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(54) **Title:** A PLANT, ITS USE AS A NUTRACEUTICAL AND THE IDENTIFICATION THEREOF

(57) **Abstract:** The present invention generally relates to a transgenic plant, which over-accumulates flavonoids in its vegetative tissues and seeds. In particular, the present invention relates to a transgenic monocotyledonous plant regenerated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

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A PLANT, ITS USE AS A NUTRACEUTICAL AND THE IDENTIFICATION THEREOF

FIELD OF THE INVENTION

The present invention generally relates to a transgenic plant, which over-accumulates flavonoids in its vegetative tissues and seeds. In particular, the present invention relates to a transgenic monocotyledonous plant, part of said plant, or progeny of said plant, wherein said plant, part or progeny comprises a transgene expressing R and C1 polynucleotides, with the proviso that the part is not a single cell.

BACKGROUND OF THE INVENTION

The consumption of foods containing elevated levels of flavonoids such as anthocyanin, resveratrol and isoliquiritigenin has been associated with health benefits in humans. For example, flavonoids have been reported to lower oxidative stress by producing beneficial effects on diseases associated with nitric oxide production (Wang and Mazza, 2002). They have also been shown to have antioxidant, anti-inflammatory, anti-carcinogenic and estrogenic activities (Hollman and Katam, 1999; Peterson and Dwyer, 1998; Robards *et al.*, 1999; Harborne and Williams, 2000). In particular, the flavonoids resveratrol and isoliquiritigenin are known to have the following activities: hair regrowth, anti-colon cancer, aorta vasorelaxant, anti-tumour promoting agent, anti-prostate cancer, anti-gout, anti-depressant activity, anti-coagulant, anti-angiogenesis, anti-neovascularisation and anti-inflammatory activities.

Accordingly, the production of flavonoids and the use of plants comprising high levels of flavonoids are of great interest to scientists and nutraceutical companies.

However, while some plants produce flavonoids by the phenylpropanoid biosynthetic pathway, studies have shown that the level of anthocyanin is inversely related to the level of revesterol in a plant. Moreover, current methods of isolating flavonoids include complex and time-consuming extraction and purification procedures. Therefore, an edible plant with a high-yield of flavonoids, such as anthocyanin, resveratrol and isoliquiritigenin would be desirable for the production of foods containing high levels of flavonoids and a desirable starting material in a process for extracting flavonoids. Furthermore, it would be highly desirable to be able to readily identify suitable plants.

The phenylpropanoid biosynthetic pathway comprises the R and C1 polynucleotides, which encode proteins that activate enzymes involved in the phenylpropanoid pathway to produce anthocyanins. It has been shown that when the maize R and C1 polynucleotides are constitutively expressed in tobacco, anthocyanin levels increase in flowers and roots (Lloyd *et al.*, 1992). Furthermore, haploid plants from immature pollen grains of wheat which are homozygous for the C1 and *bar* polynucleotides over-express anthocyanin (Schmid, 2001).

Accordingly, the introduction of genetic sequences driving expression of the R and C1 polynucleotides and consequent high levels of flavonoid accumulation would be highly desirable. However, to date this has only been achieved in the dicotyledonous plants Tobacco and Arabidopsis. No transgenic monocotyledonous plants expressing the R and C1 polynucleotides have been produced, despite the fact that monocotyledonous plants are the major food crops in the world eg wheat, rice, oats, sorghum, barley, maize, rye, or triticale. While the introduction of R and C1 polynucleotides in single cells of monocotyledonous plants has been shown to result in over-accumulation of the

flavonoid, anthocyanin, (Bower *et al.*, 1996; McKinnon *et al.*, 1996) prior to the present invention it was believed that over-expression of anthocyanin caused termination of cell division and was lethal to the cells (Chawla *et al.*, 1999). For example, Gandikota *et al.*, 2001 were unable to produce red pigmented rice using the C1 and R genes, but were able to produce varying degrees of pigmentation using C2 alone. Furthermore, this group found that those expressing the highest levels of anthocyanin died. Indeed, the only way that a monocotyledonous plant expressing the B polynucleotide, a family member of the R polynucleotide, and C1 polynucleotides has been successfully produced is by transforming the apical meristem of shoots with a transgene expressing B and C1 polynucleotides to produce a chimeric plant; that is, a plant within which some cells express the B and C1 polynucleotides (Chawla *et al.*, 1999). However, the plant was not truly transgenic as the B and C1 polynucleotides were not integrated into the germ-line of the plant, and the plant only displayed pigmentation in discrete sectors rather than pigmentation of the majority of the vegetative tissue of the plant as well as the seeds.

SUMMARY OF THE INVENTION

The inventors have unexpectedly found that it is indeed possible to generate a plant, which comprises a transgene expressing both R and C1 polynucleotides. This plant over-accumulates a number of nutraceuticals including anthocyanin, resveratrol, and isoliquiritigenin, in at least one of vegetative tissue, seeds or roots of the plant.

Accordingly, a first aspect provides a transgenic monocotyledonous plant, part of said plant, or progeny of said plant, wherein said plant, part or progeny comprises a transgene expressing R and C1 polynucleotides,

functionally active derivatives, analogs, homologs or variants thereof, with the proviso that the part is not a single cell.

A second aspect provides a method of producing a transgenic monocotyledonous plant comprising:

(i) transfecting a plant cell with a vector comprising a transgene capable of expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof; and

(ii) regenerating said cell into a plant, thereby producing a plant comprising a transgene expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof.

The monocotyledonous plant may be any variety known in the art. For example, the monocotyledonous plant may be selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat. Preferably, the plant is wheat. Most preferably, the plant can be regenerated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 on 2 July 2003 or NCIMB 41232 on 6 July 2004.

It will be appreciated by the person skilled in the art that the polynucleotides may be cDNA, RNA, or a hybrid molecule thereof. Preferably the polynucleotide is a cDNA molecule encoding an R or C1 polypeptide from any plant. More preferably, the R and C1 polynucleotides are from maize. Most preferably the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively. In a further embodiment, the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively. However, it is appreciated that functionally active variants or homologs of the R and/or C1 polynucleotides

disclosed in SEQ ID Nos 1 and 2 may be used in the methods of the invention.

The vector may be any vector known to a person skilled in the art, which is capable of expressing R and C1 polynucleotides. Preferably, the vector is a plasmid. More preferably, the plasmid is pAnth, pGBA2R or pGBAC1 as shown in Figures 5, 15 and 16 respectively.

A third aspect provides a method of identifying a transgenic plant comprising a transgene expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof comprising detecting the presence of red pigmentation in the vegetative tissue and/or seeds of the plant, wherein the absence of red pigmentation in the vegetative tissue and/or seeds indicates that a plant does not comprise a transgene expressing R and C1 polynucleotides, or functionally active derivatives, analogs, homologs or variants thereof.

A fourth aspect provides a method of identifying a transgenic plant which expresses at least one foreign polynucleotide which is operably linked to the expression of R and C1 polynucleotides, or functionally active derivatives, analogs, homologs or variants thereof, comprising the step of detecting the presence of red pigmentation in the vegetative tissue and/or seeds of the plant wherein a control plant does not have red pigmentation.

A fifth aspect provides the use of a transgenic plant, comprising a transgene expressing R and C1 polynucleotides, as a nutraceutical or in the preparation of a nutraceutical.

A sixth aspect provides a nutraceutical when obtained from a transgenic plant comprising a transgene expressing R and

C1 polynucleotides or a part or progeny of said plant, wherein said part is not a single cell.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide sequence of maize R polynucleotide (SEQ ID NO: 1).

Figure 2 shows the nucleotide sequence of maize C1 polynucleotide (SEQ ID NO: 2).

Figure 3 shows the amino acid sequence of maize R polypeptide (SEQ ID NO: 3).

Figure 4 shows the amino acid sequence of maize C1 polypeptide (SEQ ID NO: 4).

Figure 5 shows a map of pAnth.

Figure 6 shows the different degrees of red pigmentation in progeny of WRB1. Panel A shows a leaf blade from a plant homozygous for the transgenes showing dark red pigmentation. Panel B shows the equivalent leaf blade from a null segregant control plant.

Figure 7 shows the red pigmentation of seed from different WRB1 plants. Panel A shows a null segregant (normally pigmented Westonia, Pigment Rating = 1). Panel B shows a heterozygote plant (Pigment Rating = 7) and Panel C shows a homozygous plant (Pigment Rating = 10).

Figure 8 shows a total ion chromatogram showing the compounds (peaks) as they separate on the HPLC and entered the mass spectrometer.

Figure 9 shows the mass spectrogram of Peak 1 after being separated by HPLC or LC-MS.

Figure 10 shows the mass spectrogram of Peak 2 after being separated by HPLC or LC-MS.

Figure 11 shows the mass spectrogram of Peak 3 after being separated by HPLC or LC-MS.

Figure 12 shows the mass spectrogram of Peak 4 after being separated by HPLC or LC-MS.

Figure 13 shows the peak expanded view of the mass spectrogram of Peak 4 after being separated by HPLC or LC-MS.

Figure 14 shows the heat stability of antioxidant activity of 0.1g samples of seed tissue pre-treated for 20mins at the relevant temperature.

Figure 15 shows a schematic of vector pGBA2C1.

Figure 16 shows a schematic of vector pGBA2R.

Figure 17 shows the mean number of red spots per embryo (with standard deviation bar) (duplicated bombardments retain same shading)

Figure 18 shows the mean number of red spots per embryo (with standard error bar) (duplicated bombardments retain same shading).

Figure 19 shows a map of pGBA2NPTII.

Figure 20 shows a map of pGBA2Cah

Figure 21 shows the nucleotide sequence of the *Zea mays* Lc regulatory protein (SEQ ID NO: 5).

Figure 22 shows the deduced amino acid sequence of the *Zea mays* Lc regulatory protein (SEQ ID NO: 6).

Figure 23 shows the nucleotide sequence of the *Zea mays* B-Peru gene for a regulatory protein (SEQ ID NO: 7).

Figure 24 shows the deduced amino acid sequence of the *Zea mays* B-Peru regulatory protein (SEQ ID NO: 8).

DETAILED DESCRIPTION OF THE INVENTION

All publications mentioned herein are cited for the purpose of describing and disclosing the protocols and reagents which are reported in the publications and which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

The practice of the present invention employs, unless otherwise indicated, conventional molecular biology, plant biology, and recombinant DNA techniques within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature. *See,* eg., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover, Ed., 1985); "Oligonucleotide Synthesis" (M.J. Gait, Ed., 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins, eds., 1985); "Transcription and Translation" (B.D. Hames & S.J. Higgins, eds., 1984); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning: a Laboratory Manual" 12th edition (1989).

The description that follows makes use of a number of terms used in recombinant DNA technology. Unless defined

otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton, *et al.*, "Dictionary of Microbiology and Molecular Biology" (2nd ed. 1994); "The Cambridge Dictionary of Science and Technology" (Walker ed., 1988); "The Glossary of Genetics" 5th Ed., Rieger, R., *et al.* (eds.), Springer Verlag (1991); and Hale & Marham, "The Harper Collins Dictionary of Biology" (1991). Generally, the nomenclature and the laboratory procedures in plant maintenance and breeding as well as recombinant DNA technology described herein are those well known and commonly employed in the art.

It is understood that the invention is not limited to the particular materials and methods described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and it is not intended to limit the scope of the present invention which will be limited only by the appended claims. It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a polynucleotide" includes a plurality of such polynucleotides, and a reference to "an enhancer element" is a reference to one or more enhancer elements. Although any materials and methods similar or equivalent to those described herein can be used to practice or test the present invention, the preferred materials and methods are now described.

One of the broadest aspects of the present invention contemplates the use of a transgene engineered so as to produce a transgenic monocotyledonous plant which expresses R and C1 polynucleotides in vegetative tissue,

seed and/or roots. As used herein, the term "transgenic plant" is intended to refer to a plant that has incorporated therein R and C1 polynucleotide sequences, including but not limited to polynucleotides which are perhaps not normally present, DNA sequences not normally transcribed into RNA or translated into a protein ("expressed"), or any other polynucleotide sequences which one desires to introduce into the non-transformed plant, such as genes which may normally be present in the non-transformed plant, but which one desires to either genetically engineer or to have altered expression.

The term "monocotyledonous plant" as used herein, includes, for example, wheat, sorghum, rice, barley, maize, rye, triticale, oat, palms, bananas, orchids, grasses, lilies, and many garden bulbs and corms. Any of these plants may be used, because although constructs disclosed herein comprise R and C1 polynucleotides isolated from maize, homologs of the maize R and C1 polynucleotides are present in many plants. The R and C1 genes are regulatory genes of the anthocyanin pathway. They are part of a multigene family whose predicted protein products contain a region with homology to DNA-binding domains typically found in transcription factors.

Furthermore, the R and C1 polynucleotides are conserved in different species of plant (Quattrocchio *et al.*, 1988 Plant J 13:475-488). Table 1 shows that the C1 gene from *Zea mays* has significant homology with the structurally conserved DNA-binding domain of MYB proteins. MYB proteins constitute a diverse class of DNA-binding proteins of particular importance in transcriptional regulation in plants.

TABLE 1

		R2	R3
	Position	55	99
		helix	helix turn
	200 match	LRWLNYLRP--KRG-----EE---II-LH--GN--WS-IA-LPGR	
	Majority	LRW-NYLRPD-KRG-FT-EEE---II-LH-LGN-RWS-IA-LPGR	
	Zm C1	LRWLNYLRPNIRRGNISYDEEDL-IIRLHRLLGNRWSLIAGRLPGR	
	Zl C1	**********-**********	
	Zm P-type R2R3	**********M**********	
	Zd Transcript regulator 1	**********-**********	
	Zp Transcript regulator 1	**********-**********	
	P1	**********K**********-*V***K**********	
	Td C1	**********K**********-*****K**********	
	Zm R2R3MYB-domain protein	****---**********M*****R**********	
	Gh MYB-like protein	**********K*****DE****-*****K**********	
	Gh MYB150	**********K*****DE****-*****K**********	
	Gk myb-like factor 2	**********K*****DE****-*****K**********	
	Gh myb-like factor 2	**********K*****DE****-*****K**********	
	Gr myb-like factor 2	**********K*****DE****-*****K**********	
	Gh MYB124 protein	*********DIK*****H***E*-*****N**********	
	Gh MYB36	*********DIK*****H***E*-*****N**********	
	Gh MYB130 protein	*********DIK*****IE**G*-*****R**********	
	Gh MYB120b protein	*********DIK*****H***E*-*****N**********	
	Gh MYB120a	*********DIK*****H***E*-*****N**********	
	Gh MYB107b	*********DIK*****IE**E*-*****K**********	
	Gh MYB107a	*********DIK*****IE**E*-*****K**********	
	Gh MYB38	*********DIK*****IE**E*-*****K**********	
	Gh MYB126	*********DIK*****IG**E*-*****K**********	
	Gh MYB10	*********DIK*****P***E*-**K**K**********	
	Gr Myb 3	***M*******K*****-DQ*EDL*L***K**********	
	Gh Myb 3	***M*******K*****-DQ*EDL*L***K**********	

Zm = Zea mays; Zd = Zea diploperennis; Zp = Zea perennis; Td = Tripsacum dactyloides; Gh = Gossypium hirsutum; Gk = Gossypioides kirkii; Gossypium raimondii

The R gene found in plants is a transcriptional activator, which has significant homology with the helix-loop-helix domain found in a large number of transcription factors. Table 2 shows a number of helix-loop-helix domains from various plants, together with the consensus sequence and *Zea mays* R gene.

TABLE 2

Consensus Sequence	KNHVMSERKRREKLNEMFLILKSLVPSIHKVDKASILAETIAYLKELQRRVQ
Zm R gene	********************V****L****R*N*********V**********
Zm Antho regul. Lc pro	********************V****L****R*N**********************
Zm regulatory protein	********************V****L****R*N**********************
Zm Antho regul. R-S prot	********************V****L****R*N**********************
Zm transcription factor	********************V****L****R*N**********************
Zm gene SN protein	********************V****L****R*N**********************
Zm BHLH protein	*********Q*********V****L****R*N**********************
Zm regul protein B-Peru	********************V**********************************
Zm regulatory protein	*****L**************V**********************************
Os R-type h-l-h protein	********R**************V*****R*****************EK**E
Rice trans act	********R**************V***********************EK**E
Os transcription act	********R*******************D*********S*********E****
Os R-type h-l-h protein	*S******R***************L**VR************T***V*EK**K
Os	****I***R**************I************E********V*EK**K
Os	*S******R***************L**VR************T***V*EK**K
Os	****I***R**************I************E********V*EK**K
Os putative trans act	********R*W*********T********D******S***********E****
Myc-like regul R gene	*K*******************V****L****RGEQ*******************
Myc-like regul R gene	*K*******************V**********************************
Sorghum myc-like regul	*K***********I**************************T***********G**
Myc-like regul R gene	*K*****************A**********************************
Myc-like regul R gene	*K*****************V****A****RM**V****Q******D******
Myc-like regul R gene	*K*****************V************************N*******
Sorghum myc-like regul.	**********************L*****Q**A*V*L*************K**
Myc-like regul R gene	*K******R***EQ**E
At bHLH Trans factor	-**AL**K********R*MT*R*II***S*I**V***DD**E**QD**K***
At thaliana bHLH protein	-**AL**K********R*MT*R*II***S*I**V***DD**E**QD**K***
At bHLH protein	-**A*L*K********R*MT*RKII***N*I**V***DD**E**Q**E****
Rice trans act Ra hom	----****R**************V************F**********EK**E
P. frutescens	-****A**R*******QR*IV*R*M**F*T*M*******D**D***Q*KK*I*
Petunia anthocyanin 1	-***LA**R*******R*I**R****FVT*M******GD**E*V*Q*RKK**
Wild rice trans act	----****R**************V*********************D*EK**E
Oa trans act	----****R**************V*********************EK**E
Oo trans act	----****R**************V*********************EK**E
Os putative intensifier	-***LK**R*******K*I**R****FMT*M******GD**E*V*Q*RN*I*
Oe transcription act	----****R**************V*******A*******P*******EK**E
Gh	*S**L***R****I**RLM*******TNS*A**V***DD**E**QD*E***E
Rice trans act Rb	----****R*********************D**********P*P***D*E**F*
Am h-l-h protein	-R***L********I**R*M**A*****GG****V***DH**D**RG*E*K*
Petunia bHLH trans fact	RSR*I***R****I**R*ML*A*ML*AGG****I*L*D***E*****E****

Zm = Zea mays; Gh = Gossypium hirsutum; Os = Oryza sativa; Oa = Oryza australiensis; Oo = Oryza officinalis; Oe = Oryza eichingeri; At = Arabidopsis thaliana; Am = Antirrhinum majus; h-l-h = helix-loop-helix; trans - transcription; act = activator; regul = regulatory; Antho = anthocyanin

Therefore, although the present specification provides experimental evidence in respect of one species of monocotyledon plant and R and C1 polynucleotides derived from maize, it will be clearly appreciated by a person skilled in the art that the transgenic plant of the invention may be any monocotyledonous plant.

The term "transgene" as used herein refers to any polynucleotide sequence, which codes for a functional R and/or C1 polypeptide, which is introduced into the genome of a monocotyledonous plant cell by experimental manipulations. The transgene may be an "endogenous DNA sequence," or a "heterologous DNA sequence" (ie., "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (eg., a point mutation, the presence of a selectable marker gene, etc.) relative to the naturally-occurring sequence. The term "heterologous DNA sequence" refers to a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Heterologous DNA also includes an endogenous DNA sequence which contains some modification. Generally, although not necessarily, heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is expressed. Examples of heterologous DNA include mutated wild-type genes (ie., wild-type genes that have been modified such that they are no longer wild-type genes), reporter genes, transcriptional and translational regulatory sequences, selectable marker proteins (eg., proteins which confer drug resistance), etc.

Thus, once an appropriate monocotyledonous host plant has

been identified as discussed above, a transgene is constructed which comprises one or more R and one or more C1 polynucleotides. The person skilled in the art will understand that the term "polynucleotide" is synonymous with nucleic acids, and refers herein to deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in all their forms, ie., single and double-stranded DNA, cDNA, mRNA, and the like. It will be clearly understood that the term "polynucleotide" encompasses a full-length molecule encoding a polypeptide including the R and C1 polypeptides, as well as truncated molecules or altered molecules that code for functionally active derivatives, analogs, homologs or variants thereof.

The term "functionally active," when used in reference to the R or C1 polynucleotides of the present invention, refers to the paradigm in which an alteration to a nucleotide sequence does not necessarily affect the sequences ability to code for a polypeptide capable of performing substantially the same function as the unaltered "parent" polypeptide. For example, a nucleotide sequence may be truncated, elongated, or mutated in such a way that the polypeptide coded by the nucleotide sequence differs from the "parent" sequence, but still codes for a polypeptide that is capable of functioning in a substantially similar way to the "parent" molecule. Consequently, a functionally active derivative, analog, homolog or variant of the R or C1 polynucleotide of the present invention will have a nucleotide sequence which differs from the nucleotide sequences shown in Figures 1 or 2, but the polypeptide coded for by the functionally active derivative, analog, homolog or variant is capable of displaying one or more known functional activities associated with the R or C1 polypeptides, such as transcriptional activation. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these

modifications are included herein as long as the kinase activity of C1 or R is present.

It will be appreciated by those skilled in the art that a functionally active derivative, analog, homolog or variant of the R or C1 polynucleotide of the present invention can vary substantially outside regions of importance eg receptor binding sites; however, regions of high sequence conservation between R and C1 polynucleotides isolated from different plant species are likely to code for important regions such as receptor binding sites and the like. Accordingly, it is likely that mutations in these highly conserved regions will not generate functionally active derivatives, analogs, homologs or variants. For example, the nucleotide and/or amino acid sequences shown in Tables 1 and 2, respectively are likely to remain unchanged unless the changes are extremely conservative.

Accordingly, a derivative or analog of the C1 polynucleotide includes, but is not limited to, those functionally active molecules comprising regions that are substantially homologous to the *myb* binding domain or fragments thereof.

Two polynucleotide sequences are "substantially homologous" when at least about 85%, preferably at least about 90%, and most preferably at least about 95%, of the nucleotides match over the defined length of the polynucleotide sequences. Sequences that are substantially similar can be identified in a Southern hybridisation experiment, for example under high, medium or low stringency conditions as defined for that particular system. Defining appropriate hybridisation conditions is within the skill of the art. See eg., Sambrook et al., DNA Cloning, vols. I, II and III. Nucleic Acid Hybridization. However, ordinarily, "stringent conditions" for hybridisation or annealing of nucleic acid

molecules are those that

(1) employ low ionic strength and high temperature for washing, for example, 0.015M NaCl/0.0015M sodium citrate/0.1% sodium dodecyl sulfate (SDS) at 50°C, or

(2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750mM NaCl, 75mM sodium citrate at 42°C.

An example of medium stringency conditions for hybridisation is the use of 50% formamide, 5 X SSC (0.75M NaCl, 0.075M sodium citrate), 50mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 X Denhardt's solution, sonicated salmon sperm DNA (50µg/mL), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 X SSC and 0.1% SDS.

By way of further example, and not intended as limiting, low stringency conditions include those described by Shilo and Weinberg in 1981 (Proc. Natl. Acad. Sci. USA 78:6789-6792). When filters containing DNA are treated using these conditions they are usually pre-treated for 6h at 40°C in a solution containing 35% formamide, 5 X SSC, 50mM Tris-HCl (pH 7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500µg/ml denatured salmon sperm DNA. Hybridisations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridisation mixture for 18-20h at 40°C., and then washed for 1.5h at 55°C in a solution containing 2 X SSC, 25mM Tris-HCl (pH 7.4), 5mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are

washed for a third time at 65-68°C and re-exposed to film. Other conditions of low stringency, which may be used are well known in the art (eg., as employed for cross-species hybridisations).

The C1 or R polynucleotides, functionally active derivatives, analogs or variants of the invention can be produced by various methods known in the art. For example, cloned C1 or R polynucleotides can be modified by any of numerous strategies known in the art (See, for example, Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*.

Additionally, the C1 and R encoding polynucleotide sequences can be mutated *in vitro* or *in vivo*, to create or destroy functional regions or create variations in functional regions and/or form new restriction endonuclease sites or destroy pre-existing ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including, but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson *et al.*, 1978, J. Biol. Chem 253:6551).

Alternatively, polynucleotide variants of the R and C1 polynucleotides may result from degenerate codon substitutions or complementary sequences. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer, et al., Nucleic Acid Res. 19: 5081 (1991); Ohtsuka, et al., J. Biol. Chem. 260: 2605-2608 (1985); and Rossolini, et al., Mol. Cell.

Probes 8: 91-98 (1994)). Alternatively, a variant may be a polynucleotide which is substantially similar to SEQ ID Nos 1 or 2, or in which one or more nucleotides have been added, deleted or substituted, at the 3' and/or 5' end(s) of the polynucleotide, or within the polynucleotide. For example, Shieh et al (1993) altered the targeting characteristics of the R gene in a series of 24 deletion mutants of the LcR allele created by site directed mutagenesis, restriction digests and subcloning. Scheffler *et al* (1994) isolated C1 allelic mutants from mutant maize lines (C1-S, C1-1, c1-p) and one transposable element-induced mutant, c1-m1, which they used to vary the regulation of anthocyanin biosynthesis in maize tissues. Novel chimeric versions of the B genes, which are homologues of R were made by Radicella *et al* (1992) to alter anthocyanin production levels in maize aleurone. These chimeric variants were created from the B-1 and B-Peru allelic variants by standard restriction digest, cloning and ligation procedures.

Polynucleotide "homologs" refers to DNAs or RNAs and polymers thereof in either single- or double-stranded form containing known analogues of natural nucleotides, which have similar binding properties as the reference nucleic acid and are metabolised in a manner similar to naturally occurring nucleotides. The main class of homologue for C1 is P1, and that of R is B. Both of these classes contain allelic variants or mutations as outlined above, but not exclusive to those. For example four C1 alleles are ^{S}C1, c1-p, ^{Cornell}C and ^{W22C}C. Two R alleles are Lc and R-S. Two P alleles are P1-vv and P1-rr. Two B alleles are B-1 and B-Peru.

In one embodiment, the R and C1 polynucleotides are double-stranded DNA molecules having at least 85%, more preferably at least 95%, even more preferably 99% nucleotide sequence identity with SEQ ID NO 1 and 2,

respectively. In one embodiment, the R and C1 polynucleotides are double-stranded DNA molecules consisting essentially of the nucleotide sequences shown in SEQ ID NO 1 and 2. The term "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its normal, double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (eg., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (ie., the strand having a sequence homologous to the mRNA).

Australian patent Number 688297 (WO94/13822) discloses the use of B-Peru (see Figures 23 and 24) and C1; however, this patent does not demonstrate expression of both the C1 and B-Peru genes. In contrast, the present invention has demonstrated functional expression of both C1 and R genes, with a deep red colour in the leaves and seeds of plants (see Examples, *infra*).

Once the appropriate transgene has been identified and either isolated or constructed, it is incorporated into an expression vector by standard techniques. Accordingly, the present invention also contemplates an expression vector comprising the transgene of the present invention. Thus, in one embodiment an expression vector is constructed which comprises an isolated and purified DNA molecule comprising a promoter operably linked to the coding region for the C1 or R polypeptides, which coding region is operatively linked to a transcription-terminating region,

whereby the promoter drives the transcription of the coding region. The coding region may include a segment or sequence encoding C1 or R polypeptides. The DNA molecule comprising the expression vector may also contain a plant functional intron, and may also contain other plant functional elements such as sequences encoding untranslated sequences (UTL's) and sequences which act as enhancers of transcription or translation.

As used herein, the terms "operatively linked" or "operably linked" mean that a sequence which functions as a promoter is connected or linked to a coding region in such a way that the transcription of that coding region is controlled and regulated by that promoter. Means for operatively linking a promoter to a coding region to regulate both upstream and downstream are well known in the art.

Preferred plant transformation vectors include, but are not limited to, those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, eg., by Herrera-Estrella (1983), Bevan (1983), Klee (1985) and Eur. Pat. Appl. No. EP 0120516 (each specifically incorporated herein by reference).

As the expression vectors of the present invention are preferably used to transform a monocotyledonous plant, a promoter is selected that has the ability to drive expression in that particular species of plant. Promoters that function in different plant species are also well known in the art. Promoters useful in expressing the polypeptide in plants are those which are inducible, viral, synthetic, or constitutive as described (Odell *et al.*, 1985), and/or temporally regulated, spatially regulated, and spatio-temporally regulated. Preferred promoters include the enhanced CaMV35S promoters, and the

maize ubiquitin promoter (Christensen & Quail, (1996), *Transgenic Research*, 5: 213-218).

The expression of a gene which exists in double-stranded DNA form localised to the plant nuclear genome involves transcription of messenger RNA (mRNA) from the coding strand of the DNA by an RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. Transcription of DNA into mRNA is regulated by a region of DNA referred to as the "promoter". The DNA comprising the promoter is represented by a sequence of bases that signals RNA polymerase to associate with the DNA and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding strand of RNA. The particular promoter selected should be capable of causing sufficient expression of the C1 or R polypeptide coding sequences to result in the presence of red pigmentation in the vegetative tissue, seeds or roots of monocotyledonous plant.

C1 or R polynucleotides of the present invention can be driven by a variety of promoters in plant tissues. Promoters can be near-constitutive (ie. they drive transcription of the transgene in all tissue), such as the CaMV35S promoter, or tissue-specific or developmentally specific. Enhanced or duplicate versions of the CaMV35S and FMV35S promoters are particularly useful in the practice of this invention (Kay *et al.*, 1987; Rogers, U.S. Pat. No. 5,378,619).

Those skilled in the art will recognise that there are a number of promoters, which are active in plant cells, and have been described in the literature. Such promoters may be obtained from plants or plant viruses and include, but are not limited to, the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on

tumor-inducing plasmids of *A. tumefaciens*), the cauliflower mosaic virus (CaMV) 19S and 35S promoters, the light-inducible promoter from the small subunit of ribulose 1,5-bisphosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide), the rice Act1 promoter, the maize ubiquitin promoter and the Figwort Mosaic Virus (FMV) 35S promoter. All of these promoters have been used to create various types of DNA constructs which have been expressed in plants (see eg., McElroy *et al.*, 1990, U.S. Pat. No. 5,463,175, Christensen and Quail, 1996, *supra*).

In addition, it may also be preferred to bring about expression of C1 or R polynucleotides by using plant integrating vectors containing a tissue-specific promoter. Specific target tissues may include the leaf, stem, root, tuber, seed, fruit, etc., and the promoter chosen should have the desired tissue and developmental specificity. Therefore, promoter function should be optimised by selecting a promoter with the desired tissue expression capabilities and approximate promoter strength, and selecting a transformant which produces the desired level of red pigmentation in the target tissues. This selection approach from the pool of transformants is routinely employed in expression of heterologous structural genes in plants since there is variation between transformants containing the same heterologous gene due to the site of gene insertion within the plant genome (commonly referred to as "position effect"). In addition to promoters which are known to cause transcription (constitutive or tissue-specific) of DNA in plant cells, other promoters may be identified for use in the current invention by screening a plant cDNA library for genes which are selectively or preferably expressed in the target tissues, then determining the promoter regions.

Other exemplary tissue-specific promoters are corn sucrose synthetase 1 (Yang *et al.*, 1990), corn alcohol

dehydrogenase 1 (Vogel *et al.*, 1989), corn light harvesting complex (Simpson, 1986), corn heat shock protein (Odell *et al.*, 1985), pea small subunit RuBP carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (McBride and Summerfelt, 1989), Ti plasmid nopaline synthase (Langridge *et al.*, 1989), petunia chalcone isomerase (Van Tunen *et al.*, 1988), bean glycine rich protein 1 (Keller et al., 1989), CaMV 35s transcript (Odell et al., 1985) and Potato patatin (Wenzler et al., 1989) promoters. Preferred promoters are the cauliflower mosaic virus (CaMV 35S) promoter and the S-E9 small subunit RuBP carboxylase promoter.

The promoters used in the DNA constructs of the present invention may be modified, if desired, to affect their control characteristics. For example, the CaMV35S promoter may be ligated to the portion of the ssRUBISCO gene that represses the expression of ssRUBISCO in the absence of light, to create a promoter which is active in leaves but not in roots. For purposes of this description, the phrase "CaMV35S" promoter thus includes variations of CaMV35S promoter, eg., promoters derived by means of ligation with operator regions, random or controlled mutagenesis, etc. Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Examples of such enhancer sequences have been reported by Kay *et al.* (1987).

A transgenic plant of the present invention produced from a plant cell transformed with a tissue specific promoter can be crossed with a second transgenic plant developed from a plant cell transformed with a different tissue specific promoter to produce a hybrid transgenic plant that shows the effects of transformation in more than one specific tissue.

The RNA produced by a DNA construct of the present invention may also contain a 5' non-translated leader sequence (5'UTL). This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. One plant gene leader sequence for use in the present invention is the petunia heat shock protein 70 (hsp70) leader (Winter *et al.*, 1988).

5' UTL's are capable of regulating gene expression when localised to the DNA sequence between the transcription initiation site and the start of the coding sequence. Compilations of leader sequences have been made to predict optimum or sub-optimum sequences and generate "consensus" and preferred leader sequences (Joshi, 1987). Preferred leader sequences are contemplated to include those which comprise sequences predicted to direct optimum expression of the linked structural gene, ie. to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants, and in maize in particular, will be most preferred. One particularly useful leader may be the petunia HSP70 leader.

For optimised expression an intron may also be included in the DNA expression construct. Such an intron is typically placed near the 5' end of the mRNA in untranslated sequence. This intron could be obtained from, but not

limited to, a set of introns consisting of the maize heat shock protein (HSP) 70 intron (U.S. Pat. No. 5,424,412; 1995), the rice Act1 intron (McElroy *et al.*, 1990), the Adh intron 1 (Callis *et al.*, 1987), the maize ubiquitin intron (Christensen & Quail, 1996, *supra*) or the sucrose synthase intron (Vasil *et al.*, 1989).

The 3' non-translated region of the genes of the present invention which are localised to the plant nuclear genome also contain a polyadenylation signal which functions in plants to cause the addition of adenylate nucleotides to the 3' end of the mRNA. RNA polymerase transcribes a nuclear genome coding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA). Examples of preferred 3'0 regions are (1) the 3' transcribed, non-translated regions containing the polyadenylation signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene and (2) the 3' ends of plant genes such as the pea ribulose-1,5-bisphosphate carboxylase small subunit gene, designated herein as E9 (Fischhoff *et al.*, 1987). Constructs will typically include the C1 or R polynucleotides along with a 3' end DNA sequence that acts as a signal to terminate transcription and, in constructs intended for nuclear genome expression, allow for the poly-adenylation of the resultant mRNA. The most preferred 3' elements are contemplated to be those from the nopaline synthase gene of *A. tumefaciens* (nos 3'end) (Bevan *et al.*, 1983), the terminator for the T7 transcript from the octopine synthase gene of *A. tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as TMV .OMEGA. element

(Gallie, *et al.*, 1989), may further be included where desired.

Transcription enhancers or duplications of enhancers could be used to increase expression. These enhancers often are found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted in the forward or reverse orientation 5' or 3' to the coding sequence. Examples of enhancers include elements from the CaMV 35S promoter, octopine synthase genes (Ellis *et al.*, 1987), the rice actin gene, and promoter from non-plant eukaryotes (eg., yeast; Ma *et al.*, 1988).

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5' methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can

be simultaneously engineered into a cell with a single construct and a single selectable marker.

The choice of which expression vector and ultimately to which promoter the C1 or R polynucleotides are operatively linked depends directly on the functional properties desired, eg., the location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention is capable of directing the expression of the polypeptide coding region to which it is operatively linked.

Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *A. tumefaciens* described (Rogers et al., 1987). However, several other plant integrating vector systems are known to function in plants including pCaMVCN transfer control vector described (Fromm *et al.*, 1985). pCaMVCN (available from Pharmacia, Piscataway, N.J.) includes the CaMV35S promoter.

In preferred embodiments, the vector used to express the C1 or R polynucleotides includes a selection marker that is effective in a plant cell, preferably a drug resistance selection marker. One preferred drug resistance marker is the gene whose expression results in kanamycin resistance; ie. the chimeric gene containing the maize ubiquitin promoter, Tn5 neomycin phosphotransferase and maize zein terminator herein described (pGBA2NPTII).

Means for preparing expression vectors are well known in the art. Expression (transformation) vectors used to transform plants and methods of making those vectors are described in U.S. Pat. Nos. 4,971,908, 4,940,835,

4,769,061 and 4,757,011 (each of which is specifically incorporated herein by reference). Those vectors can be modified to include a coding sequence in accordance with the present invention.

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted and to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

In one embodiment double-stranded DNA coding for the C1 and R polynucleotides shown in SEQ ID No. 1 and No. 2, respectively are linked to the CaMV35S promoter and the ADH enhancer element to form an expression vector termed "pAnth", which is shown in Figure 5.

A monocotyledonous plant transformed with an expression vector of the present invention is also contemplated. A transgenic plant derived from such a transformed or transgenic cell is also contemplated. Those skilled in the art will recognise that a chimeric plant gene containing a structural coding sequence of the present invention can be inserted into the genome of a plant by methods well known in the art. Such methods for DNA transformation of plant cells include *Agrobacterium*-mediated plant transformation, the use of liposomes, transformation using viruses or pollen, electroporation, protoplast transformation, gene transfer into pollen, injection into reproductive organs, injection into immature embryos and particle bombardment. Each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant strain may not necessarily be the most effective for another plant strain, but it is

well known which methods are useful for a particular plant strain.

There are many methods for introducing transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods are believed to include virtually any method by which DNA can be introduced into a cell, such as infection by *A. tumefaciens* and related *Agrobacterium* strains, direct delivery of DNA such as, for example, by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation with silicon carbide fibres, by acceleration of DNA coated particles, etc. In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

Technology for introduction of DNA into cells is well-known to those of skill in the art. Four general methods for delivering a gene into cells have been described: (1) chemical methods (Graham and van der Eb, 1973); (2) physical methods such as microinjection (Capecchi, 1980), electroporation (Wong and Neumann, 1982; Fromm *et al.*, 1985) and the gene gun (Johnston and Tang, 1994; Fynan et al., 1993); (3) viral vectors (Clapp, 1993; Lu *et al.*, 1993; Eglitis and Anderson, 1988a; 1988b); and (4) receptor-mediated mechanisms (Curiel *et al.*, 1991; 1992; Wagner *et al.*, 1992).

The application of brief, high-voltage electric pulses to a variety of animal and plant cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of the redistribution of membrane components that accompanies closure of the pores. Electroporation can be extremely efficient and can be used

both for transient expression of cloned genes and for establishment of cell lines that carry integrated copies of the gene of interest. Electroporation, in contrast to calcium phosphate-mediated transfection and protoplast fusion, frequently gives rise to cell lines that carry one, or at most a few, integrated copies of the foreign DNA.

The introduction of DNA by means of electroporation is well-known to those of skill in the art. To effect transformation by electroporation, one may employ either friable tissues such as a suspension culture of cells, or embryogenic callus, or alternatively, one may transform immature embryos or other organised tissues directly. One would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner, rendering the cells more susceptible to transformation. Such cells would then be recipient to DNA transfer by electroporation, which may be carried out at this stage, and transformed cells then identified by a suitable selection or screening protocol dependent on the nature of the newly incorporated DNA.

A further advantageous method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like. Using these particles, DNA is carried through the cell wall and into the cytoplasm on the surface of small metal particles as described (Klein et al., 1987; Klein et al., 1988; Kawata et al., 1988). The metal particles penetrate through several layers of cells and thus allow the transformation of cells within tissue explants.

An advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming plant cells, is that neither the isolation of protoplasts (Cristou *et al.*, 1988) nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with the plant cultured cells in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the microprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more foci of cells transiently expressing a marker gene. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and average 1 to 3.

In bombardment transformation, one may optimise the pre-bombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters

for bombardment are important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such as linearised DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature plant embryos.

Accordingly, it is contemplated that one may desire to adjust various of the bombardment parameters in small scale studies to fully optimise the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance, and helium pressure. One may also minimise the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art in light of the present disclosure.

The methods of particle-mediated transformation is well-known to those of skill in the art. U.S. Pat. No. 5,015,580 (specifically incorporated herein by reference) describes the transformation of soybeans using such a technique.

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described (Fraley *et al.*, 1985; Rogers *et al.*, 1987). The genetic engineering of cotton plants using *Agrobacterium*-mediated transfer is described in U.S. Pat. No. 5,004,863 (specifically incorporated herein by reference); like transformation of lettuce plants is described in U.S. Pat. No. 5,349,124 (specifically incorporated herein by reference); and the *Agrobacterium*-mediated transformation of soybean is described in U.S. Pat. No. 5,416,011 (specifically incorporated herein by reference). Further, the integration of the Ti-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is defined by the border sequences, and intervening DNA is usually inserted into the plant genome as described (Spielmann *et al.*, 1986; Jorgensen *et al.*, 1987).

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.*, 1987), have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant

varieties where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

Agrobacterium-mediated transformation of leaf disks and other tissues such as cotyledons and hypocotyls appears to be limited to plants that *Agrobacterium* naturally infects. *Agrobacterium*-mediated transformation is most efficient in dicotyledonous plants. Few monocots appear to be natural hosts for *Agrobacterium*, although transgenic plants have been produced in asparagus using *Agrobacterium* vectors as described (Bytebier et al., 1987). Other monocots recently have also been transformed with *Agrobacterium*. Included in this group are corn (Ishida *et al.*) and rice (Cheng *et al.*).

A transgenic plant formed using *Agrobacterium* transformation methods typically contains a single gene on one chromosome. Such transgenic plants can be referred to as being heterozygous for the added gene. However, inasmuch as use of the word "heterozygous" usually implies the presence of a complementary gene at the same locus of the second chromosome of a pair of chromosomes, and there is no such gene in a plant containing one added gene as here, it is believed that a more accurate name for such a plant is an independent segregant, because the added, exogenous gene segregates independently during mitosis and meiosis.

An independent segregant may be preferred when the plant is commercialised as a hybrid, such as corn. In this case, an independent segregant containing the gene is crossed with another plant, to form a hybrid plant that is heterozygous for the gene of interest.

An alternate preference is for a transgenic plant that is homozygous for the added C1 and R polynucleotides; ie. a

transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants produced for gene of interest activity and mendelian inheritance indicating homozygosity relative to a control (native, non-transgenic) or an independent segregant transgenic plant.

Two different transgenic plants can be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes that encode a polypeptide of interest. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see eg., Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Fromm *et al.*, 1985; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

Application of these systems to different plant germplasm depends upon the ability to regenerate that particular plant variety from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (see, eg., Fujimura *et al.*, 1985; Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986).

To transform plant germplasm that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilised. For example,

regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1988).

DNA can also be introduced into plants by direct DNA transfer into pollen as described (Zhou *et al.*, 1983; Hess, 1987). Expression of polypeptide coding genes can be obtained by injection of the DNA into reproductive organs of a plant as described (Pena *et al.*, 1987). DNA can also be injected directly into the cells of immature embryos and introduced into cells by rehydration of desiccated embryos as described (Neuhaus *et al.*, 1987; Benbrook *et al.*, 1986).

After effecting delivery of exogenous R and C1 polynucleotides to recipient monocot cells, the next step to obtain the transgenic plants of the present invention generally concerns identifying the transformed cells for further culturing and plant regeneration. As mentioned herein, in order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the R and C1 polynucleotides. In this case, one would then generally assay the potentially transformed cell population by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

An exemplary embodiment of methods for identifying transformed cells involves exposing the transformed cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing. One example of a preferred marker gene confers resistance to glyphosate. When this gene is used as a selectable marker, the putatively transformed cell culture is treated with

glyphosate. Upon treatment, transgenic cells will be available for further culturing while sensitive, or non-transformed cells, will not. This method is described in detail in U.S. Pat. No. 5,569,834, which is specifically incorporated herein by reference. Another example of a preferred selectable marker system is the neomycin phosphotransferase (nptII) resistance system by which resistance to the antibiotic kanamycin is conferred, as described in U.S. Pat. No. 5,569,834 (specifically incorporated herein by reference). Again, after transformation with this system, transformed cells will be available for further culturing upon treatment with kanamycin, while non-transformed cells will not. Yet another preferred selectable marker system involves the use of a gene construct conferring resistance to paromomycin. Use of this type of a selectable marker system is described in U.S. Pat. No. 5,424,412 (specifically incorporated herein by reference. A further example is the use of the cyanamide hydratase gene conferring resistance to cyanamide (Weeks *et al.*, (2000), *Crop Science*, 40:1749-1754)

Another embodiment is the use of a positive selection gene such as that for phosphomannose isomerase (*pmi*) which allows any such transformed cells to feed from otherwise unmetabolisable mannose when supplied in the medium (Wright *et al.*, (2001), *Plant Cell Reports*, 20:429-436), so conferring selective advantage upon these transformed cells.

Another preferred selectable marker system involves the use of the genes contemplated by this invention. In particular, cells transformed with the C1 and R polynucleotides or functional equivalents or a substantially similar gene encoding an AMPA transacylase will develop red pigmentation. Plant cells which have had a recombinant DNA molecule introduced into their genome

can thus be selected from a population of cells which failed to incorporate a recombinant molecule by growing the cells and isolating cells which have red pigmentation.

It is further contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as glyphosate or kanamycin, may either not provide enough killing activity to clearly recognise transformed cells or may cause substantial non-selective inhibition of transformants and non-transformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as kanamycin would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. It is proposed that combinations of selection and screening may enable one to identify transformants in a wider variety of cell and tissue types.

The development or regeneration of plants from either single plant protoplasts or various explants is well known in the art (Weissbach and Weissbach, 1988). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualised cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

The development or regeneration of plants containing the foreign, exogenous gene that encodes a polypeptide of interest introduced by *Agrobacterium* from leaf explants

can be achieved by methods well known in the art such as described (Horsch *et al.*, 1985). In this procedure, transformants are cultured in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant strain being transformed as described (Fraley *et al.*, 1983). In particular, U.S. Pat. No. 5,349,124 (specification incorporated herein by reference) details the creation of genetically transformed lettuce cells and plants resulting therefrom which express hybrid crystal proteins conferring insecticidal activity against Lepidopteran larvae to such plants.

This procedure typically produces shoots within two to four months and those shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Shoots that rooted in the presence of the selective agent to form plantlets are then transplanted to soil or other media to allow the production of roots. These procedures vary depending upon the particular plant strain employed, such variations being well known in the art.

Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants, or pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important, preferably inbred lines. Conversely, pollen from plants of those important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

In one embodiment, a transgenic plant of this invention thus has an increased amount of a coding region encoding R and C1 polypeptides. A preferred transgenic plant is an independent segregant and can transmit these genes and their activities to its progeny. A more preferred

transgenic plant is homozygous for the R and C1 polynucleotides, and transmits these to all of its offspring on sexual mating. Seed from a transgenic plant may be grown in the field or greenhouse, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines that are evaluated for expression of the R and C1 transgene as well as for red pigmentation and/or nutraceutical production. One preferred transgenic plant may be generated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

It is contemplated that in some instances the genome of a transgenic plant will have been augmented through the stable introduction of one or more C1 and R transgenes, either native, synthetically modified, or mutated. In some instances, more than one transgene will be incorporated into the genome of the transformed host plant cell. Such is the case when more than one C1 and R-encoding DNA segments are incorporated into the genome of such a plant. In certain situations, it may be desirable to have one, two, three, four, or even more C1 and R proteins (either native or recombinantly-engineered) incorporated and stably expressed in the transformed transgenic plant.

Plants of the invention may have any intensity of red pigmentation, provided that at least 50% of the overall vegetative tissues of the plant are pigmented. The seeds and roots of the plants may also be pigmented. Preferably, at least 70% of the vegetative tissue and seeds of the plant have red pigmentation. More preferably, at least 90% of the vegetative tissue and seeds have red pigmentation. Most preferably, 100% of the vegetative tissue and seeds have red pigmentation.

It will be clearly understood by the skilled addressee that plants of the present invention may also be used to express one or more foreign polynucleotides in addition to the R and C1 polynucleotides, or functional variants or homologs thereof. For example, the present invention may be used as a marker for a transgenic plant.

The presence of red pigmentation indicates that the plant expresses the foreign polypeptide(s) as well as R and C1 polynucleotides, or functional variants or homologs thereof. The foreign polynucleotide(s) may encode a polypeptide such as a pharmaceutical. Alternatively, the specific polynucleotide(s) may be a pharmaceutical.

The transgenic plant of the invention may be used as a nutraceutical. A nutraceutical is any food or food ingredient considered to provide medical or health benefits, including the prevention and treatment of disease. For example, it is known that R and C1 polynucleotides can upregulate the biochemical pathway that results in accumulation of anthocyanins (Ludwig et al., 1990; Goff et al., 1991; Lloyd et al., 1992). The pathway diverges at the compound 4-hydroxy-cinnamyl-coA. At this point it can be converted into 3,4',5-trihydroxystilbene (resveratrol) which hydroxylated into 3,3',4',5-tetrahydroxystilbene or it can be converted into chalcone which is the precursor to a large family of flavonoids and flavinins. Many of the anthocyanins have demonstrated anti-oxidant activities (Fauconneau *et al.*, 1997; Wang *et al.*, 1997) and are thus candidates to confer health benefits when eaten. Resveratrol has also been shown to confer health benefits to humans (Holmes-McNary 2000; Bhat and Pezzuto 2002; Stewart *et al.*, 2003).

A plant of the invention may readily be identified by the red pigmentation of its vegetative tissue and seeds. This has the advantage that the plant may be readily

distinguished from "control" plants, ie, a plant of the same variety, of a similar age, and grown under similar conditions, as the plant of the invention, such that differences in the pigmentation of the plants is likely to be a result of the difference in expression of R and C1 polynucleotides.

Throughout the specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", means "including but not limited to" and is not intended to exclude other additives, components, integers or steps.

The invention will now be further described by way of reference only to the following non-limiting examples. It should be understood, however, that the examples following are illustrative only, and should not be taken in any way as a restriction on the generality of the invention described above. In particular, while the invention is described in detail in relation to the use of R and C1 genes isolated from maize and wheat plants, it will be clearly understood that the findings herein are not limited to these sources of R and C1 genes or plants.

EXAMPLE 1 CONSTRUCTION OF R AND C1 EXPRESSION VECTORS

The double-stranded DNA plasmid as used by Bower *et al* (1996), which carries the C1 (Figure 1 - SEQ ID NO: 1) and R genes (Figure 2 - SEQ ID NO: 2) on an ampicillin resistance pUC 118 plasmid of 9056bp was isolated and purified by standard techniques. Both the C1 and R genes are attached to 400bp Pin II terminator isolated from potato, alcohol dehydrogenase intron 1 and driven by a dual CaMV35S promoter in which a full-length promoter is combined with a truncated one. The two cassettes are linked in tandem.

The coding sequence for the R polynucleotide encodes the amino acid sequence shown in Figure 3 (SEQ ID NO: 3), while the coding sequence for C1 polynucleotide encodes the amino acid sequence shown in Figure 4 (SEQ ID NO: 4).

A schematic of the above described expression vector termed pAnth is shown in Figure 5.

The R and C1 genes were isolated from pAnth by PCR and independently cloned into pUC118 plasmid vectors between the maize ubiquitin (plus intron 1) promoter and zein terminator (Figures 15 and 16).

EXAMPLE 2 PRODUCTION OF TRANSGENIC WHEAT PLANTS CONTAINING INTRODUCED DNA CONSTRUCTS

Transgenic wheat plants, containing DNA constructs in which the maize C1 and R polynucleotides were linked to a promoter region were generated using the following procedures.

Target Tissues

Wheat plants (cultivars Westonia, Bobwhite) were grown at 22- 24°C in a glasshouse. Seeds containing immature embryos were harvested at 11-15 days post-anthesis and surface sterilised. Immature embryos were excised and placed on MS (Murashige and Skoog, 1962) medium containing 2.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) for five to sixteen days prior to bombardment.

Microprojectile Bombardment

Osmoticum treatments of target tissues, DNA precipitation and microprojectile bombardment were performed as described for sugarcane (Bower *et al.*, 1996) with the exception of the use of tungsten particles. Wheat tissues

were bombarded with 150µg of gold particles per bombardment, which had previously been coated with pAnth, or a combination of pGBA2R1 and pGBA2C1, with either pGBA2NPTII (Figure 19) or pGBA2cah (Figure 20). pGBA2NPTII encodes the gene for neomycin phosphotransferase which confers resistance to the antibiotic kanamycin, and pGBA2cah encodes the gene for cyanamide hydratase which confers resistance to the herbicide cyanamide.

Regeneration of Transgenic Wheat Lines

Following bombardment the embryos were placed on MS medium containing 2.5mg/l 2,4-D for two weeks at 24°C in the dark, then transferred to the same medium plus either 40mg/l cyanamide, or 150mg/l kanamycin. They were then cultured in the same way once again for two weeks except that the cyanamide selection was elevated to 50mg/l with the 2,4D levels reduced to 0.5mg/l or zero in the cases of cyanamide and kanamycin selection respectively. All plates from this time are exposed to 16hr per day fluorescent light. Transfers to the same medium with no 2,4D were carried out after a further two weeks. Multiple green shoots were regenerated among which red shoots expressing the C1 and R polynucleotides were readily identifiable. Red pigmented shoots were selectively subcultured then transferred to ½ strength MS medium for rooting under either 150mg/l kanamycin selection, or 65mg/l cyanamide selection. Red pigmented plants from both Westonia and Bobwhite were regenerated and these were grown to seed in the glasshouse under seasonal conditions, but with cooling to keep the temperature as close to 22^{0}C as possible.

8550 embryos were bombarded, and three Westonia and one Bobwhite wheat plants were regenerated with pigmented leaves.

Transgenic Phenotype

Plants from lines WRB1, WDC1, WDC2 and BWJM1 showed a high degree of pigmentation in the leaves, particularly under bright light levels in the glasshouse, and produced seed that showed a distinct red pigmentation. Figure 6 shows the typical leaf dark red pigmentation of a homozygous plant (Panel A) as compared to the light pigmentation of a null segregant control (Panel B).

One primary transgenic line WRB1 (cv Westonia) was selected for an examination of the heritability of the red pigmented phenotype. Seeds of this line, that were a result of self-pollination, were grown to seed. The seeds were all similarly pigmented. Sixteen of the seeds were planted and grown to maturity. After self-pollination seeds were produced and scored on the basis of degree of red pigmentation. It was noted that there were three distinct phenotypes based on degree of red pigmentation. These were scored on the following scale. 1 = normal Westonia wheat colour, predominantly yellow with no visible red pigmentation, and 10 = very dark red. Figure 7 shows examples of seed from each of the three classes observed. These were Panel A: null segregants (normally pigmented Westonia, Pigment Rating = 1); Panel B heterozygotes (Pigment Rating = 7) and Panel C. homozygotes (Pigment Rating = 10). To determine whether the different classes of pigmentation was due to segregation of the transgene six seeds of the dark red seeds (Pigment Rating = 10), three seeds of the moderately red seeds (Pigment Rating = 7), and seven seeds with normal Westonia cultivar pigmentation (Pigment Rating = 1) were planted and grown to maturity.

Plants from dark red seed (homozygous) produced only dark red seeds (75 seeds sampled), with no moderately red pigmented seeds and no typical non-red pigmented Westonia

seeds. Plants grown from moderately red pigmented seeds (heterozygous) produced progeny that segregated into three classes, 45 moderately red seeds, 14 dark red seeds, and no seeds lacking red pigmentation. Plants grown from seeds with normal Westonia pigmentation produced only seeds showing the same lack of red pigmentation. These segregation patterns are consistent with a high degree of red pigmentation in plants that are homozygous for the introduced C1 and R polynucleotides, while plants that are heterzygotic for the transgenes show an intermediate degree of red pigmentation and null segregants show the normal lack of red pigmentation of Westonia seeds that do not contain the C1 and R polynucleotides.

EXAMPLE 3 NUTRACEUTICAL PROPERTIES OF WHEAT SEEDS EXPRESSING THE C1 AND R POLYNUCLEOTIDES

To determine the identity of some of the compounds present at elevated levels in the seeds of transgenic plants expressing the C1 and R polynucleotides the seeds were analysed. Samples of the red wheat grain from progeny of transgenic wheat line WRB1 were ground thoroughly and extracted with ethanol. After removal of solids by centrifugation the supernatant was analysed by High Pressure Liquid Chromatography - Mass Spectrometry (HP LCMS). The following data shows the presence of elevated levels of flavonoid compounds, including anthocyanin and resveratrol.

Figure 8 shows the total ion chromatogram, in which the four main individual peaks have been labelled 1, 2, 3 and 4. The bottom scale is the time the peaks eluted. The scale on the left is the intensity of the ions. The peak heights do not represent the relative quantities. The individual peaks of interest were then isolated and studied further. These are shown in Figures 9 to 12, in which the scale on the left indicates the relative

intensities of each mass. The scale on the bottom is the mass of each fragment. This can be used to help identify the mass of the compound and the way it breaks down in the mass spectrometer.

Peak 1 (Figure 9) shows a mass and fragmentation pattern expected from 4,2,4-trihydroxychalcone (isoliquiritigenin), a major precursor of the flavonoids. There is a small peak at 118, indicating it has a similar building block to anthocyanins. Peak 2 (Figure 10) shows a large proportion of mass 118 and the base peak of 234 indicating that it is likely to be a member of the flavonoid group.

Peak 3 (Figure 11) shows a very similar structure to Peak 4 (Figure 12) based on the mass spectrum. It has a sizeable peak at 118 indicating a p-ethylphenol substituent similar to resveratrol. The masses and fragmentation patterns indicate that it is a member of the flavonoid group.

Figure 12 (Peak 4) shows a very strong peak at 228.9. This is the expected mass for the protonated version of resveratrol (mass 228). Figure 13 is an expanded view of the mass spectrum of Peak 4 with the smaller fragments showing. The peaks at 228.9 and 240.9 are now off the scale. This shows a peak at 118. One of the major fragments of resveratrol is 118 if it fragments across the ethylene bridge. The other possible fragment is 135, which is also shown on the mass spectrum. The combination of the expected mass signal for resveratrol, plus the presence of the two predicted fragments confirms that the signal seen as Peak 4 in the Mass Ion Chromatogram (above) is resveratrol.

EXAMPLE 4 ASSESSMENT OF THE HEAT STABILITY OF SEED ANTIOXIDANT ACTIVITY

Seed samples were incubated for 20 minutes at 20, 60 and 100°C. 250mg samples from each temperature treatment were ground up thoroughly, and 100mg from each taken and put into a 125ml Erlenmeyer flask with 25ml of 101µM 2,2-diphenyl-1-picrylhidrazyl (DPPH) in 50% aqueous methanol and a control flask with no seed powder was also included. They were incubated for three hours on an orbital shaker at 37°C. During this time due to the activity of antioxidants DPPH looses its free radical causing a colour loss. Then the reaction solution was filtered, and the resulting absorption measured at 515nm. After subtraction of the blank control each value was converted to a Trolox (an antioxidant used as a standard) equivalent (in terms of µmoles Trolox Equivalents/100g sample), calculated with the use of a Trolox standard curve previously prepared. Figure 14 shows that the antioxidant activity was quite stable up to 100°C.

EXAMPLE 5 SYNERGISTIC INTERACTION OF R AND C1 CONSTRUCTS

Twenty embryos were bombarded as described above with pGBA2R and pGBA2C1 constructs (Figures 15 and 16), alone or in combination, and pAnth was also included as a reference. All four bombardments were duplicated. The DNA amounts used to prepare the microprojectiles were adjusted to include in each bombardment the same number of either R or C1 genes, or R and C1 as a combined total. All formulations were calculated against the pAnth microprojectile preparation, which used 5µg of pAnth from stock at a concentration of 1µg/µl.

Table 3 and Figures 17 and 18 show the results of transient expression experiments.

TABLE 3

Transient Expression Foci Per Immature Embryo Produced With Different R Or C1 Plasmid Vectors

Bombardment no.	1	2	3	4	5	6	7	8
Vector	GBA2R	GBA2R	GBA2C1	GBA2C1	GBA2R + GBA2C1	GBA2R + GBA2C1	pAnth	pAnth
Mean spots/embryo	0.85	1.45	31.9	40.0	181.7	224.7	244.35	260.25
Std Deviation	1.09	1.32	9.88	8.35	43.53	62.84	62.14	70.67
Std Error	0.24	0.29	2.21	1.87	9.74	14.1	13.9	15.8

It can be seen that the combination of R and C1 has clearly much more effect on anthocyanin production and accumulation than either alone being 177X and 5.7X respectively to that achieved with R and C1 genes as separate bombardments. A comparison between GBAR / GBAC1 combined and pAnth is difficult as the promoters used are different and also pAnth does not require a co-transformation event as both genes are on the same plasmid whereas the two plasmids combined must probably both transform the same nucleus independently to achieve a synergistic effect. Nevertheless, combining the two elements even in a different vector background is enough to reproduce a level of expression showing a good comparison with the pAnth standard result.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A transgenic monocotyledonous plant, part of said plant, or progeny of said plant, wherein said plant, part or progeny comprises a transgene expressing R and C1 polynucleotides, functionally active derivatives, analogs, homologs or variants thereof, with the proviso that the part is not a single cell.

2. A transgenic monocotyledonous plant according to claim 1, wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

3. A transgenic monocotyledonous plant according to claim 1, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

4. A transgenic monocotyledonous plant according to any one of claims 1 to 3, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat.

5. A transgenic monocotyledonous plant according to any one of claims 1 to 3, wherein said monocotyledonous plant is wheat.

6. A transgenic monocotyledonous plant according to claim 5, wherein said monocotyledonous plant can be regenerated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

7. A transgenic monocotyledonous plant regenerated

from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

8. Part of a monocotyledonous plant which comprises a transgene expressing R and C1 polynucleotides or functional variants thereof, wherein said part is not a single cell.

9. Part of a plant according to claim 8, wherein the plant is wheat.

10. Part of a plant according to claim 9, which is deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

11. Part of a plant according to any one of claims 8 to 10, wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

12. Part of a plant according to any one of claims 8 to 10, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively

13. Part of a plant according to any one of claims 8 to 12, wherein the part is a seed.

14. Part of a plant according to any one of claims 8 to 12, wherein the part is a leaf.

15. Progeny of a monocotyledonous plant comprising a transgene expressing R and C1 polynucleotides or functional variants thereof.

16. A method of producing a transgenic monocotyledonous plant comprising:

(i) transfecting a plant cell with a vector comprising a transgene capable of expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof; and

(ii) regenerating said cell into a plant, thereby producing a plant comprising a transgene expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof.

17. A method according to claim 16, wherein said vector is a plasmid.

18. A method according to claim 16 or 17, wherein the transgene comprises the R and C1 polynucleotides and a promoter.

19. A method according to claim 18, wherein the promoter is a 35S-promoter.

20. A method according to any one of claims 16 to 19, wherein the vector further comprises an enhancer element.

21. A method according to claim 20, wherein the enhancer element is an ADH enhancer element.

22. A method according to claim 17, wherein said plasmid is pAnth, pGBA2R or pGBAC1 as shown in Figures 5, 15 and 16, respectively.

23. A method according to any one of claims 16 to 22, wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

24. A method according to any one of claims 16 to 22, wherein the R and C1 polynucleotides consist essentially

of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

25. A method according to any one of claims 16 to 24, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat.

26. A method according to any one of claims 16 to 24, wherein said monocotyledonous plant is wheat.

27. A method according to any one of claims 16 to 26, wherein the step of transfecting a plant cell comprises the steps of osmoticum treatment of target tissues, DNA precipitation and microprojectile bombardment.

28. A method according to claim 27, wherein the bombardment is with 50μg of gold particles per bombardment.

29. A method according to any one of claims 16 to 28, wherein the plant is heterozygous for the R and C1 polynucleotides or functional variants thereof.

30. A method according to any one of claims 16 to 28, wherein the plant is homozygous for the R and C1 polynucleotides or functional variants thereof.

31. A method according to any one of claims 16 to 28, wherein the plant is deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

32. A plant, part, or progeny thereof according to any one of claims 1 to 31, for use as a nutraceutical.

33. A plant, part, or progeny thereof according to

claim 32, wherein the nutraceutical is a food.

34. A plant, part, or progeny thereof according to claim 32, wherein the nutraceutical is a drink.

35. A method of identifying a transgenic plant comprising a transgene expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof comprising detecting the presence of red pigmentation in the vegetative tissue and/or seeds of the plant, wherein the absence of red pigmentation in the vegetative tissue and/or seeds indicates that a plant does not comprise a transgene expressing R and C1 polynucleotides, or functionally active derivatives, analogs, homologs or variants thereof.

36. A method of identifying a transgenic plant which expresses at least one foreign polynucleotide which is operably linked to the expression of R and C1 polynucleotides, or functionally active derivatives, analogs, homologs or variants thereof, comprising the step of detecting the presence of red pigmentation in the vegetative tissue and/or seeds of the plant wherein a control plant does not have red pigmentation.

37. A method according to claim 35 or claim 36, wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

38. A method according to claim 35 or claim 36, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

39. A method according to any one of claims 35 to 38, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize,

rye, triticale and oat.

40. A method according to any one of claims 35 to 38, wherein said monocotyledonous plant is wheat.

41. Use of a transgenic plant, comprising a transgene expressing R and C1 polynucleotides, as a nutraceutical or in the preparation of a nutraceutical.

42. Use according to claim 41, wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

43. Use according to claim 41, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

44. Use according to any one of claims 41 to 43, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat.

45. Use according to any one of claims 41 to 43, wherein said monocotyledonous plant is wheat.

46. Use according to claim 45, wherein said monocotyledonous plant can be regenerated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

47. A nutraceutical when obtained from a transgenic plant comprising a transgene expressing R and C1 polynucleotides or a part or progeny of said plant, wherein said part is not a single cell.

48. A nutraceutical according to claim 27, wherein

the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

49. A nutraceutical according to claim 47, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

50. A nutraceutical according to any one of claims 47 to 49, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat.

51. A nutraceutical according to any one of claims 47 to 49, wherein said monocotyledonous plant is wheat.

52. A nutraceutical according to claim 51, wherein said monocotyledonous plant can be regenerated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

52. A nutraceutical when obtained from a monocotyledonous plant comprising a transgene expressing R and C1 polynucleotides, or a part or progeny of said plant, wherein said part is not a single cell.

53. A nutraceutical according to claim 52, which is a food.

54. A nutraceutical according to claim 52, which is a drink

55. Use of a nutraceutical according to any one of claims 47 to 54, for the preparation of a medicament for the prevention or treatment of disease.

56. A method of preventing or treating disease comprising the step of administering to a patient in need thereof an effective amount of a nutraceutical according to any one of claims 47 to 54.

AMENDED CLAIMS

[received by the International Bureau on 29 July 2005 (29.07.05);
original claims 1-56 replaced by amended claims 1-57 (8 pages)]

1. A transgenic monocotyledonous plant, part of said plant, or progeny of said plant, wherein said plant, part or progeny comprises a transgene expressing R and C1 polynucleotides, functionally active derivatives, analogs, homologs or variants thereof in the vegetative tissue and seeds, with the proviso that the part is not a single cell.

2. A transgenic monocotyledonous plant according to claim 1, wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

3. A transgenic monocotyledonous plant according to claim 1, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

4. A transgenic monocotyledonous plant according to any one of claims 1 to 3, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat.

5. A transgenic monocotyledonous plant according to any one of claims 1 to 3, wherein said monocotyledonous plant is wheat.

6. A transgenic monocotyledonous plant according to claim 5, wherein said monocotyledonous plant can be regenerated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

7. A transgenic monocotyledonous plant regenerated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

8. Part of a monocotyledonous plant which comprises a transgene expressing R and C1 polynucleotides or functional variants thereof wherein said expression is in the vegetative tissue and/or seeds, and wherein said part is not a single cell.

9. Part of a plant according to claim 8, wherein the plant is wheat.

10. Part of a plant according to claim 9, which is deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

11. Part of a plant according to any one of claims 8 to 10, wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

12. Part of a plant according to any one of claims 8 to 10, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively

13. Part of a plant according to any one of claims 8 to 12, wherein the part is a seed.

14. Part of a plant according to any one of claims 8 to 12, wherein the part is a leaf.

15. Progeny of a monocotyledonous plant comprising a

transgene expressing R and C1 polynucleotides or functional variants thereof in the vegetative tissue and/or seeds.

16. A method of producing a transgenic monocotyledonous plant comprising:

(i) transfecting a plant cell with a vector comprising a transgene capable of expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof; and

(ii) regenerating said cell into a plant, thereby producing a plant comprising a transgene expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof in the vegetative tissue and/or seeds.

17. A method according to claim 16, wherein said vector is a plasmid.

18. A method according to claim 16 or 17, wherein the transgene comprises the R and C1 polynucleotides and a promoter.

19. A method according to claim 18, wherein the promoter is a 35S-promoter.

20. A method according to any one of claims 16 to 19, wherein the vector further comprises an enhancer element.

21. A method according to claim 20, wherein the enhancer element is an ADH enhancer element.

22. A method according to claim 17, wherein said plasmid is pAnth, pGBA2R or pGBAC1 as shown in Figures 5, 15 and 16, respectively.

23. A method according to any one of claims 16 to 22,

wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

24. A method according to any one of claims 16 to 22, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

25. A method according to any one of claims 16 to 24, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat.

26. A method according to any one of claims 16 to 24, wherein said monocotyledonous plant is wheat.

27. A method according to any one of claims 16 to 26, wherein the step of transfecting a plant cell comprises the steps of osmoticum treatment of target tissues, DNA precipitation and microprojectile bombardment.

28. A method according to claim 27, wherein the bombardment is with 50µg of gold particles per bombardment.

29. A method according to any one of claims 16 to 28, wherein the plant is heterozygous for the R and C1 polynucleotides or functional variants thereof.

30. A method according to any one of claims 16 to 28, wherein the plant is homozygous for the R and C1 polynucleotides or functional variants thereof.

31. A method according to any one of claims 16 to 28, wherein the plant is deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

32. A plant, part, or progeny thereof according to any one of claims 1 to 31, for use as a nutraceutical.

33. A plant, part, or progeny thereof according to claim 32, wherein the nutraceutical is a food.

34. A plant, part, or progeny thereof according to claim 32, wherein the nutraceutical is a drink.

35. A method of identifying a transgenic monocotyledonous plant comprising a transgene expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof comprising detecting the presence of red pigmentation in the vegetative tissue and/or seeds of the plant, wherein the absence of red pigmentation in the vegetative tissue and/or seeds indicates that a plant does not comprise a transgene expressing R and C1 polynucleotides, or functionally active derivatives, analogs, homologs or variants thereof.

36. A method of identifying a transgenic monocotyledonous plant which expresses at least one foreign polynucleotide which is operably linked to the expression of R and C1 polynucleotides, or functionally active derivatives, analogs, homologs or variants thereof, comprising the step of detecting the presence of red pigmentation in the vegetative tissue and/or seeds of the plant wherein a control plant does not have red pigmentation.

37. A method according to claim 35 or claim 36, wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

38. A method according to claim 35 or claim 36, wherein the R and C1 polynucleotides consist essentially

of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

39. A method according to any one of claims 35 to 38, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat.

40. A method according to any one of claims 35 to 38, wherein said monocotyledonous plant is wheat.

41. Use of a transgenic monocotyledonous plant, in the vegetative tissue and/or seeds comprising a transgene expressing R and C1 polynucleotides, as a nutraceutical or in the preparation of a nutraceutical.

42. Use according to claim 41, wherein the R and C1 polynucleotides are substantially those shown in SEO ID Nos 1 and 2, respectively.

43. Use according to claim 41, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

44. Use according to any one of claims 41 to 43, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat.

45. Use according to any one of claims 41 to 43, wherein said monocotyledonous plant is wheat.

46. Use according to claim 45, wherein said monocotyledonous plant can be regenerated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

47. A nutraceutical when obtained from a transgenic monocotyledonous plant comprising a transgene expressing R and C1 polynucleotides in the vegetative tissue and/or seeds or a part or progeny of said plant, wherein said part is not a single cell.

48. A nutraceutical according to claim 47, wherein the R and C1 polynucleotides are substantially those shown in SEQ ID Nos 1 and 2, respectively.

49. A nutraceutical according to claim 47, wherein the R and C1 polynucleotides consist essentially of nucleotide sequences shown in SEQ ID Nos 1 and 2, respectively.

50. A nutraceutical according to any one of claims 47 to 49, wherein said monocotyledonous plant is selected from the group consisting of wheat, sorghum, rice, barley, maize, rye, triticale and oat.

51. A nutraceutical according to any one of claims 47 to 49, wherein said monocotyledonous plant is wheat.

52. A nutraceutical according to claim 51, wherein said monocotyledonous plant can be regenerated from seed deposited at the National Collections of Industrial Food and Marine Bacteria, Aberdeen, Scotland, under Accession No. NCIMB 41182 or NCIMB 41232.

53. A nutraceutical when obtained from a monocotyledonous plant comprising a transgene expressing R and C1 polynucleotides, or a part or progeny of said plant, wherein said part is not a single cell.

54. A nutraceutical according to claim 53, which is a food.

55. A nutraceutical according to claim 53, which is a drink

56. Use of a nutraceutical according to any one of claims 47 to 55, for the preparation of a medicament for the prevention or treatment of disease.

57. A method of preventing or treating disease comprising the step of administering to a patient in need thereof an effective amount of a nutraceutical according to any one of claims 47 to 55.

```
1     GGATCCACCA TGGCGCTTTC AGCTTCCCGA GTTCAGCAGG CGGAAGAACT
51    GCTGCAACGA CCTGCTGAGA GGCAGCTGAT GAGGAGCCAG CTTGCTGCAG
101   CCGCCAGGAG CATCAACTGG AGCTACGCCC TCTTCTGGTC CATTTCAGAC
151   ACTCAACCAA GGGTGCTGAC GTGGACGGAC GGGTTCTACA ACGGCGAGGT
201   GAAGACGCGG AAGATCTCCA ACTCCGTGGA GCTGACATCC GACCAGCTCG
251   TCATGCAGAG GAGCGACCAG CTCCGGGAGC TCTACGAGGC CCTCCTGTCG
301   GGCGAGGGCG ACCGCCGCGC TGCGCCTGCG CGGCCGGCCG GCTCTCTGTC
351   GCCGGAGGAC CTCGGCGACA CCGAGTGGTA CTACGTGGTC TCCATGACCT
401   ACGCCTTCCG GCCAGGCCAA GGGTTGCCCG GCAGGAGTTT CGCGAGCGAC
451   GAGCATGTCT GGCTGTGCAA CGCGCACCTC GCCGGCAGCA AAGCCTTCCC
501   CCGCGCGCTC CTGGCCAAGA GCGCGTCCAT TCAGTCAATC CTCTGCATCC
551   CGGTTATGGG CGGCGTGCTT GAGCTTGGTA CAACTGACAC GGTGCCGGAG
601   GCCCCGGACT TGGTCAGCCG AGCAACCGCG GCTTTCTGGG AGCCGCAGTG
651   CCCGAGCTCC AGCCCGTCAG GACGAGCAAA CGAGACCGGC GAGGCCGCAG
701   CAGACGACGG CACGTTTGCG TTCGAGGAAC TCGACCACAA TAATGGCATG
751   GACGACATAG AGGCGATGAC CGCCGCCGGG GGACACGGGC AGGAGGAGGA
801   GCTAAGACTA AGAGAAGCCG AGGCCCTGTC AGACGACGCA AGCCTGGAGC
851   ACATCACCAA GGAGATCGAG GAGTTCTACA GCCTCTGCGA CGAAATGGAC
901   CTGCAGGCGC TACCACTACC GCTAGAGGAC GGCTGGACCG TGGACGCGTC
951   CAATTTCGAG GTCCCCTGCT CTTCCCCGCA GCCAGCGCCG CCTCCGGTGG
1001  ACAGGGCTAC CGCTAACGTC GCCGCCGACG CCTCAAGGGC ACCCGTCTAC
1051  GGCTCTCGCG CGACGAGTTT CATGGCTTGG ACGAGGTCCT CGCAGCAGTC
1101  GTCGTGCTCC GACGACGCGG CGCCCGCAGC AGTAGTGCCG GCCATCGAGG
1151  AGCCGCAGAG ATTGCTGAAG AAAGTGGTGG CCGGCGGCGG TGCTTGGGAG
1201  AGCTGTGGCG GCGCGACGGG AGCAGCACAG GAAATGAGTG GCACTGGCAC
1251  CAAGAACCAC GTCATGTCGG AGCGAAAGCG ACGAGAGAAG CTCAACGAGA
1301  TGTTCCTCGT CCTCAAGTCA CTGCTTCCGT CCATTCACAG GGTGAACAAA
1351  GCGTCGATCC TCGCCGAAAC GATAGTCTAC CTCAAGGAGC TTCAGAGAAG
1401  GGTGCAAGAG CTGGAGTCCA GTAGGGAACC TGCGTCGCGC CCATCCGAAA
1451  CGACGACAAG GCTAATAACA AGGCCCTCCC GTGGCAATAA TGAGAGTGTG
1501  AGGAAGGAGG TCTGCGCGGG CTCCAAGAGG AAGAGCCCAG AGCTCGGCAG
1551  AGACGACGTG GAGCGCCCCC CGGTCCTCAC CATGGACGCC GGCACCAGCA
1601  ACGTCACCGT CACCGTCTCG GACAAGGACG TGCTCCTGGA GGTGCAGTGC
1651  CGGTGGGAGG AGCTCCTGAT GACGCGAGTG TTCGACGCCA TCAAGAGCCT
1701  CCATTTGGAC GTCCTCTCGG TTCAGGCTTC AGCGCCAGAT GGCTTCATGG
1751  GGCTTAAGAT ACGAGCTCAG TTTGCTGGCT CCGGTGCCGT CGTGCCCTGG
1801  ATGATCAGCG AGGCTCTTCG CAAAGCTATA GGGAAGCGGT GACTCTAGAG
1851  GATCC
```

FIGURE 1

catgggatccccgggtaccgagctcgaattaattccgcgcgatggggaggagggcgt
gttgcgcgaaggaaggcgttaagagaggggcgtggacgagcaaggaggacgatgcct
tggccgcctacgtcaaggcccatggcgaaggcaaatggagggaagtgccccagaaag
ccggtttgcgtcggtgcggcaagagctgccggctgcggtggctgaactacctccggc
ccaacatcaggcgcggcaacatctcctacgacgaggaggatctcatcatccgcctcc
acaggctcctcggcaacaggtggtcgctgattgcaggcaggctgcctggccgaacag
acaatgaaatcaagaactactggaacagcacgctgggccggagggcaggcgccggcg
ccggcgccggcggcagttgggttgtcgtcgcgccggacaccggctcgcacgccaccc
cggccgcgacgtcgggcgcctgcgagaccggccagaatagcgccgctcatcgcgcgg
accccgactcagccgggacgacgacgacctccgcggcggcggtgtgggcgcccaagg
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cgggcgagacggcgacgccaatggccggtggaggtggaggaggaggaggagaagcag
ggtcgtcggacgactgcagctcggcggcgtcggtatcgcttcgcgtcggaagccacg
acgagccgtgcttctccggcgacggtgacggcgactggatggacgacgtgagggccc
tggcgtcgtttctcgagtccgacgaggactggctccgctgtcagacggccgggcagc
ttgcgtagacaacaagtacacgtatagatgtccaataagcacgaggcccgcgagccc
ggcacgaagcccgcttttgggcccggtccgagcccggcacggcccggttatatgca
gacccgggccggcccggcacgaataagcgggccgggctcggacaggaaattaggcac
ggtgagctagaggatccgact

FIGURE 2

```
1    MALSASRVQQ AEELLQRPAE RQLMRSQLAA AARSINWSYA LFWSISDTQP
51   RVLTWTDGFY NGEVKTRKIS NSVELTSDQL VMQRSDQLRE LYEALLSGEG
101  DRRAAPARPA GSLSPEDLGD TEWYYVVSMT YAFRPGQGLP GRSFASDEHV
151  WLCNAHLAGS KAFPRALLAK SASIQSILCI PVMGGVLELG TTDTVPEAPD
201  LVSRATAAFW EPQCPSSSPS GRANETGEAA ADDGTFAFEE LDHNNGMDDI
251  EAMTAAGGHG QEEELRLREA EALSDDASLE HITKEIEEFY SLCDEMDLQA
301  LPLPLEDGWT VDASNFEVPC SSPQPAPPPV DRATANVAAD ASRAPVYGSR
351  ATSFMAWTRS SQQSSCSDDA APAAVVPAIE EPQRLLKKVV AGGGAWESCG
401  GATGAAQEMS GTGTKNHVMS ERKRREKLNE MFLVLKSLLP SIHRVNKASI
451  LAETIVYLKE LQRRVQELES SREPASRPSE TTTRLITRPS RGNNESVRKE
501  VCAGSKRKSP ELGRDDVERP PVLTMDAGTS NVTVTVSDKD VLLEVQCRWE
551  ELLMTRVFDA IKSLHLDVLS VQASAPDGFM GLKIRAQFAG SGAVVPWMIS
601  EALRKAIGKR *
```

FIGURE 3

```
1   MGRRACCAKE GVKRGAWTSK EDDALAAYVK AHGEGKWREV PQKAGLRRCG
51  KSCRLRWLNY LRPNIRRGNI SYDEEDLIIR LHRLLGNRWS LIAGRLPGRT
101 DNEIKNYWNS TLGRRAGAGA GAGGSWVVVA PDTGSHATPA ATSGACETGQ
151 NSAAHRADPD SAGTTTTSAA AVWAPKAVRC TGGLFFFHRD TTPAHAGETA
201 TPMAGGGGGG GGEAGSSDDC SSAASVSLRV GSHDEPCFSG DGDGDWMDDV
251 RALASFLESD EDWLRCQTAG QLA*
```

FIGURE 4

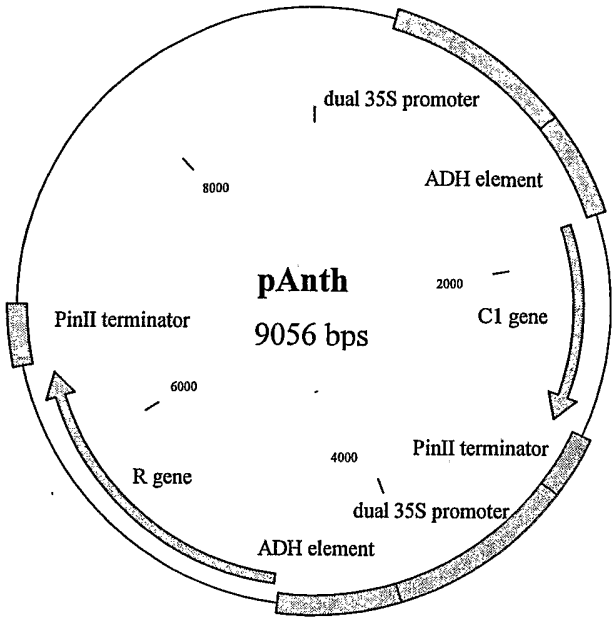

dual 35S promoter
8000
ADH element
pAnth
9056 bps
2000
C1 gene
PinII terminator
6000
4000
PinII terminator
R gene
dual 35S promoter
ADH element

FIGURE 5

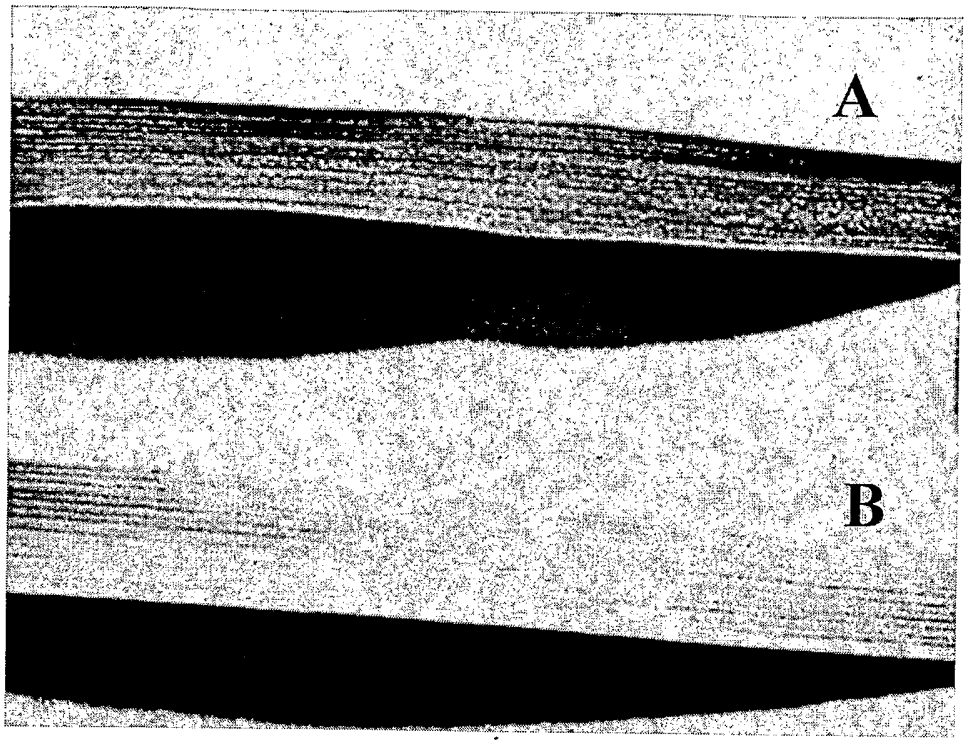


FIGURE 6

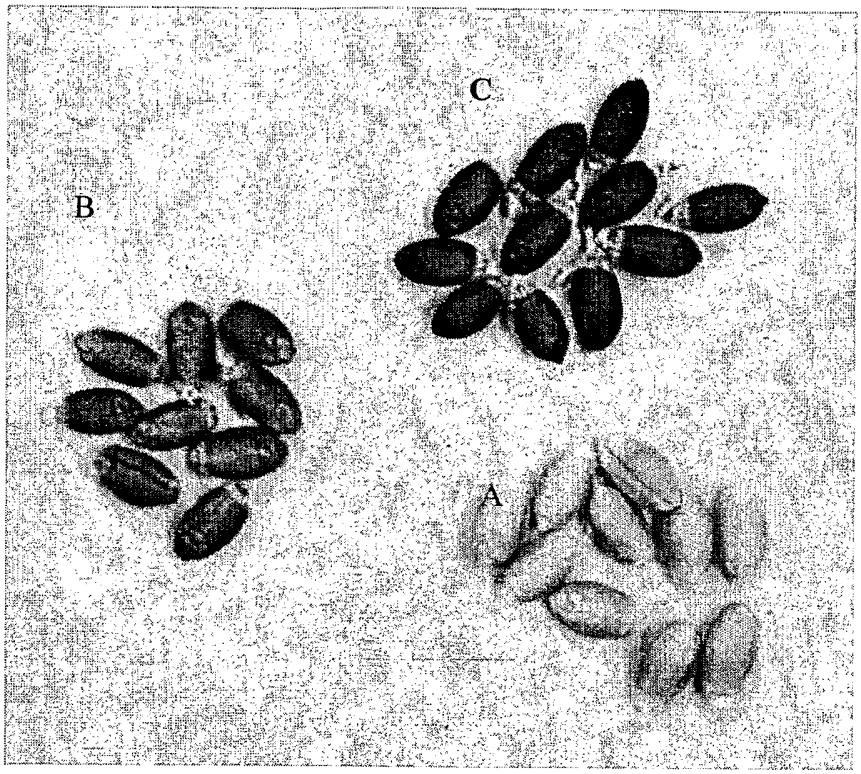


FIGURE 7

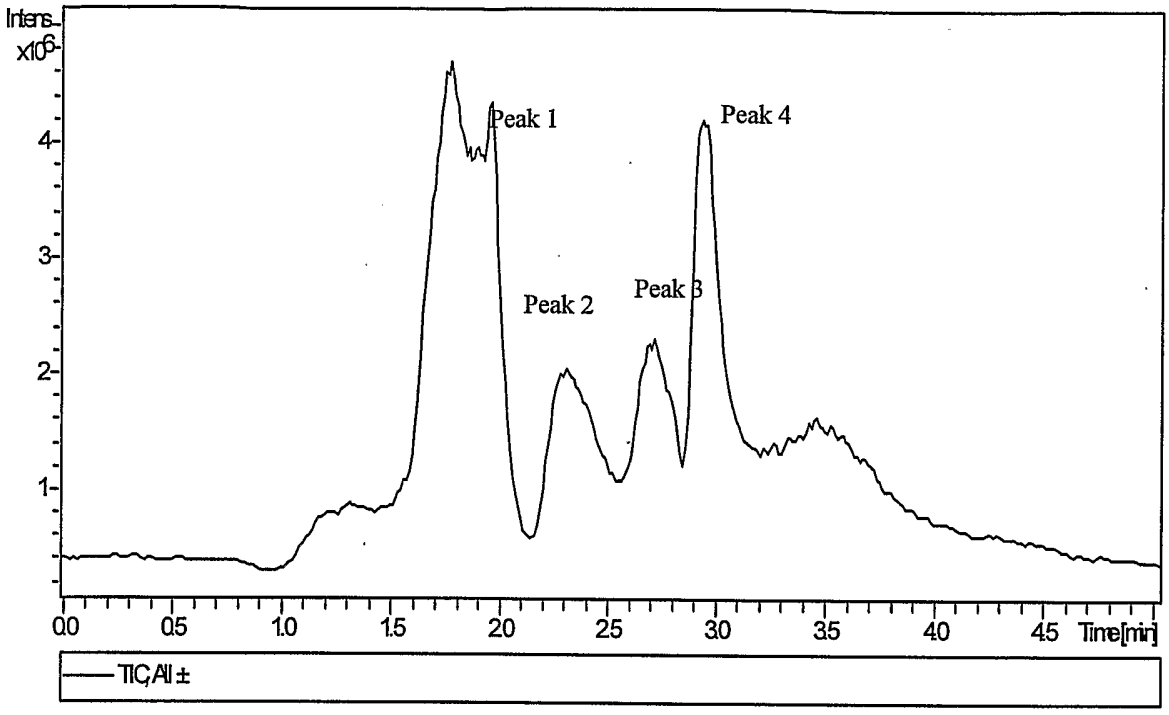

Intens.
x10^6
4
3
2
1
Peak 1
Peak 2
Peak 3
Peak 4
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time[min]
TIC All ±

FIGURE 8

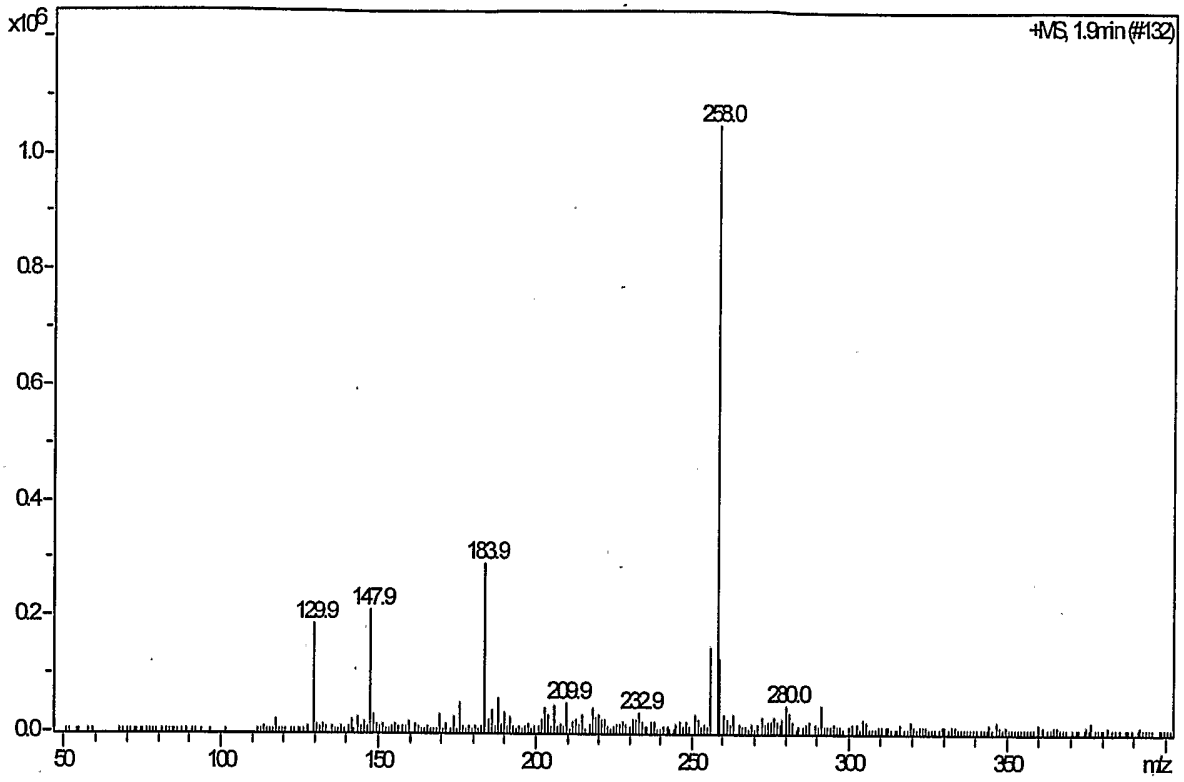
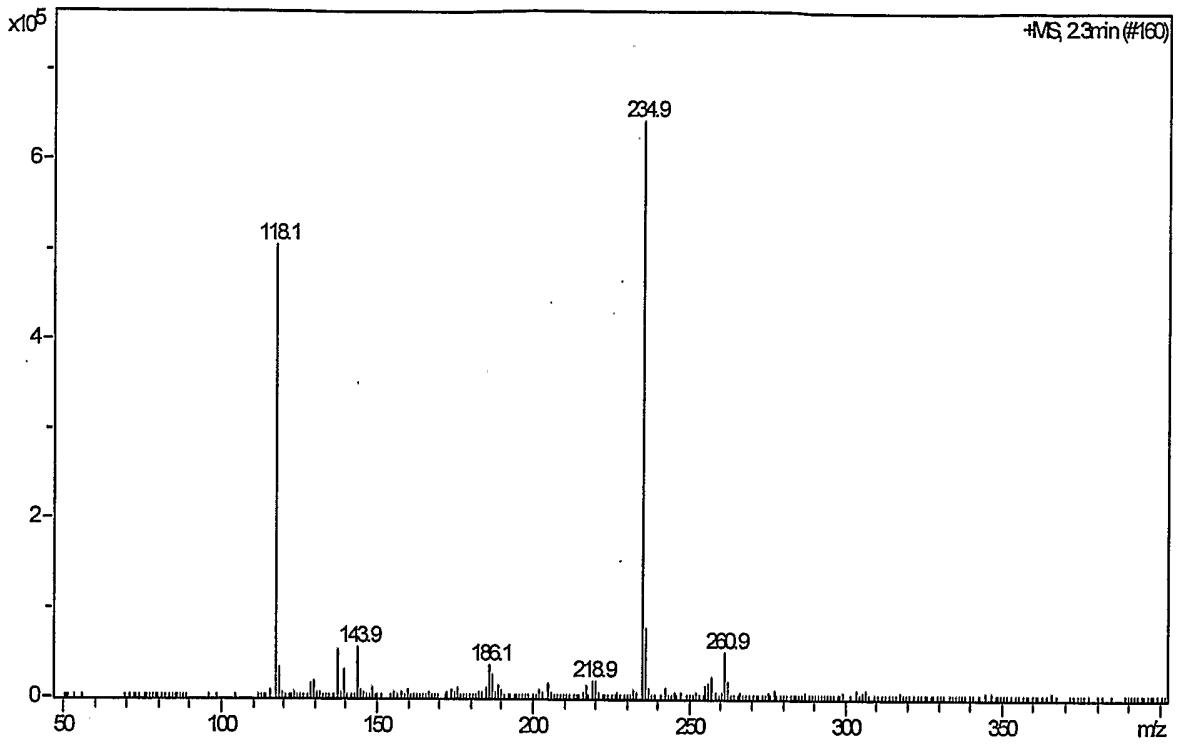

x10^6
+MS, 1.9min (#132)
1.0
0.8
0.6
0.4
0.2
0.0
258.0
183.9
147.9
129.9
209.9
232.9
280.0
50
100
150
200
250
300
350
m/z

FIGURE 9


x10^5
+MS, 2.3min (#160)
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118.1
143.9
186.1
218.9
260.9
6
4
2
0
50
100
150
200
250
300
350
m/z

FIGURE 10

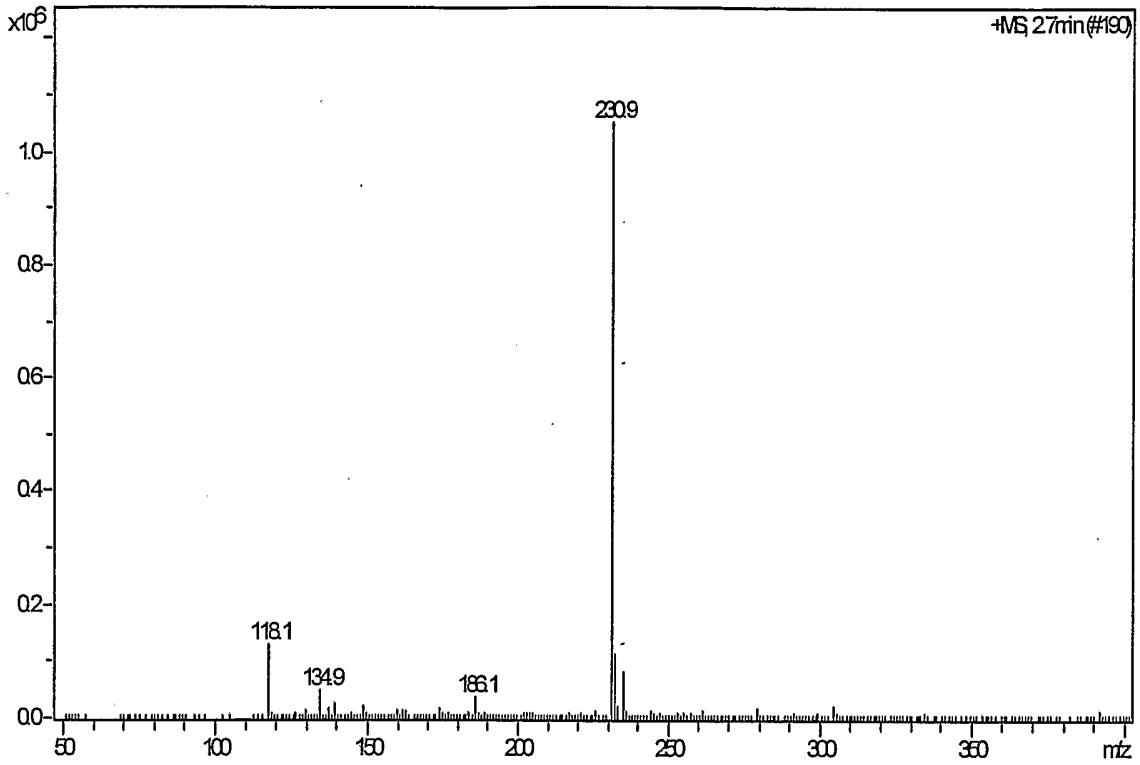
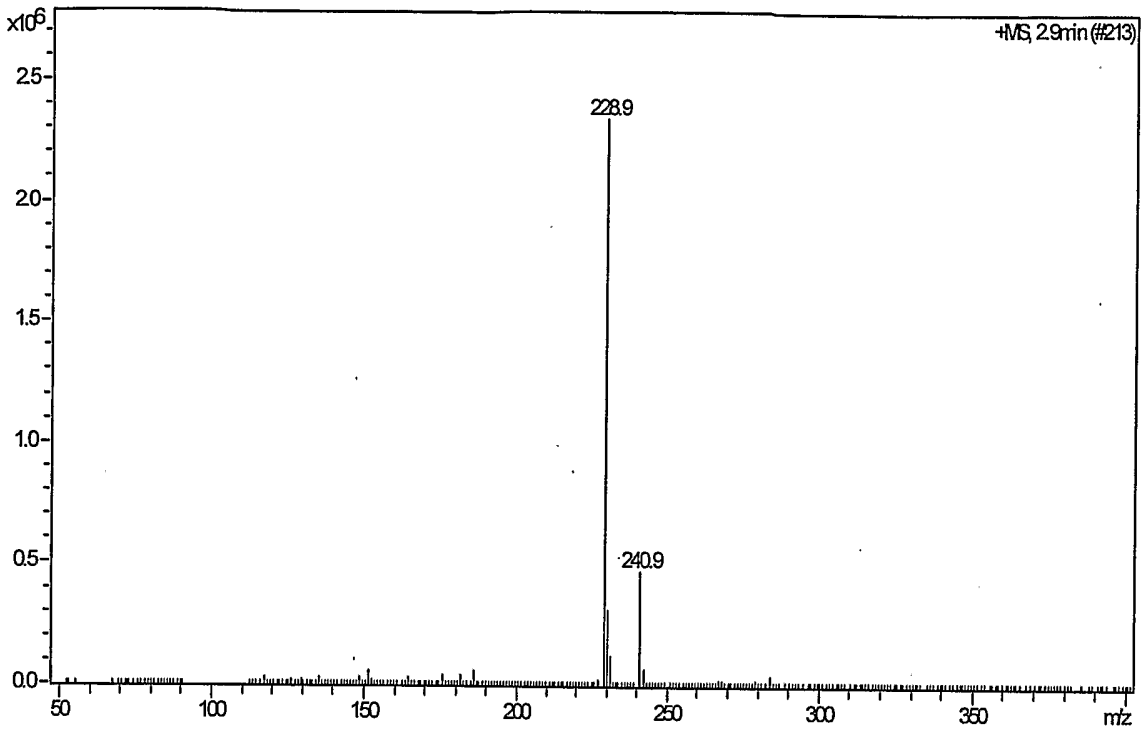

x10^6
+MS, 2.7min (#190)
230.9
118.1
134.9
186.1
1.0
0.8
0.6
0.4
0.2
0.0
50
100
150
200
250
300
350
m/z

FIGURE 11


x10^6
2.5
2.0
1.5
1.0
0.5
0.0
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240.9
50
100
150
200
250
300
350
m/z

FIGURE 12

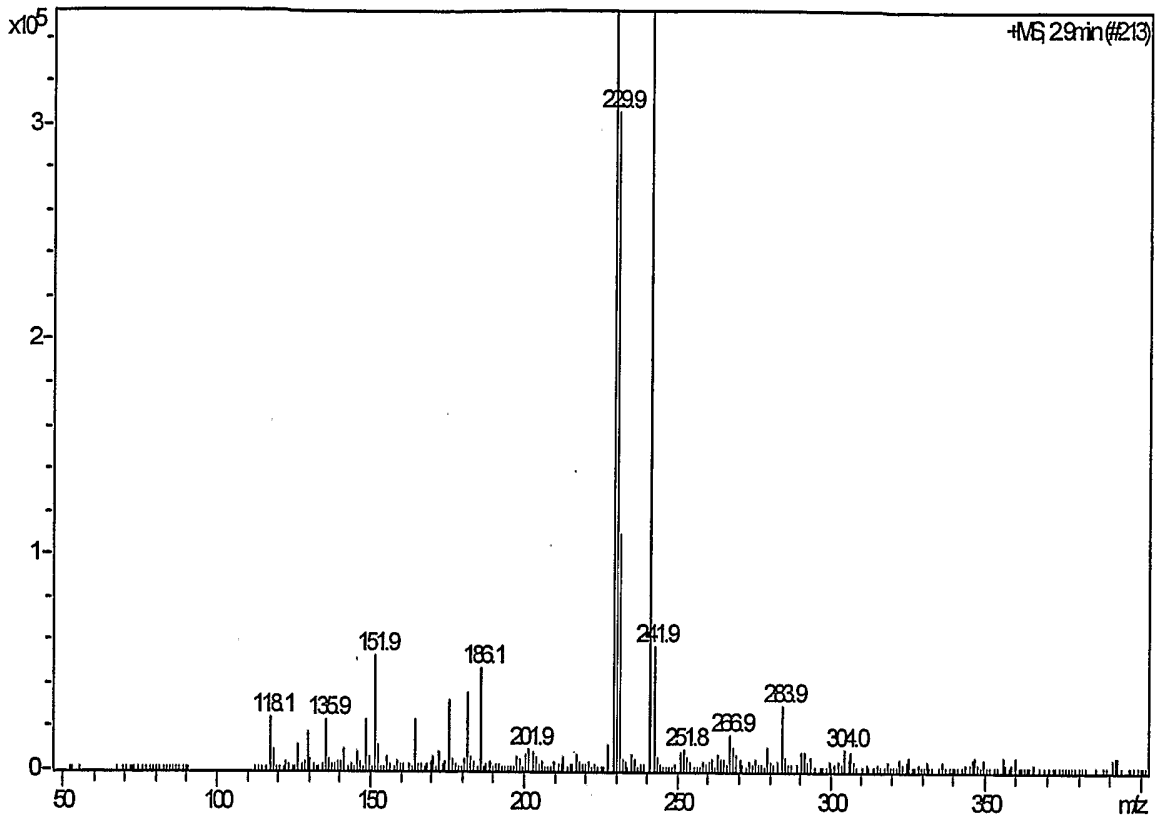

x10^5
+MS, 2.9min (#213)
229.9
241.9
151.9
186.1
118.1
135.9
201.9
251.8
266.9
283.9
304.0
0
1
2
3
50
100
150
200
250
300
350
m/z

FIGURE 13

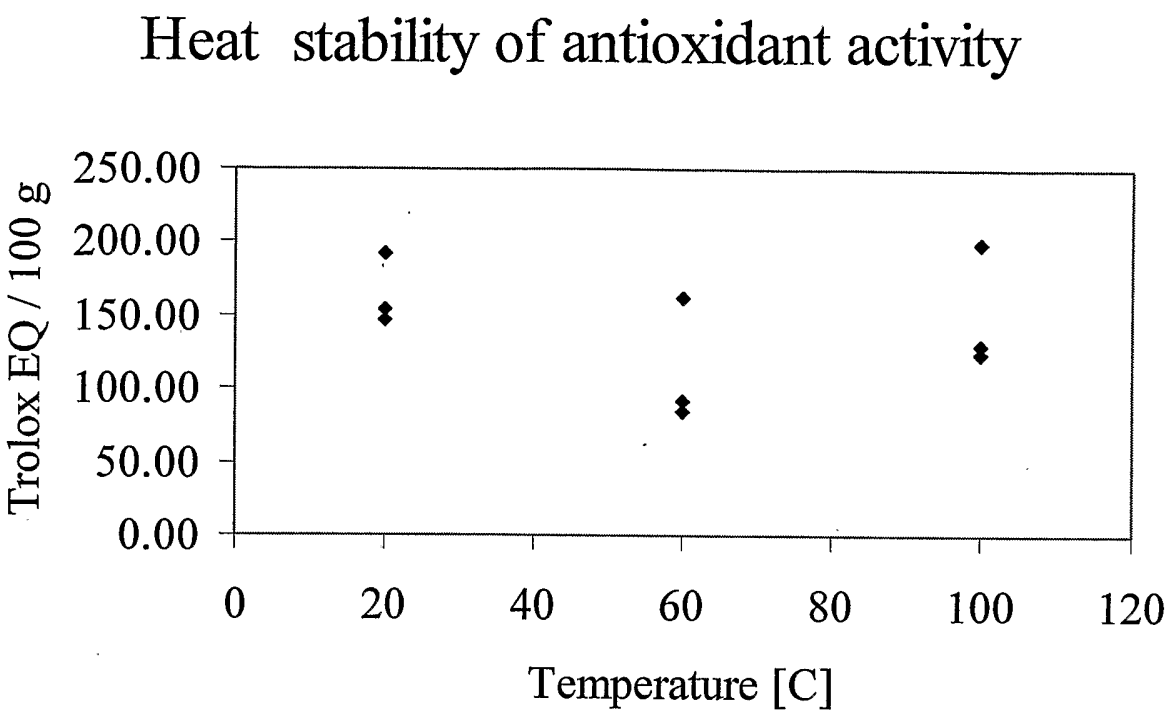

Heat stability of antioxidant activity
Trolox EQ / 100 g
250.00
200.00
150.00
100.00
50.00
0.00
0
20
40
60
80
100
120
Temperature [C]

FIGURE 14

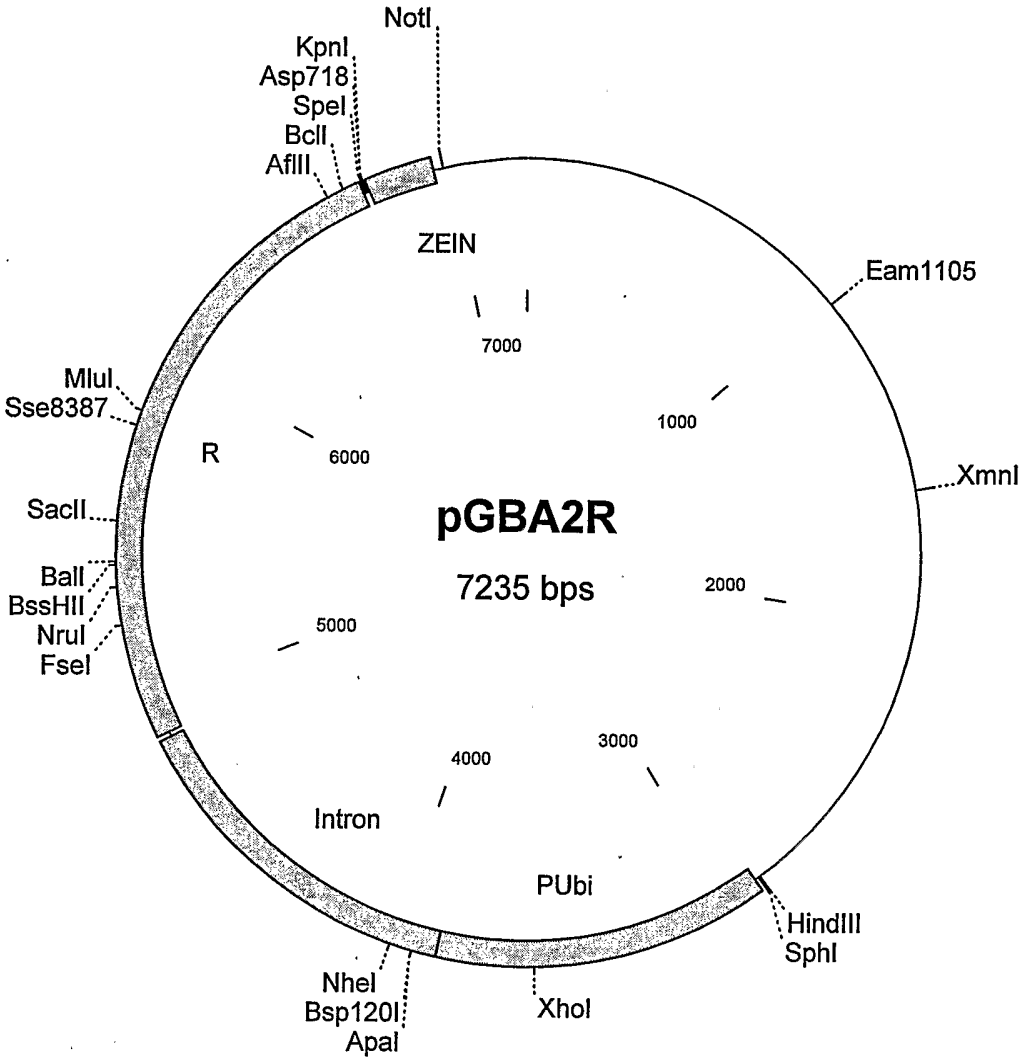
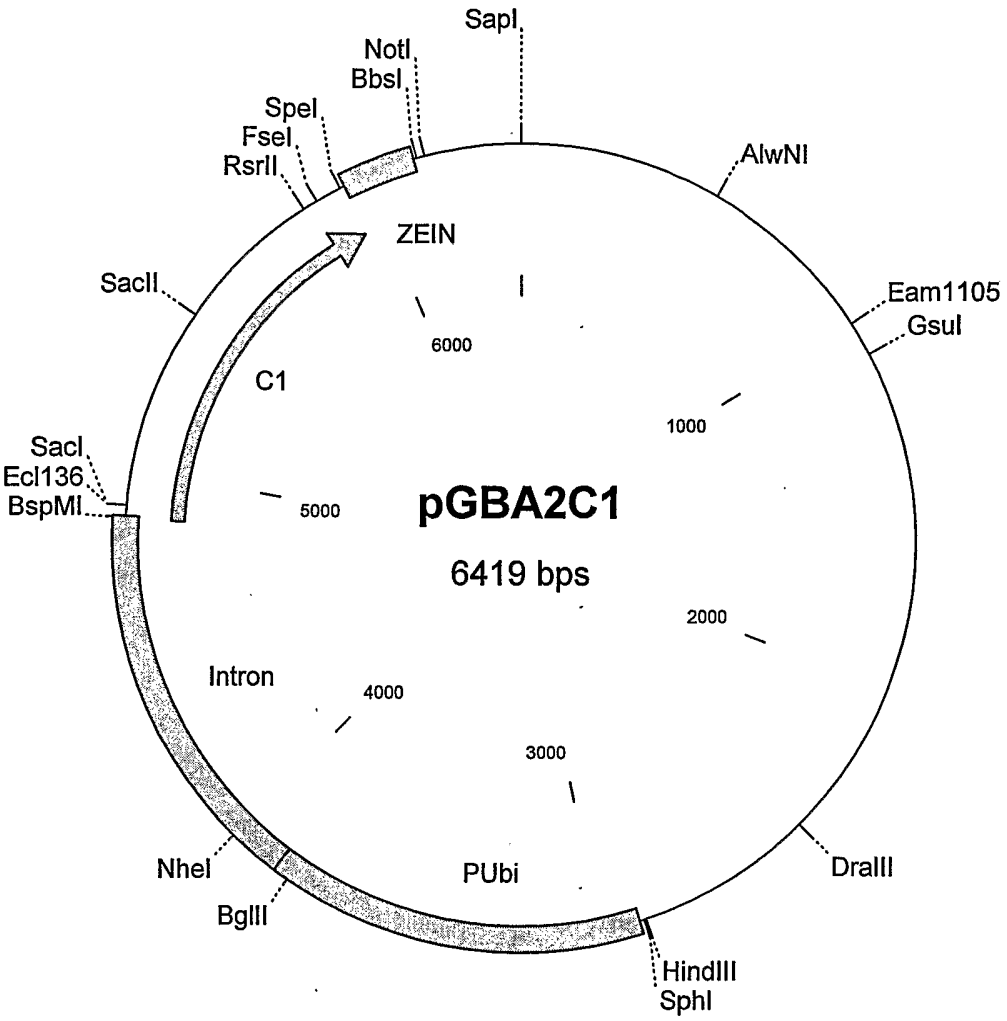

NotI
KpnI
Asp718
SpeI
BclI
AflII
ZEIN
Eam1105
7000
1000
MluI
Sse8387
R
6000
XmnI
pGBA2R
SacII
7235 bps
BalI
BssHII
NruI
FseI
2000
5000
3000
4000
Intron
PUbi
HindIII
SphI
NheI
Bsp120I
ApaI
XhoI

FIGURE 15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NotI
BbsI
SpeI
FseI
RsrII
AlwNI
ZEIN
SacII
Eam1105
GsuI
6000
C1
1000
SacI
Ecl136
BspMI
5000
pGBA2C1
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2000
Intron
4000
3000
NheI
PUbi
DraIII
BglII
HindIII
SphI

FIGURE 16

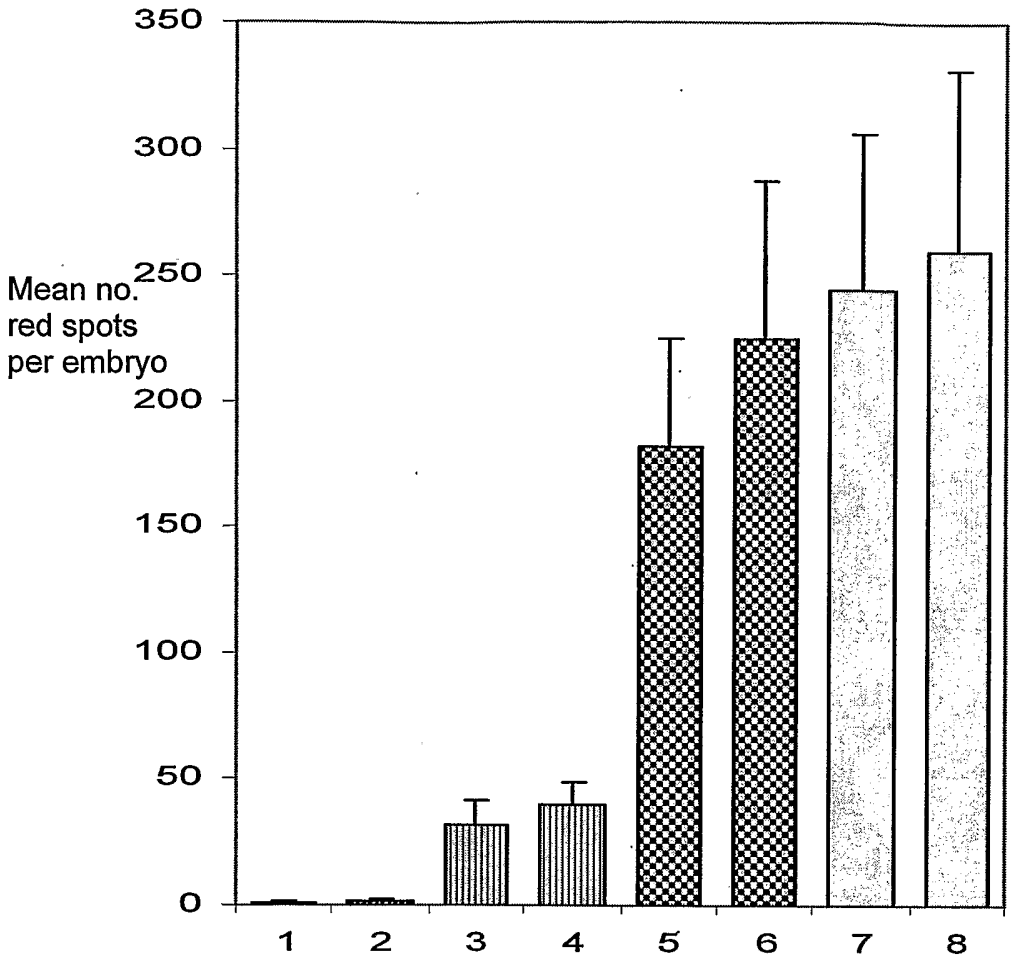

350
300
250
200
150
100
50
0
Mean no. red spots per embryo
1
2
3
4
5
6
7
8
BOMBARDMENT NO.

FIGURE 17

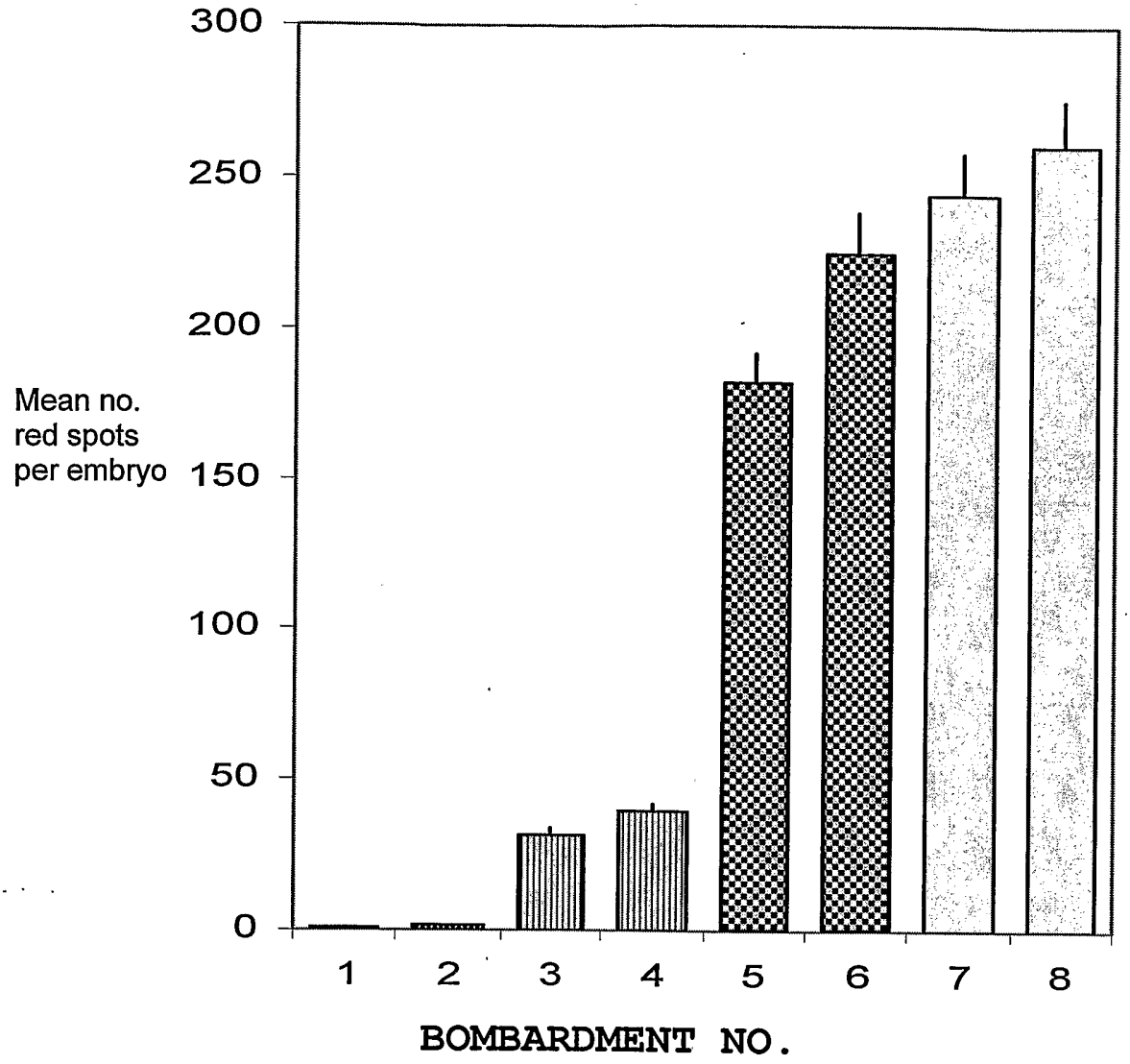

300
250
200
150
100
50
0
Mean no. red spots per embryo
1
2
3
4
5
6
7
8
BOMBARDMENT NO.

FIGURE 18

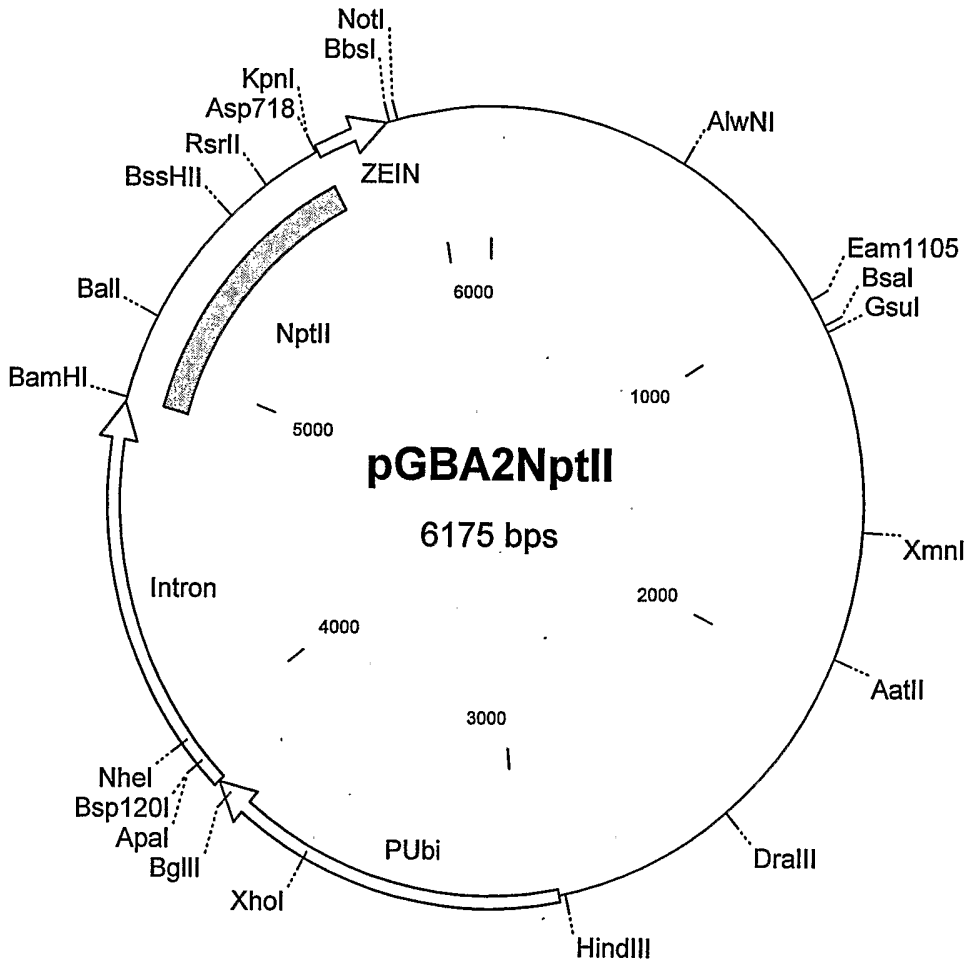
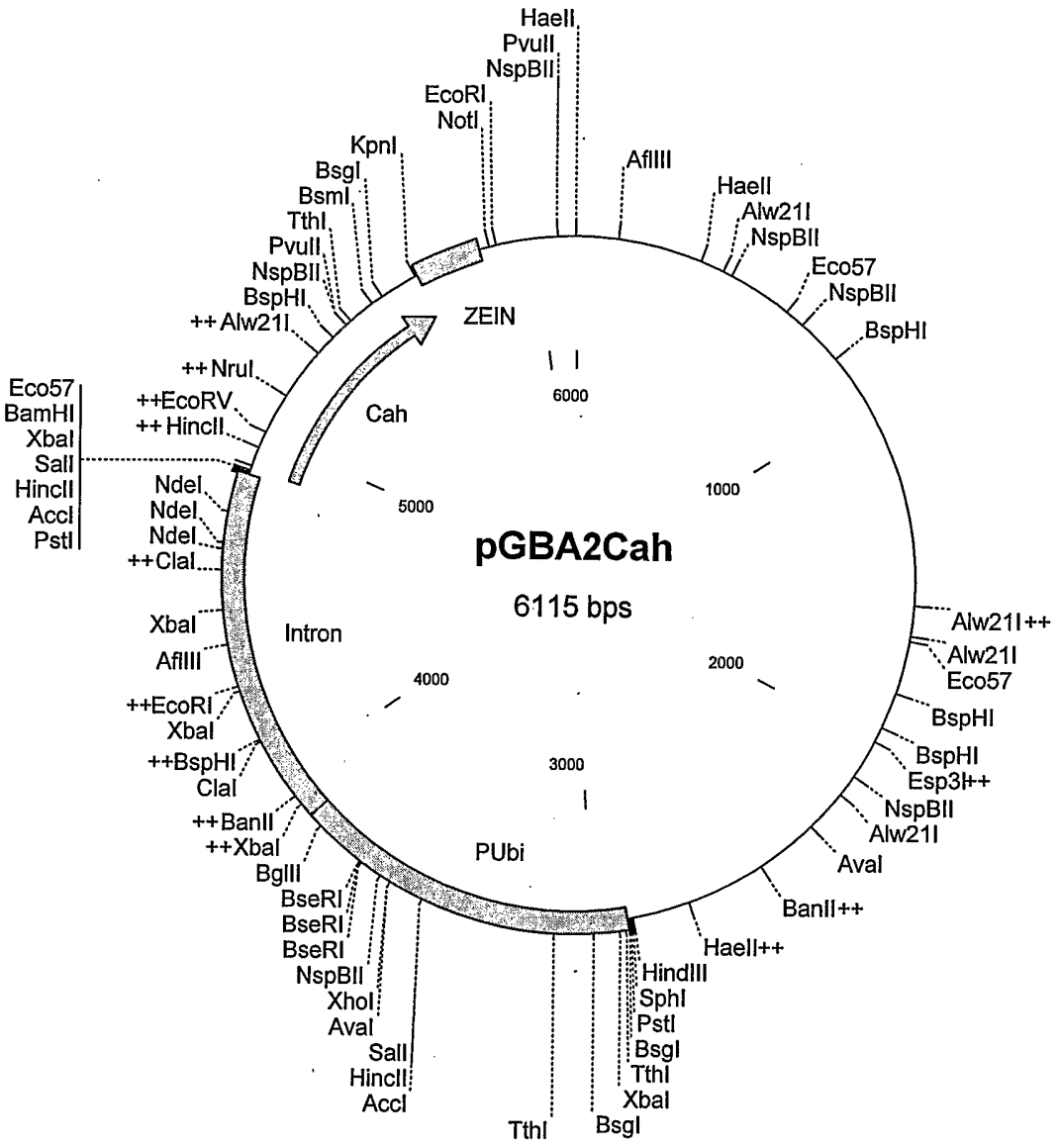

NotI
BbsI
KpnI
Asp718
RsrII
BssHII
ZEIN
AlwNI
Eam1105
BsaI
GsuI
BalI
NptII
BamHI
6000
1000
5000
pGBA2NptII
6175 bps
XmnI
Intron
2000
4000
AatII
3000
NheI
Bsp120I
ApaI
BglII
PUbi
DraIII
XhoI
HindIII

FIGURE 19


pGBA2Cah
6115 bps
ZEIN
Cah
Intron
PUbi
1000
2000
3000
4000
5000
6000
HaeII
PvuII
NspBII
EcoRI
NotI
KpnI
BsgI
BsmI
TthI
PvuII
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XhoI
AvaI
SalI
HincII
AccI
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HindIII
SphI
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BanII++
AvaI
NspBII
Alw21I
Esp3I++
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FIGURE 20

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FIGURE 21

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FIGURE 22

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FIGURE 23

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FIGURE 24

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/000545

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/29, A01H 1/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAPLUS, MEDLINE, BIOSIS, AGRICOLA: Phenylpropanoid, Anthocyanin, Flavonoid, C1 and R polynucleotide, Transgene, Transform, Genetic, Modified, regulate, Transcription factor, Plant or Plants.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5880331A (Krebbers, E. et al) 9 March 1999. (See whole document, especially abstract, pages 3-5, examples and claims).	1-5, 8-9, 11-13, 15-30 and 35-40
X	US 2002/0188964A1. (Erich Grotewold). 12 December 2002. (See whole document, especially abstract, pages 1-2, examples and claims).	1-5, 8-9, 11-30, 35-40

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

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

"E" earlier application or patent 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 to 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 when the document is taken alone

"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

Date of the actual completion of the international search	Date of mailing of the international search report
16 May 2005	- 1 JUN 2005
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **Julie Kneeshaw** Telephone No : (02) 6283

INTERNATIONAL SEARCH REPORT	International application No. PCT/AU2005/000545

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2002/101023A2. (E.I DU PONT DE NEMOURS AND COMPANY). 19 December 2002. (See whole document, especially abstract, pages 1-4, examples and claims).	35-38, 41-43 and 47-49
X	WO 2003/106633A2. (E.I DU PONT DE NEMOURS AND COMPANY). 24 December 2003. (See whole document, especially abstract, pages 1-3, examples and claims).	35-38, 41-43 and 47-49
X	WO 1999/37794 A1. (HINDUSTAN LEVER LIMITED et al). 29 July 1999. (See whole document, especially pages 3-4, 6-8 examples and claims).	35-38, and 41
X	Oliver, Yu. Et al. Metabolic engineering to increase isoflavone biosynthesis in soybean seed. Phytochemistry. 2003, 63, 753-763. (See whole document especially abstract, pages 1-2).	35-38, 41-43 and 47-49
X	de Majnik, J. et al. Anthocyanin regulatory gene expression in transgenic white clover can result in an altered pattern of pigmentation. Australian Journal of Plant Physiology. 2000, 27: 659-667. (See whole document especially abstract, introduction and results).	35-38, 41-43 and 47-49
A	Chawla, H.S et al. Expression of anthocyanin pigmentation in Wheat tissue transformed with anthocyanin regulatory genes. Current Science, 1999. Vol: 76, No: 10. Pages 1356-1370. [Retrieved on 2005-03-02]. Retrieved from the internet: <URL:http://tejas.serc.iisc.ernet.in/currsci/may25/articles27.htm. (See whole document, especially abstract, materials and methods and results).	1-56
A	Pairoba, C.F. et al. Post-transcriptional regulation of expression of the *Bronze2* gene of *Zea mays L.* Plant Molecular Biology. 2003, 53: 75-86. (See whole document especially abstract, page 76 last paragraph and materials and methods).	1-56
A	WO 2000/44909A1. (E.I. DU PONT DE NEMOURS AND COMPANY). 3 August 2000. (See whole document, especially pages 4-6, 12, 23 and claims 20-21, 23-27).	1-56
A	Grotewold, E. et al. Engineering Secondary Metabolism in Maize Cells by Ectopic Expression of Transcription Factors. The Plant Cell. 1998, Vol: 10, 721-740. (See whole document especially abstract, introduction and pages 727-731).	1-56

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Search Terms Continued:

The search was based on the following search statement:

1) A transgenic monocotyledonous plant, part of said plant, or progeny of said plant, wherein said plant, part or progeny comprises a transgene expressing R and C1 polynucleotides, functionally active derivatives, analogs, homologs or variants thereof, with the proviso that the part is not a single cell.

2) Part of a monocotyledonous plant which comprises transgene expressing R and C1 polynucleotides or functional variant thereof, wherein said part is not a single cell.

3) A method of producing a transgenic monocotyledonous plant comprising:

(i) transfecting a plant cell with a vector comprising a transgene capable of expressing R and C1 polynucleotides or functionally active derivatives, analogs, homologs or variants thereof.

(ii) regenerating said cell into a plant, thereby producing a plant comprising a transgene expressing R and C1 polynucleotides, or functionally active derivatives, analogs, homologs or variants thereof.

4) A nutraceutical when obtained from a monocotyledonous plant comprising a transgene expressing R and C1 polynucleotides, or a part or progeny of said plant, wherein said plant is not a single cell.

5) A method of identifying a transgenic plant comprising a transgene expressing R and C1 polynucleotides, or functionally active derivatives, analogs, homologs or variants thereof comprising the step of detecting the presence of red pigmentation in the vegetative tissue and/or seeds of the plant, wherein the absence of red pigmentation in the vegetative tissue and/or seeds indicates that a plant does not have the transgene expressing R and C1 polunucleotides or functionally active derivatives, analogs, homologs or variants thereof.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2005/000545

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	5880331	AU	27379/95	CA	2191441	EP	0764212
		US	6008437	WO	9534634		
US	20020188964						
WO	2002101023						
WO	2003106633						
WO	199937794						

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX