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(54) Titre : UTILISATION COMME GENE DE SELECTION D'UNE SEQUENCE D'ADN CODANT POUR UNE PROTEINE
DEGRADANT L'ACIDE OXALIQUE

(54) Title: USE OF A DNA SEQUENCE CODING FOR AN OXALIC ACID DEGRADING PROTEIN AS A SELECTION
GENE

(57) **Abrégé/Abstract:**

The invention relates to the novel use of a sequence coding for a protein capable of degrading oxalic acid to select plant cells which have integrated a gene of interest, and a novel process for selecting, on oxalic acid, cells, calluses or plants transformed by this recombinant DNA.



ABSTRACT

The invention relates to the novel use of a sequence coding for a protein capable of degrading oxalic acid to select plant cells which have integrated a gene of interest, and a novel process for selecting, on oxalic acid, cells, calluses or plants transformed by this recombinant DNA.

Use of a DNA sequence coding for a protein capable of
degrading oxalic acid as selection gene

The invention relates to a novel use of a DNA
5 sequence coding for a protein capable of degrading
oxalic acid for selecting plant cells, in particular
plant cells which have integrated a gene of interest,
and a novel process for selecting, on oxalic acid,
cells, calluses or transformed plants.

10 Since the arrival of the first transgenic
plants in 1983, the number of these has enjoyed an
accelerated growth. The transformation vectors which
have been developed in this period and which are always
used, such as, for example, the vector pBIN19
15 (M. Bevan, 1984, Nucl. Ac. Res., 12, 8711-8721) produce
a resistance gene to an antibiotic, kanamycin, as
selection gene of transformed plant cells. The use of
this mode of selection, generally easy to carry out,
cheap and applicable to numerous plant species, has
20 become very widespread in research laboratories.

Since the first field trials, thus out of
confinement, of transgenic plants took place in 1986,
the use of a resistance gene to an antibiotic as
selection gene has been the subject of numerous reviews
25 (cf. particularly F. Casse-Delbart and M. Tepfer, 1990,
Biofutur, June, 56-59 as well as J. Bryant and
S. Leather, 1992, Tibtech, 10, 274-275). The risk of
transmission of the resistance gene of the transgenic
plant to a soil bacterium and, subsequently, to a
30 bacteria which is potentially pathogenic for man,
although a priori being very slight and still never
demonstrated, is not to be neglected (J.A. Heinemann,
1991, TIG, 7, 181-185).

Numerous substitutes for the resistance gene to
35 kanamycin have been proposed (M. Ratner, 1989, Bio-
Technology, 7, 337-341) but the majority produce either
resistance to another antibiotic (such as, for example,
gentamicin, streptomycin, methotrexate or hygromycin),
or resistance to a herbicide (such as, for example,

bromoxynil or phosphinothrycin), which raises similar objections. Another approach proposed has been the elimination, after use, of the resistance gene due to a system of homologous recombination (E.C. Dale and D.W. Ow, 1991, Proc. Natl. Acad. Sci. USA, 88, 10558-10562). This system, called the cre/lox system, presents, however, the disadvantage of requiring a subsequent transformation of the transgenic plants to introduce into them the cre gene responsible for the recombination, followed by a self-fertilization of the plants in order to be able to segregate in the descendents this cre gene from the gene of interest. It is thus not simple to use. In addition, this system leaves in the transgenic plants a copy of the lox sequences, which do not have any agronomic interest.

The invention proposes, as selection gene of the transgenic plants, a gene coding for a protein capable of degrading oxalic acid, a phytotoxin produced by numerous species of fungi. This selection gene which remains in the transgenic plants has an agronomic interest because it has a phytoprotective effect with respect to these fungi.

The invention thus relates to the use of a DNA sequence coding for a protein capable of degrading oxalic acid as selection gene for plant cells.

In accordance with an aspect of the present invention, there is provided use of a DNA sequence coding for an enzyme having oxalate oxidase activity as selection agent for transformed plant cells.

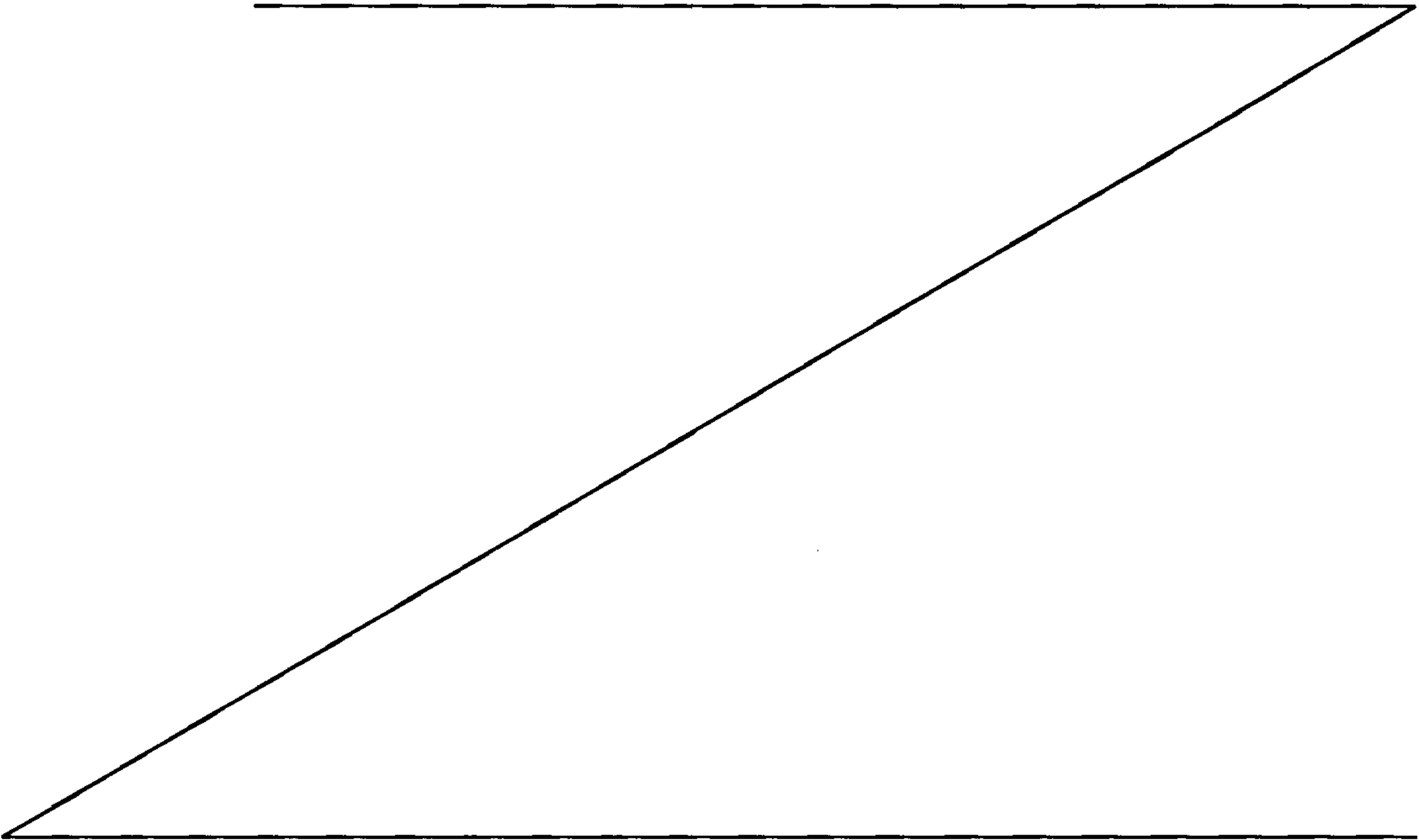
In accordance with another aspect of the present invention, there is provided a Process for selecting, on oxalic acid, transgenic calluses or transformed plants by a DNA

- 2a -

sequence coding for an enzyme having oxalate oxidase activity, wherein it consists of culturing the calluses or the plants on a culture medium containing oxalic acid and calcium in the presence of a chelating agent having an affinity for calcium higher than that of oxalic acid, said chelating agent being chosen from amongst EDTA and EGTA.

he protein capable of degrading oxalic acid can be an enzyme with decarboxylase activity, such as, especially, the oxalate decarboxylase of Aspergillus or of Collybia velutipes or preferably an enzyme with oxidase activity, such as, for example, the oxalate oxidase of barley (marketed by Boehringer, ref. 567 698), of sorghum (Chandra S. Pundier, 1991, Phytochemistry, 30, 4, p. 1065) or of moss [Mnium menziesii (M.F. Laker et al., 1980, Clinical Chemistry, 26, 7, 827)].

A particularly valued protein with oxalate oxidase activity is the protein of sequence [SEQ ID No.1].



Met	Gly	Tyr	Ser	Lys	Thr	Leu	Val	Ala	Gly	Leu	Phe	Ala	Met	Leu	Leu
1				5					10					15	
Leu	Ala	Pro	Ala	Val	Leu	Ala	Thr	Asp	Pro	Asp	Pro	Leu	Gln	Asp	Phe
				20				25					30		
Cys	Val	Ala	Asp	Leu	Asp	Gly	Lys	Ala	Val	Ser	Val	Asn	Gly	His	Thr
				35			40					45			
Cys	Lys	Pro	Met	Ser	Glu	Ala	Gly	Asp	Asp	Phe	Leu	Phe	Ser	Ser	Lys
				50			55					60			
Leu	Ala	Lys	Ala	Gly	Asn	Thr	Ser	Thr	Pro	Asn	Gly	Ser	Ala	Val	Thr
65					70					75				80	
Glu	Leu	Asp	Val	Ala	Glu	Trp	Pro	Gly	Thr	Asn	Thr	Leu	Gly	Val	Ser
				85					90					95	
Met	Asn	Arg	Val	Asp	Phe	Ala	Pro	Gly	Gly	Thr	Asn	Pro	Pro	His	Ile
				100				105						110	
His	Pro	Arg	Ala	Thr	Glu	Ile	Gly	Ile	Val	Met	Lys	Gly	Glu	Leu	Leu
				115			120					125			
Val	Gly	Ile	Leu	Gly	Ser	Leu	Asp	Ser	Gly	Asn	Lys	Leu	Tyr	Ser	Arg
				130			135					140			
Val	Val	Arg	Ala	Gly	Glu	Thr	Phe	Leu	Ile	Pro	Arg	Gly	Leu	Met	His
145					150					155				160	
Phe	Gln	Phe	Asn	Val	Gly	Lys	Thr	Glu	Ala	Ser	Met	Val	Val	Ser	Phe
				165					170					175	
Asn	Ser	Gln	Asn	Pro	Gly	Ile	Val	Phe	Val	Pro	Leu	Thr	Leu	Phe	Gly
				180				185						190	
Ser	Asn	Pro	Pro	Ile	Pro	Thr	Pro	Val	Leu	Thr	Lys	Ala	Leu	Arg	Val
				195			200					205			
Glu	Ala	Arg	Val	Val	Glu	Leu	Leu	Lys	Ser	Lys	Phe	Ala	Ala	Gly	Phe
				210			215					220			

or of sequence having a high degree of homology with
5 the sequence [SEQ ID No.1].

The sequence [SEQ ID No.1] is that of wheat
germin, a protein induced during the germination of
wheat, whose sequence has been described by
E. Dratewka-Kos, 1989, J. Biol. Chem., 264, 4896-4900
10 and B.G. Lane, 1991, J. Biol. Chem., 266, 10461-10469.

A high degree of homology here signifies a
homology (ratio between the identical amino acids and
the total number of amino acids) of at least 80% of the

amino acid sequences, when they are aligned according to maximum homology according to the optimum alignment method of the sequences of Needleman and Wunsch, 1970, J. Mol. Biol, 48, 443-453. This method is particularly
 5 used in the UWGCG software of the University of Wisconsin: Devereux et al., 1984, Nucl. Ac. Res., 12, 387-395 - GAP option.

An example of a protein having a high degree of homology with the sequence [SEQ ID No.1] is that of
 10 oxalate oxidase of barley whose sequence is described in the Patent Application WO 92/14824 (this sequence has a homology of 96% with the sequence [SEQ ID No.1], or that of other oxalate oxidases of cereals close to wheat.

15 Considering the degeneration of the genetic code, there are a large number of nucleotide sequences coding for the oxalate oxidase of sequence [SEQ ID No.1]. Among these, the sequence [SEQ ID No.2] is particularly valued

20

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ATGGGGTACT CCAAAACCCT AGTAGCTGGC CTGTTTCGCAA TGCTGTTACT AGCTCCGGCC      60
GTCTTGGCCA CCGACCCAGA CCTCTCCAG GACTTCTGTG TCGCCGACCT CGACGGCAAG      120
GCGGTCTCGG TGAACGGGCA CACGTGCAAG CCCATGTCGG AGGCCGGCGA CGACTTCCTC      180
TTCTCGTCCA AGTTGGCCAA GGCCGGCAAC ACGTCCACCC CGAACGGCTC CGCCGTGACG      240
GAGCTCGACG TGGCCGAGTG GCCCGGTACC AACACGCTGG GTGTGTCCAT GAACCGCGTG      300
GACTTTGCTC CCGGAGGCAC CAACCCACCA CACATCCACC CGCGTGCCAC CGAGATCGGC      360
ATCGTGATGA AAGGTGAGCT TCTCGTGGGA ATCCTTGGCA GCCTCGACTC CGGGAACAAG      420
CTCTACTCGA GGGTGGTGCG CGCCGGAGAG ACGTTCCTCA TCCCACGGGG CCTCATGCAC      480
TTCCAGTTCA ACGTCGGTAA GACCGAGGCC TCCATGGTCG TCTCCTTCAA CAGCCAGAAC      540
CCCGGCATTG TCTTCGTGCC CCTCACGCTC TTCGGCTCCA ACCCGCCCAT CCCAACGCCG      600
GTGCTCACCA AGGCACTCCG GGTGGAGGCC AGGGTCGTGG AACTTCTCAA GTCCAAGTTT      660
GCCGCTGGGT TT                                                                672
  
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According to a variant of the invention, the DNA sequence coding for a protein capable of degrading
 25 oxalic acid can be used in combination with a sequence of interest.

Thus, according to this variant, the invention relates to the use of the DNA sequence coding for a

protein capable of degrading oxalic acid for selecting transformed plant cells with a sequence of interest, the transformation being carried out either with the aid of two distinct vectors, one carrying the DNA
5 sequence coding for a protein capable of degrading oxalic acid, the other the sequence of interest, or with a single vector containing the two sequences above, this vector being termed recombinant DNA below.

The sequence of interest is the whole DNA
10 sequence providing an advantage to the plant cells when it is integrated into their genome. It can be, for example, an advantageous regulatory sequence. It can also be a sequence coding for a protein of interest or for a precursor of the latter.

15 According to a preferred method of carrying out the invention, the sequence of interest confers on plants resistance to pathogenic agents, such as fungi, bacteria, as well as arthropods, particularly insects and nematodes.

20 Such a sequence of interest can be, for example, a sequence coding for a protein with endochitinase activity or for a precursor of the latter. It is in fact known, as described in the Patent Application WO92/01792, that such a protein has a
25 phytoprotective effect because it is capable of degrading chitin, a polysaccharide polymer formed from units of N-acetylglucosamine associated by β -1,4 links, which is an important structural compound of the wall of most pathogenic fungi, of the exoskeleton of
30 arthropods, in particular of insects, and of the external covering of eggs and of nematode cysts.

An interesting sequence coding for a protein with endochitinase activity or for a precursor of the latter is that described in the Patent Application
35 WO92/01792, which codes for a protein comprising the sequence [SEQ ID No.3]; this sequence [SEQ ID No.3] corresponds to the sequence [SEQ ID No.1] of the Application WO92/01792.

It is particularly appreciated that this

sequence codes for a precursor of a protein with endochitinase activity, which comprises, upstream of the sequence [SEQ ID No.3], the peptide signal of sequence [SEQ ID No.4]; this peptide signal corresponds
5 to the peptide signal having the sequence [SEQ ID No.3] described in the Application WO92/01792.

It is advantageous then that the peptide signal of sequence [SEQ ID No.4] be separated from the protein with chitinase activity of sequence [SEQ ID No.3] by
10 the peptide of sequence [SEQ ID No.5]; this peptide corresponds to the peptide having the sequence [SEQ ID No.2] described in the Application WO92/01792.

Among the numerous nucleotide sequences which code for a precursor of the protein of sequence [SEQ ID
15 No.3] comprising, upstream of this, the peptide signal of sequence [SEQ ID No.4], separated from the protein of sequence [SEQ ID No.3] by the peptide of sequence [SEQ ID No.5], the DNA sequence [SEQ ID No.6] is particularly preferred. This sequence, which
20 corresponds to the sequence [SEQ ID No.4] described in the Application WO92/01792, comprises two introns in positions 443-521 and in positions 676-756.

Another sequence coding for a protein with endochitinase activity or for a precursor of the latter
25 is that of the chitinase of Aphanocladium album described in the Application EP-A1-531 218, which comprises the sequence [SEQ ID No.7]. This sequence corresponds to the sequence [SEQ ID No.1] of the Application EP-A1-531 218.

30 It is particularly appreciated that this sequence codes for a precursor of a protein with endochitinase activity, which comprises, upstream of the sequence [SEQ ID No.7], the peptide signal of sequence [SEQ ID No.8]. This peptide signal corresponds
35 to the peptide signal having the sequence [SEQ ID No.4] of the Application EP-A1-531 218.

It is advantageous then that the peptide signal of sequence [SEQ ID No.8] be separated from the protein with chitinase activity of sequence [SEQ ID No.7] by

the peptide of sequence [SEQ ID No.9]. This peptide corresponds to the peptide having the sequence [SEQ ID No.5] described in the Application EP-A1-531 218.

Among the numerous nucleotide sequences which
5 code for the protein of sequence [SEQ ID No.7], a particularly valued sequence is the DNA sequence [SEQ ID No.10] which corresponds to the sequence [SEQ ID No.6] described in the Application EP-A1-531 218.

Another advantageous sequence of interest which
10 confers on plants resistance to pathogenic agents is that which codes for a protein with β -1,3-glucanase activity or for a precursor of the latter. It is in fact known, as described in the Patent Application WO-92 16632, that such a protein has a phytoprotective
15 effect because it is capable of degrading β -1,3-glucans, polysaccharide polymers formed from glucose units associated by β -1,3 links sometimes having branchings of β -1,4 or β -1,6 type, which are an important structural compound of the wall of the
20 majority of fungi, and especially of phytopathogenic fungi.

Such an advantageous sequence is that described in the Patent Application WO 92/16 632, which codes for a protein comprising the sequence [SEQ ID No.11]. This
25 sequence corresponds to the sequence (a_1) described in the Application WO92/16 632.

It is interesting that this sequence of interest comprises, immediately downstream of the sequence coding for the sequence [SEQ ID No.11], the
30 sequence [SEQ ID No.12] optionally truncated in its carboxy-terminal part by 0 to 27 amino acids. This sequence [SEQ ID No. 12] corresponds to the sequence (a_4) described in the Application WO 92/16 632.

This sequence of interest thus preferably
35 comprises, immediately upstream of the sequence coding for the sequence [SEQ ID No.11], a CAA or CAG codon coding for Gln.

A particularly valued sequence of this type is that coding for a protein with β -1,3-glucanase activity

or a precursor of the latter which comprises the sequence [SEQ ID No.13]. This sequence corresponds to the sequence (a_5) described in the Application WO 92/16 632.

5 It is particularly appreciated that this sequence codes for a precursor of a protein with β -1,3-glucanase activity which comprises, upstream of the sequence [SEQ ID No.13] the peptide signal of sequence [SEQ ID No.14]. This peptide signal corresponds to the
10 peptide signal having the sequence (a_2) described in the Application WO 92/16 632.

 Among the numerous nucleotide sequences which code for the protein of sequence [SEQ ID No.13], an advantageous sequence is the DNA sequence [SEQ ID
15 No.15] which corresponds to the sequence (Na_1) described in the Application WO 92/16 632.

 The recombinant DNA defined above, comprising the gene coding for the oxalate oxidase flanked by signals necessary for its expression as well as a
20 sequence of interest, is introduced into the plant cells to be transformed. When the sequence of interest codes for a protein or a precursor of this, it likewise comprises the signals necessary for its expression. The construct containing these sequences can be produced in
25 a unique vector or in different vectors which will be used for the transformation.

 The promoter is preferably a strong constitutive promoter, for example the 35S promoter of the cauliflower mosaic virus, or a promoter controlling a
30 specific tissue or organ expression such as the promoter of the small sub-unit of the ribulose 1,5-biphosphate carboxylase-oxygenase which is expressed preferentially in the leaves and very particularly in the tissues of the mesophyll (Kuhlemeier et al., 1987, Ann. Rev. Plant Physiol, 38, 221-257). It is likewise
35 possible to use a specific promoter controlling, for example, an expression in the seeds or in the course of a precise stage of the development of the plant, or a promoter inducible following a thermal shock, a wound

or the interaction between the plant and parasites (Kuhlemeier et al., 1987, reference cited above), if an expression of recombinant DNA is sought after in these situations.

5 The terminator sequence is used, comprising polyadenylation sites, which can be isolated from plant genes or from genes which are expressed in plants, such as, for example, the terminator of the gene of nopaline synthase of Agrobacterium tumefaciens.

10 A bacteria, for example of the type Escherichia coli, which contains recombinant DNA defined above with the means allowing its replication can serve for the cloning of this recombinant DNA, and a bacteria capable of infecting a plant with transference of genetic
15 material, for example of one of the type Agrobacterium rhizogenes and Agrobacterium tumefaciens, which contains this DNA in a context allowing its replication, can serve to transform plant cells. The transformation of plant cells by the recombinant DNA
20 above can likewise be effected by another biological method such as the pollen tube route (Zhong-xun Luo et al., Plant Molec. Biol. Rep., 1988, 6, 165-176), the direct transformation of germinating seeds (Toepfer R. et al., 1989, The Plant Cell., 1, 133-139) or by a
25 physical method such as the use of polyethylene glycol, of electroporation (Chistou P. et al., 1987, Proc. Ntl. Acad. Sci. USA, 84, 3662-3699) or of bombardment with the aid of microprojectiles (Klein T.M. et al., 1988, Proc. Ntl. Acad. Sci. USA, 85, 8502-8505).

30 The invention thus also relates to a plant cell, characterized in that it is transformed by recombinant DNA defined above, with the necessary means for the expression of the protein capable of degrading oxalic acid and of the protein of interest or of the
35 precursor of the latter; such a cell can be selected on a medium containing oxalic acid. This plant cell can arise from a type of large crop, such as, for example, maize, soya, beetroot, wheat, barley, poppy, rape, sunflower, lucerne and sorghum, from a flower species,

such as, for example, the rose, carnation or gerbera or from a culinary species, such as the carrot, tomato, lettuce, chicory, pepper, melon and cabbage. Species particularly valued are the rape Brassica napus, the
5 sunflower Helianthus annuus and the tobacco Nicotiana tabacum.

The transformation stage which affects one or more cells is followed by a multiplication stage of these transformed cells in such a way as to obtain
10 calluses, which can give rise to transformed plants by organogenesis or embryogenesis processes.

The invention thus also relates to a plant or a plant part, characterized in that it contains recombinant DNA defined above, with the necessary means for
15 the expression of the gene coding for the protein capable of degrading oxalic acid and of the gene coding for the protein of interest or of the precursor of the latter and in that this has been selected on a medium containing oxalic acid. A plant part particularly
20 valued is the part capable of forming a complete new plant, especially after sowing, digging in or transplanting, or of producing seeds. Such a part is, for example, a grain, a kernel, a seed, a cutting or a layer. These plants can be, more particularly, of the
25 species Nicotiana tabacum, Helianthus annuus and Brassica napus.

The protein capable of degrading oxalic acid can be an enzyme with decarboxylase activity, such as, especially, the oxalate decarboxylase of Aspergillus or
30 of Collybia velutipes or preferably an enzyme with oxidase activity, such as, for example, the oxalate oxidase of barley (marketed by Boehringer, ref. 567 698), of sorghum (Chandra S. Pundier, 1991, Phytochemistry, 30, 4, p.1065) or of moss (Mnium
35 menziesii) (M.F. Laker et al., 1980, Clinical Chemistry, 26, 7, 827). A particularly valued protein with oxalate oxidase activity is the protein of sequence [SEQ ID No.1], or a sequence having a high degree of homology with the sequence [SEQ ID No.1].

This is advantageously coded by the DNA sequence [SEQ ID No.3].

Oxalic acid is a phytotoxin produced by numerous pathogenic fungi, such as, especially,
5 Sclerotinia sclerotiorum (B. Grezes-Besset, 1988, Doctoral Thesis, Université Paul Sabatier, Toulouse, as well as G. Goday et al., 1990, Physiological and Molecular Plant Pathology, 37, 179-191), Sclerotium
10 rolfsii (D.F. Bateman et al., 1965, Phytopathology, 68, 1597-1599), Aspergillus niger (I.A.S. Gibson, 1953, Transactions British Mycological Society, 36, 198-209),
Cristulariella pyramidalis (P. Kurian et al., 1979, Phytopathology, 69, 712-714) and Cryphonectria
15 parasitica (A.R. Bennett et al., 1990, Mycologia, 358-363).

The invention likewise relates to a process for selecting, on oxalic acid, cells, calluses or plants transformed by a recombinant DNA defined above, characterized in that, in the selection medium, the
20 calcium is in soluble form.

The plants can arise from a species of large crop, such as, for example, maize, soya, beetroot, wheat, barley, poppy, rape, sunflower, lucerne and sorghum, from a flower species, such as the rose,
25 carnation or gerbera or from a culinary species, such as the carrot, tomato, lettuce, chicory, pepper, melon and cabbage. Particularly valued species are the rape Brassica napus, the sunflower Helianthus annuus and the tobacco Nicotiana tabacum.

30 The selection medium comprises oxalic acid and all the elements necessary for the multiplication and the differentiation of plant cells and especially of calcium, which is indispensable for their development, which must then remain available. In the presence of
35 oxalic acid, calcium has a tendency to associate with the latter to form an insoluble oxalate salt, which makes it unavailable to the plant cells. It is thus necessary for the selection medium to contain agents allowing calcium to be kept in soluble form.

Preferably, these agents are chelating agents having an affinity for calcium which is higher than that of oxalic acid. Of course these must not, in addition, be toxic to the cells.

Examples of chelating agents having an affinity for calcium higher than that of oxalic acid are EDTA and EGTA. In the case of the sunflower, EGTA is a particularly valued chelating agent.

Thus, according to another aspect, the invention relates to a process for selecting, on oxalic acid, calluses or plants transformed by a DNA sequence coding for a protein capable of degrading oxalic acid, which consists in cultivating the calluses or the plants on a medium containing oxalic acid and calcium in the presence of a chelating agent having an affinity for calcium which is higher than that of oxalic acid.

According to a preferred variant, the plants are transformed with a DNA sequence coding for a protein capable of degrading oxalic acid associated with a sequence of interest such as that defined above, in particular a sequence coding for a protein of interest.

The invention will be better understood with the aid of the account below, divided into sections, which comprises experimental results and a discussion of these. Certain of these sections relate to experiments carried out with the aim of accomplishing the invention, others to examples of accomplishment of the invention, given, of course, in a purely illustrative capacity.

In this experimental part, the clone gf-2.8 of wheat germin described by B.G. Lane et al., 1991, J. Biol. Chem., 226- 10461-10469 is used; the genomic DNA sequence of this clone is the sequence [SEQ ID No.16] and the peptide sequence translated is the sequence [SEQ ID No.17].

A large part of the whole of the techniques below, which are well known to the person skilled in the art, is set out in detail in the works of Sambrook

et al.: "Molecular Cloning : a Laboratory Manual",
published in 1969 by the publishers Cold Spring Harbor
Press in New York (2nd edition), and in the work of
Gelvin et al. "Plant Molecular Biology Manual",
5 published in 1988 by the publishers Kluwer Academics.

**SECTION 1 : Purification and partial
characterization of oxalate oxidase of
barley**

10 1) Purification of oxalate oxidase of barley

An oxalate oxidase of barley has been
purified to homogeneity starting from a commercial
preparation enriched in oxalate oxidase activity
(Boehringer, ref. S67 696) prepared starting from
15 germinating barley grains. The protein is purified
according to the protocol described below:

Stage 1:

The lyophilized commercial preparation is
solubilized in water and then equilibrated in a 10mM
20 acetate buffer of pH 5.2 by passage through a ready-to-
use Sephadex™ G25 mini-column (NAP 10-Pharmacia). This
extract is fractionated by chromatography on a
synthetic polymer ion-exchange column (Mono S™HR5/5
column from Pharmacia). After applying the sample, the
25 unretained proteins are eluted by the 10mM sodium
acetate buffer of pH 5.2. The proteins retained on the
column are eluted by a 10 to 500mM linear gradient of
sodium acetate buffer of pH 5.2.

The eluate is analysed on line by its absor-
30 bance at 280 nm and the collected fractions are
characterized : protein contents measured by the
colorimetric technique of Bradford (1976, Anal.
Biochem., 72, 248-252), oxalate oxidase activity
measured according to the technique of Suguira et al.,
35 1979, Chem. Pharm. Bull., 79, 2003-2007, described at
point 2) below. Each fraction is characterized, after
electrophoresis under denaturing conditions (SDS) and
coloration with silver, by its electrophoretic mobility

compared with reference proteins.

Stage 2:

5 The fractions have oxalate oxidase activity
and1 eluted at a sodium acetate concentration of
between 200mM and 275mM, are collected and then
concentrated by centrifugation in a Centricon-10™
system (Amicon-ref. 4205). The extract is then
10 fractionated by exclusion chromatography on a
Superdex™ 75 column (Pharmacia). The fractions
collected are analysed according to the methods
described in Stage 1.

At the end of the purification, a
unique protein is obtained which has an oxalate
15 oxidase activity and an apparent molecular weight of 26
± 3 kDa (molecular weight determined after
electrophoresis on 15% polyacrylamide gel in the
presence of SDS and visualisation with silver).

2) Measurement of the oxalate oxidase activity

20 The oxalate oxidase activity is measured
according to the method described by Sugaira et al.,
1979, Chem. Pharm. Bull., 79, 2003-2007, and summarized
below. The enzymatic extract is incubated in the
presence of 75 µl of sodium oxalate (0.18% in a 100 mM
25 succinate buffer of pH 4.0) and of 100mM succinate
buffer of pH 4.0, in quantities sufficient to have a
volume of reaction mixture equal to 1.5 ml.

After incubation of the reaction mixture at
37°C for 10 minutes, 100 µl of 1 M tris buffer of pH
30 8.9, then 1 ml of reagent prepared at the same time and
made up of : 8 trig of 4-aminoantipyrine, 6 trig
of horseradish peroxidase (Sigma, ref. P8250) and 80 µl
of dimethylaniline prepared in 100 ml of 0.1 M
phosphate buffer of pH 7.0 are added successively.

35 The enzymatic activity is estimated by the
spectrophotometric measurement of the absorbance at
530 nm. It is expressed in units of oxalate oxidase/mg
of protein (one unit of oxalate oxidase (U oxox) is the
quantity of enzyme which converts 1 µmol of oxalate

into hydrogen peroxide in 1 minute at 37°C and at pH 3.8).

3) Characterization of the purified protein

5

a) Preparation of polyclonal antibodies

25 µg of the barley protein purified to homogeneity and having an oxalate oxidase activity are injected into a rabbit in 500 µl of complete Freund's
10 adjuvant (Sigma, ref. F5881).

Three booster injections of 25 µg in incomplete Freund's adjuvant (500 µl) (Sigma ref. F5506) were carried out at 3 week intervals. The immune serum was taken 3 weeks after the last injection.

15 This immune serum specifically recognises oxalate oxidase. It allows this protein to be revealed by the Western blot technique (described in Section 2 4) b) starting from a total protein extract of germinating barley embryos.

20

b) Determination of the partial sequence of the oxalate oxidase

A sample of the protein of 26 ± 3 kDa having an oxalate oxidase activity is treated with cyanogen
25 bromide and the oligopeptides liberated are separated by inverse phase HPLC on a C4 Brownlee column. The N-terminal sequence of the protein as well as that of an internal peptide are determined using a protein
sequencer (Model 470A, Applied Biosystems, USA)
30 equipped with a chromatograph (Model 120A, Applied Biosystems) which continuously analyses the phenylthiohydantoic derivatives formed after each degradation cycle.

The amino-terminal sequence determined is the
35 following sequence [SEQ ID No.18]:

Thr Asp Pro Asp Pro Leu Gln Asp Phe Xaa Val Ala Asp Leu Asp Gly
Lys Ala Val Ser Val Asn Gly His Thr Xaa Lys Pro Met Ser Glu Ala
Gly Asp Asp Phe Leu Phe

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Xaa being an undetermined amino acid.

The sequence of an internal peptide is the following sequence (SEQ ID No.19]:

5 Ala Gly Glu Thr Phe Val Ile Pro Arg

After comparison with the bank of known protein sequences (Swiss-Prot bank) using the GAP option of the UWGCG software of the University of Wisconsin : Devereux et al., 1984, Nucl. Acids Res., 12, 387-395, a homology of at least 94% is found with the sequence of a wheat protein induced in the course of germination, germin, described by Lane et al., 1991, J. Biol. Chem., 226, 10461-10469.

15

SECTION 2 : Transformation of tobacco by the wheat germin gene, selection an oxalic acid of the calluses and of transgenic plants

20 1) Construction of a transformation vector
a) Preparation of the sequence coding for wheat germin

The 745 base pair DNA fragment HindIII-SphI of the clone gf-2.8 described by B.G. Lane et al., 1991, J. Biol. Chem., 226, 10461-10469) carrying the sequence coding for wheat germin was purified by electrophoresis on agarose gel followed by extraction by means of the Geneclean™ kit (Bio 101, ref. 3105) according to the procedure of the manufacturer. This fragment comprises 19 base pairs upstream of the ATG initiator as well as 54 base pairs downstream of the stop codon. This fragment was inserted with the aid of the DNA T4 ligase between the sites HindIII and SphI of the multiple cloning site of a PTZ19R vector (marketed by Pharmacia), whose Ba,HI site had been destroyed by filling using Klenow polymerase, according to the methods which are well known to the person skilled in the art. The plasmid thus created is called plasmid

pPH096. The HindIII site present in this plasmid is then opened, and a new BamHI site is recreated by addition of an oligonucleotide of following sequence [SEQ ID No.20] : AGCTGGATCC

5 The vector obtained, called plasmid pPH098, is cloned in the strain E.coli JM 109 (Clontech). After verification of the nucleotide sequence of the cloned fragment, the coding part is repurified in the form of the restriction fragment BamHI-SacI of 789 base pairs.
10 This fragment contains the sequence coding for the peptide signal, as well as that coding for the mature germin, such as are described by B. Lane et al., 1991, J. Biol. Chem., 226, 10461-10469.

15 b) Preparation of the promoter sequence comprising the 35S promoter of the cauliflower mosaic virus

 Starting from the plasmid pBI121 (Clontech), by cleavage with the aid of the endonucleases HindIII and
20 BamHI, then electrophoresis on agarose gel, the HindIII-BamHI fragment of approximately 900 base pairs, containing the 35S promoter of the cauliflower mosaic virus, is isolated. This fragment is cut again by Hind-III. The fragment of approximately 410 base pairs,
25 carrying the BamHI site, is treated by DNA T4 ligase in the presence of a HindIII linker (synthetic sequence containing a HindIII site). After cutting by the endonuclease HindIII and electrophoresis on agarose gel, the resulting HindIII-BamHI fragment, of
30 approximately 420 base pairs, is isolated and purified.

 c) Preparation of the terminator sequence comprising the terminator of the nopaline synthase (NOS) gene of Agrobacterium
35 tumefaciens

 Starting from the plasmid pBI121 (Clontech), by cutting with the aid of the restriction enzymes SacI and EcoRI, then electrophoresis on agarose gel, a fragment of approximately 250 base pairs, containing

the terminator of the nopaline synthase gene, was isolated.

d) Cloning in the binary vector pBIN19

The promoter sequence (cf. above 1)b)), the sequence coding for germin (cf. above 1) a)) and the terminator sequence (cf. above 1)c)) were ligated into the binary vector pBIN19 (Bevan, 1984, Nucl. Acid Res., 12, 8711-8721) with the aid of DNA T4 ligase, and opened with the aid of the endonucleases HindIII and EcoRI. This vector carries two resistance genes to kanamycin, one being able to express itself in bacteria, the other situated immediately upstream of the complete recombinant gene being able to be transferred to plant cells. This resistance gene to kanamycin will serve to verify that the regenerated plantlets obtained after selection on oxalic acid with the aid of the gene coding for oxalate oxidase are effectively transformed.

The vector obtained, called pPH100, is cloned in the strain *E. coli* HB101 (Clontech).

2) Transformation of *Agrobacterium tumefaciens*

The transformation is carried out according to the freezing-thawing method described in Plant Molecular Biology Manual (Gelvin et al., op. cit.) and summarized below.

Competent cells of *Agrobacterium tumefaciens* (LBA 4404 strain, Clontech) are prepared by rapid cooling in ice of a culture in the exponential growth phase. The bacteria are then resuspended in a 20mM solution of CaCl₂. Aliquot parts of this suspension are distributed in Eppendorf tubes, then frozen in liquid nitrogen.

1 µg of pPH100 plasmid is added to the frozen cells, contained in an Eppendorf tube. The suspension is then incubated at 37°C for 5 min; 1 ml of Luria medium (Gibco) is then added and the tube is incubated

at 28°C for 4h. Aliquot parts are spread on Petri dishes containing a minimum agar medium, described in Plant Molecular Biology Manual (op. cit.) in the presence of 100 mg of rifampicin and 25 mg/l of kanamycin. Under these conditions, only the colonies of Agrobacterium tumefaciens which have integrated the plasmid pPH100 grow. These contain the chimeric gene in a context allowing its replication.

The resistance to the two antibiotics of the selected colonies is verified by transplanting these on the same selection medium twice in succession. The presence of the chimeric gene associating the 35S promoter with the coding part of wheat germin in Agrobacterium tumefaciens is verified by the Southern Blot method on a total DNA preparation (lysis of the cells, purification of DNA by extraction with the aid of the mixture phenol/chloroform, according to the procedure described by Gelvin in the work cited above, cutting of the purified DNA with the aid of restriction enzymes, electrophoresis on agarose gel, transfer to membrane and hybridization, according to the techniques which are well known to the person skilled in the art).

3) Transformation of the tobacco

Nicotiana tabacum tobacco cultivated in vitro was infected by Agrobacterium tumefaciens containing the plasmid pPH100 according to the procedure of Horsch et al., which is well known to the specialist (Horsch R.B. et al., 1985 Science 227, 1229-1231), whose principle stages are explained below.

Axenic plant leaf discs of Nicotiana tabacum tobacco (Wisconsin Havana 38 variety) are incubated in a culture of A. tumefaciens containing the plasmid pPH100. The discs, drained on Whatman paper, are cultured on culture media in Petri dishes in order to multiply the transformed cells so as to obtain calluses. These calluses are then transferred onto medium containing cefotaxime at 500 µg/ml which is

intended to decontaminate the plant tissues (elimination of Agrobacterium tumefaciens) and kanamycin at 100 µg/ml to select the transgenic material. Transformed shoots develop from these calluses; the plants which result from them are transferred to greenhouses.

4) Demonstration of the expression of the germin gene in transgenic tobacco

a) Preparation of protein extracts of transformed tobacco and of control tobacco

The tissue fragments (calluses and plant leaves) were frozen in liquid nitrogen, powdered and stored at -20°C.

To carry out electrophoreses, the oxalate oxidase is extracted directly from the plant powder by the Laemmli loading buffer (reference below).

For the determinations of oxalate oxidase activity, the enzymatic extract is prepared by suspending plant powder in a 0.05M succinate buffer, pH 4.

For the protein determinations, the plant extract, suspended in the above succinate buffer, is centrifuged at 10,000 g for 5 min.

The concentration of total proteins is determined on the supernatants, below called crude protein extracts, following the technique of Bradford (Bradford M.M., 1976, Anal. Biochem., 72, 248-254).

b) Immunodetection of oxalate oxidase
(Western blot) in the calluses and in the plants

The crude protein extracts are submitted to a Western blot, a technique, which is well known by the

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person skilled in the art and described by H. Towbin et al., Proc. Ntl. Acad. Sci. USA 76, 1979, 4350-4354, which comprises the following stages:

- 5 • denaturation by heating to 100°C for 10 min in a buffer, termed loading buffer, made up of 0.125M Tris, pH 6.8, 4% SDS, 0.002% Bromophenol Blue, 20% glycerol and 10% β -mercaptoethanol (according to the procedure described by Laemmli U.K., 10 1970, Nature, 227, 680-685), followed by centrifugation at 10,000 g;
- 15 • electrophoretic separation of the different proteins contained in the solubilizate according to the procedure described by Laemmli (ref. above);
- 20 • electrotransfer of the said proteins contained in the gel onto a PVDF membrane (according to the technique of H. Towbin et al., 1979, Proc. Natl. Acad. Sci. USA 4350-4354).

Immunodetection is carried out according to a procedure which comprises the following stages:

- 25 • saturation of the PVDF (polyvinylidene fluoride) membrane onto which the proteins have been transferred by incubation for at least 2 h at 37°C in a 3% gelatin solution in phosphate-buffered saline containing 0.05% of Tween 20™ detergent.
- 30 • Incubation (for 1 h at 37°C) in the presence of the immune serum prepared above (containing the polyclonal antibodies recognizing the recombinant protein), diluted to 1/10,000 in phosphate-buffered saline.
- 35 • 3 washes in phosphate-buffered saline containing 0.05% of Tween 20™ detergent.

The antigen-antibody complex is then visualised with the aid of a streptavidin-biotin system conjugated to alkaline phosphatase with the Amersham RPN 23 kit ("Blotting detection kit"), used according to the 40 instructions of the manufacturer.

The blot obtained shows, for the calluses and

for the tobacco plant leaves transformed by the plasmid pPH100, the presence of a protein of apparent molecular weight of approximately 26 ± 3 kDa, recognized by the polyclonal antibodies prepared in Section 1 3)a) and
5 absent from the calluses and from the control tobacco plant leaves. This protein has the same apparent molecular weight as the purified oxalate oxidase obtained in Section 1.

10 c) Demonstration of the oxalate oxidase activity of wheat germin expressed in tobacco

The oxalate oxidase activity of 6 extracts of
15 calluses and of tobacco plant leaves transformed by the plasmid pPH100 is measured according to the method of Sugaira et al., described in Section 1. The results are gathered together in Table I below:

20 Table I: Oxalate oxidase activity measured in different transgenic tobaccos

	Control	Calluses					Leaves	
		Transgenic					Control	Trans- genic
No. of sample	W38	2	26	65	81	86	W38	400
Activity (U oxox/ml of extract)	1.0	10.5	10.1	16.0	2.8	3.0	0.0	5.4

W38 = untransformed control tobacco

25

It is noted by reading the table above that the transgenic tobacco (calluses or leaves) has a significantly higher oxalate oxidase activity than that of the control tobacco.

5) Selection on oxalic acid of the regenerants

a) Selection procedure

5

Axenic plant leaf discs of Nicotiana tabacum tobacco (Wisconsin Havana 38 variety) are incubated in a culture of A. tumefaciens containing the plasmid pPH100. The discs, which are drained on Whatman paper, are cultured on culture media in Petri dishes in order to multiply the transformed cells so as to obtain calluses. After three days, the leaf discs are rinsed in 80% ethanol and then in Murashige and Skoog agar medium (1962, *Physiol. Plant.* 15, 473) containing 500 $\mu\text{g/ml}$ of cefotaxime. They are then transferred to a medium containing oxalic acid in order to select the cellular aggregates expressing oxalate oxidase.

b) Method of preparation of the culture media

20

As oxalic acid associates with calcium to form an insoluble calcium oxalate salt, the selection medium must be prepared according to a procedure allowing the calcium to be kept in a soluble form which can be used by the plant cells despite the presence of oxalic acid.

Preparation of solution A:

For each concentration, 50 ml of a solution of oxalic acid concentrated 20 times with respect to the final concentration expected, are adjusted to pH 5.8 with a 3M solution of potassium hydroxide (KOH). The solution is adjusted to 100 ml with deionized water, sterilized by filtration through a 0.45 μm filter, then maintained at a temperature of 50°C until use.

Preparation of solution B:

258 mg of EGTA (ethylene glycol bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid) (Sigma, ref. E. 4378) are dissolved in 100 ml of deionized water. The EGTA is solubilized by adjusting the pH to 10.0 with a solution of 10M KOH. 100 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ are then added

and the pH of the solution is adjusted to 5.8 with a 2M HCl solution. The volume is adjusted to 150 ml and then the solution is sterilized by passing through a 0.45 μ m filter.

5 Incorporation of the calcium and the oxalate in the culture medium:

750 ml of Murashige and Skoog agar culture medium (1962, Physiol. Plant. 15, 473) without calcium chloride, concentrated 1.33 times, are autoclaved for 10 20 min at 120°C. The temperature is then lowered to 50°C and the solutions A and B are incorporated into this medium. After homogenization of the solution, the medium is poured into Petri dishes.

15 c) Determination of the amount of oxalic acid to be used for selection

A range of sensitivity to oxalic acid of non-transgenic tobacco calluses (Wisconsin Havana 38) was established. The culture media were prepared according to the procedure described above. For each 20 concentration of oxalic acid, 4 dishes containing 25 calluses of 2 mm diameter and an average weight of 10 mg were cultured. The results, expressed in % by weight inhibition of growth with respect to control calluses, are shown in Table II below:

25

Table II: Inhibition of the growth in weight of tobacco calluses by oxalic acid

Oxalic acid conc. (μ g/ml)	0	40	60	80	120	180	270
Growth in weight inhibition	0%	0%	12%	19%	51%	89%	99%

30 Taking the above results into account, the selection dose chosen is 270 μ g/ml (3mM).

d) Use of the gene coding for oxalate oxidase as selection gene of the transformants

35

After transformation and induction of callogenesis, the tobacco leaf discs are transferred to

a medium containing 270 µg/ml of oxalic acid. At this concentration, the calluses expressing oxalate oxidase do not show a different growth from the control growing on a medium devoid of oxalic acid and are capable of surviving and of producing transgenic plants. The fact that the regenerated plantlets are indeed transgenic is verified by their resistance to kanamycin (second selection gene carried by the plasmid pPH100). Of 29 plants selected on oxalic acid, 28 are equally resistant to kanamycin.

The selection on oxalic acid thus allowed transformant calluses as well as transgenic plants to be selected.

SECTION 3: Transformation of the sunflower by the wheat germin gene, selection of the calluses on oxalic acid

1) Transformation of the sunflower

Obtainment of transformed sunflower calluses.

Immature sunflower seeds are selected on the flower head of sunflower plants of the well known HA89 line, which has been studied especially by the P.J. Goyne et al., Journal Article No. 1534 of the North Dakota State Univ. Agric. Exp. Stn. Fargo ND-58105 and by M.F. Geriani, Plant Cell Physiol, 33, 2, 157-164. These seeds are sterilized on the surface for 30 min in a solution of 2% calcium hypochlorite, then rinsed with sterile distilled water.

The immature embryos are selected on these seeds and cultured on the medium I (Table III) for 14 days at 25°C and in darkness. These embryos are then cultured for 10 days on the medium II at 25°C with a 16 h day/8 h night photoperiod.

The embryos are then cut in two at the level of the embryonal axis and soaked for 10 min in a suspension of Agrobacterium tumefaciens containing the binary vector pPH100 (cf. Sections 2.1 and 2.2). This

suspension is obtained by culture of this bacteria for 15 h in the Luria liquid medium.

The embryos are then drained on sterile filter paper and then recultured on the medium II in darkness for 3 days. The embryos are then briefly rinsed with the Murashige and Skoog liquid medium (Murashige and Skoog, 1962, *Physiol. Plant* 15 : 473) containing 500 mg/l of the antibiotic cefotaxime. They are then drained on sterile filter paper and cultured on the medium III containing 250 mg/l of cefotaxime, 250 mg/l of carbenicillin and 50 mg/l of paromomycin. This culture takes place at 25°C with a 16 h day/8 h night photoperiod; the plant tissues as well as the calluses developing on their surface are transplanted every 21 days on the same medium.

Table III: Composition of the different media used to obtain transformed sunflower plants

Medium	I	II	III
Composition mg/l			
KNO ₃	2500	2500	1900
NH ₄ NO ₃	-	-	1650
CaCl ₂ .2H ₂ O	150	150	440
MgSO ₄ .7H ₂ O	250	250	370
KH ₂ PO ₄	-	-	170
(NH ₄) ₂ SO ₄	134	134	-
NaH ₂ PO ₄ .H ₂ O	150	150	-
MnSO ₄ .H ₂ O	10	10	-
ZnSO ₄ .7H ₂ O	2	2	8.6
H ₃ BO ₃	3	3	6.2
KI	0.75	0.75	0.83
CuSO ₄ .5H ₂ O	0.025	0.025	0.025
Na ₂ MoO ₄ .2H ₂ O	0.25	0.25	0.25
CaCl ₂ .6H ₂ O	0.025	0.025	0.025
MnSO ₄ .4H ₂ O	-	-	22.3
Na ₂ EDTA	37.3	37.3	37.3
FeSO ₄ .7H ₂ O	27.8	27.8	27.8

Medium	I	II	III
Composition mg/l			
Nicotinic acid	1	1	0.5
Thiamine HCl	10	10	0.1
Pyridoxine HCl	1	1	0.1
Myo-inositol	4000	4000	100
L-Alanine	1000	1000	-
L-Glutamine	800	800	-
L-Serine	160	160	-
L-Tryptophan	50	50	-
L-Cysteine	10	10	-
Ca D-pantothenate	-	-	0.8
Folic acid	-	-	0.1
Choline chloride	-	-	0.1
4-Aminobenzoic acid	-	-	0.05
Riboflavin	-	-	0.05
Sucrose	120,000	60,000	30,000
2,4-Dichlorophenoxyacetic acid	2	-	-
6-Benzylaminopurine	-	0.4	-
Kinetinn	-	-	1
Agar	7000	7000	7000
pH	5.7	5.8	5.7

2) Demonstration of the expression of the
germin gene in the transgenic sunflower

5

a) Preparation of the protein extracts

Takes place in a manner identical to that
described in Section 2 4)a).

10

b) Immunodetection of the oxalate oxidase
(Western blot) in calluses

Immunodetection, carried out according to the
15 procedure described in Section 2 4)b), demonstrates, in
sunflower calluses and leaves transformed by the

plasmid pPH100, the presence of a supernumerary protein of apparent molecular weight of approximately 26 ± 3 kDA. This protein, which is absent from calluses and leaves of control plants, has the same apparent molecular weight as the purified oxalate oxidase obtained in Section 1.

c) Oxalate oxidase activity of the wheat germin expressed in the sunflower

10

The oxalate oxidase activity of 5 protein extracts of sunflower calluses transformed by the plasmid pPH100 is measured according to the method of Sugaira et al., described in Section 1. The results show a significantly higher oxalate oxidase activity to that of the control extract.

15

Table IV: Oxalate oxidase activity of transgenic sunflower calluses

20

No. of the callus	Untransformed control	17	18	19	20	21
Units OxOx/mg prot/min	0.0	9.5	0.7	0.8	0.6	1.4

3) Selection of the transgenic calluses on oxalic acid

25

The selection of the transgenic calluses is carried out by culturing the plant material resulting from the medium III (cf. above 1) on a Murashige and Skoog selection medium prepared according to the procedure described in Section 2 5)b).

30

The determination of the selection dose to be applied in the course of the culture of sunflower calluses is made according to the method described in Section 2 5)c), this method being applied to non-transgenic sunflower calluses obtained from immature embryos. The results are shown in Table V:

35

Table V: Inhibition of the growth in weight of the sunflower by oxalic acid

Oxalic acid conc. ($\mu\text{g/ml}$)	0	40	70	90	140	180	235	270
Growth in weight inhibition	0%	0%	0%	0%	42%	92%	97%	99%

5 The dose of oxalic acid allowing the selection to be carried out is 270 $\mu\text{g/ml}$ (3mM). At this concentration, the calluses expressing oxalate oxidase do not show inhibition of growth.

10 **SECTION 4: Use of the selection on oxalic acid to obtain transgenic plants possessing a gene of interest coding, for example, for a protein with endochitinase activity.**

15 1) Construction of the transformation vector

a) Preparation of the fragment carrying the gene coding for oxalate oxidase

20 The fragment HindIII - EcoRI of approximately 1420 bp of the plasmid pPH100 described in Section 2 d) is purified and recloned in a pUC19 vector according to the methods well known to the person skilled in the art. This plasmid is then linearized using the
25 restriction endonuclease EcoRI and the sticky end is made up by means of the Klenow fragment. Then, after cutting by the HindIII endonuclease, the HindIII - free end fragment is purified.

30 b) Preparation of the fragment carrying a hybrid gene coding for a protein with endochitinase activity

35 The fragment HindIII - EcoRI arising from the plasmid pBR1 described in the Patent Application WO92/01792 Example 1 and containing a chimeric gene coding for a protein with endochitinase activity which

comprises the 35S promoter, a sequence coding for a tomato-tobacco hybrid chitinase and the NOS terminator is purified, recloned in the vector pUC19, then the HindIII site is destroyed in a conventional manner. The
5 free end - EcoRI fragment is purified.

c) Preparation of a transformation vector of plants not comprising a resistance gene to kanamycin

10

The fragment NheI - HindIII comprising the part coding for the resistance gene to kanamycin is eliminated from the T-DNA of the plasmid pBIN19 (cf. Section 2 1 d). The use, according to the methods well
15 known by the person skilled in the art, of the oligonucleotides CTAGCA and AGCTTG allows the plasmid to be recircularized recreating the restriction sites NheI and HindIII. The resulting plasmid is then linearized by the restriction endonucleases HindIII and
20 EcoRI.

c) Assembly of the transformation vector

With the aid of the DNA ligase T4, the gene
25 coding for oxalate oxidase (obtained in a) above) and the chimeric gene coding for a protein with chitinase activity (obtained in b) above) were ligated in a binary vector pBIN19 in which the resistance gene to kanamycin expressed in plants (obtained in c) above)
30 has been eliminated.

The vector obtained, called pPH106, is cloned in the strain E.Coli HB101 (Clontech).

2) Transformation of *Agrobacterium tumefaciens*

35

The transformation is carried out according to the method described in Section 2 2).

3) Transformation of the tobacco

Nicotiana tabacum tobacco cultured in vitro is infected with Agrobacterium tumefaciens containing the plasmid pPH106 according to the procedure of Horsch et al., which is well known to the person skilled in the art, (Horsch R.B. et al., 1985 Science 227, 1229-1231), whose principle stages are explained below.

Axenic plant leaf discs of Nicotiana tabacum tobacco (Wisconsin Havana 38 variety) are incubated in a culture of A. tumefaciens containing the plasmid pPH106. The discs, which are drained on Whatman paper, are cultured on culture media in Petri dishes in order to multiply the transformed cells so as to obtain calluses. After 48 hours, the discs are rinsed in 80% ethanol and then in Murashige and Skoog agar medium (1962, Physiol. Plant. 15, 473) containing 500 µg/ml of cefotaxime. They are then transferred for 3 days to medium containing 500 µg/ml of cefotaxime which is intended to decontaminate the plant tissues (elimination of the Agrobacterium tumefaciens).

4) Selection on oxalic acid of the regenerants

After transformation and induction of callogenesis, the tobacco leaf discs are transferred onto a medium containing 270 µg/ml of oxalic acid prepared according to the method described in Section 2 5)b). Only the transgenic calluses expressing the oxalate oxidase gene, thus capable of degrading oxalic acid, are capable of surviving and of producing transgenic plants.

5) Demonstration of the expression of the
protein with endochitinase activity in
transgenic tobaccos selected on oxalic acid

- 5 a) Preparation of the crude protein extracts
 of transformed tobacco

 This preparation is carried out according to
the method described in Section 2 4)a).

10

- b) Demonstration of the chitinase hybrid by
 immuno-blot (Western blot)

 The crude protein extracts are subjected to a
15 Western blot, a technique well known to the person
skilled in the art and described by H. Towbin et al.,
Proc. Ntl. Acad. Sci. USA, 76, 1979, 4350-4354, which
especially comprises the stages mentioned in Section
2.4)b).

20 The immunodetection of the protein of interest
is carried out using an immune serum containing
polyclonal antibodies recognizing the hybrid protein
with chitinase activity (cf. W092/01792 Example 5).

 The antigen-antibody complex is then visualized
25 with the aid of a streptavidin-biotin system conjugated
to alkaline phosphatase using the Amersham RPN 23 kit
("Blotting detection kit"), used according to the
instructions of the manufacturer.

 The blot obtained shows, for the leaves of
30 tobacco plants transformed by the plasmid pPH106, the
presence of a protein of apparent molecular weight of
approximately 26 ± 6 kDa recognized by the polyclonal
antibodies and absent from leaves of control tobacco
plants. This protein has the same apparent molecular
35 weight as the hybrid protein with chitinase activity
described in the Application W092/01792.

c) Demonstration of the chitinolytic activity
of the recombinant protein

The chitinolytic activity of the crude protein
5 extracts of leaves of 5 tobacco plants transformed by
the plasmid pPH106 (plants nos. 463, 464, 465, 468 and
469) and of crude protein extract of untransformed
tobacco plant leaves (plant W38) is measured according
to the following method:

10 The endochitinase activity of the protein is
measured by a radiochemical method allowing the
quantity of monomers or oligomers liberated by the
enzyme from a substrate (tritiated chitin) to be
estimated. This method, described by Molano et al.,
15 (1977, Anal. Biochem., 83, 648-656), is summarized
below.

50 μ l of a suspension of tritiated chitin of
specific activity 0.58 MBq/ml is added to a volume of
protein extract of 10 μ l. The final volume is adjusted
20 to 300 μ l with 0.2M sodium acetate buffer of pH 5.0.
After incubation at 30°C for 90 min, the hydrolysis
reaction of the chitin is stopped by 100 μ l of 20%
trichloroacetic acid. The reaction tubes are then
centrifuged for 10 min at 12,000 g. An aliquot part of
25 100 μ l of the supernatant containing the soluble
oligomers of chitin is taken and the corresponding
radioactivity is measured by liquid scintillation in
the presence of 5 ml of scintillation mixture. The
specific chitinolytic activity is expressed in dpm/ μ g
30 of protein.

For the 5 plants selected on oxalic acid, the
following values are obtained:

Genotype	W 38	463	464	465	468	469
Specific activity						
DPM/ μ g prot	95	149	348	318	301	320

35 (W 38 = untransformed control tobacco)

It is confirmed in the table above that the extracts of tobacco plants transformed by the plasmid pPH106 have a chitinolytic activity which is significantly higher than that of the extract of
5 control tobacco plants. Selection on oxalic acid thus allows plants expressing a gene of interest to be obtained on occurrence of the hybrid gene coding for a protein with chitinase activity described in the Patent Application WO92/01792.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Use of a DNA sequence coding for a protein
capable of degrading oxalic acid as selection gene

(iii) NUMBER OF SEQUENCES: 20

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
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(A) APPLICATION NUMBER: FR 92 14721
(B) FILING DATE: 07-DEC-1992

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 224 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Met	Gly	Tyr	Ser	Lys	Thr	Leu	Val	Ala	Gly	Leu	Phe	Ala	Met	Leu	Leu				
1				5					10					15					
Leu	Ala	Pro	Ala	Val	Leu	Ala	Thr	Asp	Pro	Asp	Pro	Leu	Gln	Asp	Phe				
			20					25					30						
Cys	Val	Ala	Asp	Leu	Asp	Gly	Lys	Ala	Val	Ser	Val	Asn	Gly	His	Thr				
		35				40						45							
Cys	Lys	Pro	Met	Ser	Glu	Ala	Gly	Asp	Asp	Phe	Leu	Phe	Ser	Ser	Lys				
	50					55					60								
Leu	Ala	Lys	Ala	Gly	Asn	Thr	Ser	Thr	Pro	Asn	Gly	Ser	Ala	Val	Thr				
65					70					75				80					
Glu	Leu	Asp	Val	Ala	Glu	Trp	Pro	Gly	Thr	Asn	Thr	Leu	Gly	Val	Ser				
			85					90					95						
Met	Asn	Arg	Val	Asp	Phe	Ala	Pro	Gly	Gly	Thr	Asn	Pro	Pro	His	Ile				
			100					105					110						
His	Pro	Arg	Ala	Thr	Glu	Ile	Gly	Ile	Val	Met	Lys	Gly	Glu	Leu	Leu				
		115					120					125							
Val	Gly	Ile	Leu	Gly	Ser	Leu	Asp	Ser	Gly	Asn	Lys	Leu	Tyr	Ser	Arg				
	130					135					140								
Val	Val	Arg	Ala	Gly	Glu	Thr	Phe	Leu	Ile	Pro	Arg	Gly	Leu	Met	His				
145					150					155				160					
Phe	Gln	Phe	Asn	Val	Gly	Lys	Thr	Glu	Ala	Ser	Met	Val	Val	Ser	Phe				
			165					170					175						
Asn	Ser	Gln	Asn	Pro	Gly	Ile	Val	Phe	Val	Pro	Leu	Thr	Leu	Phe	Gly				
		180						185					190						
Ser	Asn	Pro	Pro	Ile	Pro	Thr	Pro	Val	Leu	Thr	Lys	Ala	Leu	Arg	Val				
	195					200						205							
Glu	Ala	Arg	Val	Val	Glu	Leu	Leu	Lys	Ser	Lys	Phe	Ala	Ala	Gly	Phe				
	210					215					220								

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 672 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

ATGGGGTACT CCAAACCCT AGTAGCTGGC GTGTTGCAA TGCTGTTACT AGCTCCGGCC	60
GTCTTGGCCA CCGACCCAGA CCCTCTCCAG GACTTCTGTG TCGCCGACCT CGACGGCAAG	120

GCGGTCTCGG TGAACGGGCA CACGTGCAAG CCCATGTCGG AGGCCGGCGA CGACTTCCTC 180
 TTCTCGTCCA AGTTGGCCAA GGCCGGCAAC ACGTCCACCC CGAACGGCTC CGCCGTGACG 240
 GAGCTCGACG TGGCCGAGTG GCCCGGTACC AACACGCTGG GTGTGTCCAT GAACCGCGTG 300
 GACTTTGCTC CCGGAGGCAC CAACCCACCA CACATCCACC CGCGTGCCAC CGAGATCGGC 360
 ATCGTGATGA AAGGTGAGCT TCTCGTGGGA ATCCTTGGCA GCCTCGACTC CGGGAACAAG 420
 CTCTACTCGA GGGTGGTGCG CGCCGGAGAG ACGTTCCTCA TCCCACGGGG CCTCATGCAC 480
 TTCCAGTTCA ACGTCGGTAA GACCGAGGCC TCCATGGTCG TCTCCTTCAA CAGCCAGAAC 540
 CCCGGCATTG TCTTCGTGCC CCTCACGCTC TTCGGCTCCA ACCCGCCCAT CCCAACGCCG 600
 GTGCTCACCA AGGCACTCCG GGTGGAGGCC AGGGTCGTGG AACTTCTCAA GTCCAAGTTT 660
 GCCGCTGGGT TT 672

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 254 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Gly	Gly	Asp	Leu	Gly	Ser	Val	Ile	Ser	Asn	Ser	Met	Phe	Asp	Gln	Met	1	5	10	15
Leu	Lys	His	Arg	Asn	Glu	Asn	Ser	Cys	Gln	Gly	Lys	Asn	Asn	Phe	Tyr	20	25	30	
Ser	Tyr	Asn	Ala	Phe	Ile	Thr	Ala	Ala	Arg	Ser	Phe	Pro	Gly	Phe	Gly	35	40	45	
Thr	Ser	Gly	Asp	Ile	Asn	Ala	Arg	Lys	Arg	Glu	Ile	Ala	Ala	Phe	Phe	50	55	60	
Ala	Gln	Thr	Ser	His	Glu	Thr	Thr	Gly	Gly	Trp	Pro	Ser	Ala	Pro	Asp	65	70	75	80
Gly	Pro	Phe	Ala	Trp	Gly	Tyr	Cys	Phe	Leu	Arg	Glu	Arg	Gly	Asn	Pro	85	90	95	
Gly	Asp	Tyr	Cys	Ser	Pro	Ser	Ser	Gln	Trp	Pro	Cys	Ala	Pro	Gly	Arg	100	105	110	
Lys	Tyr	Phe	Gly	Arg	Gly	Pro	Ile	Gln	Ile	Ser	His	Asn	Tyr	Asn	Tyr	115	120	125	
Gly	Pro	Cys	Gly	Arg	Ala	Ile	Gly	Val	Asp	Leu	Leu	Asn	Asn	Pro	Asp	130	135	140	
Leu	Val	Ala	Thr	Asp	Pro	Val	Ile	Ser	Phe	Lys	Thr	Ala	Ile	Trp	Phe	145	150	155	160
Trp	Met	Thr	Pro	Gln	Ser	Pro	Lys	Pro	Ser	Cys	His	Asp	Val	Ile	Ile	165	170	175	

Gly Arg Trp Asn Pro Ser Ala Gly Asp Arg Ser Ala Asn Arg Leu Pro
 180 185 190
 Gly Phe Gly Val Ile Thr Asn Ile Ile Asn Gly Gly Leu Glu Cys Gly
 195 200 205
 Arg Gly Asn Asp Asn Arg Val Gln Asp Arg Ile Gly Phe Tyr Arg Arg
 210 215 220
 Tyr Cys Gly Ile Leu Gly Val Ser Pro Gly Asp Asn Leu Asp Cys Gly
 225 230 235 240
 Asn Gln Arg Ser Phe Gly Asn Gly Leu Leu Val Asp Thr Met
 245 250

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Arg Arg Thr Ser Lys Leu Thr Thr Phe Ser Leu Leu Phe Ser Leu
 1 5 10 15
 Val Leu Leu Ser Ala Ala Leu Ala
 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 51 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gln Asn Cys Gly Ser Gln Gly Gly Gly Lys Val Cys Ala Ser Gly Gln
 1 5 10 15
 Cys Cys Ser Lys Phe Gly Trp Cys Gly Asn Thr Asn Asp His Cys Gly
 20 25 30
 Ser Gly Asn Cys Gln Ser Gln Cys Pro Gly Gly Gly Pro Gly Pro Gly
 35 40 45
 Pro Val Thr
 50

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1153 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 443..521

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 676..756

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATGAGGCGAA CTTCTAAATT GACTACTTTT TCTTTGCTGT TTTCTCTGGT TTTGCTGAGT	60
GCTGCCTTGG CACAGAATTG TGGTTCACAG GGCGGAGGCA AAGTTTGTGC GTCGGGACAA	120
TGTTGCAGCA AATTCGGGTG GTGCGGTAAC ACTAATGACC ATTGTGGTTC TGGCAATTGT	180
CAAAGTCAGT GTCCAGGTGG CGGCCCTGGT CCTGGTCCTG TTAAGTGGTGG GGACCTCGGA	240
AGCGTCATCT CAAATTCTAT GTTTGATCAA ATGCTTAAGC ATCGTAACGA AAATTCTTGT	300
CAAGGAAAGA ATAATTTCTA CAGTTACAAT GCCTTTATTA CTGCTGCTAG GTCTTTTCCT	360
GGCTTTGGTA CAAGTGGTGA TATCAATGCC CGTAAAGGG AAATTGCTGC TTTCTTTGCC	420
CAAACCTCCC ATGAAACTAC TGGTATGTGT ATAACCATTC ACATCGAACC ATTAAAATAT	480
AATTTCAATT TATTTTATTT AGTAATTGAT TATATATGTA GGAGGATGGC CTTCCGCACC	540
TGATGGACCA TTCGCATGGG GTTACTGTTT CCTTAGAGAA CGAGGTAACC CCGGTGACTA	600
CTGTTACCA AGTAGTCAAT GGCCTTGTGC ACCTGGAAGG AAATATTTTCG GACGAGGCCC	660
AATCCAAATT TCACAGTAAG CTACATAAAT CTATATATGG TAAAATTTGA TGAACCTGTA	720
GTGTCTAATT ACGTGTATTT TGACATTTCA AAACAGCAAC TACAACTATG GGCCATGTGG	780
AAGAGCCATC GGAGTGGACC TTTTAAACAA TCCTGATTTA GTAGCCACAG ACCCAGTCAT	840
CTCATTCAAG ACTGCTATCT GGTTCCTGGAT GACCCCTCAA TCACCAAAGC CTTCTTGCCA	900
CGATGTCATC ATTGGAAGAT GGAACCCATC TGCCGGTGAC CGATCAGCCA ATCGTCTTCC	960
TGGATTTGGT GTCATCACAA ACATCATCAA TGGGGGCCTG GAATGTGGTC GTGGCAATGA	1020
CAATAGGGTC CAGGATCGCA TTGGGTTTTA CAGGAGGTAT TGCGGTATTC TTGGTGTTAG	1080
TCCTGGTGAC AATCTTGATT GCGGAAACCA GAGATCTTTT GGAAACGGAC TTTTAGTCGA	1140
TACTATGTAA TGA	1153

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 389 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Gly	Ser	Gly	Phe	Ala	Asn	Ala	Val	Tyr	Phe	Thr	Asn	Trp	Gly	Ile	Tyr	1	5	10	15
Gly	Arg	Asn	Phe	Gln	Pro	Ala	Asp	Leu	Pro	Ala	Ser	Glu	Ile	Thr	His	20	25	30	
Val	Leu	Tyr	Ser	Phe	Met	Asn	Val	Arg	Ala	Asp	Gly	Thr	Ile	Phe	Ser	35	40	45	
Gly	Asp	Thr	Tyr	Ala	Asp	Tyr	Glu	Lys	His	Tyr	Ala	Gly	Asp	Ser	Trp	50	55	60	
Asn	Asp	Val	Gly	Thr	Asn	Ala	Tyr	Gly	Cys	Val	Lys	Gln	Leu	Tyr	Leu	65	70	75	80
Leu	Lys	Lys	Gln	Asn	Arg	Asn	Met	Lys	Val	Met	Leu	Ser	Ile	Gly	Gly	85	90	95	
Trp	Thr	Trp	Ser	Thr	Asn	Phe	Pro	Ala	Ala	Ala	Ser	Ser	Ala	Ala	Thr	100	105	110	
Arg	Lys	Thr	Phe	Ala	Gln	Ser	Ala	Val	Gly	Phe	Met	Lys	Asp	Trp	Gly	115	120	125	
Phe	Asp	Gly	Ile	Asp	Ile	Asp	Trp	Glu	Tyr	Pro	Ala	Asp	Ala	Thr	Gln	130	135	140	
Ala	Gln	Asn	Met	Val	Leu	Leu	Leu	Gln	Ala	Val	Arg	Ser	Glu	Leu	Asp	145	150	155	160
Ser	Tyr	Ala	Ala	Gln	Tyr	Ala	Lys	Gly	His	His	Phe	Leu	Leu	Ser	Ile	165	170	175	
Ala	Ala	Pro	Ala	Gly	Pro	Asp	Asn	Tyr	Asn	Lys	Leu	Lys	Phe	Ala	Glu	180	185	190	
Leu	Gly	Lys	Val	Leu	Asp	Tyr	Ile	Asn	Leu	Met	Ala	Tyr	Asp	Tyr	Ala	195	200	205	
Gly	Ser	Trp	Ser	Asn	Tyr	Thr	Gly	His	Asp	Ala	Asn	Ile	Tyr	Ala	Asn	210	215	220	
Pro	Gln	Asn	Pro	Asn	Ala	Thr	Pro	Tyr	Asn	Thr	Asp	Asp	Ala	Val	Gln	225	230	235	240
Ala	Tyr	Ile	Asn	Gly	Gly	Val	Pro	Ala	Asn	Lys	Ile	Val	Leu	Gly	Met	245	250	255	
Pro	Ile	Tyr	Gly	Arg	Ser	Phe	Gln	Gln	Thr	Glu	Gly	Ile	Gly	Lys	Pro	260	265	270	

Tyr Asn Gly Ile Gly Ser Gly Ser Trp Glu Asn Gly Ile Trp Asp Tyr
 275 280 285
 Lys Ala Leu Pro Lys Ala Gly Ala Thr Val Lys Cys Asp Asp Thr Ala
 290 295 300
 Lys Gly Cys Tyr Ser Tyr Asp Pro Ser Thr Lys Glu Leu Ile Ser Phe
 305 310 315 320
 Asp Thr Pro Ala Met Ile Ser Thr Lys Val Ser Trp Leu Lys Gly Lys
 325 330 335
 Gly Leu Gly Gly Ser Met Phe Trp Glu Ala Ser Ala Asp Lys Lys Gly
 340 345 350
 Ser Asp Ser Leu Ile Ser Thr Ser His Gln Gly Leu Gly Ser Gln Asp
 355 360 365
 Ser Thr Gln Asn Tyr Leu Asp Tyr Pro Asn Ser Lys Tyr Asp Asn Ile
 370 375 380
 Lys Lys Gly Met Asn
 385

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

- (B) CLONE: signal peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met Leu Ser Phe Val Lys Lys Ser Ile Ala Leu Val Ala Ala Leu Gln
 1 5 10 15
 Ala Val Thr Ala Leu Ala
 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Thr	Pro	Ile	Ser	Ser	Glu	Ala	Gly	Val	Glu	Lys	Arg
1				5					10		

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1167 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGTAGTGGTT TTGCAAATGC CGTCTACTTC ACCAACTGGG GCATTTATGG CCGCAACTTC	60
CAGCCTGCCG ACCTTCCTGC CTCGGAGATT ACTCACGTAC TCTACTCCTT CATGAATGTC	120
CGCGCAGATG GCACCATCTT TTCCGGTGAT ACCTATGCCG ACTACGAGAA GCACTACGCT	180
GGTGACTCTT GGAACGATGT GGGCACGAAC GCTTACGGTT GTGTTAAGCA ACTTTATCTT	240
CTCAAGAAGC AGAACCGCAA CATGAAGGTG ATGCTGTCTG TTGGTGGTTG GACATGGTCT	300
ACCAACTTCC CCGCTGCCGC CAGCTCGGCT GCTACCCGAA AGACTTTTGC TCAGTCTGCT	360
GTTGGCTTCA TGAAGGACTG GGGTTTCGAC GGTATTGATA TCGACTGGGA GTACCCCGCC	420
GATGCCACTC AGGCTCAGAA TATGGTTCTC TTGCTACAGG CTGTCCGCAG TGAGCTCGAC	480
TCCTACGCTG CCCAGTACGC CAAGGGTCAC CACTTCCTGC TTTCAATTGC CGCCCCTGCT	540
GGACCTGACA ATTATAACAA GCTGAAGTTT GCTGAGCTTG GCAAGGTTCT CGATTACATT	600
AACCTCATGG CTTACGATTA CGCTGGATCT TGGAGCAACT ACACTGGCCA CGATGCCAAC	660
ATATACGCAA ACCCGCAGAA CCCCACGCC ACCCCTTACA ACACGGACGA TGCTGTCCAG	720
GCCTATATCA ACGGCGGCGT CCCTGCCAAC AAGATCGTCC TTGGTATGCC AATCTACGGC	780
CGATCCTTCC AGCAAACCGA GGGTATCGGT AAGCCTTACA ATGGTATTGG CTCTGGTAGC	840
TGGGAGAACG GTATCTGGGA CTACAAGGCT CTCCCCAAGG CTGGTGCCAC CGTCAAGTGC	900
GACGATACCG CCAAGGGATG CTACAGCTAC GATCCAAGCA CTAAGGAGCT TATTTCTTTC	960
GATACGCCGG CTATGATCAG CACCAAAGTT AGCTGGCTCA AGGGCAAGGG CCTTGGCGGC	1020
AGCATGTTCT GGGAGGCTTC TGCCGACAAG AAGGGCTCGG ACTCTCTTAT TAGCACCAGC	1080
CACCAAGGTC TCGGTAGCCA GGACAGCACT CAGAACTACC TCGACTACCC TAACTCCAAG	1140
TACGACAACA TCAAGAAGGG CATGAAC	1167

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 309 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ile	Gly	Val	Cys	Tyr	Gly	Met	Leu	Gly	Asn	Asn	Leu	Pro	Ser	Ala	Asn	1	5	10	15
Asp	Val	Ile	Gly	Leu	Tyr	Arg	Ser	Asn	Asn	Ile	Lys	Arg	Met	Arg	Leu	20	25	30	
Tyr	Asp	Pro	Asn	Gln	Ala	Ala	Leu	Glu	Ala	Leu	Arg	Asn	Ser	Gly	Ile	35	40	45	
Glu	Leu	Ile	Leu	Gly	Val	Pro	Asn	Ser	Asp	Leu	Gln	Gly	Leu	Ala	Thr	50	55	60	
Asn	Pro	Asp	Thr	Ser	Arg	Gln	Trp	Val	Gln	Lys	Asn	Val	Leu	Asn	Phe	65	70	75	80
Trp	Pro	Ser	Val	Lys	Ile	Lys	Tyr	Val	Ala	Val	Gly	Asn	Glu	Val	Ser	85	90	95	
Pro	Val	Gly	Gly	Ser	Ser	Ser	Val	Ala	Gln	Tyr	Val	Leu	Pro	Ala	Ile	100	105	110	
Gln	Asn	Val	Tyr	Gln	Ala	Ile	Arg	Ala	Gln	Gly	Leu	His	Asp	Gln	Ile	115	120	125	
Lys	Val	Ser	Thr	Ser	Ile	Asp	Met	Thr	Leu	Ile	Gly	Asn	Ser	Phe	Pro	130	135	140	
Pro	Ser	Gln	Gly	Ser	Phe	Arg	Gly	Asp	Val	Arg	Ser	Tyr	Leu	Asp	Pro	145	150	155	160
Ile	Ile	Gly	Tyr	Leu	Val	Tyr	Ala	Asn	Ala	Pro	Leu	Leu	Val	Asn	Val	165	170	175	
Tyr	Pro	Tyr	Phe	Ser	Tyr	Thr	Gly	Asn	Pro	Arg	Asp	Ile	Ser	Leu	Pro	180	185	190	
Tyr	Ala	Leu	Phe	Thr	Ala	Pro	Asn	Val	Val	Val	Trp	Asp	Gly	Gln	Tyr	195	200	205	
Gly	Tyr	Gln	Asn	Leu	Phe	Asp	Ala	Met	Leu	Asp	Ser	Val	His	Ala	Ala	210	215	220	
Ile	Asp	Asn	Thr	Lys	Ile	Gly	Tyr	Val	Glu	Val	Val	Val	Ser	Glu	Ser	225	230	235	240
Gly	Trp	Pro	Ser	Asp	Gly	Gly	Phe	Ala	Ala	Thr	Tyr	Asp	Asn	Ala	Arg	245	250	255	
Val	Tyr	Leu	Asp	Asn	Leu	Val	Arg	Arg	Ala	Asn	Arg	Gly	Ser	Pro	Arg	260	265	270	
Arg	Pro	Ser	Lys	Pro	Thr	Glu	Thr	Tyr	Ile	Phe	Ala	Met	Phe	Asp	Glu	275	280	285	
Asn	Gln	Lys	Asn	Pro	Glu	Ile	Glu	Lys	His	Phe	Gly	Leu	Phe	Asn	Pro	290	295	300	

Asn Lys Gln Lys Lys
305

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Tyr	Pro	Phe	Gly	Phe	Gly	Gly	Lys	Arg	Leu	Gly	Lys	Val	Val	Ile	Asp
1				5					10					15	
Asp	Phe	Asn	Ala	Thr	Thr	Ser	Ile	Lys	Ser	Asp	Val				
			20					25							

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 338 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Gln	Ile	Gly	Val	Cys	Tyr	Gly	Met	Leu	Gly	Asn	Asn	Leu	Pro	Ser	Ala
1				5					10					15	
Asn	Asp	Val	Ile	Gly	Leu	Tyr	Arg	Ser	Asn	Asn	Ile	Lys	Arg	Met	Arg
			20					25					30		
Leu	Tyr	Asp	Pro	Asn	Gln	Ala	Ala	Leu	Glu	Ala	Leu	Arg	Asn	Ser	Gly
		35				40					45				
Ile	Glu	Leu	Ile	Leu	Gly	Val	Pro	Asn	Ser	Asp	Leu	Gln	Gly	Leu	Ala
	50				55					60					
Thr	Asn	Pro	Asp	Thr	Ser	Arg	Gln	Trp	Val	Gln	Lys	Asn	Val	Leu	Asn
65				70					75					80	
Phe	Trp	Pro	Ser	Val	Lys	Ile	Lys	Tyr	Val	Ala	Val	Gly	Asn	Glu	Val
			85					90				95			
Ser	Pro	Val	Gly	Gly	Ser	Ser	Ser	Val	Ala	Gln	Tyr	Val	Leu	Pro	Ala
		100						105				110			
Ile	Gln	Asn	Val	Tyr	Gln	Ala	Ile	Arg	Ala	Gln	Gly	Leu	His	Asp	Gln
		115					120					125			

Ile	Lys	Val	Ser	Thr	Ser	Ile	Asp	Met	Thr	Leu	Ile	Gly	Asn	Ser	Phe	130	135	140	
Pro	Pro	Ser	Gln	Gly	Ser	Phe	Arg	Gly	Asp	Val	Arg	Ser	Tyr	Leu	Asp	145	150	155	160
Pro	Ile	Ile	Gly	Tyr	Leu	Val	Tyr	Ala	Asn	Ala	Pro	Leu	Leu	Val	Asn	165	170	175	
Val	Tyr	Pro	Tyr	Phe	Ser	Tyr	Thr	Gly	Asn	Pro	Arg	Asp	Ile	Ser	Leu	180	185	190	
Pro	Tyr	Ala	Leu	Phe	Thr	Ala	Pro	Asn	Val	Val	Val	Trp	Asp	Gly	Gln	195	200	205	
Tyr	Gly	Tyr	Gln	Asn	Leu	Phe	Asp	Ala	Met	Leu	Asp	Ser	Val	His	Ala	210	215	220	
Ala	Ile	Asp	Asn	Thr	Lys	Ile	Gly	Tyr	Val	Glu	Val	Val	Val	Ser	Glu	225	230	235	240
Ser	Gly	Trp	Pro	Ser	Asp	Gly	Gly	Phe	Ala	Ala	Thr	Tyr	Asp	Asn	Ala	245	250	255	
Arg	Val	Tyr	Leu	Asp	Asn	Leu	Val	Arg	Arg	Ala	Asn	Arg	Gly	Ser	Pro	260	265	270	
Arg	Arg	Pro	Ser	Lys	Pro	Thr	Glu	Thr	Tyr	Ile	Phe	Ala	Met	Phe	Asp	275	280	285	
Glu	Asn	Gln	Lys	Asn	Pro	Glu	Ile	Glu	Lys	His	Phe	Gly	Leu	Phe	Asn	290	295	300	
Pro	Asn	Lys	Gln	Lys	Lys	Tyr	Pro	Phe	Gly	Phe	Gly	Gly	Lys	Arg	Leu	305	310	315	320
Gly	Lys	Val	Val	Ile	Asp	Asp	Phe	Asn	Ala	Thr	Thr	Ser	Ile	Lys	Ser	325	330	335	
Asp	Val																		

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Met	Pro	Ser	Leu	Phe	Ala	Arg	Asn	Gln	Arg	Phe	Ser	Leu	Ala	Thr	Leu	1	5	10	15
Leu	Leu	Leu	Leu	Glu	Leu	Leu	Thr	Gly	Asn	Leu	Arg	Met	Ala	Asp	Ala	20	25	30	

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 927 base pairs.
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

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ATTGGTGTGT GTTATGGCAT GCTGGGCAAC AATCTACCGT CAGCAAACGA TGTTATAGGT      60
CTTTATAGAT CAAATAACAT AAAGAGAATG AGACTCTATG ATCCTAATCA AGCTGCTCTA      120
GAAGCACTTA GAAATTCTGG CATTGAACTC ATTCTTGGGG TGCCAAACTC TGACCTTCAA      180
GGCCTTGCCA CCAATCCTGA CACTTCTCGT CAATGGGTGC AAAAAACGT GTTGAAC TTT      240
TGGCCTAGTG TCAAAATCAA GTACGTGGCA GTTGGAAATG AAGTGAGTCC CGTTGGAGGC      300
TCTTCTTCGG TAGCCCAATA TGTTCTACCT GCCATCCAAA ATGTATACCA AGCAATAAGA      360
GCTCAAGGCC TTCATGATCA AATCAAGGTT TCAACATCTA TTGACATGAC CCTAATAGGA      420
AACTCTTTCC CTCCATCGCA AGGTTCTTC AGGGGTGATG TGAGATCATA CCTAGATCCC      480
ATAATTGGGT ACTTGGTATA TGCAAATGCA CCATTACTAG TCAATGTGTA CCCTTATTTT      540
AGTTACACTG GTAACCCCGG TGACATATCA CTTCCCTATG CTCTTTTCAC AGCACCAAAT      600
GTTGTGGTAT GGGATGGTCA ATATGGGTAC CAAAATTTGT TTGATGCTAT GTTGGATTCA      660
GTACATGCAG CCATTGATAA CACTAAGATT GGTTATGTGG AGGTTGTTGT ATCCGAGAGT      720
GGGTGGCCAT CAGATGGAGG ATTTGCTGCC ACTTATGACA ACGCACGCGT GTACTTAGAC      780
AATTTGGTTC GTCGTGCTAA TAGAGGAAGC CCAAGAAGGC CTTCGAAGCC CACTGAGACT      840
TATATATTTG CCATGTTCTGA TGAAAATCAA AAAAATCCAG AGATAGAGAA ACATTTTGGG      900
CTCTTCAATC CCAACAAACA AAAAAAA                      927

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(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 751 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: gf-2.8 of wheat germin

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:21..692

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AAGCTTATTA CATAGCAAGC ATG GGG TAC TCC AAA ACC CTA GTA GCT GGC	50
Met Gly Tyr Ser Lys Thr Leu Val Ala Gly	
1 5 10	
GTG TTC GCA ATG CTG TTA CTA GCT CCG GCC GTC TTG GCC ACC GAC CCA	98
Val Phe Ala Met Leu Leu Leu Ala Pro Ala Val Leu Ala Thr Asp Pro	
15 20 25	
GAC CCT CTC CAG GAC TTC TGT GTC GCC GAC CTC GAC GGC AAG GCG GTC	146
Asp Pro Leu Gln Asp Phe Cys Val Ala Asp Leu Asp Gly Lys Ala Val	
30 35 40	
TCG GTG AAC GGG CAC ACG TGC AAG CCC ATG TCG GAG GCC GGC GAC GAC	194
Ser Val Asn Gly His Thr Cys Lys Pro Met Ser Glu Ala Gly Asp Asp	
45 50 55	
TTC CTC TTC TCG TCC AAG TTG GCC AAG GCC GGC AAC ACG TCC ACC CCG	242
Phe Leu Phe Ser Ser Lys Leu Ala Lys Ala Gly Asn Thr Ser Thr Pro	
60 65 70	
AAC GGC TCC GCC GTG ACG GAG CTC GAC GTG GCC GAG TGG CCC GGT ACC	290
Asn Gly Ser Ala Val Thr Glu Leu Asp Val Ala Glu Trp Pro Gly Thr	
75 80 85 90	
AAC ACG CTG GGT GTG TCC ATG AAC CGC GTG GAC TTT GCT CCC GGA GGC	338
Asn Thr Leu Gly Val Ser Met Asn Arg Val Asp Phe Ala Pro Gly Gly	
95 100 105	
ACC AAC CCA CCA CAC ATC CAC CCG CGT GCC ACC GAG ATC GGC ATC GTG	386
Thr Asn Pro Pro His Ile His Pro Arg Ala Thr Glu Ile Gly Ile Val	
110 115 120	
ATG AAA GGT GAG CTT CTC GTG GGA ATC CTT GGC AGC CTC GAC TCC GGC	434
Met Lys Gly Glu Leu Leu Val Gly Ile Leu Gly Ser Leu Asp Ser Gly	
125 130 135	
AAC AAG CTC TAC TCG AGG GTG GTG CGC GCC GGA GAG ACG TTC CTC ATC	482
Asn Lys Leu Tyr Ser Arg Val Val Arg Ala Gly Glu Thr Phe Leu Ile	
140 145 150	

CCA CGG GGC CTC ATG CAC TTC CAG TTC AAC GTC GGT AAG ACC GAG GCC 530
 Pro Arg Gly Leu Met His Phe Gln Phe Asn Val Gly Lys Thr Glu Ala
 155 160 165 170

TCC ATG GTC GTC TCC TTC AAC AGC CAG AAC CCC GGC ATT GTC TTC GTG 578
 Ser Met Val Val Ser Phe Asn Ser Gln Asn Pro Gly Ile Val Phe Val
 175 180 185

CCC CTC ACG CTC TTC GGC TCC AAC CCG CCC ATC CCA ACG CCG GTG CTC 626
 Pro Leu Thr Leu Phe Gly Ser Asn Pro Pro Ile Pro Thr Pro Val Leu
 190 195 200

ACC AAG GCA CTC CGG GTG GAG GCC AGG GTC GTG GAA CTT CTC AAG TCC 674
 Thr Lys Ala Leu Arg Val Glu Ala Arg Val Val Glu Leu Leu Lys Ser
 205 210 215

AAG TTT GCC GCT GGG TTT TAATTTCTAG GAGCCTTCCC TGAAATGATA 722
 Lys Phe Ala Ala Gly Phe
 220

ATTATATAAT TCCATATATG CATGCTAGC 751

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 224 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Met Gly Tyr Ser Lys Thr Leu Val Ala Gly Val Phe Ala Met Leu Leu
 1 5 10 15
 Leu Ala Pro Ala Val Leu Ala Thr Asp Pro Asp Pro Leu Gln Asp Phe
 20 25 30
 Cys Val Ala Asp Leu Asp Gly Lys Ala Val Ser Val Asn Gly His Thr
 35 40 45
 Cys Lys Pro Met Ser Glu Ala Gly Asp Asp Phe Leu Phe Ser Ser Lys
 50 55 60
 Leu Ala Lys Ala Gly Asn Thr Ser Thr Pro Asn Gly Ser Ala Val Thr
 65 70 75 80
 Glu Leu Asp Val Ala Glu Trp Pro Gly Thr Asn Thr Leu Gly Val Ser
 85 90 95

Met	Asn	Arg	Val	Asp	Phe	Ala	Pro	Gly	Gly	Thr	Asn	Pro	Pro	His	Ile
			100					105					110		
His	Pro	Arg	Ala	Thr	Glu	Ile	Gly	Ile	Val	Met	Lys	Gly	Glu	Leu	Leu
		115					120					125			
Val	Gly	Ile	Leu	Gly	Ser	Leu	Asp	Ser	Gly	Asn	Lys	Leu	Tyr	Ser	Arg
	130					135					140				
Val	Val	Arg	Ala	Gly	Glu	Thr	Phe	Leu	Ile	Pro	Arg	Gly	Leu	Met	His
145					150					155					160
Phe	Gln	Phe	Asn	Val	Gly	Lys	Thr	Glu	Ala	Ser	Met	Val	Val	Ser	Phe
			165						170					175	
Asn	Ser	Gln	Asn	Pro	Gly	Ile	Val	Phe	Val	Pro	Leu	Thr	Leu	Phe	Gly
		180					185						190		
Ser	Asn	Pro	Pro	Ile	Pro	Thr	Pro	Val	Leu	Thr	Lys	Ala	Leu	Arg	Val
	195					200					205				
Glu	Ala	Arg	Val	Val	Glu	Leu	Leu	Lys	Ser	Lys	Phe	Ala	Ala	Gly	Phe
210						215					220				

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Thr	Asp	Pro	Asp	Pro	Leu	Gln	Asp	Phe	Xaa	Val	Ala	Asp	Leu	Asp	Gly
1				5				10					15		
Lys	Ala	Val	Ser	Val	Asn	Gly	His	Thr	Xaa	Lys	Pro	Met	Ser	Glu	Ala
			20					25					30		
Gly	Asp	Asp	Phe	Leu	Phe										
			35												

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Ala Gly Glu Thr Phe Val Ile Pro Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AGCTGGATCC

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CLAIMS

1. Use of a DNA sequence coding for an enzyme
having oxalate oxidase activity as selection agent for
5 transformed plant cells.

2. Use according to Claim 1, wherein the
transformation of the plant cells is carried out with
said DNA sequence in combination with a gene of
10 interest.

3. Use according to any one of Claims 1 and 2,
wherein the enzyme with oxalate oxidase activity is the
oxalate oxidase of sequence [SEQ ID No. 1] below or a
15 sequence having at least 80% of identity with the
sequence [SEQ ID No. 1] below:

	Met	Gly	Tyr	Ser	Lys	Thr	Leu	Val	Ala	Gly	Leu	Phe	Ala	Met	Leu	Leu	
	1				5					10					15		
20	Leu	Ala	Pro	Ala	Val	Leu	Ala	Thr	Asp	Pro	Asp	Pro	Leu	Gln	Asp	Phe	
				20					25					30			
	Cys	Val	Ala	Asp	Leu	Asp	Gly	Lys	Ala	Val	Ser	Val	Asn	Gly	His	Thr	
				35				40					45				
	Cys	Lys	Pro	Met	Ser	Glu	Ala	Gly	Asp	Asp	Phe	Leu	Phe	Ser	Ser	Lys	
25		50					55				60						
	Leu	Ala	Lys	Ala	Gly	Asn	Thr	Ser	Thr	Pro	Asn	Gly	Ser	Ala	Val	Thr	
	65					70				75					80		
	Glu	Leu	Asp	Val	Ala	Glu	Trp	Pro	Gly	Thr	Asn	Thr	Leu	Gly	Val	Ser	
				85					90				95				
	Met	Asn	Arg	Val	Asp	Phe	Ala	Pro	Gly	Gly	Thr	Asn	Pro	Pro	His	Ile	
30				100					105				110				
	His	Pro	Arg	Ala	Thr	Glu	Ile	Gly	Ile	Val	Met	Lys	Gly	Glu	Leu	Leu	
				115				120					125				
	Val	Gly	Ile	Leu	Gly	Ser	Leu	Asp	Ser	Gly	Asn	Lys	Leu	Tyr	Ser	Arg	
		130					135					140					
35	Val	Val	Arg	Ala	Gly	Glu	Thr	Phe	Leu	Ile	Pro	Arg	Gly	Leu	Met	His	
	145					150				155					160		

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7. Use according to Claim 6, wherein the protein of interest is a protein with endochitinase activity.

8. Process for selecting, on oxalic acid,
5 transgenic calluses or transformed plants by a DNA sequence coding for an enzyme having oxalate oxidase activity, wherein it consists of culturing the calluses or the plants on a culture medium containing oxalic acid and calcium in the
10 presence of a chelating agent having an affinity for calcium higher than that of oxalic acid, said chelating agent being chosen from amongst EDTA and EGTA.

9. Process according to Claim 8, wherein the said
15 DNA sequence is associated with a sequence of interest.

10. Process according to any one of Claims 8 to 9, wherein the calluses or the plants belong to one of
20 the species *Nicotiana tabacum*, *Helianthus annuus* and *Brassica napus*.