypeptide sequence (starting at position 3020) there would be a short hydrophilic region with a charged amino acid residue (Fig. 1). This would be followed by a hydrophobic core which would become more hydrophilic towards the COOH-terminal region of the putative signal peptide. Helix-breaking proline and glycine residues would be located close to the 3' end of this exon and therefore close to the protein processing site (von Heijne 1983) located before the fourth amino acid (isoleucine) on the large exon of the gene (Fig. 1). The length of the intron between these exons would be 55 bp, which is smaller than a value of 70 to 73 bp recently reported to be the minimal functional length of plant introns; however, shorter introns have been recorded (Goodall and Filipowicz 1990). The sequence analysis of the 5' part of the gene therefore implies the existence of two promoter sequences, the proximal promoter being within the large intron of the region transcribed from the distal promoter. However,

biological activity was confirmed by the existence of a corresponding cDNA clone only for the distal promoter. In order to investigate the activity of the two promoters, primer extension experiments were carried out with oligonucleotide primers complementary to the putative signal peptide (nucleotides 3058-3077, Fig. 1) of the proximal promoter or the 5' untranslated region (nucleotides 499-515, Fig. 1) of the distal promoter. In both instances the oligonucleotides primed synthesis of cDNA from scutellum RNA corresponding to the predicted transcripts of the two promoters: the products of the distal primer were cDNAs of 41 and 38 nucleotides (Fig. 2a) corresponding to transcriptional start points 54 and 57 nucleotides 3' of the TATA box sequence of the putative distal promoter. When the proximal primer was annealed to scutellum RNA there was clear termination of cDNA product of 241 nucleotides at position 2836 (Fig. 2b). This transcriptional start point is situated 38 bp downstream from the putative TATA box in the proximal promoter (Table 2). The primer extension experiments therefore confirm the activity of the two promoters in the isoenzyme EI gene, although it is likely that the proximal promoter was the weaker, as more RNA was required than with the distal primer to produce a detectable amount of primer-extended cDNA (Fig. 2).

Promoter sequences

The secretion of the barley (1→3,1→4)-ß-glucanase isoenzyme EI from isolated scutella appears to be enhanced by the phytohormone gibberellic acid (GA), although high levels of endogenous hormone in the tissue fragments mask the effects of added GA (Stuart et al. 1986). In isolated aleurone layers, levels of secreted (1→3,1→4)-ß-glucanase enzyme (Stuart et al. 1986) and

translatable mRNA (Mundy and Fincher 1986) are significantly enhanced by GA treatment. Accordingly, the promoter region of the barley (1→3,1→4)-ß-glucanase isoenzyme EI gene (Fig. 1) was examined for sequence similarities with the 5' regions of other genes expressed under the influence of GA in the aleurone layer of germinating cereal grains (Baulcombe et al. 1987a; Huttly et al. 1988; Whittier et al. 1987; Rahmatullah et al. 1989; Ou-Lee et al. 1988; Huang et al. 1990). Although direct and inverted repeat elements are present, we are unable to identify nucleotide sequences that are unique to GA-responsive genes. A detailed functional analysis using a transient assay system (Huttly and Baulcombe 1989) will be used to dissect cis-acting elements in the isoenzyme EI gene promoter and to ask whether the α-amylase and other GA-responsive genes are regulated in the same way as the (1→3,1→4)-ß-glucanase gene.

Codon Usage

The region of the gene that encodes the mature (1→3,1→4)-ß-glucanase enzyme has an overall (G+C) content of 67% and this is mainly attributable to a strong bias towards the use of G and C in the wobble base position of codons. Of the 306 codons in this region, only 13 have A or T in the third nucleotide position. In the two possible signal peptide regions of the gene codon usage is not strongly biased towards the use of G and C at the wobble base positions (Fig. 1). Similar strong preferences for G- and C-rich codons have been observed in cDNAs encoding barley (1→3,1→4)-ß-glucanase isoenzyme EII (Fincher et al. 1986), barley (1→3)-ß-glucanase isoenzyme GII (Hoj et al. 1989) and in other cereal genes that are expressed at high levels in germinating grains and are enhanced by GA (Baulcombe et al. 1987a).

The bias in codon usage may be related to mRNA stability or to translational efficiency, both of which would contribute to the high levels of expression of these genes in the aleurone cells of germinating cereal grains, where hydrolytic enzymes are required for rapid endosperm mobilization (Fincher 1989).

Intron location

It is noteworthy that the position of the intron close to the processing site of the signal peptide in the barley (1→3,1→4)-ß-glucanase gene corresponds exactly to the position of a single intron in a barley gene encoding (1→3)-ß-glucan endohydrolase (EC 3.2.1.39) which splits the same codon at precisely the same site (P. Xu, J. Wang and G.B. Fincher, unpublished data). This supports an earlier suggestion that the barley (1→3,1→4)-ß-glucanase and (1→3)-ß-glucanase genes share a common ancestry (Hoj et al. 1989).

The position of the intron also marks the transition point in codon usage in the gene. Barely (1→3)-ß-glucanase genes (Hoj et al. 1989; P. Xu, J. Wang and G.B. Fincher, unpublished data), a wheat (1→3,1→4)-ß-glucanase cDNA (D. Lai and G.B. Fincher, unpublished data), and wheat α-amylase genes (Baulcombe et al. 1987b) exhibit a similar abrupt change from (A+T) to (G+C) in the wobble base position at the site of intron insertion near the 5' end of the genes. This may reflect a function for introns in exon "shuffling", whereby exons encoding signal peptides or other sequences responsible for targetting gene products to specific cellular locations have been appended to the 5' region of the coding region of the enzyme (Gilbert 1985).

Signal peptides

Many of the signal peptide-like features of the NH_2-terminal sequence derived from the isoenzyme EI genomic and cDNA clones have been discussed above. Similar features are also found in the sequence from the isoenzyme EII cDNA (Fig. 4a). A further noteworthy feature of the sequences is a (Val-Glu-Ser) sequence that precedes the signal peptide cleavage site (Fig. 1) which is conserved in both wheat and barley (1→3,1→4)-ß-glucanases (Fincher et al. 1986; unpublished data) and is similar to the (Val-Gln-Ser) sequence observed for the barley (1→3)-ß-glucanase isoenzyme GII (Hoj et al. 1989).

Comparison of the sequence of the isoenzyme EI and EII cDNAs and proteins

The nucleotide sequence of the gene (Fig. 1) has permitted the complete primary structure of isoenzyme EI to be defined. The coding region for the mature enzyme encodes a polypeptide of 306 amino acids. The calculated pI for the polypeptide is 8.7, which may be compared with a value of 8.5 reported for purified (1→3,1→4)-ß-glucanse isoenzyme EI and a value of greater than 10 for isoenzyme EII (Woodward and Fincher 1982a; Fincher et al 1986). The primary structure of isoenzyme EII derived from the cDNA is also 306 amino acids long, but has a calculated pI value of 10.6. The two protein sequences are compared in Fig. 3. There are 25 amino acid substitutions in the two enzymes and an overall positional identity of 92% at the amino acid level (Fig. 3). Of the 25 substitutions, 7 involve more basic amino acids in isoenzyme EII and this explains its higher isoelectric point. Comparison of the gene sequence of isoenzyme EI (Fig. 1) and the nucleotide sequence of a

cDNA encoding isoenzyme EII (Fincher et al. 1986) reveals that the two genes also show 92% positional identity at the nucleotide level within the coding sequence. Furthermore, there is extensive similarity between the isoenzyme EI and EII genes in the 5' non-translated sequence of the transcribed region (Fig. 4a). However in the 3' non-translated region, the sequence are completely different (Figs. 1 & 4b).

Northern Analyses

To obtain a general profile of (1→3,1→4)-ß-glucanase mRNA in barley, a 1.4kb HinfI fragment from the coding region of the genomic clone was used to probe various RNA samples on a Northern blot. The probe includes regions of similarity between the isoenzyme EI and EII genes and would therefore detect transcripts from both. No expression was detected in roots, coleoptiles, stems or mature leaves but an mRNA of approximately 1550 nucleotides was detected in the RNA of aleurones from germinated grains (Fig. 5a) and of young leaves. A relatively faint signal could also be detected with scutellar RNA (Fig. 5a).

Use of oligonucleotide probes complementary to the 3' untranslated region of the isoenzyme EI and EII mRNAs allowed separate detection of these two types of mRNA and showed that the aleurone cells expressed both isoenzyme EI and EII mRNAs whereas the scutellar and leaf RNA contained only the EI type (Figs. 5b, c).

Amplification of Specific mRNAs by PCR

In order to detect transcription of the (1→3,1→4)-ß-glucanase isoenzyme EI gene from the two possible promoters shown in Figure 1, oligonucleotides were

designed (Fig. 6) to specifically amplify first strand cDNAs synthesized from leaf, scutellum and aleurone RNA preparations. Synthesis of first strand cDNA by reverse transcriptase was primed with oligonucleotide 12 (3347 to 3366; Figs. 1 and 6), which was complementary to part of the protein-coding region. In subsequent PCR incubations the cDNAs were amplified with oligonucleotides 13 (500 to 519, Figs. 1 and 6) and 12 to detect cDNAs corresponding to mRNA transcribed from the distal promoter, or with oligonucleotides 14 (2973 to 2993, Fig. 1) and 12 to detect transcription from the proximal promoter (Fig. 6). The expected sizes of PCR fragments were 362 bp for oligonucleotides 13/12 and 347 bp for oligonucleotides 14/12.

In each of the 3 RNA preparations, cDNAs corresponding to transcription from the distal promoter were clearly seen (Fig. 7). Faint bands were also detectable in the PCR products amplified with oligonucleotides 14/12, but these were approximately 400 bp in length and presumably represent products from low levels of unprocessed pre-mRNA in the total RNA preparations. These experiments therefore confirm that the distal promoter is the more active of the two promoters in the EI gene. It is not possible at this stage to determine whether the unprocessed RNA was derived from the proximal or distal promoters and further experiments are needed to clarify the activity of the proximal promoter.

Southern Analyses

In earlier work, Loi et al. (1988) reported that barley (1→3,1→4)-ß-glucanase genes were located on chromosomes 1 and 5, but their evidence for the chromosome 5 location was indirect and they were unable to assign chromosome locations to individual genes. The

availability of wheat aneuploid lines has enabled the (1→3,1→4)-ß-glucanase gene locations to be defined more precisely. When DNA samples from wheat nullisomic-tetrasomic lines, each lacking one of the 21 wheat chromosomes, were subjected to Southern analysis, DNA fragments that hybridized to the (1→3,1→4)-ß-glucanase probe were absent from nullisomic lines of the homoeologous group 1 and group 7 wheat chromosomes (Fig. 8a). This indicates that the genes for (1→3,1→4)-ß-glucanase are located on these chromosomes. Similar analyses of DNA from ditelosomic lines, which lack specific chromosome arms, showed that the genes were carried on the long arms of each of the 6 chromosomes (not shown). Restriction fragment length polymorphisms (RFLPs) were observed with restriction digests of DNA prepared from 13 barley varieties, 13 wheat varieties and 2 rye varieties (Fig. 8b and further data not shown.)

DISCUSSION

There are two genes encoding (1→3,1→4)-ß-glucan endohydrolases in barley (Woodward et al. 1982; Loi et al. 1988). Here we have isolated and sequenced a gene encoding isoenzyme EI and characterized cDNA clones of isoenzyme EI and EII mRNAs. No clones carrying the gene for isoenzyme EII were detected during the initial or subsequent screening of the genomic library.

The primary structure of the isoenzyme EI gene and its corresponding protein are typical of plant genes and proteins in terms of content, size and position of sequence motifs for transcription, RNA processing, translation (Table 2) and protein processing. However, the presence of sequence motifs in the gene and primer extension data (Figs. 1 and 2) imply that there are two

promoters. In young leaves and aleurones the distal promoter was the more active, as shown by the primer extension (Fig. 2) and PCR (Fig. 7) data. However we cannot yet rule out that the proximal promoter is expressed in specialized tissues or under physiological conditions not tested here. Until the use of the proximal promoter is investigated further, we can only speculate on the reason for the existence of two promoters. One plausible hypothesis involves tissue-specific and developmentally-regulated transcription from different promoters, coupled with alternative splicing of an associated intron. These phenomena are well-documented in mammalian and plant gene expression (Young et al. 1981; Breithart et al. 1987; Brown and Feix 1990; Chelly et al. 1990). Furthermore, there is evidence that the (1→3,1→4)-ß-glucanase genes are indeed subject to tissue-specific and developmental regulation (Stuart et al. 1986; McFadden et al. 1988).

The two barley (1→3,1→4)-ß-glucanase isoenzymes are probably derived from single copy genes that arose by duplication of a common ancestral gene (Loi et al. 1988; Woodward et al. 1982). Using euplasmic wheat-barley addition lines, Loi et al. (1988) concluded that the genes are now located on different chromosomes. These disomic addition lines contain a full complement of wheat chromosomes together with barley chromosomes 1, 2, 3, 4, 6 or 7, in a wheat cytoplasm background. A disomic form of addition line 5 is not available because plants carrying this chromosome are self-sterile (Islam and Shepherd 1981). One of the barley genes was shown to be located on chromosome 1, but the other could not be detected in the available addition lines and, by a process of elimination, was assumed to be located on chromosome 5 (Loi et al. 1988). Here we have used aneuploid and ditelosomic wheat lines that lack specific

chromosomes or chromosome arms to show that the two (1→3,1→4)-ß-glucanase genes are carried on the group 1 and group 7 chromosomes of wheat (Fig. 8a); these are homoeologous to barley chromosomes 5 and 1, respectively (Islam and Shepherd 1981). Furthermore, the 3.8kb HindIII fragment observed by Loi et al. (1988) and seen also in Fig. 8b has been cloned and sequenced, and corresponds to the isoenzyme EI gene. Thus, it can be concluded that the gene encoding (1→3,1→4)-ß-glucanase isoenzyme EI is carried on barley chromosome 5, while the isoenzyme EII gene is located on barley chromosome 1. Examination of DNA from 13 barley varieties, cut with a number of restriction enzymes, shows that RFLPs occur, but that these appear to be restricted to one gene (Fig. 8 and other data not shown here). The same effect is observed in 13 hexaploid wheat varieties. Because the conserved 3.8kb HindIII fragment (Fig. 8b) is known to be the isoenzyme EI gene, and within the constraints imposed by the relatively small number of barley varieties and restriction enzymes examined, it appears that the isoenzyme EI gene is conserved while the isoenzyme EII gene exhibits significant polymorphism. The RFLPs may prove useful in the construction of detailed genetic maps for barley and wheat, which may in turn be of value in breeding programs through the identification of linkages between marker genes and genes of agronomic or commercial importance (Beckmann and Soller 1986; Sharp et al. 1988). For example, the ability of barley grain to rapidly produce high levels of (1→3,1→4)-ß-glucanase activity is closely correlated with malting performance and is therefore an important selection criterion in breeding programs (Stuart et al. 1988).

It is likely that the evolution of multiple genes for barley (1→3,1→4)-ß-glucanase is associated with some

functional specialization of the different types of protein. This specialization is reflected in the differential expression of the EI and EII genes and also in differences in the properties to the two proteins. For example, the mature barley (1→3,1→4)-ß-glucanases exhibit detectable differences in mobility during sodium dodecyl sulphate polyacrylamide gel electrophoresis. Early estimations indicated that isoenzymes EI and EII have apparent M_r values of 28000 and 30000, respectively (Woodward and Fincher 1982a), while more recent determinations yield apparent values of 30000 and 32000, respectively (Hoj et al. 1989). Actual molecular weights calculated from deduced amino acid sequences are 32151 for isoenzyme EI and 32097 for isoenzyme EII. Thus, the differences in electrophoretic mobility of the two isoenzymes may result from differences in their isoelectric points or in their degree of glycosylation. The purified isoenzyme EI carries only traces of associated carbohydrate, while isoenzyme EII has 3.6% by weight of carbohydrate, of which 3 residues are probably N-acetylglucosamine (Woodward and Fincher 1982a). It may be significant in regard to differences in their degree of glycosylation that isoenzyme EII has a single potential N-glycosylation site at amino acid residue 190 (Fig. 3) where an AAC codon (asparagine) is found in the isoenzyme EII cDNA (Fincher et al. 1986; Fig. 3). At the corresponding position in the isoenzyme EI gene, an ACC (threonine) codon is present (Fig. 3) and, as a result, isoenzyme EI has no N-glycosylation site. It has been suggested that the differences in carbohydrate content of the (1→3,1→4)-ß-glucanase isoenzymes are responsible for dramatic differences in their respective thermostabilities, which have been observed in both unpurified extracts of germinating barley (Loi et al 1987) and with highly purified enzyme preparations (Woodward and Fincher 1982b). In each case isoenzyme EI

is far less stable at elevated temperatures than the more heavily glycosylated isoenzyme EII. The availability of cDNA clones encoding the two (1→3,1→4)-ß-glucanase isoenzymes opens the way for an examination of the effects of glycosylation on thermostability using site-directed mutagenesis.

The differential expression of the isoenzyme EI and EII genes in the scutellum and aleurone cells, as suggested previously by analysis of enzyme accumulation (Stuart et al. 1986) and which is now confirmed using specific oligonucleotide probes complementary to the 3' non-translated regions of their mRNAs (Fig. 5). Presumably, isoenzyme EI and EII proteins with slightly different properties fulfil complementary functions in the various tissues examined. A third isoenzyme, designated isoenzyme EIII, was also secreted from isolated scutella (Stuart et al. 1986) but this is now believed to be a (1→3)-ß-glucanase that cross-reacted with polyclonal antibody preparations used in the Western blot analyses (unpublished data). Specific monoclonal antibodies (Hoj et al. 1990) are available to further investigate this possibility.

Clearly there are several factors affecting expression of the different (1→3,1→4)-ß-glucanase genes in barley. In isolated aleurone layers, and possibly also in the scutellum, expression of (1→3,1→4)-ß-glucanase mRNA is enhanced by GA (Mundy and Fincher 1986; Stuart et al. 1986). However there must also be other unidentified stimuli that control the expression of isoenzyme EI but not EII mRNAs in the scutellum and young leaf tissues (Fig. 5).

The two (1→3,1→4)-ß-glucanases in germinated barley grain clearly function to depolymerize the (1→3,1→4)-ß-

glucans of the endosperm cell walls, which represent a physical barrier between hydrolytic enzymes secreted from peripheral tissues and their substrates within the cells of the starchy endosperm. Northern analyses (Fig. 5) also indicate that the isoenzyme EI gene is transcribed at relatively high levels in young leaves, as they emerge from the coleoptiles approximately 10 days after the initiation of germination. The role of (1→3,1→4)-ß-glucanase isoenzyme EI produced at this time may be to loosen the cell wall matrix during cell expansion and elongation in growing tissues (Hoson and Nevins 1989; Sakurai and Matsuda 1978). However, one might anticipate that the levels of enzyme required for such loosening would be low, whereas the observed levels of isoenzyme EI mRNA in young leaves is higher than in germinating grain, where extensive degradation of endosperm cell walls is achieved. If enzyme activity in young leaves is commensurate with (1→3,1→4)-ß-glucanase mRNA levels, and if (1→3,1→4)-ß-glucans are major constituents of walls in young barley leaves, the results shown in Fig. 5 would suggest that large scale, rapid cell wall degradation or turnover is occuring in the leaves at this time. The obvious explanation for the high levels of (1→3,1→4)-ß-glucanase mRNA is that the enzyme is participating in the formation of intercellular airspaces that are necessary for diffusion of carbon dioxide, oxygen and water vapour in parts of the leaf that initially have no direct access to the atmosphere. These airspaces develop by selective cell dissolution (lysogeny) or more often by cell separation (schizogeny) (Fincher and Stone 1981). Extensive erosion of cell walls has been observed during the formation of airspaces between palisade cells in developing leaves of Phaseolus vulgaris (Jeffree et al. 1986). It will be interesting to determine whether (1→3,1→4)-ß-glucanase production is involved in other stages in plant development where intercellular spaces

develop, for example in the roots of paddy rice.

TABLE 1: Comparison of directly determined amino acid composition of (1→3,1→4)-ß-glucanase isoenzyme EI and the composition deduced from the genomic clone

	Number of Residues			Number of Residues	
	Protein*	DNA		Protein*	DNA
Asx	32	32	Val	31	31
Thr	16	16	Met	11	11
Ser	25	25	Ile	10	10
Glx	19	19	Leu	17	17
Pro	19	18	Tyr	17	17
Gly	31	31	Phe	12	12
Ala	42	42	His	5	5
Cys	1	2	Lys	7	7
Trp	3	4	Arg	7	7
			Total	305	306

*The numbers of residues were calculated from the molar composition (Woodward and Fincher 1982a) assuming a total of 306 residues. The total number of calculated amino acids is 305 because of errors introduced by approximating individual values to the nearest integer.

TABLE 2: Sequence Motifs in the Barley (1→3,1→4)-ß-Glucanase Gene

Motif	Region in gene	Sequence	Position
TATA Box	consensus	ACTATATATAG	-25 to -39
	proximal promoter	AGTATATAGAG	-38 to TSP
	distal promoter	CCTATATAAGG	-54 to TSP
Transcription start point (TSP)	consensus	CTCATCA	1
	proximal promoter	ACTAGTT	1 (2836)
	distal promoter	AAGAGCT	1 (457)
Translation start point	consensus	TAAACAATGGCT	1st ATG 3' TSP
	proximal promoter	GCCATCATGGGA	not 1st ATG
	distal promoter	AAGAGAATGGCA	1st ATG 3' TSP
Intron 5' junction	consensus	AG↓GTAAG	
	proximal promoter	TG↓GTGCA	(3077)
	distal promoter	AA↓GTGAT	(619)
Intron 3' junction	consensus	TTTGCAG	
	both promoters	ATTGCAG	(3132)

Consensus sequences for plant TATA boxes, TSP and translation start from Joshi (1987), and for intron splice sites from Hanley and Schuler (1988). Numbers in parentheses show the position of the motif in the nucleotide sequence in Fig. 1.

LEGENDS TO FIGURES

Fig. 1 Complete nucleotide sequence and derived amino acid sequence for a 4643 bp barley genomic DNA fragment carrying the gene for (1→3, 1→4)-β-glucanase isoenzyme EI. Possible TATA boxes and transcription start points are underlined for both the distal promoter (nucleotides 420 and 474) and a putative proximal promoter (2797 and 2836), and a potential polyadenylation signal is shown (4399). The intron boundaries are indicated by arrows, as is the NH_2-terminal isoleucine residue of the mature protein.

Fig. 2 Polyacrylamide gel electrophoresis of cDNAs generated by primer extension experiments from oligonucleotides complementary to the distal (a) and proximal (b) promoters. For the distal promoter (a), 5µg scutellar (upper lane) or leaf (lower lane) RNA was used and in each case major termination products of 41 and 38 nucleotides are seen. The nucleotide sequence is derived from M13. For the proximal promoter (b) the product was derived from 50µg scutellar RNA, is 241 nucleotides in length and is shown with nucleotide sequence generated from the isoenzyme EI gene itself with the same oligonucleotide used for the primer extension. Details of oligonucleotide sequences and locations are described in the text.

Fig. 3 Amino acid sequence similarities between barley (1→3,1→4)-ß-glucanase isoenzyme EI and isoenzyme EII. Only the residues of isoenzyme EII that differ from the corresponding residue in isoenzyme EI are shown. The underlined residues indicate the N-glycosylation site in isoenzyme EII.

Fig. 4(a) Comparison of the nucleotide sequences of cDNAs encoding (1→3,1→4)-ß-glucanase isoenzymes EI and EII in the region of the signal peptides and their 5' flanking sequences. The vertical lines indicate nucleotide identity and standard one letter abbreviations are used to show the deduced amino acid sequences of the signal peptides. Arrows indicate the processing point of the signal peptide, adjacent to the NH_2-terminal residue of the mature protein. (b) The nucleotide sequence of the 3' untranslated region of the cDNA encoding (1→3,1→4)-ß-glucanase isoenzyme EII. Comparison of this sequence with the corresponding 3' region of the genomic clone for isoenzyme EI (Fig. 1,

beginning at nucleotide 4062) reveals no sequence similarity.

Fig. 5 Northern analyses of total RNA preparations from aleurone (A), scutellum (S), young leaves (YL), mature leaves (ML), roots (R), coleptiles (C) and stems (St). Filters were probed with a DNA fragment that binds to both isoenzyme EI and isoenzyme EII mRNA (panel a), with an oligonucleotide specific for the 3' untranslated region of isoenzyme EI mRNA (panel b), and with an oligonucleotide specific for the 3' region of isoenzyme EII mRNA (panel c).

Fig. 6 Diagramatic representation of the barley (1→3,1→4)-ß-glucanase isoenzyme EI gene, showing certain restriction enzyme sites, distal and proximal promoter regions, and exons. The arrows indicate the approximate positions and orientations of oligonucleotides 12, 13 and 14, which were used in PCR experiments to investigate transcriptional activity of the two potential promoters (Fig. 7). The sequences and precise locations of the oligonucleotides are described in the text.

Fig. 7 Agarose electrophoresis of PCR products from aleurone (A) scutellum (S) and leaf (L). For each preparation, first strand cDNA was primed either with oligonucleotides 13/12 to detect transcription from the distal promoter (loaded in the left lane of the two lanes for each tissue) and oligonucleotides 14/12 for transcription from the putative proximal promoter (loaded in the right lane of each pair). Little or no DNA is detected with oligonucleotides 14/12, in each case. The exact positions and sequences of the oligonucleotides are indicated in the text. The mobility of 344 bp and 394 bp DNA size markers are

indicated. Controls to check the oligonucleotides include the isoenzyme EI cDNA (E), which results from transcription from the distal promoter and is 362 bp in length; as expected it has no sequence primed by oligonucleotide 14. The other control DNA is a 3.8 kb HindIII fragment of the gene that encompasses the proximal promoter but not the distal promoter (cf. Fig. 6); as observed, this cDNA should yield a PCR product of 402 bp from oligonucleotides 14/12, but has no sequence corresponding to oligonucleotide 13.

Fig. 8 (a) Southern analyses of wheat aneuploid line DNA cut with DraI and probed with a (1→3,1→4)-ß-glucanase cDNA. In each wheat aneuploid group (1 to 7) there are three lines that are nullisomic for that particular chromosome from the A, B and D genomes. The absence of bands in the wheat nullisomic lines from groups 1 and 7 indicate that the (1→3,1→4)-ß-glucanase genes are located on these chromosomes. Size markers are shown in the left lane and are 23.1 kb, 9.4 kb, 6.6 kb, 4.3 kb, 2.3 kb and 2.0 kb in length. (b) Southern blots of DNA from 9 barley varieties cut with HindIII and probed with a (1→3,1→4)-ß-glucanase cDNA. The 3.8 kb HindIII fragment, which corresponds to the isoenzyme EI gene, appears to be conserved, while the other fragment exhibits polymorphism.

Fig. 9 Nucleotide sequence for the isolated 1436 bp c-DNA of barley (1→3,1→4)-ß-glucanase isoenzyme EI. The putative signal peptide is highlighted; the stop codon is marked by an asterisk; and the putative polyadenylation signal is underlined.

Fig. 10 Nucleotide sequence for the isolated 1229 bp c-DNA of barley (1→3,1→4)-ß-glucanase isoenzyme EII. The putative signal peptide is highlighted; the stop

codon is marked by an asterisk; and the putative polyadenylation signal is underlined.

The matter contained in each of the following claims is to be read as part of the general description of the present invention.

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CLAIMS:

1. DNA sequence which encodes for barley (1→3,1→4)-ß-glucanase isoenzyme I, said DNA sequence having a nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

2. DNA sequence which encodes for barley (1→3,1→4)-ß-glucanase isoenzyme I, said DNA sequence being the isolate in accordance with the GenBank/EMBL Date Bank accession number X56260 dated 5th September 1990.

3. DNA sequence which encodes for barley (1→3,1→4)-ß-glucanase isoenzyme I, said DNA sequence having from nucleotides 3144 to 4061 of the nucleotide sequence according to Fig. 1 of the accompanying drawings.

4. DNA sequence according to claim 3 which is the sequence from nucleotides 3144 to 4061 of the nucleotide sequence according to Fig. 1 of the accompanying drawings, modified at nucleotides 3711 to 3719 so as to improve the heat stability of the barley (1→3,1→4)-ß-glucanase isoenzyme I translated therefrom.

5. DNA sequence according to claim 4 which is modified at nucleotide 3712 by the substitution of cytosine with adenine so as to improve the heat stability of the barley (1→3,1→4)-ß-glucanase isoenzyme I translated therefrom.

6. DNA sequence which encodes for a promotor region of barley (1→3, 1→4)-ß-glucanase isoenzyme I, said promoter region being the sequence from nucleotides 1 to 3000 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

7. DNA sequence according to claim 6 which is the sequence from nucleotides 1 to 620 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

8. DNA sequence according to claim 6 which is the sequence from nucleotides 1 to 480 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

9. DNA sequence according to claim 6 which is the sequence from nucleotides 1 to 474 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

10. DNA sequence according to claim 6 which is the sequence from nucleotides 620 to 2840 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

11. DNA sequence according to claim 6 which is the sequence from nucleotides 1800 to 2840 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

12. DNA sequence according to claim 6 which is the sequence from nucleotides 1800 to 2836 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

13. DNA sequence which encodes for an intron region of barley (1→3,1→4)-ß- glucanase isoenzyme I, said intron region being the sequence from nucleotides 619 to 3132 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

14. DNA sequence which encodes for an enhancer region of barley (1→3, 1→4)-ß-glucanase isoenzyme I, said enhancer region being the sequence from nucleotides 4060 to 4643 of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings.

15. DNA sequence which encodes for a modified barley (1→3,1→4)-ß-glucanase isoenzyme I, said DNA sequence being a modification of the nucleotide sequence conforming with Fig. 1 of the accompanying drawings by the omission or addition or substitution or rearrangement of any functionally non-critical (a) nucleotide constituents thereof, or (b) fragments thereof, or (c) multiple fragments thereof, which do not significantly affect the (1→3, 1→4)-ß-glucanase isoenzyme I activity of proteins encoded by the modified nucleotide sequence, or which do not significantly affect the promotor region(s) of the DNA sequence, or which do not significantly affect the enhancer region of the DNA sequence.

16. A synthetic or copy DNA (c-DNA) sequence which encodes for barley (1→3, 1→4)-ß- glucanase isoenzyme I, said DNA sequence having a nucleotide sequence conforming with Fig. 9 of the accompanying drawings.

17. DNA sequence which encodes for a modified barley (1→3,1→4)-ß-glucanase isoenzyme I, said DNA sequence being a modification of the nucleotide sequence conforming with Fig. 9 of the accompanying drawings by the omission or addition or substitution or rearrangement of any functionally non-critical (a) nucleotide constituents thereof, or (b) fragments thereof, or (c) multiple

fragments thereof, which do not significantly affect the (1→3, 1→4)-ß-glucanase isoenzyme I activity of proteins encoded by the modified nucleotide sequence.

18. A synthetic or copy DNA (c-DNA) sequence which encodes for barley (1→3,1→4)-ß- glucanase isoenzyme II, said DNA sequence having a nucleotide sequence conforming with Fig. 10 of the accompaning drawings.

19. DNA sequence which encodes for a modified barley (1→3,1→4)-ß-glucanase isoenzyme II, said DNA sequence being a modification of the nucleotide sequence conforming with Fig. 10 of the accompanying drawings by the omission or addition or substitution or rearrangement of any functionally non-critical (a) nucleotide constituents thereof, or (b) fragments thereof, or (c) multiple fragments thereof, which do not significantly affect the (1→3, 1→4)-ß-glucanase isoenzyme II activity of proteins encoded by the modified nucleotide sequence.

20. Plasmid or phage or other vectors embodying an isolated or synthetic DNA sequence as defined in any one of claims 1 to 19.

21. Microorganisms embodying an isolated or synthetic DNA sequence as defined in any one of claims 1 to 19.

22. Yeasts embodying an isolated or synthetic DNA sequence as defined in any one of claims 1 to 19.

23. Method for the preparation of a plasmid or phage or other vector according to claim 20, which comprises isolating the specified DNA sequence from barley plant material by digesting an appropriate DNA sample of the barley plant material with a restriction endonuclease; locating genomic DNA fragments carrying (1→3,1→4)-ß-glucanase sequences using fragments of a (1→3,1→4)-ß-glucanase copy DNA (cDNA); then excising and inserting the appropriate DNA sequence into the plasmid or phage or other vector.

24. Synthetic polypeptides having barley (1→3,1→4)-ß-glucanase activity, obtained by protein engineering or recombinant DNA expression, employing an isolate or synthetic DNA sequence as defined in any one of claims 1 to 19.

25. Synthetic polypeptides having barley (1→3,1→4)-ß-glucanase activity, obtained by protein engineering or recombinant DNA expression, employing a plasmid or phage or other vector as defined in claim 20.

26. Synthetic polypeptides having barley (1→3,1→4)-ß-glucanase activity, obtained by protein engineering or recombinant DNA expression, employing a microorganism as defined in claim 21.

27. Synthetic polypeptides having barley (1→3,1→4)-ß-glucanase activity, obtained by protein engineering or recombinant DNA expression, employing a yeast as defined in claim 22.

28. Synthetic polypeptides having barley (1→3,1→4)-ß-glucanase isoenzyme I or II activity, said polypeptides having an amino acid sequence in general conformity with any one of Figs. 1, 9 and 10 of the accompanying drawings but in a which a portion(s) of the respective amino acid sequence has been substituted with: (i) one or more amino acids of those in the amino acid sequences shown for barley (1→3,1→4)-ß-glucanase isoenzyme I or II, which provides barley (1→3,1→4)-ß-glucanase isoenzyme I- or II-like activity with improved thermal stability and is readily reproducible via recombinant DNA (rDNA) in bacterial or other expression systems; or (ii) one or more larger peptides having an amino acid sequence in general conformity with any one of Figs. 1, 9 and 10 of the accompanying drawings but in which a portion(s) of the respective amino acid sequence optionally has a deletion and/or substitution variant thereof and/or one or more additional amino acids directly attached to its amino terminus or carboxy terminus, or optionally is modified by glycosylation or phosphorylation or acetylation such that the resultant peptide can be used with or without further processing to provide a biological activity of barley (1→3,1→4)-ß-glucanase isoenzyme I or II in substantial measure.

29. Process for producing a synthetic polypeptide according to claim 24 exhibiting barley (1→3,1→4)-ß-glucanase I activity, comprising the steps of:

a) providing a vector comprising the isolated or synthetic nucleotide sequence according to claim 20 wherein the nucleotide sequence is capable of being expressed by a host containing the vector;

b) incorporating the vector into the host; and

c) maintaining the host containing the vector under conditions suitable for transcription and translation of the nucleotide sequence into said synthetic polypeptide.

30. Use of an isolated or synthetic DNA sequence as defined in any one of claims 1 to 19 in the production of barley grains having enhanced barley (1→3,1→4)-ß-glucan degradation quality for application in the malting or brewing industries.

31. Use of a plasmid or phage or other vector as defined in claim 20 in the production of barley grains having having enhanced barley (1→3,1→4)-ß-glucan degradation quality for application in the malting or brewing industries.

32. Use of a microorganism as defined in claim 21 in the production of polypeptides that degrade barley (1→3,1→4)-ß-glucan for application in the malting or brewing industries.

33. Use of a yeast as defined in claim 22 in the production of polypeptides that degrade barley (1→3,1→4)-ß-glucan for application in the malting or brewing industries.

34. Use of a synthetic polypeptide as defined in any one of claims 24 to 28 that degrades barley (1→3,1→4)-ß-glucan for application in the malting or brewing industries.

35. Malting process or brewing process involving the use of barley grains embodying an isolated or synthetic DNA sequence as defined in any one of claims 1 to 19 so as to enhance the degradation of barley (1→3,1→4)-ß-glucan.

36. Malting process or brewing process involving the use of barley grains embodying an isolated or synthetic DNA sequence introduced via a plasmid or phage or other vector as defined in claim 20 so as to enhance the degradation of barley (1→3,1→4)-ß-glucan.

37. Malting process or brewing process involving the use of a synthetic polypeptide as defined in any one of claims 24 to 28 so as to enhance the degradation of barley (1→3,1→4)-ß-glucan.

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   1  cgttgtgtcaattcttcttacgtaagttatgtcggattccaccgaacacggtccttgcgtgaaaaaaccgccgccgaatgtcgttgagtaagacgtgaacatatccgacgcggcgcgacc  120

 121  catcattgacctagaaacttcacttcatggtacatcatgggtggagtccaaaattcaaactatttttcaaaagttgtttggtaccacttatgaatgaattatcccccttcccctac  240

 241  ctgcaacaacaacctggtgtaccggatacctctgcccaccaccagacacacactgtgagaaggcggtgacgcatgcaaaccggcctaggtagtcaatcgcaacaggctaaataaatgtc  360

 361  gctggaggcgttgggcctgcgctcccgagtggattggaccgaactatgtctcctcggatcctatataagggcctgcaccccgttgtggcctcaccagaaaagaaacacaacaagagctttt  480

 481  acagagagccttggcatcacccaccacccacccacccctcaccctccaacgcagctagagagaagaaagaatggcaggccaaggcgttgcctccatgttggctctggcattgctcctcggagcct  600
                                                                                    M  A  G  Q  G  V  A  S  M  L  A  L  L  L  L  G  A  F
                                                                                    ↓
 601  tcgcctccatcccacaaagtgattccccttcctccctcctctcctcttgaagtgattgggtgcagttttttttttttcatgcagggagtttctttgaagatagtaatacgtacgtt  720
                                                                                    A  S  I  P  Q  S

 721  gatcttagcttttcattaagagaaggcattagggagctagctaggtagccggcggccatggttgtacccatgctcacataaacctctgtgggacgtcacatagcataccttcctctgtttagg  840

 841  tgcatgtcgctttcgttctgcgttttatttcttcccgcgccatcagtcgcatggtaaagcatgaacggccggcgcccgccggttctcattatttacctcgatgcgtccatcaatgcatggga  960

 961  gcacagtaacttttcaaaaaaaaaaattcgaaactaaacacggtaaatttttgtaaaaaaaatcgtttcactcgctaaaagaatcaggagtcttgggtttttgctgcggagtatatacgtatc 1080

1081  aaaatatatgcacaccgcacgctccaagataaaagtgcacgtcagcacgtgtgatcggccttcattgtgtttcttctatggaagaaaacgttgcaagaagtaggtattccgcaggaaaatta 1200

1201  atatcttaagaacaacaaaaaatgatatttttctagataaaacattgaaagaggaagtaagcagtgaaaacgatgcttgtagatagtttcaaactgttttaaccataaatttaatctagttgat 1320

1321  cgtacgcaaaagcatcaagcaaaagcttgcaaatgaagtctcgaaatgtttggtgaccatgcatgtcagttagctagctgcatcttttttacaaagtcagaaggtttaactaacgacctac 1440

1441  aagtagctagctagggccagtgtcttctttctccagtgtcatgctaatcacctagaaaggttttttcttcttcctcctcttccctgcatgcaaagctgtgcatgcatgtctccgacag 1560

1561  tgtcgccctagcactgtagacacgagccctaccacttaattggtggtttgttccctgattaattcggtgcaactactgctgaaccacccgtcgctaaccatcttttttctttcttccttttggg 1680

1681  tccgcatcttgacgttttttcagcgatggacgatgccgtaccggccagtccagtggtcctactcgtttcagctagccactgttccatttctttaagcttaacgaagacgattacaccagc 1800

1801  tgctagctagccagtaactataactcaactgcagcctttgtgtagtcatcatatcgacttgaaagtgcaaccggtggatccatggtacatgacgccatcgatcaccaggcactaactaa 1920

1921  ccatgcataacccaggtgttggttctgctggccttccaagtttggtctgcctgaaaagaattgtgatataatagattgtaggttagtgtggtactagctagtagttggcacttga 2040

2041  tcggccgggcagcaagttaagaagaggattaagtcgtgtacttatatggagtacttttgtcatgcacgtgaagctagaccgtttattggagcttagctgggagcagccggaggagggcatgca 2160
```

FIG. 1

```
2161 tgcatgcacgccatggcgatccacatcgatcgtatgtggactatccaacggccgtgctgcggacgttggaccagaaactttggtttgtcctagcatgtatgtatgcatgaggtctctaca 2280

2281 cccccctgtatttctaggcttcctgccaattgccatctacgtgtggtcgacctccattctgccctacgatattcctggcccgtttagtttaataaatccagctagctagtgccgtgcaag 2400

2401 tactacacaatttatgccataccattgcttggcgatggcactcttaagtgtacacgtactgtaaaaatacatgtattctttctagcataattgatgtaccaactatcaagtgtaacactt 2520

2521 taatagagtagattggtgtagagttagttggtaaaccaagcaaatcagggagggtaaaagattcactgcactggaagctagacaaagttcattccttcctttaaggttatctagttcctt 2640

2641 tttctccttgctcgtggtagagtagctagtagctagtgacaagtcggtcaaggcgccggccgtgaaaatagcaatgttcctcggccgtgtgcgtgcatctgacaccaactcgtgactgta 2760

2761 aaaaaaacaatatttaagtggtgcatcagccaccaaagtatatagagagaaagagataaaaaaatgtgcaaaactagttagctagaggcttacacgcatgcatccatgcatgcgtacaag 2880

2881 aatttcagttaaacgtcctttttcggggagttagtattatccctcgccaacaggtcaaaaggctctgtgtgcatgtgtgttcatgcatcgccgccattcttgcttattggtttcttttttta 3000

3001 tattccatcacatgccatcatgggagaattttttaatttctactatggcaatggaacagtgctactactctacctggtgcaaataattgatttgtgaaggttaactaaccgaggttat 3120
                          M  G  E  F  F  N  F  L  L  W  Q  W  N  S  A  T  T  L  P  G

3121 attacattgcaggcgtggagtccatcggggtgtgctacggcatgagcgccaacaatctgccggcggcgagcaccgtggtcaacatgttcaagtccaacgggatcaactccatgcggctgt 3240
               V  E  S  I  G  V  C  Y  G  M  S  A  N  N  L  P  A  A  S  T  V  V  N  M  F  K  S  N  G  I  N  S  M  R  L  Y

3241 acgctcccgaccaggcggcgctgcaggcggtcggcggcacgggcgtgaacgttgttgtgggcgcgcccaacgacgtgctctccaacctcgccgccagtcccgcagcggctgcatcgtggg 3360
      A  P  D  Q  A  A  L  Q  A  V  G  G  T  G  V  N  V  V  V  G  A  P  N  D  V  L  S  N  L  A  A  S  P  A  A  A  A  S  W  V

3361 tgaggagcaacatccaggcgtaccccaaggtctccttccggtatgtctgcgtgggcaacgaggtcgccggcggcgccacccagaaccttgtccccgccatgaagaacgtgcagggcgcgc 3480
      R  S  N  I  Q  A  Y  P  K  V  S  F  R  Y  V  C  V  G  N  E  V  A  G  G  A  T  Q  N  L  V  P  A  M  K  N  V  Q  G  A  L

3481 tggcctccgccgggctgggccacatcaaggtgaccacgtcggtgtcgcaggccatcctgggggtgtacagcccgccgtccgccgggtccttcaccggagaggcggacgcgttcatgggcc 3600
      A  S  A  G  L  G  H  I  K  V  T  T  S  V  S  Q  A  I  L  G  V  Y  S  P  P  S  A  G  S  F  T  G  E  A  D  A  F  M  G  P

3601 ccgtggtgcagttccttgcccgcaccggcgcgccgctcatggccaacatctacccgtacctggcctgggcctacaacccgagcgccatggacatgagctacgcgctcttcaccgcctccg 3720
      V  V  Q  F  L  A  R  T  G  A  P  L  M  A  N  I  Y  P  Y  L  A  W  A  Y  N  P  S  A  M  D  M  S  Y  A  L  F  T  A  S  G

3721 gcaccgtggtccaggacggctcctacgggtaccagaacctgttcgacaccaccgtggacgccttctacacggccatggccaagcacggcggctccaacgtgaagctggtggtgtccgaga 3840
      T  V  V  Q  D  G  S  Y  G  Y  Q  N  L  F  D  T  T  V  D  A  F  Y  T  A  M  A  K  H  G  G  S  N  V  K  L  V  V  S  E  S

3841 gcgggtggccgtcagccggcggcacggcggcgaccccggccaacgccaggatctacaaccagtacctcatcaaccacgtcggcgcggcaccccccgccacccggcgccatcgagacct 3960
      G  W  P  S  A  G  G  T  A  A  T  P  A  N  A  R  I  Y  N  Q  Y  L  I  N  H  V  G  R  G  T  P  R  H  P  G  A  I  E  T  Y

3961 acgtcttctccatgttcaacgagaaccagaaggacaacggcgtggagcagaactggggcctcttctaccccaacatgcagcacgtctaccccatcagcttctgatgaggtagcagctacc 4080
      V  F  S  M  F  N  E  N  Q  K  D  N  G  V  E  Q  N  W  G  L  F  Y  P  N  M  Q  H  V  Y  P  I  S  F  *
```

FIG. 1 contd.

```
4081  tagtgcccgtatgtccgtacgtacgcgcgcgcgtataagagcgtgtacgccgtacgtatgcgcacattatgtattgtacagggcttgggttgggaacttgggatgcgaccgctgaggcag  4200

4201  ctcagatgcgtacgcgagtagtggcttgctatactagtgtaccagtacgtatgatttcgatggaagggaagcatatgcaaacgctccccctccttcctcgattgatcatgcacttgatacgt  4320

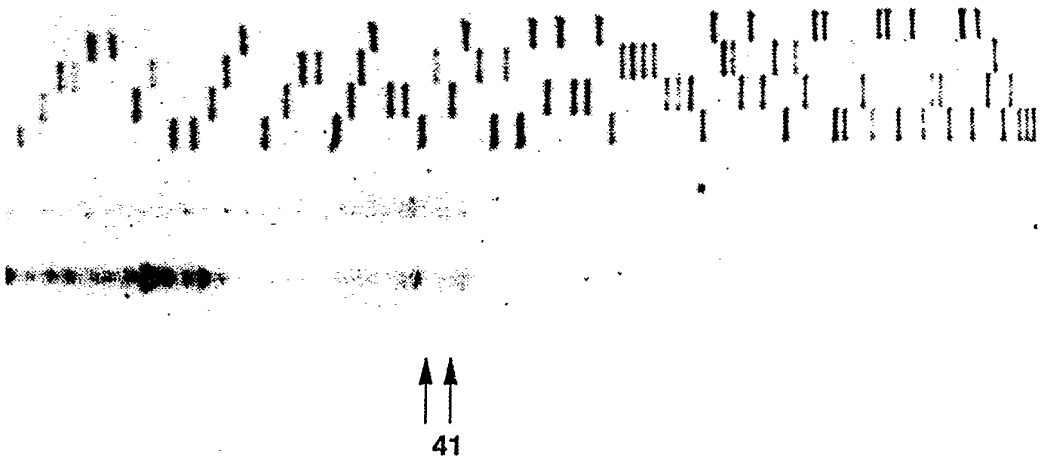
4321  acacgcatgtgtgcgtacctaggaactatattgtagggttcaaaatttcgtcaaaacttgacgaaatttgtccaaaataataaagtatgaaatatatcggcgaagaccgaatattcctat  4440

4441  atataatttttccatgtgtctatataagccctgaccacactaacatataacttccaagatgtgcaagtcggaagaaacccaattaatcgcgtcctacttaagattcaaatgagaaaaatcaca  4560

4561  tggatgacatgggcatcgtacatgagctcgtaaatggacaccagccggacacgtgtcctcagaaagggatgatgtagacgatc  4643
```

FIG. 1 contd.

a distal primer



b proximal primer

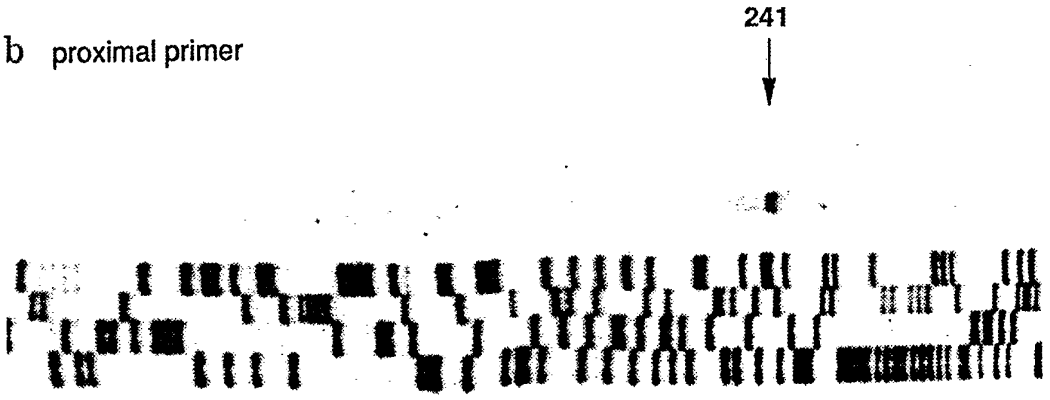


FIG. 2

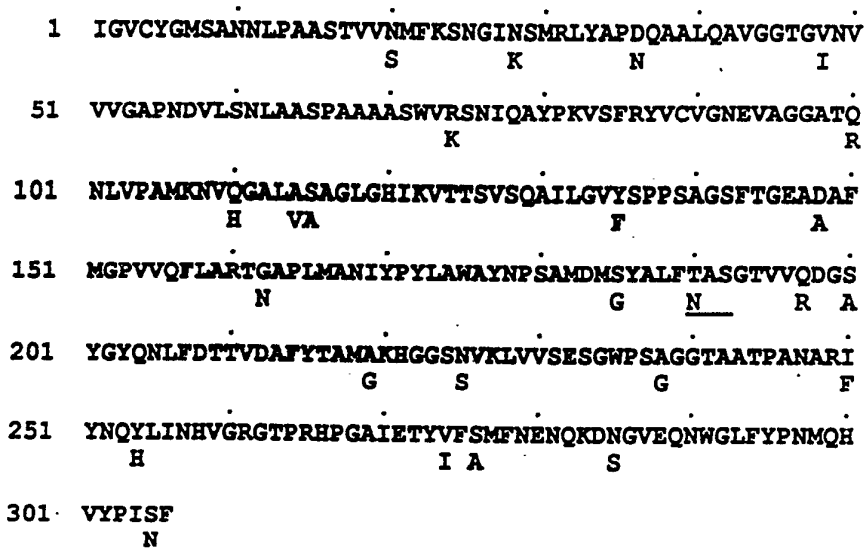

1 IGVCYGMSANNLPAASTVVNMFKSNGINSMRLYAPDQAALQAVGGTGVNV
S K N I
51 VVGAPNDVLSNLAASPAAAASWVRSNIQAYPKVSFRYVCVGNEVAGGATQ
K R
101 NLVPAMKNVQGALASAGLGHIKVTTSVSQAILGVYSPPSAGSFTGEADAF
H VA F A
151 MGPVVQFLARTGAPLMANIYPYLAWAYNPSAMDMSYALFTASGTVVQDGS
N G N R A
201 YGYQNLFDTTVDAFYTAMAKHGGSNVKLVVSESGWPSAGGTAATPANARI
G S G F
251 YNQYLINHVGRGTPRHPGAIETYVFSMFNENQKDNGVEQNWGLFYPNMQH
H I A S
301 VYPISF
N

FIG. 3

a

```
EI : CATCACCCACCCACACCCTCACCCTCCAACGCAGCTAGAGAGAGAAAGAG   50
                  |   | ||||||||||||||| |||||||| | ||||
EII:              CTTGCCCCCTCCAACGCAGGTAGAGAGAAAGAGAG    35

                  M  A  G  Q  G  V  A  S  M  L  A  L
EI : .............AATGGCAGGCCAAGGCGTTGCCTCCATGTTGGCTCTG   87
                  ||||||  ||||||||||||||||||||||||  ||||
EII: AGTTTCGAGACCCAATGGCGAGCCAAGGCGTTGCCTCCATGTTCACTCTC   85
                  M  A  S  Q  G  V  A  S  M  F  T  L

      A  L  L  L  G  A  F  A  S  I  P  Q  S  V  E  S ↓
EI : GCATTGCTCCTCGGAGCCTTCGCCTCCATCCCACAAAGCGTGGAGTCC    135
     ||||||||  ||||||||||||||  ||  |||||||  ||||||||||||
EII: GCATTGCTTCTCGGAGCCTTCGCGTCTATCCCACCAAGCGTGGAGTCG    133
      A  L  L  L  G  A  F  A  S  I  P  P  S  V  E  S ↓
```

b

```
EII: TGACGGAGCTCGTGCTCGTTAAGTCCCTACTTGTTCTTGTTAACGAGT   1099

     AAAAAGTCATGTTACGCGAACTTGACGAGCTACTCGTTTGGAGAGCCT   1147

     CTTAATTACCTCCTCTTTCCACATGAGGGATGAGAACGTATGAGTTAA   1195

     TAACCAGACCCAAAAAAAAAAAAAAAAAAAAA                   1229
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FIG. 4

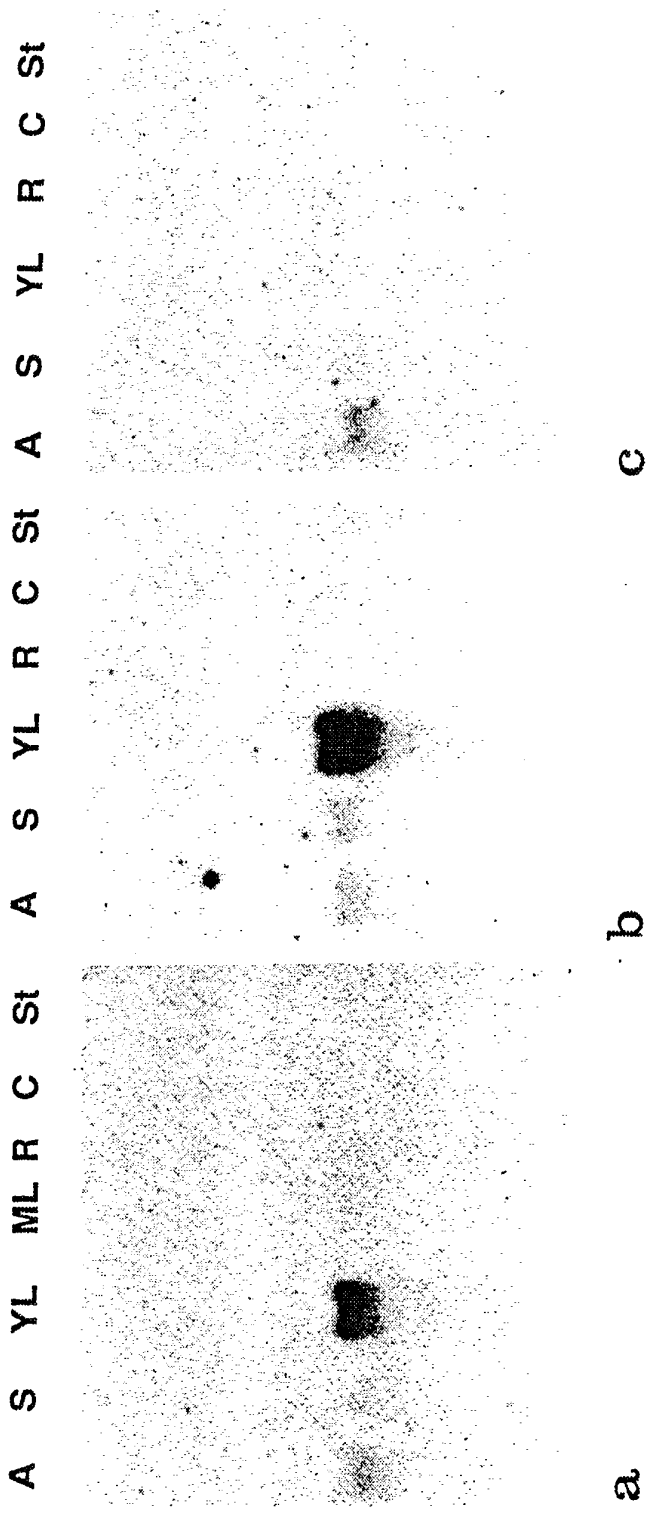

FIG. 5

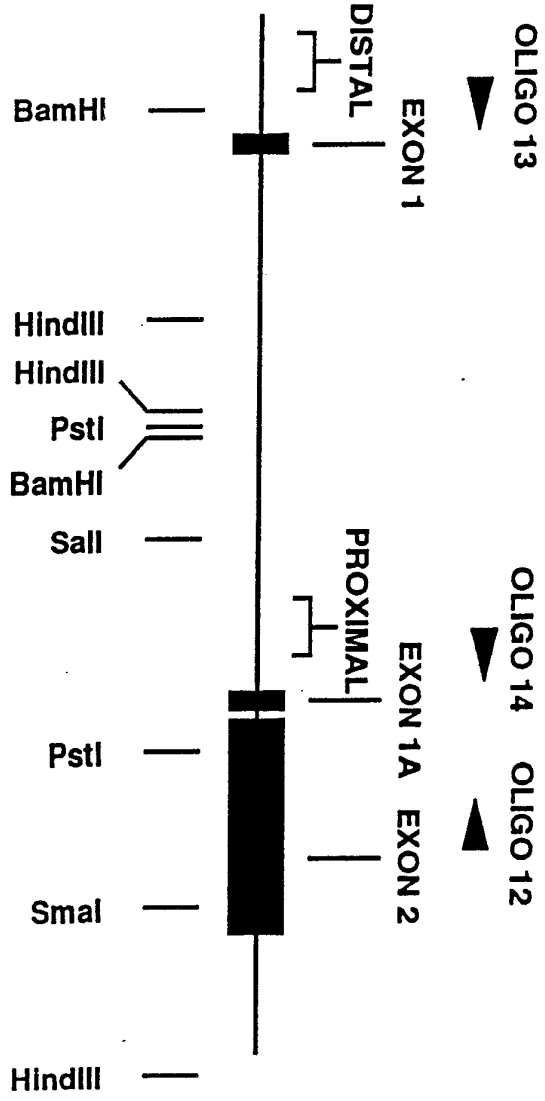
BamHI
HindIII
HindIII
PstI
BamHI
SalI
PstI
SmaI
HindIII
DISTAL
EXON 1
OLIGO 13
PROXIMAL
EXON 1A
EXON 2
OLIGO 14
OLIGO 12

FIG. 6

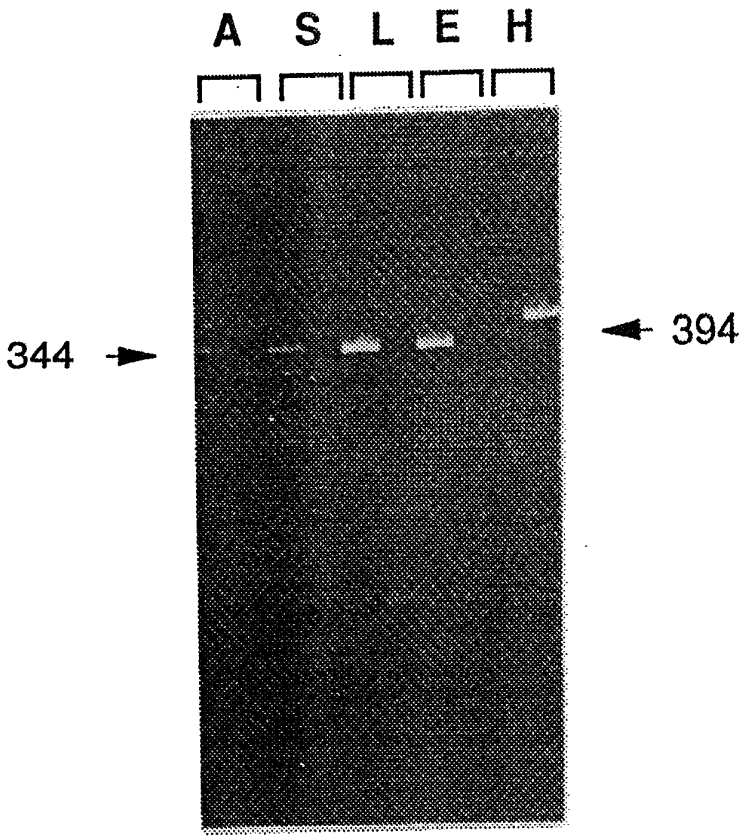

FIG. 7

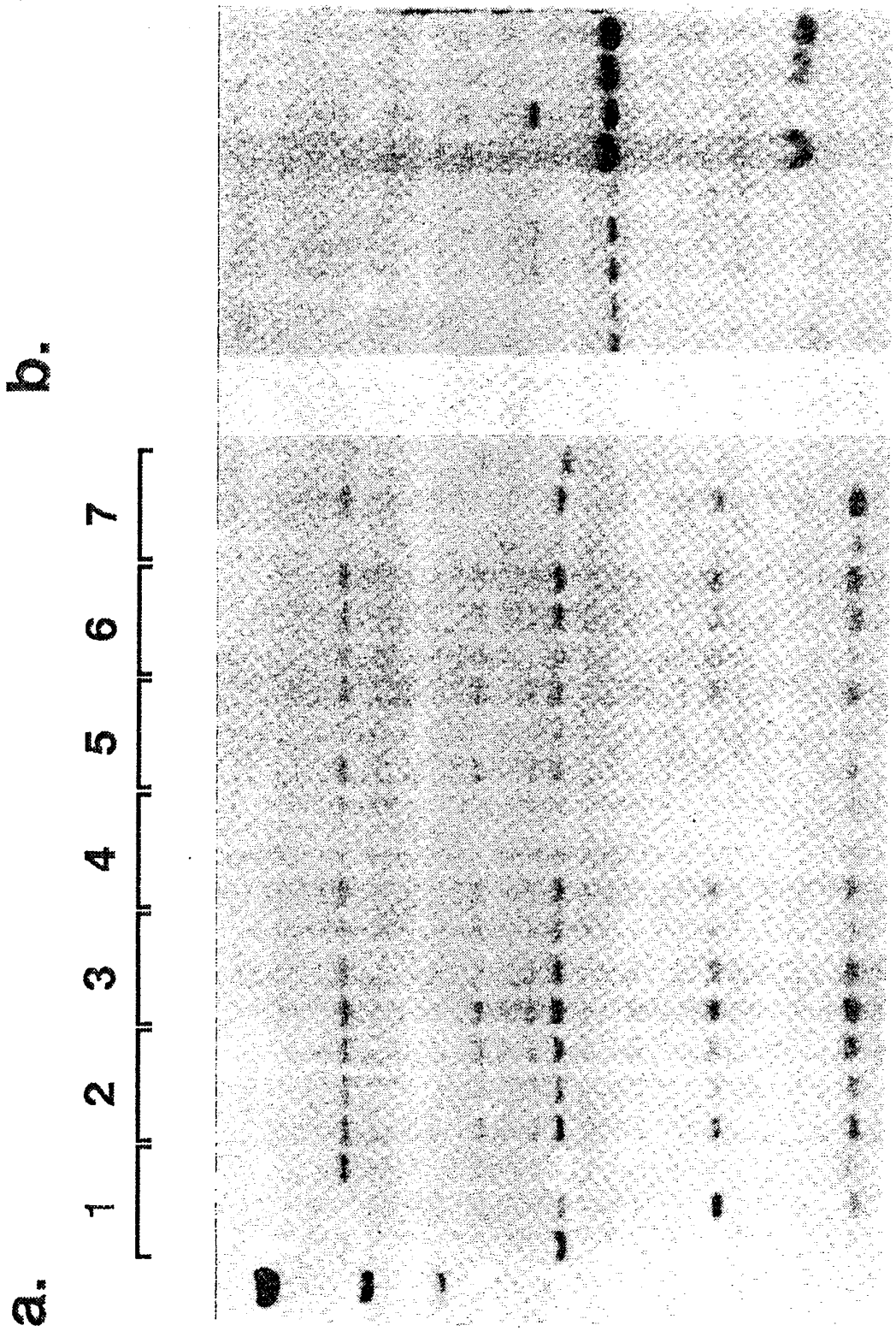

FIG. 8

```
a att ccg ttg ctg tcg CAT CAC CCA CCC ACA CCC TCA CCC TCC AAC GCA GCT AGA GAG AGA AAG AGA ATG GCA
                                                                                      Met Ala

GGC CAA GGC GTT GCC TCC ATG TTG GCT CTG GCA TTG CTC CTC GGA GCC TTC GCC TCC ATC CCA CAA AGC GTG GAG
Gly Gln Gly Val Ala Ser Met Leu Ala Leu Ala Leu Leu Leu Gly Ala Phe Ala Ser Ile Pro Gln Ser Val Glu

TCC ATC GGG GTG TGC TAC GGC ATG AGC GCC AAC AAT CTG CCG GCG GCG AGC ACC GTG GTC AAC ATG TTC AAG TCC
Ser Ile Gly Val Cys Tyr Gly Met Ser Ala Asn Asn Leu Pro Ala Ala Ser Thr Val Val Asn Met Phe Lys Ser

AAC GGG ATC AAC TCC ATG CGG CTG TAC GCT CCC GAC CAG GCG GCG CTG CAG GCG GTC GGC GGC ACG GGC GTG AAC
Asn Gly Ile Asn Ser Met Arg Leu Tyr Ala Pro Asp Gln Ala Ala Leu Gln Ala Val Gly Gly Thr Gly Val Asn

GTT GTT GTG GGC GCG CCC AAC GAC GTG CTC TCC AAC CTC GCC GCC AGT CCC GCA GCG GCT GCA TCG TGG GTG AGG
Val Val Val Gly Ala Pro Asn Asp Val Leu Ser Asn Leu Ala Ala Ser Pro Ala Ala Ala Ala Ser Trp Val Arg

AGC AAC ATC CAG GCG TAC CCC AAG GTC TCC TTC CGG TAC GTC TGC GTG GGC AAC GAG GTC GCC GGC GGC GCC ACC
Ser Asn Ile Gln Ala Tyr Pro Lys Val Ser Phe Arg Tyr Val Cys Val Gly Asn Glu Val Ala Gly Gly Ala Thr

CAG AAC CTT GTC CCC GCC ATG AAG AAC GTG CAG GGC GCG CTG GCC TCC GCC GGG CTG GGC CAC ATC AAG GTG ACC
Gln Asn Leu Val Pro Ala Met Lys Asn Val Gln Gly Ala Leu Ala Ser Ala Gly Leu Gly His Ile Lys Val Thr

ACG TCG GTG TCG CAG GCC ATC CTG GGG GTG TAC AGC CCG CCG TCC GCC GGG TCC TTC ACC GGA GAG GCG GAC GCG
Thr Ser Val Ser Gln Ala Ile Leu Gly Val Tyr Ser Pro Pro Ser Ala Gly Ser Phe Thr Gly Glu Ala Asp Ala

TTC ATG GGC CCC GTG GTG CAG TTC CTT GCC CGC ACC GGC GCG CCG CTC ATG GCC AAC ATC TAC CCG TAC CTG GCC
Phe Met Gly Pro Val Val Gln Phe Leu Ala Arg Thr Gly Ala Pro Leu Met Ala Asn Ile Tyr Pro Tyr Leu Ala

TGG GCC TAC AAC CCG AGC GCC ATG GAC ATG AGC TAC GCG CTC TTC ACC GCC TCC GGC ACC GTG GTC CAG GAC GGC
Trp Ala Tyr Asn Pro Ser Ala Met Asp Met Ser Tyr Ala Leu Phe Thr Ala Ser Gly Thr Val Val Gln Asp Gly

TCC TAC GGG TAC CAG AAC CTG TTC GAC ACC ACC GTG GAC GCC TTC TAC ACG GCC ATG GCC AAG CAC GGC GGC TCC
Ser Tyr Gly Tyr Gln Asn Leu Phe Asp Thr Thr Val Asp Ala Phe Tyr Thr Ala Met Ala Lys His Gly Gly Ser

AAC GTG AAG CTG GTG GTG TCC GAG AGC GGG TGG CCG TCA GCC GGC GGC ACG GCG GCG ACC CCG GCC AAC GCC AGG
Asn Val Lys Leu Val Val Ser Glu Ser Gly Trp Pro Ser Ala Gly Gly Thr Ala Ala Thr Pro Ala Asn Ala Arg

ATC TAC AAC CAG TAC CTC ATC AAC CAC GTC GGG CGC GGC ACC CCC CGC CAC CCG GGC GCC ATC GAG ACC TAC GTC
Ile Tyr Asn Gln Tyr Leu Ile Asn His Val Gly Arg Gly Thr Pro Arg His Pro Gly Ala Ile Glu Thr Tyr Val

TTC TCC ATG TTC AAC GAG AAC CAG AAG GAC AAC GGC GTG GAG CAG AAC TGG GGG CTC TTC TAC CCC AAC ATG CAG
Phe Ser Met Phe Asn Glu Asn Gln Lys Asp Asn Gly Val Glu Gln Asn Trp Gly Leu Phe Tyr Pro Asn Met Gln

CAC GTC TAC CCC ATC AGC TTC TGA TGA GGT AGC AGC TAC CTA GTG CCC GTA TGT CCG TAC GTA CGC GCG CGC GTA
His Val Tyr Pro Ile Ser Phe ***

TAA GAG CGT GTA CGC CGT ACG TAT GCG CAC ATT ATG TAT TGT ACA GGG CTT GGG TTG GGA ACT TGG GAT GCG ACC

GCT GAG GCA GCT CAG ATG CGT ACG CGA GTA GTA GTG GCT TGC TAT ACT AGT GTA CCA GTA CGT ATG ATT TTC GAT

GGA AGG GAA GCA TAT GCA AAC GCT CCC CCT TCC TCG ATT GAT CAT GCA CTT GAT ACG TAC ACG CAT GTG TGC GTA

CCT AGG AAC TAT ATT GTA GGG TTC AAA ATT TCG TCA AAA CTT GAC GAA ATT TGT CCA AAA TAA TAA AGT ATG AAA

TAT ATC GGC GAA GAC CGA ATA TTC cga cag caa cgg aat t
```

FIG. 9

```
aa ttc cgt tgc tgt cgC TTG CCC CCT CCA ACG CAG GTA GAG AGA AAG AGA GAG TTT CGA GAC CCA ATG GCG AGC
                                                                                Met Ala Ser

CAA GGC GTT GCC TCC ATG TTC ACT CTC GCA TTG CTT CTC GGA GCC TTC GCG TCT ATC CCA CCA AGC GTG GAG TCG
Gln Gly Val Ala Ser Met Phe Thr Leu Ala Leu Leu Leu Gly Ala Phe Ala Ser Ile Pro Pro Ser Val Glu Ser

ATC GGG GTG TGC TAC GGC ATG AGC GCC AAC AAC CTG CCG GCG GCG AGC ACC GTC GTG AGC ATG TTC AAG TCC AAC
Ile Gly Val Cys Tyr Gly Met Ser Ala Asn Asn Leu Pro Ala Ala Ser Thr Val Val Ser Met Phe Lys Ser Asn

GGG ATC AAA TCG ATG CGG CTG TAC GCT CCC AAC CAG GCG GCG CTG CAG GCC GTC GGC GGC ACG GGC ATC AAC GTC
Gly Ile Lys Ser Met Arg Leu Tyr Ala Pro Asn Gln Ala Ala Leu Gln Ala Val Gly Gly Thr Gly Ile Asn Val

GTC GTC GGG GCT CCT AAC GAC GTC CTC TCC AAC CTC GCC GCC AGC CCG GCA GCG GCC GCC TCG TGG GTC AAG AGC
Val Val Gly Ala Pro Asn Asp Val Leu Ser Asn Leu Ala Ala Ser Pro Ala Ala Ala Ala Ser Trp Val Lys Ser

AAC ATC CAG GCG TAC CCC AAG GTT TCC TTC CGG TAC GTC TGC GTC GGA AAC GAG GTC GCC GGC GGC GCC ACC CGG
Asn Ile Gln Ala Tyr Pro Lys Val Ser Phe Arg Tyr Val Cys Val Gly Asn Glu Val Ala Gly Gly Ala Thr Arg

AAC CTC GTC CCG GCA ATG AAG AAC GTG CAT GGC GCG CTC GTC GCC GCT GGG CTG GGC CAC ATC AAG GTG ACC ACG
Asn Leu Val Pro Ala Met Lys Asn Val His Gly Ala Leu Val Ala Ala Gly Leu Gly His Ile Lys Val Thr Thr

TCG GTG TCG CAG GCC ATC CTC GGC GTG TTC AGC CCG CCC TCC GCC GGG TCC TTC ACC GGG GAG GCG GCC GCG TTC
Ser Val Ser Gln Ala Ile Leu Gly Val Phe Ser Pro Pro Ser Ala Gly Ser Phe Thr Gly Glu Ala Ala Ala Phe

ATG GGC CCC GTG GTG CAG TTC CTT GCC CGC ACC AAC GCG CCG CTC ATG GCC AAC ATT TAC CCG TAC CTG GCC TGG
Met Gly Pro Val Val Gln Phe Leu Ala Arg Thr Asn Ala Pro Leu Met Ala Asn Ile Tyr Pro Tyr Leu Ala Trp

GCT TAC AAC CCG AGC GCC ATG GAC ATG GGC TAC GCT CTC TTC AAC GCG TCC GGC ACC GTG GTC AGG GAC GGC GCC
Ala Tyr Asn Pro Ser Ala Met Asp Met Gly Tyr Ala Leu Phe Asn Ala Ser Gly Thr Val Val Arg Asp Gly Ala

TAC GGG TAC CAG AAC CTG TTC GAC ACC ACC GTG GAC GCC TTC TAC ACG GCC ATG GGC AAG CAC GGC GGC TCC AGC
Tyr Gly Tyr Gln Asn Leu Phe Asp Thr Thr Val Asp Ala Phe Tyr Thr Ala Met Gly Lys His Gly Gly Ser Ser

GTG AAG CTG GTG GTG TCG GAG AGC GGG TGG CCG TCG GGC GGC GGC ACG GCG GCG ACT CCG GCC AAC GCT AGG TTC
Val Lys Leu Val Val Ser Glu Ser Gly Trp Pro Ser Gly Gly Gly Thr Ala Ala Thr Pro Ala Asn Ala Arg Phe

TAC AAC CAG CAC CTC ATC AAC CAC GTC GGG CGC GGC ACC CCA CGC CAC CCG GGC GCC ATC GAG ACC TAC ATC TTC
Tyr Asn Gln His Leu Ile Asn His Val Gly Arg Gly Thr Pro Arg His Pro Gly Ala Ile Glu Thr Tyr Ile Phe

GCC ATG TTC AAC GAG AAC CAG AAG GAC AGC GGC GTG GAG CAG AAC TGG GGA CTC TTC TAC CCC AAC ATG CAG CAC
Ala Met Phe Asn Glu Asn Gln Lys Asp Ser Gly Val Glu Gln Asn Trp Gly Leu Phe Tyr Pro Asn Met Gln His

GTC TAC CCC ATC AAC TTC TGA CGG AGC TCG TGC TCG TTA AGT CCC TAC TTG TTC TTG TTA ACG AGT AAA AAG TCA
Val Tyr Pro Ile Asn Phe ***

TGT TAC GCG AAC TTG ACG AGC TAC TCG TTT GGA GAG CCT CTT AAT TAC CTC CTC TTT CCA CAT GAG GGA TGA GAA

CGT ATG AGT TAA TAA CCA GAC CCA AAA AAA AAA AAA AAA AAA AAA Acg aca gca acg g
```

FIG. 10

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C12N 15/56, 1/19, 1/21, 9/42, 15/11, C12C 1/00, 11/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	WPAT (Derwent Database): Keywords: GLUCANASE, GLUCAN, ENDOHYDROLASE, LICHENASE, BARLEY, HORDEUM, VUGARIS Chemical Abstracts: Keywords: as above

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU : IPC : C12N 15/11, 15/42, 15/56

Bioitechnology Abstracts: Keywords: as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
X	Fincher, G.B. et al. Proceedings of the National Academy of Science, USA. Volume 83, April 1986, "Primary structure of the the (1→3, 1→4)-ß-D-glucan 4-glucohydrolase from barley aleurone" see pages 2081-2085	(18-28, 30-37)
P,X	Chemical Abstracts, Volume 115, no. 7, issued 19 August 1991 (Columbus, Ohio, USA), Litts, J.C et al. "The isolation and characterization of a barley 1,3-1,4-ß-glucanase gene" see pages 211-2, column 2, abstract no. 65758r, Eur. J. Biochem., 1990, 194(3), 831-8	(1-37)

(continued)

* Special categories of cited documents : [10]

"A" Document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior t the international filing date but later than the priority date claimed

"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 December 1991	3 January 92
International Searching Authority	**Signature of Authorized Officer**
AUSTRALIAN PATENT OFFICE	J. ASHMAN J.W. Ashman

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Chemical Abstracts, Volume 109, no. 17, issued 24 October 1988 (Columbus, Ohio, USA), Von Wettstein, D., "Genetic engineering: barley", see page 157, column 1, abstract no. 143348z, Proc. Congr.-Eur. Brew. Conv., 1987, 21st., 189-96	(1-37)
X,Y	Chemical Abstracts, Volume 109, no. 15, issued 10 October 1988 (Columbus, Ohio, USA), Thomsen, K.K. et al., "Genetic engineering of yeast: construction of strains that degrade ß-glucans with the aid of a barley gene" see page 192, column 2, abstract no. 123732s, J. Am. Soc. Brew. Chem. 1988, 46 (2), 31-6. (Also note Chemical Abstracts, Volume 110, no. 5, issued 30 January 1989, page 155, abstract no. 34743z)	(1-37)

(continued)

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4a

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
X	Chemical Abstracts, Volume 109, no. 9, issued 29 August 1988 (Columbus, Ohio, USA), Loi, L. et al, "Chromosomal location of genes encoding barley (1 3, 1 4)-ß-glucan 4-glucanohydrolases" see page 191, column 1, abstract no. 68050r, Plant Physiol. 1988, 87(2) 300-2	(1-37)
Y	Chemical Abstracts, Volume 108, no. 21, issued 23 May 1988 (Columbus, Ohio, USA), McFadden, G.I. et al., "Expression sites and developmental regulation of genes encoding (1 3, 1 4)-ß-glucanases in germinated barley" see page 423, column 1, abstract no. 183754q, Planta, 1988, 173(4), 500-8	(1-37)
Y	Chemical Abstracts, Volume 104, no. 3, issued 20 January 1986 (Columbus, Ohio, USA), Mundy, J. et al. "Messenger RNAS from the scutellum and aleurone of germinating barley encode (1 3, 1 4)-ß-D-glucanase, α-amylase and carboxypeptidase", see page 317, column 2, abstract no. 17764r, Plant Physiol., 1985, 79(3), 867-71	(1-37)
X	Biotechnololgy Abstracts, Volume 90, Olsen, O. and K.K. Thomsen "Processing and secretion of barley (1-3, 1-4)-beta glucanase in yeast", abstract no. 3506, class K1, Curr. Microbiol (1990) 21(4), 267-71	
X	Biotechnology Abstracts, Volume 87, Jackson, E.A. et al., "Construction of a yeast vector directing the synthesis and release of barley (1-3,1-4)-beta-glucanase", abstract no 02367, Class A1, Carlsberg Res. Comm., (1986) 51(6), 445-8	(1-37)