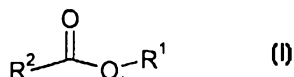




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(21) International Application Number: PCT/GB99/04348 (22) International Filing Date: 22 December 1999 (22.12.99) (30) Priority Data: 9902234.5 1 February 1999 (01.02.99) GB (71) Applicant (for all designated States except US): ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): CLARKE, Eric, Daniel [GB/GB]; Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET (GB). CHRYSTAL, Ewan, James, Turner [GB/GB]; Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET (GB). JEPSON, Ian [GB/GB]; Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET (GB). PAINE, Jacqueline, Ann, Mary [GB/GB]; Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET (GB). (74) Agents: HOUGHTON, Malcolm, John et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell RG42 6YA (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report.	

(54) Title: INDUCIBLE EXPRESSION SYSTEM FOR USE IN PLANTS



(57) Abstract

The use of an agriculturally acceptable hydrolysable ester such as a compound of formula (I) in which R¹ and R² are defined in the application, in the control of expression of a plant gene, said control being effected by an inducible promoter which requires for activation, the presence of an exogenous chemical which may comprise an alcohol, wherein hydrolysis of said agriculturally acceptable ester results in the production of said alcohol. Formulations of these esters are also described and claimed.

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INDUCIBLE EXPRESSION SYSTEM FOR USE IN PLANTS

The present invention relates to an expression system for use in plants, in particular to an expression system which utilises an exogenous chemical agent as a control mechanism and to the use of certain chemicals as said control agent.

Gene expression is controlled by regions upstream (5') of the protein encoding region, commonly referred to as the "promoter". A promoter may be constitutive, tissue-specific, developmentally-programmed or inducible.

Manipulation of crop plants to improve characteristics (such as productivity or quality) requires the expression of foreign or endogenous genes in plant tissues. Such genetic manipulation therefore relies on the availability of means to control gene expression as required; for example, on the availability and use of suitable promoters which are effective in plants. It is advantageous to have the choice of a variety of different promoters so that the most suitable promoter may be selected for a particular gene, construct, cell, tissue, plant or environment. A range of promoters are known to be operative in plants.

Particularly useful promoters in certain instances are promoters which are inducible by the application of an exogenous chemical inducer. This allows particular gene expression to be controlled at particular stages of plant growth or development, by the presence or absence of a chemical which can be applied to the plants or seeds, for example by spraying or using known seed coating techniques. This is sometimes known as a gene "switch".

The gene which is under the control of the inducible promoter may be the gene which gives rise to the desired characteristic or phenotype itself, or the inducible promoter may control expression of a repressor protein which inhibits expression of a target gene, for example by interacting with an operator sequence upstream of the target gene so as to prevent expression of the gene (for example as known in the bacterial tet and lac operator/repressor systems). In a further alternative, the gene under the control of the inducible promoter may express a protein which interacts with another protein to inhibit the activity thereof, as for example in the barnase/barstar system which barnase will inhibit or kill cells in the absence of barstar.

Gene switches of this type are known in a wide variety of applications. These include the production of reversible male sterility, a feature which is highly desirable

in hybrid plant production as described for instance in WO 90/08830. Other applications of such promoters include in germplasm protection, where containment of particular crop plants, in particular transgenic plants, and the control of volunteers is necessary and also in the prevention of pre-harvesting sprouting as described in WO 94/03619.

5 Many organisms have mechanisms which allow them to metabolise chemicals such as alcohols or ketones, for example by the production of alcohol dehydrogenase enzymes. The promoters of these systems may be useful in gene switches as the promoters may be inducible by the presence of the target alcohol or ketone.

One such example can be found in the fungal organism A nidulans which expresses
10 the enzyme alcohol dehydrogenase I (ADH1) encoded by the gene alcA only when it is grown in the presence of various alcohols and ketones. The induction is relayed through a regulator protein encoded by the alcR gene and constitutively expressed. In the presence of inducer (alcohol or ketone), the regulator protein activates the expression of the alcA gene. The regulator protein also stimulates expression of itself in the presence of inducer. This
15 means that high levels of the ADH1 enzyme are produced under inducing conditions (ie when alcohol or ketone are present). Conversely, the alcA gene and its product, ADH1, are not expressed in the absence of inducer. Expression of alcA and production of the enzyme is also repressed in the presence of glucose.

Thus the alcA gene promoter is an inducible promoter, activated by the alcR regulator
20 protein in the presence of inducer (ie by the protein/alcohol or protein/ketone combination). The alcR and alcA genes (including the respective promoters) have been cloned and sequenced (Lockington RA et al, 1985, Gene, 33:137-149; Felenbok B et al, 1988, Gene, 73:385-396; Gwynne et al, 1987, Gene, 51:205-216).

Alcohol dehydrogenase (adh) genes have been investigated in certain plant species.
25 In maize and other cereals they are switched on by anaerobic conditions. The promoter region of adh genes from maize contains a 300 bp regulatory element necessary for expression under anaerobic conditions. However, no equivalent to the alcR regulator protein has been found in any plant. Hence the alcR/alcA type of gene

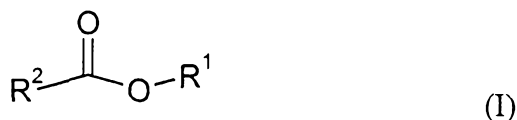
regulator system is not known in plants. Constitutive expression of alcR in plant cells does not result in the activation of endogenous adh activity.

WO 93/21334 describes the production of transgenic plants which include such as system as a gene switch. This document specifically describes a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator
 5 sequence which encodes a regulator protein, and an inducible promoter operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer whereby application of the inducer causes expression of the target gene. In particular, the alcR/alcA system is utilised in the constructs. Exogenous
 10 chemical inducers which are applied in this case include those described by Creaser et al., J. Biochem. (1984) 225, 449-454) such as butan-2-one (ethyl methyl ketone), cyclohexanone, actone, butan-2-ol, 3-oxobutyric acid, propan-2-ol and ethanol

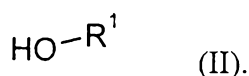
For agricultural purposes, alcohols are generally used as the exogenous chemical inducer. However, such chemicals are often volatile and therefore difficult to handle in an
 15 agricultural context, as large volumes of chemical may be lost during spraying.

The present invention provides the use of an agriculturally acceptable hydrolysable ester in the control of expression of a plant gene, said control being effected by an inducible promoter which requires for activation, the presence of an exogenous chemical which may comprise an alcohol, wherein hydrolysis of said agriculturally acceptable ester results in the
 20 production of said alcohol.

In particular the agriculturally acceptable ester comprises a compound of formula (I)



in which R^1 is a lower alkyl, lower alkenyl or lower alkynyl group, and R^2 is a organic group such that R^2COOH is an agriculturally acceptable acid. Hydrolysis of a compound of
 25 formula (I) yields an alcohol of formula (II)



The term "agriculturally acceptable" as used herein means that the compounds may be applied to a particular soil or crop situation without causing unacceptable levels of soil damage or phytotoxicity in the crop.

The expression "lower alkyl" as used herein includes C₁₋₆ alkyl groups, preferably from C₁₋₄ alkyl groups which may be straight or branched chain. The expression "lower alkenyl" and "lower alkynyl" as used herein includes C₂₋₆ alkenyl and C₂₋₆ alkynyl groups respectively, preferably from C₂₋₄ alkenyl or C₂₋₄ alkynyl groups which may be straight or branched chain.

Agriculturally acceptable esters for use in the invention such as those of formula (I) are suitably translocated into the target plant in which the gene control system is in place and/or hydrolysed either under environmental conditions or in the presence of a suitable catalytic moiety such as an enzyme or catalytic antibody, at rates which are appropriate to provide sufficient quantities of the activating alcohol at the required time in the necessary parts of the plant. These may vary depending upon the nature of the plant species being treated, the gene being expressed and the timing of the application of the ester.

Esters such as the compounds of formula (I) are advantageous in that they are easier to handle than the corresponding alcohols. It has been found that these compounds can produce the desired effect in terms of gene activation.

The compound should be applied at a sufficient period of time prior to the required gene activation to allow hydrolysis to occur and this should be reasonable depending upon factors such as the growth stage of the plant at which activation is required. If the rate of hydrolysis is relatively slow, the time of application may be earlier in order to ensure that sufficient hydrolysis has occurred by the time the plant is at the growth stage at which gene activation is required. Where this is difficult, more rapidly hydrolysing esters may be selected.

Alternatively, more than one ester, with differing rates of hydrolysis may be applied in a single treatment. By selecting combinations of esters with different rates of hydrolysis, an effective "slow release" of activating alcohol can be achieved so that gene expression may be prolonged over the desired period. This means that repeated applications of chemical may be avoided and "one-shot" treatments are possible.

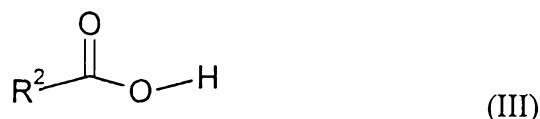
Particular examples of alcohols of formula (II) include methanol, ethanol, propan-1-ol, propan-2-ol, butan-2-ol or but-3-en-2-ol.

Suitably, the alcohol of formula (II) is a lower alkyl alcohol wherein the alkyl group has from 1 to 4 carbon atoms and may be either branched, or linear. Preferred groups for R¹ include ethyl, n-propyl and n-butyl.

A particularly preferred example of a compound of formula (II) is ethanol.

The precise nature of the R² group is immaterial provided that it gives rise to an agriculturally acceptable acid at an appropriate rate in the particular target plant to which it is applied. Rates of hydrolysis can be determined using routine methods for example as described by G. Mitchell et al., *Pestic. Sci* (1995) 44:49-58. and preferably by testing against whole plant systems. What is appropriate in any particular instance will depend upon a variety of factors including the nature of the gene expression of which is being controlled, the particular plant in which the gene is expressed and other external conditions. The rate of hydrolysis should be sufficient to allow the desired effect, for example, reversible male sterility, to be seen at an appropriate period of time after application of the chemical inducer.

R² however may be selected such that the resultant acid of formula (III)



has some useful agrochemical effect. In particular, it may itself be able to act as an inducer of the inducible promoter. For example, it has been found that a number of acids including 3-hydroxybutyric acid, 2-hydroxybutyric acid, pyruvic acid and 3-oxobutyric acid can act as an inducer of the *alcR/alcA* system (Creaser et al., *supra.*).

Particular examples of R² include optionally substituted alkyl, optionally substituted cycloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl or optionally substituted heterocyclyl.

As used herein the term "alkyl" includes straight or branched alkyl chains, suitably containing up to 10 carbon atoms, preferably from 1 to 6 carbon atoms. The terms "alkenyl" and "alkynyl" includes unsaturated straight or branched chains containing up to 10 carbon atoms, preferably from 2 to 6 carbon atoms. The term "aryl" includes phenyl and naphthyl. The term "heterocyclic" includes rings containing up to 10, preferably up to 7 atoms, up to three of which are selected from

oxygen, sulphur or nitrogen. These rings may be single rings or may be in the form of fused ring systems and these may be aromatic or non-aromatic in nature. The term "halo" or "halogen" includes chlorine, fluorine, bromine and iodine. The term "alkoxy" relates to an alkyl group as defined above, linked with an oxygen atom.

5 Suitably R^2 is an optionally substituted C_{1-10} alkyl group which may be linear or branched. Preferred alkyl groups R^2 are linear and contain 3 to 8 carbon atoms, in particular 5 carbon atoms.

Suitable optional substituents for alkyl, alkenyl and alkynyl groups R^2 include one or more groups selected from halo, nitro, cyano, oxo, optionally substituted aryl,
 10 optionally substituted heterocyclyl, OR^3 , $C(O)_pR^3$, $S(O)_mR^3$, $OCOR^3$, $-NR^4C(O)_pR^3$, $=NOH$, NR^5R^6 , $C(O)NR^5R^6$, $C(O)NR^3NR^5R^6$, $-CH=NOR^3$, $P(O)R^7R^8$ or $P(O)OR^7OR^8$, $NR^3CONR^5R^6$, $-N=CR^5R^6$, $S(O)_mNR^5R^6$ or $-NR^3S(O)_mR^4$, $-N=NR^3$ where each R^3 , R^4 , R^5 , R^6 , R^7 and R^8 are independently selected from hydrogen, alkyl, alkenyl, alkynyl, aryl or heterocyclyl, any of which may be optionally substituted by a
 15 functional group and in the case of aryl and heterocyclic groups, may also be substituted by alkyl, alkenyl or alkynyl groups; or R^5 and R^6 together with the atom to which they are attached, may additionally form, together with the atom to which they are attached, a ring which may be carbocyclic or heterocyclic; p is 1 or 2 and m is 0, 1, 2 or 3.

As used herein the term "functional group" refers to include halo, cyano, nitro, oxo,
 20 hydroxy, $=NOR^{11}$, $C(O)_pR^{11}$, OR^{11} , $S(O)_mR^{11}$, $NR^{12}R^{13}$, $C(O)NR^{12}R^{13}$, $OC(O)NR^{12}R^{13}$, $-CH=NOR^{11}$, $-NR^{12}C(O)_mR^{11}$, $-NR^{11}CONR^{12}R^{13}$, $-N=CR^{12}R^{13}$, $S(O)_mNR^{12}R^{13}$ or $-NR^{12}S(O)_mR^{11}$ where R^{11} , R^{12} and R^{13} are independently selected from hydrogen or optionally substituted hydrocarbyl, or R^{12} and R^{13} together form an optionally substituted ring which optionally contains further heteroatoms such as oxygen and nitrogen or $S(O)R^{14}$,
 25 where p is an integer of 1 or 2, m is 0 or an integer of 1-3 and R^{14} is hydrogen or alkyl.

Suitable optional substituents for hydrocarbyl groups R^{11} , R^{12} or R^{13} include halo, perhaloalkyl such as trifluoromethyl, mercapto, hydroxy, alkoxy, oxo, heteroaryloxy, alkenyloxy, alkynyloxy, alkoxyalkoxy, aryloxy (where the aryl group may be substituted by halo, nitro, or hydroxy), cyano, nitro, amino, mono- or di-alkyl
 30 amino, alkylamido or $S(O)_pR^{14}$ where m and R^{14} are as defined above.

Examples of optional substituents on alkyl, alkenyl or alkynyl groups R^2 are one or more groups selected from oxo; alkoxycarbonyl in particular lower alkoxycarbonyl; cyano; halo such as chloro, fluoro or bromo; phenyl optionally substituted with amino or mono- or dialkyl amino or alkyl such as methyl; OR^3 where R^3 is alkyl or heterocyclyl optionally substituted by halo or alkyl; $S(O)_mR^{11}$ where m is 0 or 2 and R^{11} is alkyl or phenyl optionally substituted by alkyl; NR^5R^6 or $C(O)NR^5R^6$ where R^5 is hydrogen, methyl or methoxyethyl and R^6 is alkyl such as methyl, phenyl or benzyl optionally substituted with halo such as fluoro or chloro, alkyl such as methyl or trifluoromethyl or alkoxycarbonyl where the alkyl moiety may carry a further alkoxycarbonyl group, or R^6 is heterocyclyl such as thiazinyl optionally substituted by alkyl and/or acetyl; $-NR^4C(O)_pR^3$ where p is 2, R^3 is alkyl and R^4 is alkyl optionally substituted with alkoxy carbonyl such as ethoxyl carbonyl alkyl; $-NR^3S(O)_mR^4$ where R^3 is hydrogen, R^4 is phenyl optionally substituted by halo such as chloro, and m is 2; $C(O)NR^3NR^5R^6$ where R^3 and R^5 are hydrogen and R^6 is phenyl optionally substituted by halo or alkoxy such as methoxy; $S(O)_mNR^5R^6$ where m is 2, R^5 is hydrogen and R^6 is alkyl optionally substituted by one or more alkoxycarbonyl groups; heterocyclyl such as furyl, pyridyl, pyridinyl or pyrazinyl, triazinyl, any or which may be optionally substituted by alkyl, halo, trihalomethyl, phenyl, halophenyl, cyano or oxo,

Particularly suitable substituents for alkyl, alkenyl or alkynyl groups R^2 include alkoxycarbonyl in particular where the alkoxy group is a lower alkyl group; alkoxy and in particular two alkoxy groups in the form of a dialkyl acetal; cyano or optionally substituted heterocyclyl. Preferred substituents include, but are not limited to lower alkoxycarbonyl groups and dialkyl acetals. Alkoxycarbonyl groups and dialkyl acetals are of particular interest when the alkyl group of the substituent is the same as R^1 in the compound of formula (I) since on hydrolysis these give rise to more inducer chemical of formula (II).

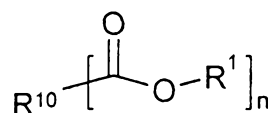
A particular aryl group for R^2 is phenyl.

Suitable optional substituents for cycloalkyl, aryl and heterocyclyl groups R^2 and for aryl or heterocyclyl substituents on the above-mentioned alkyl, alkenyl or alkynyl groups R^2 include halo; haloalkyl; cyano; nitro; amino or mono- or di-alkyl amino; hydroxy; alkoxy, thioalkyl, alkyl or alkoxycarbonyl wherein the alkyl

moiety of any of these may be optionally substituted with for example one or more groups selected from halo, alkoxy, cyano, alkoxycarbonyl, amino, mono- or di-alkyl amino, aryl or carboxylate or salts or esters thereof; cycloalkyl; or heterocyclyl.

Particularly suitable substituents for aryl or heterocyclyl groups R^2 include alkoxy in particular lower alkoxy such as methoxy, alkyl in particular lower alkyl, alkoxycarbonyl in particular lower alkoxycarbonyl and halogen.

A particular sub-group of compounds of formula (I) are compounds of formula (IA)



(IA)

where R^1 is as defined above in relation to formula (I), n is an integer of from 2 to 4 and R^{10} is an alkyl, alkenyl or alkynyl group any of which may be optionally interposed with a heteroatom, a cycloalkyl, heterocyclic group or aryl group, or R^{10} is a cycloalkyl or aryl group of valency n .

In particular R^{10} is an alkyl or aryl group of valency n .

Particularly preferred compounds of formula (I) include:-

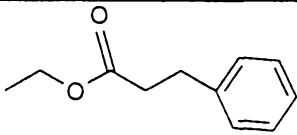
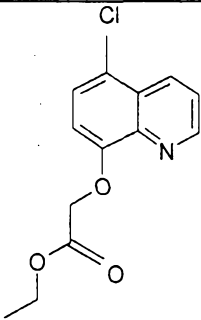
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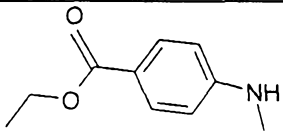
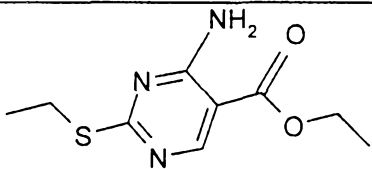
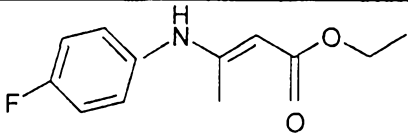
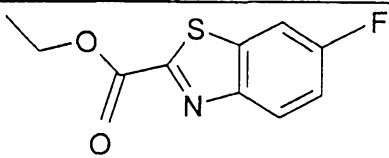
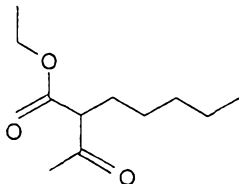
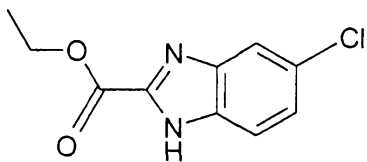
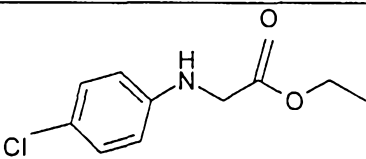
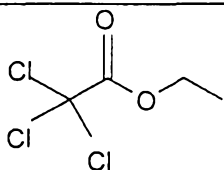
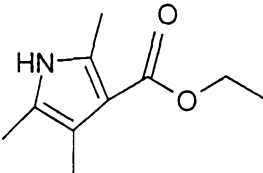
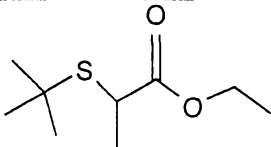
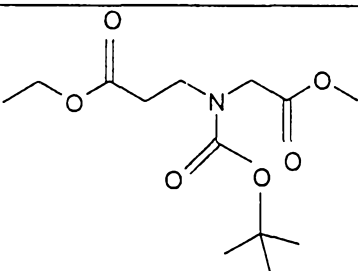
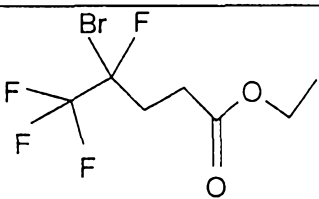
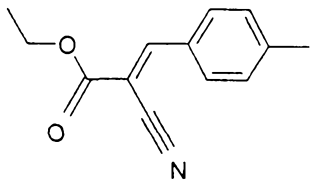
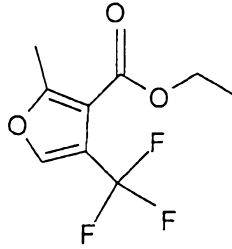
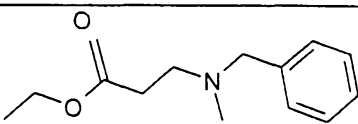
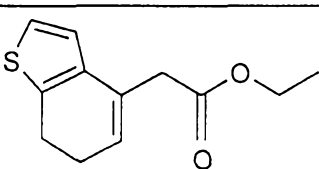
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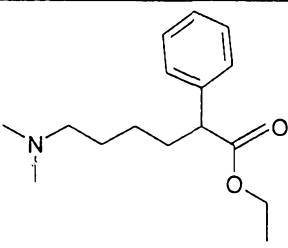
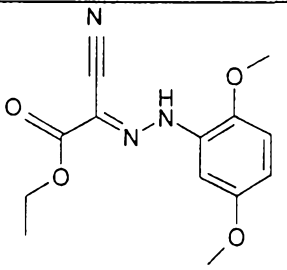
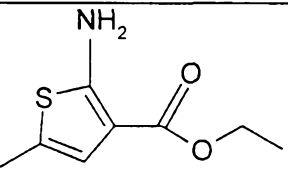
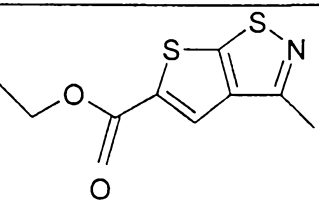
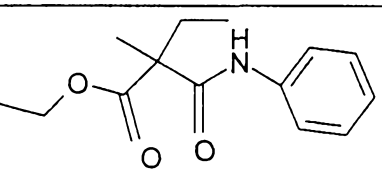
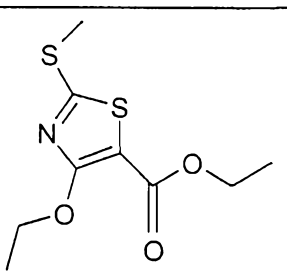
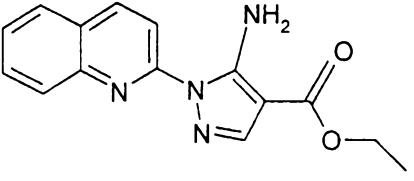
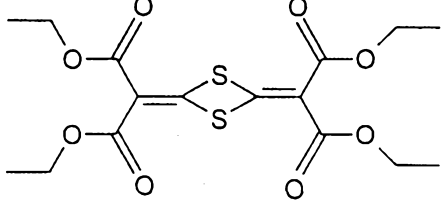
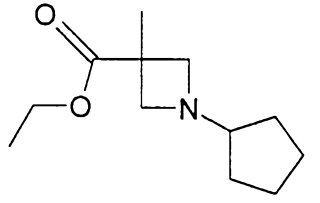
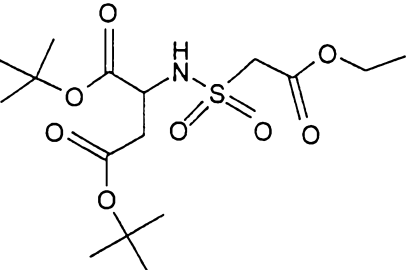
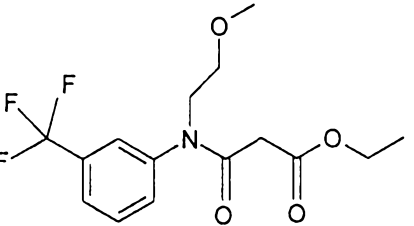
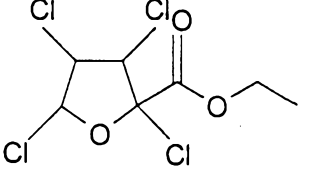
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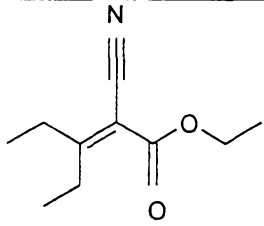
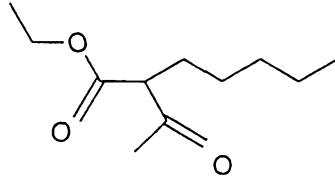
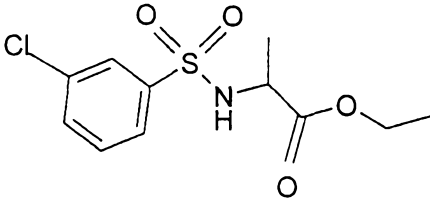
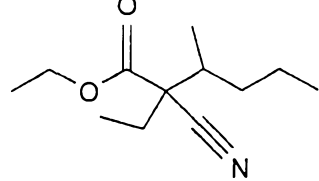
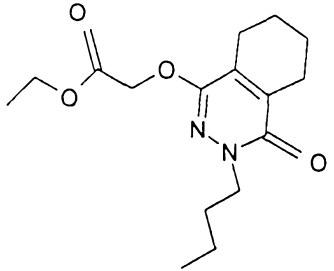
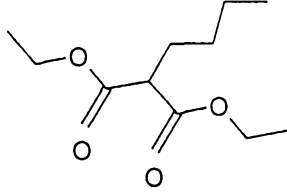
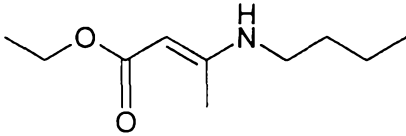
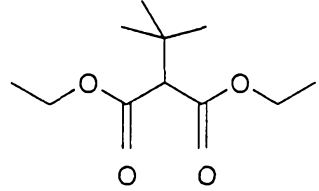
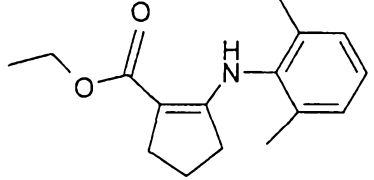
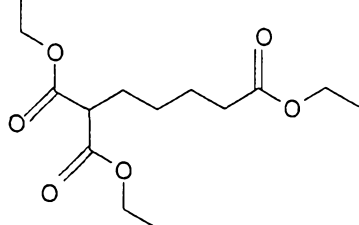
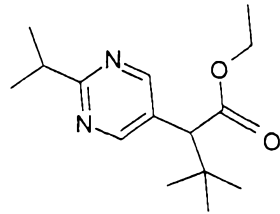
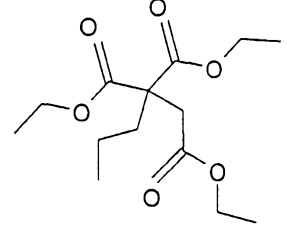
Examples of compounds of formula (I) are ethyl esters as shown in Table 1.

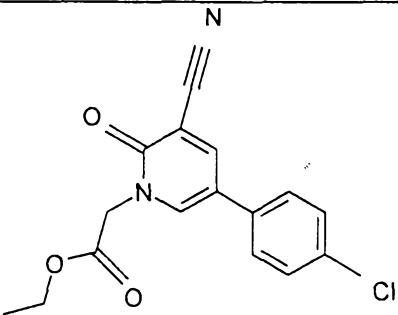
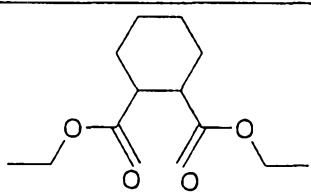
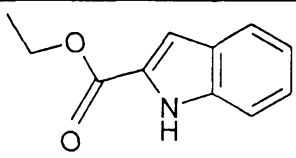
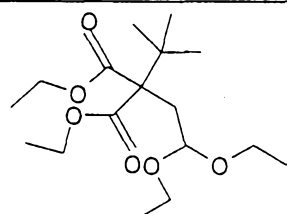
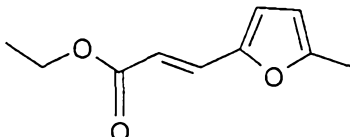
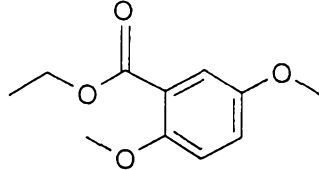
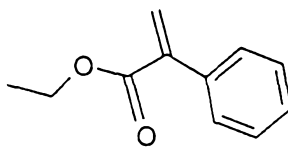
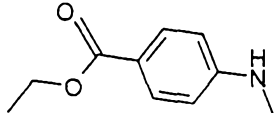
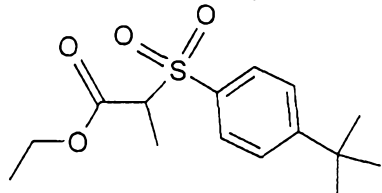
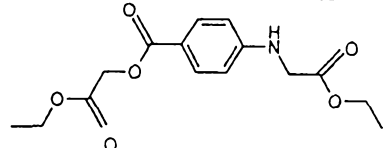
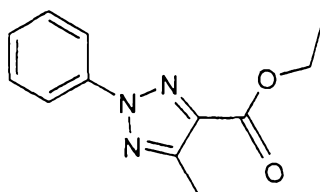
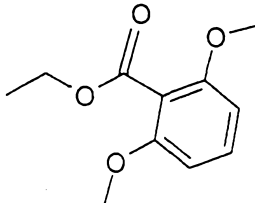
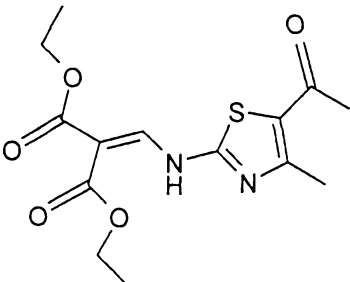
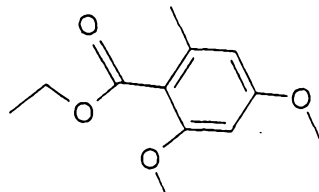
Table 1

Compd. No.	Formula	Compd No.	Formula
1		34	

2		35	
3		36	
4		37	
5		38	
6		39	
7		40	
8		41	
9		42	

10	 <chem>CN(C)CCCC(=O)Oc1ccccc1</chem>	43	 <chem>CCOC(=O)C#N=NNc1cc(OC)cc(OC)cc1</chem>
11	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>	44	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>
12	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>	45	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>
13	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>	46	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>
14	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>	47	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>
15	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>	48	 <chem>CCOC(=O)C1=CC=C(C=C1)S</chem>

16		49	
17		50	
18		51	
19		52	
20		53	
21		54	

22		55	
23		56	
24		57	
25		58	
26		59	
27		60	
28		61	

29		62	
30		63	
31		64	
32		65	
33		66	

Compounds of formula (I) are either known compounds or they can be prepared from known compounds using conventional methods.

Compounds of formula (I) may be hydrolysed in the target plant either chemically, or enzymatically by a naturally occurring enzyme in the target plant or by an enzyme

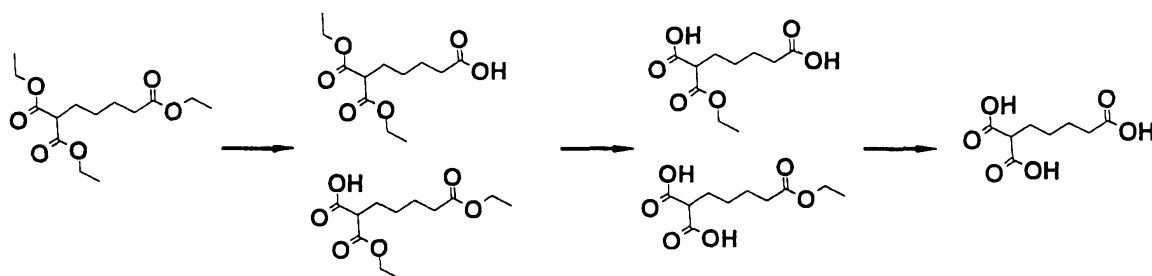
- 5 introduced by genetic engineering into the plant and expressed within the plant, or by an appropriate catalytic antibody, or catalytically active portion of a

catalytic antibody introduced by genetic engineering into the plant and expressed within the plant.

Suitable enzymes include, but are not limited to, esterases and lipases.

Suitable catalytic antibodies may be generated by standard techniques from analogues of a tetrahedral ester hydrolysis transition state, e.g. as for the hydrolysis of the pro-drug ester of chloramphenicol, when appropriate phosphonates were used, Ole K et al., 1998, J. Mol. Biol., 281:501-511, and for the detoxification of cocaine by methyl ester hydrolysis, Mets B et al., 1998, Proc. Nat. Acad. Sci. USA, 95:10176-10181.

Metabolism of the compound of invention has been investigated further and the results of these investigations are reported in the Examples hereinafte. Without being limited to mechanistic considerations, it is believed for example that a representative compound of the invention, Compound 53, is metabolised in accordance with the following scheme:



Triester

Mono Acids

Di Acids

Tri acid

A product of this metabolism is ethanol which can act as a chemical inducer as described above.

In a further aspect, the invention provides a method for controlling expression of a target gene in a plant, wherein said plant is transformed with a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which encodes a regulator protein, and an inducible promoter operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an alcohol such as a compound of formula (II) as defined above, said method comprising applying to said plant an ester which hydrolyses to form said alcohol such as a compound of formula (I) as defined above, so as to cause expression of the target gene.

Suitably the regulator sequence encodes the alcR protein as described above and the inducible promoter is the alcA promoter sequence.

Where necessary or desired, the plant may also be transformed so that it expresses or overexpresses an enzyme or catalytic antibody or catalytically active fragment thereof,
5 which hydrolyses the compound of formula (I) to form a compound of formula (II).

Enzymes which are inactive in the absence of enzymes or other moieties which must be engineered into the plant may be preferable in some circumstances since they will be effective only in the target transformed seed.

The nucleic acid sequences which encode the hydrolytic enzyme, antibody or
10 antibody fragment may be included in the construct containing the regulator protein and/or the target gene operatively linked to the inducible promoter or they may be present on a separate construct which is used to co-transform the plant. Such systems however are novel.

Thus in a further aspect there is provided a plant gene expression system comprising

(i) a first promoter operatively linked to a regulator sequence which encodes a
15 regulator protein,

(ii) an inducible promoter operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer of formula (I) as defined above, whereby application of the inducer causes expression of the target gene; and

20 (iii) a sequence which encodes a protein which effects hydrolysis of an ester such as a compound of formula (I) to the corresponding alcohol under the control of a further promoter which allows its expression in plant tissue.

The target gene may comprise any gene which is required to be introduced into a plant in order to modify the characteristics thereof as outlined above. The target gene may
25 be an endogenous plant gene or a foreign gene, and may be a single gene or a series of genes. The target gene sequence encodes at least part of a functional protein or an antisense sequence.

Any transformation method suitable for the target plant or plant cells may be employed, including infection by Agrobacterium tumefaciens containing recombinant

Ti plasmids, electroporation, microinjection of cells and protoplasts, microprojectile transformation and pollen tube transformation. The transformed cells may then in suitable cases be regenerated into whole plants in which the new nuclear material is stably incorporated into the genome. Both transformed monocot and dicot plants may be obtained
5 in this way.

Examples of genetically modified plants which may be produced include field crops, cereals, fruit and vegetables such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, onion.

10 The invention further provides a plant cell containing a gene expression system according to the invention. The gene expression system may be stably incorporated in the plant's genome by transformation. The invention also provides a plant tissue or a plant comprising such cells, and plants or seeds derived therefrom.

Preferred examples of compounds of formula (I) used in this method are those
15 described above.

Agriculturally acceptable esters of the invention such as compounds of formula (I) are suitably applied in the form of an agriculturally acceptable composition, in combination with a diluent or carrier. Such compositions form a further aspect of the invention.

The concentration of the agriculturally acceptable ester in the formulation is
20 preferably at a concentration of about 5% wt/wt or less. It is preferably at a concentration between about 2% and 5% wt/wt.

Suitable carriers or diluents will be apparent to the skilled person and will vary depending upon the particular nature of the compounds of formula (I) employed. For instance, where the compound of formula (I) is an oil, it may require the presence of an
25 emulsifier in order to allow it to be sprayed in aqueous solution. Emulsifiers are well known in the art, and a particular example is partially hydrolysed polyvinyl acetate (PVA) or Tween™.

Organic solvents or diluents such as acetone may also be present.

Thus a preferred composition comprises an agriculturally acceptable ester such as a
30 compound of formula (I), an emulsifier such as PVA and a diluent such as water. The relative amounts of the components will be determined to a large extent by

the mutual miscibility of the various components. Suitably however, the emulsifier will be present in the composition in amounts of from 1-5%w/w, preferably at about 2.5%w/w.

Thus examples of formulations of the invention include the following:

Formulation 1

- 5 1.5% Compound of the invention (e.g. Compound 53)
 2.5% PVA
 Balance water

Formulation 2

- 10 1.5% Compound of the invention (e.g. Compound 53)
 5% acetone
 0.05% Tween-20™
 Balance water

15 Formulation 3

- 3.0% Compound of the invention (e.g. Compound 53)
 2.5% PVA
 Balance water

20 Formulation 4

- 3.0% Compound of the invention (e.g. Compound 53)
 5% acetone/H₂O
 0.05% Tween-20™
 Balance water

- 25 Alternative compositions which may be employed are similar to those described in our copending British Patent application No. 9902236.0. In particular, the compositions will comprise the components:

- (a) an agriculturally acceptable ester such as a compound of formula (I);
 (b) a polyethoxylated C₁₀-C₂₀ alcohol or a trisiloxane polyethoxylate and
30 (c) a diluent.

 The diluent (c) may be, for example, water.

Component (b) of the formulation described above is preferably, a polyethoxylated oleyl, lauryl, stearyl or cetyl alcohol. It is more preferably a polyoxyethylene-oleyl alcohol having a mean molar ethylene oxide content in the range of 0 to 35 and more preferably in the range of 2 to 20. It is most preferably a polyoxyethylene-(2)-oleyl alcohol, a
5 polyoxyethylene-(10)-oleyl alcohol or a polyoxyethylene-(20)-oleyl alcohol. Component (b) is, however, preferably a polyoxyethylene-(20)-oleyl alcohol (the number in brackets indicates the mean ethylene oxide content per molecule). Such products are commercially available as BRIJ 92™, BRIJ 97™ and BRIJ 98™.

Preferably, component (b) of the formulation is at a concentration of about 0.5%
10 wt/wt or less. It is preferably at a concentration between about 0.2% wt/wt and 0.5% wt/wt.

In an alternative embodiment, the formulation includes as component (b), a hydrogen or methyl end-capped trisiloxane polyethoxylate. In particular, component (b) is a methyl end-capped trisiloxane polyethoxylate. The methyl end-capped trisiloxane polyethoxylate preferably has a mean molar ethylene oxide content of between 4 and 12 per molecule and is
15 most preferably 8 per molecule. Such products are commercially available as SILWET 77™ (SILWET is a trademark of Witco).

Preferably, the methyl end-capped trisiloxane polyethoxylate is at a concentration of about 0.5% wt/wt or less. It is preferably at a concentration between about 0.2% and 0.5% wt/wt.

20 Component (c) of the formulation is preferably at a concentration between about 90% and 98% wt/wt.

Other additives which may be included in the formulations include dispersants, antibacterial compounds, wetter compounds and anti-evaporants may also be added.

The invention will now be particularly described by way of example.

25 Example 1

Testing chemicals on soil grown plants

Following a preliminary screen on hydroponic solution, the compounds were used in a test carried out on 4 week old alcat tobacco homozygous line 30 plants in 1½" pots in the glasshouse. Leaf samples were removed from the plants as an "uninduced"

control. The leaf pieces were ground in 200ul of 250mM Tris pH8.0, centrifuged and the supernatant recovered and stored at 4°C overnight. The compounds were dissolved in 50% acetone 50% dH₂O with 0.05% tween-20 as a 200mg/ml solution. This was then diluted 1/10 for a 2% solution. unless otherwise stated, 5mls of the solution was applied to the soil of each of 2 plants to be replica treatments as a root drench. After 22 and 44 hours leaf samples were removed, extracted as described above and the supernatants stored at 4°C. The samples were analysed for cat protein quantification by using a Boehringer Mannheim CAT ELISA kit and the total protein level determined by a Bradford determination. The results are shown in Table 2 where a CAT value of 1 equates to the detection of 0-5000ng/, 2 equates to the detection of 5001-10,000 ng/g, 3 equates to 10,001-15,000ng/g .

Table 2

	Compound No	CAT score		
		0 hours	22 hours	44 hours
	1	0	1	0
(2.5mls)	2	0	1	0
	3	0	1	2
(2.5mls)	4	0	0	1
	5	0	0	3
	8	1	1	1
	9	1	0	2
	10	2	0	1
	12	1	0	2
	13	0	0	0
	14	0	1	0
	15	0	1	1
	16	1	1	6
	17	1	2	1
	18	0	1	1
	19	0	1	1
	20	0	0	1
	21	0	0	1
	22	0	0	1
	23	0	0	1
	24	1	0	1
	25	0	0	1
(2.5mls)	26	0	0	0
	28	0	0	1
	30	0	2	2
	32	0	0	2

	33	1	1	1
(2.5mls)	35	0	0	1
	36	0	0	1
	38	0	0	3
	39	0	0	1
	40	0	0	0
(2.5mls)	41	1	0	1
	42	2	1	1
	43	0	0	2
	44	1	0	0
	45	1	0	2
	46	2	0	1
	47	3	0	2
	5mls formulation	0	0	0
	2.5mls formulation	0	0	0
	5% ethanol	1	8	10
	5% butan-1-ol	1	1	3
	dH ₂ O	1	0	0

Example 2

Further treatment of soil grown plants

- 5 In this experiment, compounds were dissolved in 50% acetone 50% dH₂O with 0.05% tween-20 as a 200mg/ml solution. These were diluted to a 1.5% and 0.1% solution. Leaf samples were removed from the seedlings as an “uninduced” control. The leaf pieces were ground in 200ul of 250mM Tris pH8.0, centrifuged and the supernatant recovered and stored at 4°C overnight. 5mls of the solution was applied to the soil of each of 2 plants as replica
- 10 treatments as a root drench. After 22 and 65 hours leaf samples were removed from each of two plants treated, extracted as described in Example 1 and the supernatants stored at 4°C. The samples were analysed for cat protein quantification by using a Boehringer Mannheim CAT ELISA kit and the total protein level determined by a Bradford determination. The compounds tested and results are shown in Table 3, where the CAT scores represent the
- 15 values set out in respect of Table 2.

Table 3

Compound No.	CAT protein Score		
	0 hrs	22 hrs	65 hrs
1.5% root drench			
17	0	3	1
50	0	2	1
51	0	1	3

51	0	1	3
52	0	2	1
53	0	3	8
54	0	1	2
55	0	1	1
56	0	2	1
57	0	2	5
58	0	1	0
59	0	1	1
61	0	1	1
62	0	1	2
63	0	1	1
64	0	1	1
65	0	0	1
ethanol	0	3	6
butan-1-ol	0	3	5
formulation	0	0	0
dH ₂ O	0	0	0

Example 3

Determination of the time course of expression of the switch after induction

In order to determine the time course of expression of the alc switch after induction from the esters several compounds were applied to the soil or leaves of 5 week old alccat homozygous line 30 plants in 1½" pots in the glasshouse. The compounds were dissolved in 50% acetone 50% dH₂O with 0.05% tween-20 as either a 150mg/ml or 50mg/ml solution. They were then diluted 1/10 for a 1.5% or 0.5% solution. Leaf samples were removed from the seedlings as an "uninduced" control. The leaf pieces were frozen in dry ice/ethanol and stored at -70°C.

5mls of the solution was applied as either a root drench or a leaf spray to up to 8 plants.

Leaf samples were removed at various time points and frozen in dry ice/ethanol and stored at -70°C. When all the samples were harvested and stored they were extracted in 200ul of 250mM Tris pH8.0, centrifuged and the supernatant recovered and stored at 4°C overnight.

The samples were analysed for cat protein quantification by using a Boehringer Mannheim CAT Elisa kit and the total protein level determined by a Bradford determination. The compounds tested and results are shown on Tables 4, 5, 6, 7 8, 9 and 10. The CAT units are as set out above in Example 1 with each successive number representing an incremental 5,000ng/g range. Results shown in shaded form were obtained in separate but similar trials.

Table 4

Hours	Compound 51	CAT score
0	0.5% 49 Spray	0
5	0.5% 49 S	0
10	0.5% 49 S	0
26	0.5% 49 S	0
48	0.5% 49 S	0
98	0.5% 49 S	0
144	0.5% 49 S	0
193	0.5% 49 S	0
0	1.5% 49 S	0
5	1.5% 49 S	0
10	1.5% 49 S	0
26	1.5% 49 S	0
48	1.5% 49 S	1
98	1.5% 49 S	0
144	1.5% 49 S	0
193	1.5% 49 S	0
0	0.5% 49 Root drench	0
5	0.5% 49 R	0
10	0.5% 49 R	1
26	0.5% 49 R	2
48	0.5% 49 R	1
98	0.5% 49 R	4
144	0.5% 49 R	4
193	0.5% 49 R	0
0	1.5% 49 R	0
10	1.5% 49 R	1
22 hours	1.5% root	1
26	1.5% 49 R	1
48	1.5% 49 R	3
65 hours	1.5% root	3
98	1.5% 49 R	4

Table 5

Hours	Compound No 57	CAT score
t=0	.5%30 Spray	0
t=5	.5%30 S	0
t=10	.5%30 S	0
t=26	.5%30 S	1
t=48	.5%30 S	1
t=0	1.5% 30S	0
t=5	1.5% 30S	0
t=10	1.5% 30S	0
t=26	1.5% 30S	0
t=48	1.5% 30S	0
t=0	.5%30 Root drench	0
t=5	0.5%30 R	0
t=10	.5%30 R	1
t=26	.5%30 R	2
t=48	.5%30 R	2
t=98	.5%30 R	4
t=0	1.5% 30R	0
t=10	1.5% 30R	1
22 hours	1.5% root	2
t=26	1.5% 30R	1
t=48	1.5% 30R	2
65 hours	1.5% root	5
t=98	1.5% 30R	3

Table 6

Hours	Compound No 53	CAT Score
0	0.5%51 Spray	0
5	0.5%51 S	0
10	0.5%51 S	1
26	0.5%51 S	1
48	0.5%51 S	1
98	0.5%51 S	1
144	0.5%51 S	1
193	0.5%51 S	0
0	1.5% 51 S	0
5	1.5% 51 S	1
10	1.5% 51 S	2
26	1.5% 51 S	1
48	1.5% 51 S	1
98	1.5% 51 S	1
144	1.5% 51 S	0
193	1.5% 51 S	1
0	0.5%51 Root drench	0
5	0.5%51 R	1
10	0.5%51 R	1
26	0.5%51 R	1
48	0.5%51 R	1
98	0.5%51 R	3
144	0.5%51 R	2
193	0.5%51 R	0
0	1.5% 51 R	0
10	1.5% 51 R	1
22 hours	1.5% root	4
26	1.5% 51 R	4
48	1.5% 51 R	8
65 hours	1.5% root	8
98	1.5% 51 R	5

Table 7

Hours	Compound No 60	CAT Score
t=0	0.5% 57 Root drench	0
t=10	0.5% 57 R	1
t=26	0.5% 57 R	1
t=48	0.5% 57 R	2
t=98	0.5% 57 R	5
t=144	0.5% 57 R	8
t=0	1.5%57 R	0
t=10	1.5%57 R	1
22 hours	1.5% root	1
t=26	1.5%57 R	1
t=48	1.5%57 R	1
65 hours	1.5% root	1
t=98	1.5%57 R	4

Table 8

Hours	Compound 52	CAT score
t=0	0.5%50Spray	0
t=10	0.5%50S	0
t=26	0.5%50S	0
t=48	0.5%50S	1
t=98	0.5%50S	0
t=144	0.5%50S	0
t=193	0.5%50S	0
t=0	1.5% 50S	0
t=10	1.5% 50S	0
t=26	1.5% 50S	0
t=48	1.5% 50S	0
t=98	1.5% 50S	0
t=144	1.5% 50S	0
t=193	1.5% 50S	0
t=0	0.5%50Root drench	0
t=10	0.5%50R	1
t=26	0.5%50R	1
t=48	0.5%50R	2
t=98	0.5%50R	1
t=144	0.5%50R	1
t=193	0.5%50R	1
t=0	1.5% 50R	0
t=10	1.5% 50R	0
22 hours	1.5% root	2
t=26	1.5% 50R	1
t=48	1.5% 50R	2
65 hours	1.5% root	1
t=98	1.5% 50R	1

Table 9

Hours	Ethanol	CAT score
0	Etoh 0.5% Spray	0
5	Etoh 0.5% S	2
10	Etoh 0.5% S	2
26	Etoh 0.5% S	2
48	Etoh 0.5% S	1
98	Etoh 0.5% S	1
144	Etoh 0.5% S	1
193	Etoh 0.5% S	1
0	Etoh 1.5% S	0
5	Etoh 1.5% S	2
10	Etoh 1.5% S	4
26	Etoh 1.5% S	5
48	Etoh 1.5% S	4
98	Etoh 1.5% S	2
144	Etoh 1.5% S	2
193	Etoh 1.5% S	1
0	Etoh 0.5% Root drench	0
5	Etoh 0.5% R	2
10	Etoh 0.5% R	4
26	Etoh 0.5% R	6
48	Etoh 0.5% R	4
98	Etoh 0.5% R	4
144	Etoh 0.5% R	4
193	Etoh 0.5% R	3
0	Etoh 1.5% R	0
10	Etoh 1.5% R	4
26	Etoh 1.5% R	9
48	Etoh 1.5% R	7
98	Etoh 1.5% R	6

Table 10

Hours	dH ₂ O	CAT Score
0	dH ₂ O Root drench	0
5	dH ₂ O R	0
10	dH ₂ O R	0
26	dH ₂ O R	0
48	dH ₂ O R	0
98	dH ₂ O R	0
144	dH ₂ O R	0
193	dH ₂ O R	0
Formulation hours		
0	Spray	0
5	S	0
10	S	0
26	S	0
48	S	0
98	S	0
144	S	0
193	S	0
0	Root drench	0
5	R	0
10	R	0
26	R	0
48	R	1
98	R	1
144	R	1
193	R	0

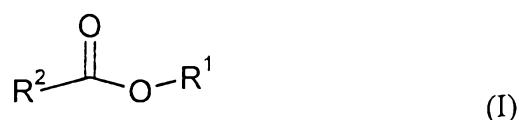
These results show that the time course of the induction can be altered by using esters in

5 accordance with the invention, as compared to alcohols. Thus the appropriate selection of ester would lead to a favoured induction profile.

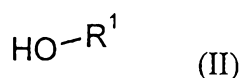
CLAIMS

1. The use of an agriculturally acceptable hydrolysable ester in the control of expression of a plant gene, said control being effected by an inducible promoter which requires for activation, the presence of an exogenous chemical which may comprise an alcohol, wherein hydrolysis of said agriculturally acceptable ester results in the production of said alcohol.

2. The use according to claim 1 where the agriculturally acceptable hydrolysable ester is a compound of formula (I)



in which R^1 is a lower alkyl group, and R^2 is an organic group which yields an agriculturally acceptable acid of formula R^2COOH on hydrolysis; and said promoter requires the presence of an alcohol of formula (II)



- for activation thereof.
3. The use according to claim 2 wherein the compound of formula (II) is a lower alkyl alcohol wherein the alkyl group R^1 has from 1 to 4 carbon atoms and may be either branched, or linear.
4. The use according to claim 2 or claim 3 wherein R^2 is optionally substituted alkyl, optionally substituted cycloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl or optionally substituted heterocyclyl.

5. The use according to claim 4 wherein R^2 is an optionally substituted C_{1-10} alkyl group.
6. The use according to claim 5 wherein R^2 is substituted by an alkoxycarbonyl group or dialkyl acetal..
- 5 7. The use according to any one of the preceding claims wherein the compound of formula (I) is ethyl 2-n-pentyl-3-oxobutanoate; triethyl 2-carboxyheptan-1,7-dioate; or ethyl 2,4-dimethoxybenzoate
- 10 8. A method for controlling expression of a target gene in a plant, wherein said plant is transformed with a chemically-inducible plant gene expression cassette comprising a first promoter operatively linked to a regulator sequence which encodes a regulator protein, and an inducible promoter operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an alcohol, said method comprising applying to said plant an agriculturally acceptable hydrolysable ester which yields said alcohol on hydrolysis thereof, so as to cause expression of the target gene.
- 15 9. A method according to claim 8 wherein the regulator sequence encodes the alcR protein as described above and the inducible promoter is the alcA promoter sequence.
- 20 10. A method according to claim 8 or claim 9 wherein the plant is transformed so that it expresses or overexpresses an enzyme or catalytic antibody or catalytically active fragment thereof, which hydrolyses the ester to form the said alcohol..
- 25 11. A plant gene expression system comprising
(i) a first promoter operatively linked to a regulator sequence which encodes a regulator protein,

(ii) an inducible promoter operatively linked to a target gene, the inducible promoter being activated by the regulator protein in the presence of an effective exogenous inducer alcohol as defined above, whereby application of the alcohol causes expression of the target gene; and

(iii) a sequence which encodes a protein which effects hydrolysis of an ester above under the control of a further promoter which allows its expression in plant tissue.

12. A plant cell containing a gene expression system according to claim 11.

13. Plant tissue or a plant comprising cells according to claim 12, and plants or seeds derived therefrom.

14. An agricultural composition, comprising an agriculturally acceptable ester as defined in claim 1 in combination with a diluent or carrier.

15. A composition according to claim 14 wherein the agriculturally acceptable ester is a compound of formula (I) as defined in claim 2.

16. A composition according to claim 14 or claim 15 which further comprises an emulsifier.

17. A composition according to claim 16 wherein the emulsifier is PVA.