METHOD OF INTRODUCING PATHOGEN RESISTANCE IN PLANTS

Abstract

Variegated plants have increased pathogen resistance: cells of the plant express a phenotype, which may comprise necrosis and/or a plant defence response, and other cells not expressing this phenotype have increased pathogen resistance. Embodiments of the invention employ various genes, including *Cladosporium fulvum* pathogen resistance genes, which are inactivated, for example as a result of insertion of a transposable genetic element, and then reactivated in plant cells to result in necrosis and/or a plant defence response, leading to increased pathogen resistance. Cells, plants and other compositions of matter are provided comprising various combinations of genes involved in this system.
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METHOD OF INTRODUCING PATHOGEN RESISTANCE IN PLANTS

The present invention relates to a method of introducing pathogen resistance in plants, particularly broad spectrum pathogen resistance, and plants which may be obtained by said method and which show resistance to at least one but preferably more than one pathogen.

Plants are constantly challenged by potentially pathogenic microorganisms. Crop plants are particularly vulnerable, because they are usually grown as genetically uniform monocultures; when disease strikes, losses can be severe. However, most plants are resistant to most plant pathogens. To defend themselves, plants have evolved an array of both preexisting and inducible defences which include barriers to pathogen entry such as thickened or chemically crosslinked cell wall components or toxic chemicals derived from complex plant biosynthetic pathways. Pathogens must specialize to circumvent the defence mechanisms of the host, especially those biotrophic pathogens that derive their nutrition from an intimate association with living plant cells. If the pathogen can cause disease, the interaction is said to be compatible, but if the plant is resistant, the interaction is said to be incompatible.

Induced resistance is strongly correlated with the hypersensitive response (HR), an induced response
associated with localized cell death at sites of attempted pathogen ingress. It is hypothesized that by HR the plant deprives the pathogen of living host cells but there is no certainty about whether localised cell death results from or induces plant defence mechanisms.

Many plant defence mechanisms are strongly induced in response to a challenge by an unsuccessful pathogen. Such an induction of enhanced resistance can be systemic (hereinafter referred to as systemic acquired resistance (SAR)) (Ross, 1961; Ryals et al., 1992). Acquired resistance can also be local (hereinafter referred to as LAR) (Ryals et al., 1992). Acquired resistance has been extensively researched and various facts have been established. For example, biotic stimuli are required to provoke the HR resulting in areas of dead plant cells on the leaf. Cell death resulting from wounding or other abiotic stresses will not suffice. (Ryals et al., 1992; Enyedi et al., 1992). In addition, SAR is correlated with the induction of a large array of pathogenesis-related (PR) proteins, some of which have demonstrated anti-fungal activity (Ward et al., 1991).

A variety of examples of SAR have been studied and include challenging of tobacco carrying the N gene for resistance to tobacco mosaic virus (TMV) with TMV (Ross, 1961) and challenging cucumber seedlings with tobacco necrosis virus or Colletotrichum lagenarium.
Results show that a challenge with one pathogen leads to enhanced resistance to a wide variety of other pathogens (Ryals et al., 1992).

SAR has also been correlated with increased levels of salicylic acid in plants which have been challenged by pathogens (Malamy et al., 1990; Metraux et al., 1990) which has been confirmed by studies that show that a supply of exogenous salicylic acid to unchallenged plants can result in SAR (Ward et al., 1991; Hennig et al., 1993). Transgenic plants designed so that salicylic acid accumulation is prevented by expression of a salicylate hydroxylase gene show reduced SAR compared to non-transgenic plants where salicylic acid accumulation is not prevented (Gaffney et al., 1993). SAR can also be induced by many chemicals manufactured by Ciba-Geigy such as 2,6-dichloroisonicotinic acid (INA) (Uknes et al., 1992).

SAR is an attractive method by which broad spectrum disease control can be achieved. However, two major drawbacks hinder its commercial exploitation: SAR is not a heritable trait and so the phenomenon has to be successfully induced into every plant in the crop stand; to be effective throughout the crop's life, the SAR phenotype has to be re-boosted at regular intervals.

Although the mechanisms causing SAR are not fully understood, it is believed that when a plant is
challenged by a pathogen to which it is resistant, it undergoes an HR at the site of attempted ingress of the incompatible pathogen. The induced HR leads to a systemic enhancement and acquisition of plant resistance to virulent pathogens that would normally cause disease in the unchallenged plant.

It has long been known that HR-associated disease resistance is often (though not exclusively) specified by dominant genes (R genes). Flor showed that when pathogens mutate to overcome such R genes, these mutations are recessive. Flor concluded that for an R gene to function, there must also be a corresponding gene in the pathogen, an "avirulence gene" (Avr gene). To become virulent, pathogens must thus stop making a product that activates R gene-dependent defence mechanisms (Flor, 1971). A broadly accepted working hypothesis, often termed the elicitor/receptor model, is that R genes encode products that enable plants to detect the presence of pathogens, provided said pathogens carry the corresponding AVR gene (Gabriel and Rolfe, 1990). This recognition is then transduced into the activation of a defence response.

The mlo allele of the Mlo gene of barley is the one example of a recessive disease resistance gene currently widely used in plant breeding. Lines that are homozygous for the recessive allele of this gene activate the defence response (comprising formation of
cell wall appositions) even in the absence of the pathogen (Wolter et al, 1993). Thus the mlo mutation causes a defence mimic phenotype, also known as a necrotic or disease lesion mimic phenotype, and appears to deregulate the defence response, so that it is activated precociously, or is regulated on more of a "hair trigger". A number of examples of such disease lesion mimic mutants exist in maize (Johal et al, 1994, Pryor, 1987, Walbot, 1983). Recently, such mutants have been sought in Arabidopsis. The characterization of one such mutant, acd1, has been reported (Greenberg and Ausubel, 1993). Further mutants of this type have been reported at scientific meetings (the Arabidopsis acd2 mutation by F.M. Ausubel at a meeting at Rutgers University, New Jersey, USA, April 1993; Arabidopsis mutations now known as lsd (for lesions simulating defence response) mutations by R. Dietrich and J. Dangl at the ARAPANET ((Arabidopsis Pathology Network) workshop in Wye College, Kent, UK in April 1993).

Manuscripts describing the acd2 and lsd mutations are Dietrich et al. and Greenberg et al. (1994). It is highly likely that the recessive mutations identified in such mutant screens that leave the defence response more constitutively on, or more rapidly activated, or less easily inactivated, are in genes that normally dampen down the defence response to prevent it becoming so severe that it is deleterious to the plant.
Conceivably, such gene could be cloned, expressed in an antisense or sense configuration to reduce expression of the corresponding gene (Hamilton, 1990, Napoli et al, 1989).

5 Pathogen avirulence genes are still poorly understood. Several bacterial Avr genes encode hydrophilic proteins with no homology to other classes of protein, while others carry repeating units whose number can be modified to change the range of plants on which they exhibit avirulence (Keen, 1992; Long and Staskawicz, 1993). Additional bacterial genes (hrp genes) are required for bacterial Avr genes to induce HR, and also for pathogenicity (Keen, 1992; Long and Staskawicz, 1993). It is not clear why pathogens make products that enable the plant to detect them. It is widely believed that certain easily discarded Avr genes contribute to but are not required for pathogenicity, whereas other Avr genes are less dispensable (Keen, 1992; Long and Staskawicz, 1993). The characterization of two fungal avirulence genes, Avr 4 and Avr 9 (De Wit et al., 1992; Joosten et al., 1994), has also been reported. Research is also being undertaken to clone rice blast avirulence genes from the causal organism Magnaporthe grisea and the avirulence genes (NIP proteins) of the barley pathogen Rhynchosporium secalis. Two viral avirulence genes have also previously been cloned. Culver and Dawson, 1991, have
shown that tobacco mosaic virus coat protein is the avirulence determinant for the \( N' \) gene product. In addition, the potato virus X coat protein appears to be the avirulence determinant for the \( R_x \) and \( N_x \) genes (Kavanagh et al., 1992; Santa-Cruz et al., 1993; Köhm et al., 1993; Goulden et al., 1993).

Recently the map based cloning of the tomato \( Pto \) gene that confers "gene-for-gene" resistance to the bacterial speck pathogen \( Pseudomonas syringae pv \) tomato (\( Pst \)) has been reported (Martin et al., 1993). It has also been recently reported that the \( Arabidopsis \) \( Rps2 \) gene (which confers \( Pseudomonas syringae \) resistance) and the tobacco \( N \) gene (which confers virus resistance) have been cloned (Keystone Symposium, January 1994). Even more recently, the \( Rps2 \) and features of the \( Cf-9 \) gene sequences have been revealed at the 13th Annual Symposium in Columbia, Missouri, April 13th-16th 1994, on the Biology of Communication in Plants.


The technology for gene isolation based primarily on genetic criteria has improved dramatically in recent years, and many workers are currently attempting to clone a variety of \( R \) genes. Targets include (amongst others) rust resistance genes in maize, \( Antirrhinum \) and flax (by transposon tagging); downy mildew resistance
genes in lettuce and Arabidopsis (by map based cloning and T-DNA tagging); Cladosporium fulvum (Cf) resistance genes in tomato (by tagging, map based cloning and affinity labelling with avirulence gene products); virus resistance genes in tomato and tobacco (by map based cloning and tagging); nematode resistance genes in tomato (by map based cloning); and genes for resistance to bacterial pathogens in Arabidopsis and tomato (by map based cloning).

Tomato (Lycopersicon esculentum) is susceptible to disease caused by the leaf mould fungal pathogen Cladosporium fulvum. According to De Wit, 1992, the Avr9 gene of C. fulvum, which confers avirulence on C. fulvum races that attempt to attack tomato varieties that carry the Cf-9 gene, encodes a secreted cysteine-rich peptide with a final processed size of 28 amino acids. However, its role in compatible interactions is not clear. The R genes (Cf-genes) that act against C. fulvum have been identified and bred into cultivated varieties, often from related species of tomato (Dickinson et al., 1993; Jones et al., 1993).

It has been shown that C. fulvum contains Avr genes that confer recognition by plants which contain the Cf-genes, leading to activation of host defence mechanisms to attack the disease (incompatibility). The Avr4 and Avr9 genes encode small peptides that are secreted by the pathogen into the intercellular spaces
of infected leaves, from which they can be extracted. This has enabled the purification and sequencing of these peptides and the isolation of the genes that encode them (De Wit, 1992; Joosten et al., 1994).

Experiments have shown that when the Avr9 gene is transformed into a race of pathogen that lacks Avr9, then the race of pathogen becomes avirulent on plants which are carrying the Cf-9 gene. In addition, it has been shown that disruption of the Avr9 gene in a pathogen race which is avirulent on plants carrying Cf-9 gene confers compatibility on the Cf-9 containing plants (Van Den Ackerveken et al., 1992, Marmeisse et al., 1993).

In addition, De Wit and colleagues have further shown that the secreted peptide encoded by the Avr9 gene can be injected into Cf-9 containing tomato leaves to elicit a necrotic response in the injected area. The necrotic response is consistent with local and vigorous activation of a defence response (De Wit, 1992; WO 91/15585). International Patent Application No. PCT/GB94/02812 describes the transgenic expression of the Avr9 gene using the strong cauliflower mosaic virus 35S plant promoter to cause lethality in Cf-9 plants. This transgenic expression has been used to select mutants in which the Cf-9 gene has been inactivated by transposon insertion in order to isolate the Cf-9 gene and perform DNA sequence analysis of this
Various pathogen races that overcome these Cf-genes have emerged and are named after the Cf-gene which they can overcome. For example, C. fulvum race 4 can overcome Cf-4; C. fulvum race 5 can overcome Cf-5 and C. fulvum race 2.4.5.9 can overcome Cf-2, Cf-4, Cf-5 and Cf-9.

WO 91/15585 describes a hypothetical method whereby if a Cf-9 gene and/or an Avr9 gene were expressed under the control of a promoter that is induced by a broad range of pathogens, then a general defence response could be induced. However, there is a lack of enabling disclosure regarding which polynucleotide sequences could be used either as the resistance gene or as an actual promoter which would be suitably affected by a broad range of pathogens. A further problem with this proposed method is that necrosis induced by the Cf-9 and Avr9 gene combination could lead to further induction of Avr9 and/or Cf-9 leading to spreading of the necrosis and severe reduction in the yield of the plant. This problem may arise since promoters such as promoters for plant defence genes and other genes involved in the defence response such as PR genes (pathogenesis related genes), are induced in both a compatible and an incompatible interaction. Therefore, even if a promoter exists which is effectively induced by a broad range of
pathogens, the method would not be viable unless the promoter is only induced by the appearance of a compatible pathogen. If the defence response provides further induction of the promoter the plant might experience spreading necrosis.

The present invention has resulted from experiments involving transposon tagging of resistance genes, the first one being Cf-9. Numerous alleles of the Cf-9 gene (Cf-9*Ds) were isolated that had been inactivated by the maize element Dissociation (Ds). These inactive Cf-9*Ds genes did not give rise to a constitutive and lethal activation of defence mechanisms in response to constitutively expressed Avr9 transgene (35S:SP:Avr9). On backcrossing plants that carried the Cf-9*Ds and 35S:SP:Avr9 genes to tomato plants carrying an Activator (Ac) transposase gene (sAc) in the homozygous state but lacking Cf-9, a quarter of the resultant progeny carried sAc, 35S:SP:Avr9 and Cf-9*Ds. These plants showed somatic excision of Ds from the Cf-9*Ds gene, somatically restoring Cf-9 function and giving rise to localised activation in cells of plant defence responses due to recognition of the constitutively expressed Avr-9 peptide. These cells died and gave rise to small necrotic sectors, the plants phenotypically showing variegation for a defence-related necrosis, similar to somatic flecks of necrosis that are associated with the
induction of SAR in plants challenged with necrotising pathogens. Further work showed that plants that variegate for somatic sectors of plant defence response in this way have increased resistance to a range of pathogens.

Thus, a first aspect of the present invention relates to a method of providing pathogen resistance, in particular broad spectrum pathogen resistance, in plants by induction of variegation in which genes are expressed or suppressed resulting in the activation of necrosis. A method according to the present invention comprises: (i) inactivating a nucleotide sequence which contributes to plant cell necrosis or inactivating one or more nucleotide sequences forming part of a combination of nucleotide sequences which contribute to plant cell necrosis; (ii) introducing said nucleotide sequence or sequences into the genome of a plant; and (iii) restoring said nucleotide sequence or sequences to a functional form to yield a level of necrosis resulting in pathogen resistance. The plant cell necrosis is preferably defence-related plant cell necrosis.

A second aspect of the present invention relates to a method of providing pathogen resistance in plants by induction of variegation in which genes are expressed or suppressed resulting in the activation of a plant defence response which comprises: (i)
inactivating a nucleotide sequence which contributes to the plant defence response or inactivating one or more nucleotide sequences forming part of a combination of nucleotide sequences which contribute to the plant defence response; (ii) introducing said nucleotide sequence or sequences into the genome of a plant; and (iii) restoring said inactivated nucleotide sequence or sequences to a functional form to result in pathogen resistance.

The variegation will generally be for somatic sectors. Pathogen resistance will generally be increased compared with wild-type.

The nucleotide sequence or sequences comprise one or more genes. The plant defence response and/or plant cell necrosis occurs on expression of the gene or genes. The defence response and/or plant cell necrosis can be conditional or unconditional on the expression of one or more interacting genes. A substance or a combination of substances may result in increased pathogen resistance. Examples are discussed further below.

For example, the nucleotide sequence or sequences may comprise a gene encoding either a substance which leads to necrosis, e.g. through activation of the plant defence response, or a substance which leads to a plant defence response with no sign of necrosis. For example, the sequence or sequences may comprise a plant
pathogen resistance gene (R), an avirulence gene (Avr)
or other elicitor or ligand gene (L) of an R gene, or
both and R gene and an L gene.

The inactivation of the nucleotide sequence or
sequences which contribute to the plant defence
response and/or plant cell necrosis is preferably
effected by insertion of a transposable genetic element
into the nucleotide sequence or one or more of the
nucleotide sequences forming a combination of
nucleotide sequences. The transposable genetic element
is preferably a transposon or a nucleotide sequence
flanked by specific nucleotide sequences so that
transposon excision gives rise to activation of the
plant defence response and/or necrosis. Thus,

insertion of a genetic lesion into the nucleotide
sequence disrupts the gene to prevent expression of a
product able to function in contributing to the plant
defence response and/or plant cell necrosis. In the
absence of the lesion, e.g. following excision of a
transposable element such as a transposon, the gene may
be expressed to produce a functional product, i.e. gene
function is restored. The lesion may be inserted into
the part of the gene coding for the expression product,
or may be in a regulatory sequence such as a promoter
required for expression of the product.

In this form of the invention, re-activation
within the plant is preferably carried out by
restoration of the inactivated nucleotide sequence or sequences resulting in activation of a plant defence response and/or necrosis. Such restoration may be caused or allowed by culturing of the plant. Where the nucleotide sequence is inactivated by virtue of insertion of a transposable element therein, the plant genome should contain at least one nucleotide sequence coding for a corresponding transposon activation system (for example, comprising a transposase).

Alternatively, the inactive form could be flanked by recombinase recognition sequences that are acted on by a site specific recombination system (comprising a specific recombinase) so that recombination activates the inactive form of the gene. Hence, when the inactivated nucleotide sequence or sequences are introduced into the plant genome somatic excision of the transposon or recombination of the nucleotide sequence occurs in some cells leading to activation of the plant defence response and/or necrosis in specific clones of cells.

The number of cells in which restoration of function occurs may vary. As discussed further below, certain measures are available for optimising the system, e.g. by controlling the frequency of spontaneous excision of a transposable element which is caused or allowed upon cultivation of a plant with the requisite nucleotide sequence or sequences within its
genome.

The present invention further provides transgenic plants having increased pathogen resistance obtainable by the method of the present invention, and any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. Derivatives of plants are also provided by the present invention. A derivative is any functional unit derived therefrom howsoever achieved (e.g. functional allele of gene made by mutagenesis, recombinant DNA, synthesis, or plant which could not have been produced without the use or manufacture of the plant from which it is derived.)

Transgenic plants in accordance with the present invention may demonstrate increased pathogen resistance since the induced plant defence response and/or necrosis of plant cells may cause other cells, such as adjacent cells, to acquire pathogen resistance. The activation of, for example, a plant resistance gene in a plant cell is inherited by the progeny and descendants of that cell. The expression of this plant resistance gene leads to initiation of the defence response in cells which may eventually lead to the
death of the participating plant cells resulting in an area of plant cell necrosis. So, plants may have variegation for small somatic sectors in which defence-related plant cell necrosis is activated. This response may induce pathogen resistance in other cells. In an alternative, operating on the same general principle, the expression of one or more plant pathogen resistance gene may either lead to initiation of the defence response only resulting in variegation for small somatic sectors in which the plant defence response is activated or of plant cell necrosis which is not related to the plant defence response resulting in variegation for small somatic sectors in which plant cell necrosis is activated.

Hence, the plant may acquire resistance to a broad range of pathogens and not only to the pathogen associated with the gene or genes contributing to necrosis, for example, *C. fulvum* in the case of the *Cf-9/Avr* gene combination. For example, a transgenic tomato plant according to the present invention may demonstrate resistance against a broad range of pathogens such as one or more bacterial plant pathogens (for example, *Xanthomonas campestris*, *Pseudomonas syringae*), fungal plant pathogens (for example, *Phytophthora infestans*, *Fusarium oxysporum*, *Botrytis cinerea*, *Verticillium dahliae*, *Altenaria solani*, *Rhizoctonia solani*) and viral pathogens (for example,
TMV, PVX, PVY, TSWV). Similarly, other transgenic plants such as transgenic tobacco, Arabidopsis and potato plants may display resistance to a large number of major diseases of important crop species such as, Peronospora, Phytophthora, Puccinia, Erysiphe and Botrytis.

Thus, according to a further aspect of the invention there is provided a plant, or any part thereof, which is phenotypically variegated, with clones of cells expressing a first phenotype and other cells expressing a second phenotype which is increased pathogen resistance compared with wild-type. The first phenotype is preferably necrosis and/or a plant defence response phenotype. As discussed, plants variegated by somatic sector for such a phenotype may have enhanced pathogen resistance as a result of a second phenotype in cells, which may be adjacent to the cells with the first phenotype which are necrotic and/or in which a plant defence response is activated. The phenotypic variegation is likely to result from expression in cells with the first phenotype of a gene or gene, or nucleic acid comprising a gene or genes, which contributes to such phenotype, whereas other cells without such phenotype lack such gene expression. As discussed herein, this may result from reactivation of a previously inactivated gene, such as a resistance gene, for example by random excision of a transposable
element such as a transposon.

In a further aspect, the present invention provides a host cell, such as a plant or microbial cell, or a plant comprising at least one such cell, containing (i) nucleic acid encoding one or more nucleotide sequences which cause or contribute to the plant defence response and/or cell necrosis, at least one of the nucleotide sequences being reversibly inactivated, for example by insertion of a transposable element such as a transposon, and (ii) nucleic acid encoding a molecule able to reverse the inactivation, such as, in the case of a transposon, a transposase. Thus, the cell may comprise a plant resistance gene or other gene involved in the plant defence response or able to kill a cell when expressed therein (either alone or in combination with one or more sequences, for example in the case of an R gene the corresponding elicitor), the gene being inactivated by insertion therein of a transposon, and the cell further comprising a gene encoding a transposase.

In an exemplary embodiment, the genome of the cell comprises the gene Cf-9, or a mutant, derivative, variant or allele thereof which retains Cf-9 function, inactivated by insertion therein of a transposon, the genome also comprising the Avr-9 gene, or a mutant, derivative, variant or allele thereof which retains Avr-9 function, and a gene encoding a transposase able
to excise the transposon from the Cf-9 gene or functional equivalent. Other resistance genes may be employed, as may genes which do not require the presence of an elicitor molecule to cause cell necrosis, as discussed further elsewhere herein.

The cell may comprise the nucleic acid encoding the various genes by virtue of introduction into the cell or an ancestor thereof of the nucleic acid, e.g. by transformation, using any suitable technique available to those skilled in the art. Furthermore, plants which comprise such cells, and seed therefore, may be produced by crossing suitable parents to create a hybrid whose genome contains the required nucleic acid, in accordance with any available plant breeding technique. For example, a parent strain comprising within its genome a plant resistance gene containing a transposon or other inactivating lesion may be crossed with a second strain comprising within its genome a gene encoding the elicitor molecule for the plant resistance gene and a suitable transposase for excision of the transposon. At least a proportion of the hybrid progeny of the parents, i.e. seed or plants grown therefrom, will comprise the required nucleic acid for activation in the plant of, in this example, the plant resistance gene and, following interaction with the elicitor, the plant defence response and/or plant cell necrosis.
Plants according to this aspect of the present invention will be variegated genetically. Clones of cells will have one or more nucleotide sequences which cause or contribute to the plant defence response and/or cell necrosis reactivated by removal of the inactivating lesion such as a transposon, so that a first phenotype such as necrosis is shown, while in other cells the sequence or sequences will remain inactivated so these cells will not show the first phenotype.

Within the cell or cells, the nucleic acid may be incorporated within the chromosome. A gene stably incorporated into the genome of a plant is passed from generation to generation to descendants of the plant, so such descendants should show the desired phenotypic variegation and so may have enhanced pathogen resistance.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on.

A further aspect of the present invention provides a method of making such a cell involving introduction of nucleic acid (e.g. a vector) comprising
(i) nucleic acid encoding one or more nucleotide sequences which cause or contribute to the plant defence response and/or cell necrosis, at least one of the nucleotide sequences being reversibly inactivated, for example by insertion of a transposable element such as a transposon, and/or (ii) nucleic acid encoding a molecule able to reverse the inactivation, such as, in the case of a transposon, a transposase into a plant cell. Introduction of nucleic acid (i) may be accompanied, preceded or followed by introduction of nucleic acid (ii). Such introduction may be followed by recombination between the nucleic acid and the plant cell genome to introduce the sequence of nucleotides into the genome. Descendants of cells into which nucleic acid has been introduced are included within the scope of the present invention.

The level of the plant defence response and/or plant cell necrosis in the small somatic sectors should be sufficient to result in the induction of acquired resistance or the induction of other defence mechanisms. Since this method leads to activation of acquired resistance but is inherited it is referred to as Genetic Acquired Resistance (GAR). Hence, any system which gives rise to a variegation leading to GAR is applicable to the present invention.

Methods and plants etc. according to the present invention are particularly beneficial since the
nucleotide sequence or sequences which contribute to the plant defence response and/or plant cell necrosis, for example the avirulence and plant resistance genes, may be under control of any suitable promoter, such as a constitutive promoter or, in the case of R genes, their own endogenous promoter, or a cell type specific promoter. Furthermore, the restoration of the nucleotide sequence or sequences, for example by the somatic excision of a transposon, gives rise to recurrent and widespread induction of the plant defence response in many small clones of cells throughout the plant, irrespective of whether or not there has been a challenge by pathogen. The resistance conferred on the plant is therefore constitutive and broad.

The present invention may be used for many applications and is suitable for deployment in F1 hybrid seed production system. In such a system, one of the parents should be homozygous, for example, for the transposase or recombinase gene. In addition, in a system where two components are required for inducing the necrosis such as in the Avr9/Cf-9 gene combination for example, this parent should also be homozygous for the constitutively expressed genes. The other parent should be homozygous for the gene that encodes the non-autonomous inactivation system, such as the transposon or recombinase-recognition sequences. After making a cross between parents of this genetic constitution, on
somatic excision or recombination, the function of the
gene or genes which give rise to the defence response
and/or plant cell necrosis is restored in somatic
sectors in the resulting progeny.

It will be clear to the person skilled in the art
that any gene or combination of genes which contributes
to variegation for the plant defence response and/or
plant cell necrosis may be used in the method of the
present invention. Furthermore, any system which gives
rise to inactivation of the nucleotide sequence or
sequences and subsequent restoration of functional
sequence or sequences may be used.

The present invention also provides in further
aspects various compositions of matter comprising
combinations of nucleotide sequences encoding various
substances employed herein. Such combinations of
nucleotide sequences which may be introduced into cells
in accordance with the present invention follow:

(X): represents a nucleotide sequence with one
or more genes of type X

(XY): represents a nucleotide sequence with one
or more genes of type X and one ore more
genes of type Y etc.

R: receptor gene

L: ligand gene (capable of interacting with the R
gene)
I: genetic insert
A: activator of transposition of genetic insert.

R may encode a substance whose presence in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, with I being a genetic insert able to inactivate R and A encoding a substance able to reactivate R inactivated by I:

(1) Any combination of:
1. (R), (I) and (A);
2. (R) and (IA);
3. (I) and (AR); or
4. (A) and (RI);
5. (RIA).

Alternatively, R and L may encode substances whose presence together in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I being a genetic insert able to inactivate R and/or L and A encoding a substance able to reactivate R and/or L inactivated by I:

(2) Any combination of:
1. (R), (L), (I) and (A);
2. (R), (LI) and (A);
3. (R), (LA) and (I);
4. (R), (IA) and (L);
5. (L), (IR) and (A).
6. (L), (AR) and (I)
7. (I), (LR) and (A)
8. (R) and (LIA)
9. (L) and (IAR)
10. (I), and (ARL); or
11. (A) and (RLI);
12. (RLIA)

If genetic insert (I) is coupled with either the R or the L gene, the number of possible combinations will then be

(1): (RI) and (A); or
   (RIA)

(2): (RI)(L) and (A)
   (R),(LI) and (A)
   (RI) and (LA)
   (RA).and (LI)
   (RLIA)

Also provided by the present invention is a method of producing a plant, or a part, propagule, derivative or descendant thereof, containing nucleic acid comprising a nucleotide sequence or nucleotide sequences encoding R, I and A, wherein R encodes a substance whose presence in a plant results in a plant
defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and A encodes a substance able to reactivate R inactivated by I, comprising crossing plant lines whose genomes comprise any of R, I, A and combinations thereof, to produce the plant or an ancestor thereof.

A further aspect provides a method of producing a plant, or a part, propagule, derivative or descendant thereof, containing nucleic acid comprising a nucleotide sequence or nucleotide sequences encoding R, L, I and A, wherein R and L encode substances whose presence together in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and/or L and A encodes a substance able to reactivate R and/or L inactivated by I, comprising crossing plant lines whose genomes comprise any of R, L, I, A and combinations thereof, to produce the plant or an ancestor thereof.

Said plant lines may contain nucleic acid comprising any of R, L, I, A and combinations thereof as a result of transformation of cells of the plant or an ancestor thereof.

Herein, unless context demands otherwise, a "receptor" is a product encoded by a gene capable of interacting with another product, the ligand.

Various embodiments of the present invention are
now described in more detail below, by way of example and not limitation.

*Nucleotide Sequence or Sequences contributing to the Plant Defence Response and/or Necrosis*

The nucleotide sequence or combination of nucleotide sequences in which at least one of the sequences is inactivated are numerous and may include an engineered allele of a ubiquitin conjugating enzyme (Becker et al., 1993), the CaMV gene VI protein (Takashashi et al., 1989), a viral coat protein in the presence of the appropriate viral resistance gene, for example Tobacco Mosaic Virus Elicitor Coat Protein and the gene N' (Culver and Dawson, 1991), a bacterial harpin protein (Wei et al., 1992; He et al., 1993), the gene N (see e.g. Whitham et al. (1994) and a ToMV-Ob gene cloned by Padgett and Beachy (1993), the potato virus X coat protein and its avirulence determinant, (Kavanagh et al., 1992; Santa-Cruz et al., 1993; Köhm et al., 1993; Goulden et al., 1993), Pto and avrPto (see e.g. Rommens et al., 1995), RPS2 of Arabidopsis thaliana and the avirulence gene avrRpt2 (Bent et al., Mindrinos et al.), and genes of Arabidopsis such as those identified by Greenberg et al. (1994), Dietrich et al., (1994) and Bowling et al., (1994).

Genes coding for substances leading to rapid cell death, such as BARNASE (Mariani et al., 1990) or
diphtheria toxin (Thorsness et al., 1993) may be usable to induce the changes that lead to GAR even though cell death in these latter examples is not caused by activation of the defence response. It is widely believed amongst researchers in this field that cell death arises from local induction of the defence response and that this cell death can activate adjacent cells to give rise to the defence response. However, the precise cause and effect relationship between these events is not clear at the present time. It is also not clear whether the defence response in plants is necessarily coupled to necrosis. Hence, cells may respond to for example the BARNASE-induced death of adjacent cells by activating a wound-inducible defence response, such as that leading to the activation of protease inhibitors or alkaloid biosynthesis (Ryan 1990). Other genes which may be employed in this way include a proton pump such as a bacterial proton pump like the one expressed by Mittler et al (1995) in transgenic tobacco plants.

A preferred example of the present invention is the use of the Cf-9/Avr9 gene system. This can involve the matching of a transposon inactivated allele of the Cf-9 gene to constitutive expression of the Avr9 gene. This system can be replaced by similar combinations of related genes for example the Avr4 and Cf-4 gene, sequence provided herein (cloning of Cf-4 is described
in a co-pending GB application filed simultaneously
with the present application); the Avr2 and the Cf-2
gene, sequence provided herein (cloning of Cf-2 is
described in GB 9506658.5, priority from which is
claimed herein); the Avr5 and the Cf-5 gene, or by
cloning resistance genes and corresponding avirulence
genes from other systems, such as RPP5, sequence
provided herein (cloning of RPP5 is described in GB
9507232.8, priority from which is claimed herein). It
certain cases it may be possible to provoke a suitable
response in plant cells expressing an R gene in the
absence of corresponding Avr, for instance by
overexpression.

It should also be noted that complete Avr or
other elicitor gene may not be required. Instead a
fragment may be employed, representing a part of the
elicitor molecule which interacts to provoke a plant
defence response and/or plant cell necrosis.

It is possible that the nucleotide sequence
comprises the inactivated R gene, the inactivated Avr
gene or both, or comprises both the R and Avr gene
wherein one of the genes is inactivated. Depending of
the genes used, the plant defence response and/or plant
cell necrosis may be dependent on the expression of
both genes and so one example would be that the R gene
could be constitutively expressed and the Avr gene
could exhibit somatic variegation for expression due to
somatic excision and restoration of Avr9 gene
expression, or vice versa.

Nucleotide sequences employed in the present
invention may encode a wild-type sequence (e.g. gene)
selected from those available, or a mutant, derivative,
variant or allele, by way of insertion, addition,
deletion or substitution of one or more nucleotides, of
such a sequence. An alteration to or difference in a
nucleotide sequence may or may not be reflected in a
change in encoded amino acid sequence, depending on the
degeneracy of the genetic code. Preferred mutants,
derivatives and alleles are those which retain a
functional characteristic of the protein encoded by the
wild-type gene, in the present context the ability to
contribute to a plant defence response and/or plant
cell necrosis. Of course, changes to the nucleic acid
which make no difference to the encoded amino acid
sequence are included.

Similarly, homologues of the various genes whose
use is disclosed herein from other species or races may
be employed, as may mutants, variants and derivatives
of such homologues.

Inactivation and Reactivation of the nucleotide
Sequence or Sequences Contributing to the Plant Defence
Response and/or Necrosis

A method according to the present invention may
employ any of a variety of transposon systems known to the skilled person, including the maize Activator/Dissociation (hereinafter referred to Ac/Ds system) (Fedoroff, 1989); the maize Enhancer/Suppressor mutator (En/Spm) system (Fedoroff, 1989); and the Antirrhinum Tam1 and Tam3 systems (Coen et al., 1989).

In addition, any modified recombination systems which are engineered to yield the appropriate results may be employed, such as, the bacterial Cre-Loxp (Odell et al., 1990) or the "FLP/FRT" system (Lloyd and Davis, 1994).

It will be apparent to the skilled person that the particular choice of transposon, recombination or other system used to inactivate the nucleotide sequence or sequences which encode substances leading to the plant defence response and/or plant cell necrosis is not essential to or a limitation of the present invention.

In some systems, a transposon or recombination system might be so active that an unacceptable level of necrosis is seen. If encountered, this may be overcome by engineering alleles of the transposon or recombinase recognition sequence in which the frequency at which activated nucleotide sequences arise is reduced, such as with Ac(C1a) (Keller et al., 1993). Alternatively, chemical or site-directed mutagenesis may be used to recover alleles of the necrosis-inducing genes which are less active and therefore result in less severe
levels of plant cell necrosis (Hammond-Kosack et al., 1994).

In other systems, transposition or recombination may be inefficient resulting in too few activated nucleotide sequences leading to an insufficient level of plant cell necrosis. This may be overcome by constructing suitable promoter fusions to the transposase or recombinase gene in the plant gene (Swinburne et al., 1992) to increase the frequency of excision or recombination to efficient levels. The most suitable promoter might give rise predominantly to late small sectors of necrosis during organ development rather than early large sectors.

Many other variations are possible as mechanisms for activating the defence response and/or necrosis after transposon excision or recombination. A form of the Cf-9 gene may be constructed so that it activates the defence response even in the absence of its ligand. For example, the Drosophila receptor sevenless (involved in eye development) can be mutated so that it is activated in the absence of its ligand (Basler et al, 1991). For example, high level expression of a disease resistance gene, or expression of a disease resistance gene in another species, may lead to activation of the defence response and/or necrosis even in the absence of an avirulence product. Bonneus, et al (1995). In an alternative, the original disease
resistance gene may be mutated so that it binds to a defined chemical such as an agrichemical and this chemical activates Cf-9 to initiate the defence response and/or necrosis. Hence, genotypic variegation for excision activating the gene may occur, without initiation of the somatic necrotic reaction due to the defence response. The defence response would be initiated when the agrichemical is applied and recognised by the resistance gene triggering the same reaction as if the avirulence gene product were present.

Introducing the Nucleotide Sequence or Sequences which Contribute to Variegation for the Plant Defence Response and/or Necrosis into the Plant Genome

The inactivated nucleotide sequence, or combination of nucleotide sequences at least one of which is inactivated, codes for a substance or substances which when expressed in the plant activates the defence response and/or leads to plant cell necrosis resulting in broad spectrum pathogen resistance.

The nucleic acid may be in the form of a recombinant vector, for example a plasmid or agrobacterium binary vector (Van den Elzen et al., 1985). The nucleic acid may be under the control of an appropriate promoter and regulatory elements for
expression in a plant cell. In the case of genomic DNA, this may contain its own promoter and regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter and regulatory elements for expression in the host cell.

Those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference.

When introducing a chosen gene or gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The
nucleic acid to be inserted may be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell.

Once the construct is within the cell membrane, integration into the endogenous chromosomal material may or may not occur according to different embodiments of the invention. In a preferred embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. Finally, as far as plants are concerned the target cell type should be such that cells can be regenerated into whole plants.

Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Although Agrobacterium has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828), microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, e.g. bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention.

Selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones
and glyphosate (Herrera-Estrella et al, 1983; van den Elzen et al, 1985).

The present invention is particularly beneficial for use in crop and amenity plants. Examples of suitable plants include tobacco, potato, pepper, cucurbits, carrot, vegetable brassicas, lettuce, strawberry, oil seed brassicas, sugar beet, wheat, barley, maize, rice, soybeans, peas, sunflower, carnation, chrysanthemum, other ornamental plants, turf grass, poplar, eucalyptus and pine.

Still further details of embodiments of the present invention are described in the following non-limiting examples, with reference to the accompanying drawings. In the drawings:

Figure 1 schematically depicts the Cf-9 gene, showing tagged alleles. X marks a probable promoter.

Figure 2 illustrates genetic acquired resistance to C. fulvum induced following necrotic sector formation caused by the excision of a Ds element from the Cf-9 resistance gene in an Avr9 expressing tomato plant. The number of C. fulvum pustules per leaf is indicated, 14 days after inoculation.

Figure 3 illustrates genetic acquired resistance to Phytophthora infestans (late blight of tomato and potato). GAR+ and GAR- plants from Cf-9*Ds, mutant
lines M31 and M50 and Cf0 plants spray inoculated with 10,000 sporangiospores/mL. In panel A the appearance of leaves from the mutant 50 experiment 7 days after inoculation is shown. In panel B the rate of leaf abscission (in days after inoculation) in the various genotypes inoculated is given.

Figure 4 illustrates genetic acquired resistance to Phytophthora infestans (late blight of tomato and potato). GAR+ and GAR- plants from Cf-9*Ds, mutant lines M31 and M50 and Cf0 plants were spray inoculated with 100 sporangiospores/mL. In panel A the appearance of leaves from the mutant 50 (GAR+ - right-hand) experiment 7 days after inoculation is shown, compared with GAR- (left-hand). In panel B the rate of sporulating lesion formation on the various plant genotypes inoculated is given, with the mean number of sporulating lesions/leaflet given at 5, 7, 10, 13 and 16 days after inoculation.

Figure 5 shows genetic acquired resistance to Oidium lycopersici (powdery mildew disease). GAR+ and GAR- plants from Cf-9*Ds, mutant lines M31 and M50 and Cf0 plants were painted with equivalent numbers of spores. In panel A the appearance of leaves 14 days after inoculation is shown, GAR- on the left, GAR+ on the right. In B, the rate of chlorotic lesion (upper panel) and sporulating lesion (lower panel) formation on the various plant genotypes is given for Mutant 31:
mean number of lesions given at 7, 10, 14, 21, 24 and 30 days after inoculation. C shows equivalent results for Mutant 50.

Figure 6 shows the appearance of tomato fruits on GAR⁺ (sAc, Cf-9*Ds - right-hand) and GAR⁻ (sAc, Cf-9*Ds, Avr-9 - left-hand) plants from mutant line M23 at 2, 3, 4, 5, 6 and 7 weeks after flower pollination. Dark green sectors formed on the GAR⁺ but not GAR⁻ fruits by 5 weeks. These dark green sectors were not visible on the red fruit.

Figure 7 shows levels of defence-related gene expression in GAR⁺ and GAR⁻ plants from Cf-9*Ds mutant lines M23, M31 and M50 just prior to the pathogen inoculation experiments. Northern analysis shows in panel A the levels of a basic β-1,3 glucanase gene transcript and in panel B the levels of an anionic peroxidase gene transcript.

Figure 8 illustrates functional expression of the Cf-9 gene under the control of its own promoter in tobacco and potato. In panel A is shown a tobacco leaf that has been injected with intercellular fluid (IF) either containing the Avr9 peptide or lacking the Avr9 peptide. Avr9⁺ IF was obtained from transgenic tobacco or a compatible C. fulvum - tomato interaction involving race 5. Avr9⁻ IF was obtained from untransformed tobacco or a compatible C. fulvum - tomato interaction involving race 2,4,5,9. Grey
necrosis was visible 3-4 h after injection only in the leaf panels that had received the Avr+ IF. In panel B four separate potato leaves are shown that have each been injected with a single type of IF. Only the two leaves that received the Avr9+IF developed grey necrosis by 24 h.

Figure 9 shows development of the necrotic lethal phenotype in seedlings from the tobacco cross cv. Petite Havana 6201A (35S:SP:Avr9)homozygote x cos 34.1 (genomic Cf-9) heterozygote. A time course for the period 5-12 days after seed planting (dsp) is shown. 50% of the seedlings become chlorotic and die within 2 days of seed germination.

Figure 10 shows development of the necrotic lethal phenotype in seedlings from the Arabidopsis cross 6201B4 (35S:SP:Avr9)heterozygote x cos 138 (genomic Cf-9) heterozygote. Appearance of seedlings 19 days after the majority of seedlings had germinated. One seedling has died and another has necrotic cotyledons.

Figure 11 shows a single T-DNA construct systems to apply GAR to potato plants. The T-DNA contains a Cf-9 gene sequence under the control of its own promoter which has been inactivated by an autonomous Ac element that is only capable of a low level of excision, the Ac (Cla) element (Keller et al. 1993; Schofield et al. 1994) and the 35S:SP:Avr9 transgene.
Figure 12 shows a photograph of three leaves, two of which are diseased with C. fulvum and one which is expressing GAR and is resistant to the same inoculum of C. fulvum.

Figure 13 illustrates how GAR⁺ plants may be made by crossing stable lines (1) comprising a Cf-9 gene, inactivated by insertion of a Ds transposon, and an Avr-9 gene and (2) an Ac transposase gene, as described in Example 1.

Figure 14 illustrates basic simplified haploid crossing schemes to produce plants with increased disease resistance.

T: transgenic line
P: offspring of transgenic line

T₁/P₁: line comprising in its genome at least one of each of the four genes, R, L, I or A
T₁,²/P₁,²: line comprising in its genome at least one of each of two of the four genes R, L, I or A
T₃/P₃: line comprising in its genome at least one of each of the four genes R, L, I or A not present in T₁,²
T₃,₄/P₃,₄: line comprising in its genome at least one of two of the four genes R, L, I or A not present in T₁,²
T₁,₂,₃/P₁,₂,₃: line comprising in its genome at
least one of each of three of the
four genes R, L, I or A

T₄/P₄

line comprising in its genome at
least one of each of the four genes
R, L, I or A not present in T₁,₂,₃

SEQ ID NO. 1 shows the genomic DNA sequence of
the Cf-9 gene. Features: Nucleic acid sequence -
Translation start at nucleotide 898; translation stop
at nucleotide 3487; polyadenylation signal (AATAAA) at
nucleotide 3703-3708; polyadenylation site at
nucleotide 3823; a 115 bp intron in the 3’ non-coding
sequence from nucleotide 3507/9 to nucleotide 3622/4.
Predicted Protein Sequence - primary translation
product 863 amino acids; signal peptide sequence amino
acids 1-23; mature peptide amino acids 24-863.

SEQ ID NO. 2 shows Cf-9 protein amino acid
sequence.

SEQ ID NO. 3 shows the sequence of one of the Cf-
9 cDNA clones. Translation initiates at the ATG at
position +58. Cf-9 genomic sequence

SEQ ID NO. 4 shows the amino acid sequence and
DNA sequence of the preferred form of the chimaeric
Avr9 gene used as described herein.

SEQ ID NO. 5 shows the genomic DNA sequence of
the Cf-2.1 gene. Features: Nucleic acid sequence -
Translation start at nucleotide 1677; translation stop
at nucleotide 5012; no consensus polyadenylation
signal (AATAAA) exists in the characterised sequence
downstream of the translation stop. Predicted Protein
Sequence - primary translation product 1112 amino
acids; signal peptide sequence amino acids 1-26;
mature peptide amino acids 27-1112.

SEQ ID NO. 6 shows Cf-2 protein amino acid
sequence, designated Cf-2.1.

SEQ ID NO. 7 shows the amino acid sequence
encoded by the Cf-2.2 gene. Amino acids which differ
between the two Cf-2 genes are underlined.

SEQ ID NO. 8 shows the sequence of an almost full
length cDNA clone which corresponds to the Cf2-2 gene.

SEQ ID NO. 9 shows the genomic DNA sequence of
the RPP5 gene. Anticipated introns are shown in non-
capitalised letters. Features: Nucleic acid sequence -
Translation start at nucleotide 966; translation stop
at nucleotide 5512.

SEQ ID NO. 10 shows predicted RPP5 protein amino
acid sequence.

SEQ ID NO. 11 shows genomic DNA sequence of Cf-4.
Features of this sequence include: translation start
site at nucleotide 201, translation stop beginning at
nucleotide 2619, consensus polyadenylation sequence
beginning at nucleotide 2835, splice donor sequence in
3' untranslated sequence at 2641, splice acceptor
sequence ending at nucleotide 2755, proposed site of
polyadenylation at nucleotide 2955.

SEQ ID NO. 12 shows the predicted Cf-4 amino acid sequence. The predicted protein sequence is composed of a primary translation product of 806 amino acids, signal peptide sequence amino acids 1-23, mature peptide amino acids 24-806.

SEQ ID NO. 13 shows double-stranded nucleic acid and deduced amino acid sequence of a ClaI/SalI DNA fragment encoding the PR1a signal peptide sequence fused to a sequence proposed to encode the mature processed form of C. fulvum AVR4. Translation initiation codon at nucleotide 5, termination codon beginning at nucleotide 413. Amino acids 1-30 represent the signal peptide and amino acids 31-136 the mature AVR4 peptide.

EXAMPLE 1

GENETIC ACQUIRED RESISTANCE (GAR) USING Cf-9

(i) Establishing a stock from which gametes carrying a mutagenised Cf-9 gene may be obtained and identified

During experiments to isolate the Cf-9 gene by transposon tagging, alleles of the Cf-9 gene (Cf-9*Ds) were isolated that had been inactivated by insertion of the transposon Ds (See International Patent Application No. PCT/GB94/02812 for further details). This inactivated Cf-9*Ds gene did not give rise to a
constitutive and lethal activation of defence mechanisms in response to the constitutively expressed 35S:SP:Avr9 gene.

We have established the capacity to carry out transposon tagging in tomato using the maize transposon Activator (Ac) and its Dissociation (Ds) derivatives (Scofield et al 1992; Thomas et al 1993; Carroll et al 1993). The strategy is founded on the fact that these transposons preferentially transpose to linked sites. Various lines that carry Ds at positions are useful, including FT33 (Rommens et al 1992), carrying a Ds linked to Cf-9, and lines that carry a construct SLJ10512 (Scofield et al 1992) which contains (a) a beta-glucuronidase (GUS) gene (Jefferson et al 1987) to monitor T-DNA segregation and (b) stable Ac (sAc) that expresses transposase and can trans-activate a Ds, but which will not transpose (Scofield et al 1992).

The line FT33 did not carry a Cf-9 gene. We had to obtain recombinants that placed Cf-9 in cis with the T-DNA in FT33 in order to carry out linked targeted tagging. Two strategies were pursued simultaneously:

(a) FT33 was crossed to Cf9, a stock that carries the Cf-9 gene. The resulting F1 was then back crossed to Cf0 (a stock that carries no Cf- genes). Progeny that carry the FT33 T-DNA are kanamycin resistant. Kanamycin resistant progeny were tested for the
presence of Cf-9; 5 C. fulvum resistant individuals were obtained among 180. We also generated progeny that were homozygous for Cf-9 and carried that sAc T-DNA of SLJ10512. These were crossed to the recombinants in which Cf-9 and FT33 were in cis. In the FT33 T-DNA, a transposable Ds element is cloned into a hygromycin resistance gene, preventing its function. The somatic transactivation of this Ds element, which only occurs in the presence of transposase gene expression, results in activation of the hygromycin resistance. Thus from crossing the recombinants between Cf-9 and FT33, to the sAc-carrying Cf-9 homozygotes, hygromycin resistant individuals could be obtained which carry sAc and FT33, and are likely to be homozygous for Cf-9. 140 individuals of this genotype were thus obtained.

(b) To accelerate obtaining individuals that carried sAc, FT33, and were Cf-9 homozygotes, the FT33/Cf-9 F1 was crossed to a line that was heterozygous for Cf-9 and sAc. 25% of the resulting progeny carried both T-DNAs and were hygromycin resistant, and of those, slightly more than 50% were disease resistant because they carried at least one copy of the Cf-9 gene. An RFLP marker was available, designated CP46, that enabled us to distinguish between homozygotes and heterozygotes for the Cf-9 gene (Balint-kurti et al 1993). In this manner two individuals that were Cf-9 homozygotes, and that
carried both the FT33 T-DNA and sAc, were obtained. These two individuals were multiplied by taking cuttings so that more crosses could be made onto this genotype.

(ii) Establishing a tomato stock that expresses functional mature AVR9 protein

A likely frequency for obtaining any desired mutation in a gene tagging experiment is less than 1 in 1000, and often less than 1 in 10,000 (Döring, 1989).

To avoid screening many thousands of plants for mutations to disease sensitivity, we established a selection for such mutations based on expressing the fungal Avr9 gene in plants.

The sequence of the 28 amino acids of the mature Avr9 protein is known (van Kan et al 1991). It is a secreted protein and can be extracted from intercellular fluid of leaves infected with Avr9-carrying races of C. fulvum. For secretion from plant cells, we designed oligonucleotides to assemble a gene that carried a 30 amino acid plant signal peptide, from the Prla gene (Cornelissen et al 1987) preceding the first amino acid of the mature Avr9 protein (see SEQ ID NO. 4). The preferred Avr9 gene sequence depicted in SEQ ID NO. 4 shows a chimaeric gene engineered from the Pr-1a signal peptide sequence (Cornelissen et al, 1987) and the Avr9 gene sequence (van Kan et al, 1991). This
reading frame was fused to the 355 promoter of
cauliflower mosaic virus (Odell et al 1984), and the 3’
terminator sequences of the octopine synthase gene
(DeGreve et al 1983), and introduced into binary
plasmid vectors for plant transformation, using
techniques well known to those skilled in the art, and
readily available plasmids (Jones et al 1992). We
obtained transformed Cf0 tomato lines that expressed
this gene.

(iii) Crossing AVR9 expressing stock with Cf-9
expressing stock

The transformed lines obtained in (ii) were
crossed to plants that carried the Cf-9 gene. When the
resulting progeny were germinated, 50% exhibited a
necrotic phenotype, that culminated in seedling death.
This outcome was only observed in seedlings that
contained the Avr9 gene. When the same transformants
were crossed to Cf0 plants, the resulting progeny were
all fully viable.

From selfing the primary transformants,
individuals were identified that were homozygous for
the Avr9 transgene. When Avr9 homozygotes were crossed
to Cf-9, all progeny died. This system thus provides a
powerful selection for individuals that carry mutations
in the Cf-9 gene.
(iv) Tagging and inactivating Cf-9

Individuals that were homozygous for the Avr9 gene (section (iv)) were used as male parents to pollinate individuals that were homozygous for Cf-9, and carried both sAc and the Ds in the FT33 T-DNA (section (iiiia) and (iiib)). Many thousands of progeny resulting from such a cross were germinated. Most died, but some survived.

DNA was obtained from survivors and subjected to Southern blot analysis using a Ds probe. It was observed that several independent mutations were correlated with insertions of the Ds into a BglII fragment of a consistent size. This suggested that several independent mutations were a consequence of insertion of the Ds into the same DNA fragment.

Using primers to the Ds sequence, DNA adjacent to the Ds in transposed Ds-carrying mutant #18 was amplified using inverse PCR (Triglia et al 1988). This DNA was used as a probe to other mutants, and proved that in independent mutations, the Ds had inserted into the same 6.7 kb BglII fragment.

The Ds in FT33 contains a bacterial replicon and a chloramphenicol resistance gene as a bacterial selectable marker (Rommens et al 1992). This means that plant DNA carrying this transposed Ds can be digested with a restriction enzyme that does not cut within the Ds (such as BglII), the digestion products
can be recircularized, and then used to transform *E. coli*. Chloramphenicol resistant clones can be obtained that carry the *Ds* and adjacent plant DNA. This procedure was used to obtain a clone that carried 1.8 kb of plant DNA on the 3' side of the *Ds*, and 4.9 kb of plant DNA on the 5' side of the *Ds*.

Our present understanding of the *Cf-9* gene is depicted schematically in Figure 1. The *Cf-9* gene sequence and the deduced amino acid sequence are shown in the sequence listing.

A series of primers (F1, 2, 3, 4, 5, 6, 7, 12, 13, 10, 26, 27 and 25, indicated in Figure 1) was used to characterise a large number of independent mutations by PCR analysis in combination with primers based on the sequence of *Ds*. Therefore, these primers were used in polymerase chain reactions with primers based on the maize Ac/Ds transposon sequence, to characterise the locations of other mutations of *Cf-9* that were caused by transposon insertion. Eighteen independent insertions have been characterized and are located as shown. Mutants E, #55, #74 and #100 gave incomplete survival and showed a necrotic phenotype, and based on the available sequence information, they are 5' to the actual reading frame and might permit enough Cf9 protein expression to activate an incomplete defence response.

Using the sequence obtained of the gene,
oligonucleotide primers were designed that could be used in polymerase chain reactions in combination with primers based on the sequence of the *Ds* element, to characterize both the location and the orientation of other transposon insertions in the gene. These are shown on Figure 1. Based on the results of such experiments, the map positions of 17 other *Ds* insertions have been reliably assigned (as shown in Figure 1).

(v) **Production of GAR plants**

On backcrossing plants that carried the *Cf-9*:*Ds* and *35S:SP:Avr9* gene to tomato plants that carried an Ac transposase gene (*sAc* that lacked the GUS gene) in the homozygous state, but lacked *Cf-9*, one quarter of the resulting progeny carried *sAc*, *35S:SP:Avr9* and *Cf-9*:*Ds* (see Figure 13) plants showed somatic excision of *Ds* from the *Cf-9*:*Ds* gene, somatically restoring *Cf-9* function, and giving rise to necrotic somatic sectors in which the defence response was activated.

Phenotypically, these plants thus showed a variegation for a defence-related necrosis, in the same manner that plants challenged with necrotizing pathogens show somatic flecks of HR that are associated with the induction of SAR.

Necrotic sectors were visible on cotyledons, leaves, stems, petioles, sepals, and green fruits throughout plant development. Also, the necrotic
sectors formed in both the lower and upper epidermis, in all mesophyll layers and in the cells surrounding the vascular tissue. The size of the necrotic sector and the frequency of their formation was determined by both the position of the Ds element in the Cf-9 sequence and the orientation of the Ds.

The plants that variegated for necrosis were tested to assess if they were more resistant to C. fulvum than their unvariegated siblings that either carried Cf-9*Ds or carried no Cf-9 gene. Plants from five independent Cf-9*Ds pedigrees were tested in which the Ds had independently inserted into five different locations in the Cf-9 gene. These five independent insertions were between Cf-9 amino acids, 7 and 8 (<M23), 28 and 29 (<M18), 47 and 48 (>M50), 56 and 57 (>M31) and 789 and 790 (>M30) The arrows (< or >) indicates the direction of transcription of the Ds element. F1 plants that developed somatic necrotic sectors were more resistant to C. fulvum than sibling offspring that did not develop necrotic sectors. On the plants with necrotic sectors an average of 1-2 small pustules per leaf developed, 14 days after inoculation with $5 \times 10^5$ spores/ml. The plants lacking a Cf gene and the non variegating individuals all showed on average 38 large sporulating pustules per leaf. A example of this is shown in Figure 2.

Nine variegated Cf-9*Ds #20 plants, fifteen
variegated Cf-9*Ds #23 plants, eighteen variegated Cf-9*Ds #30 plants and twenty-eight variegated Cf-9*Ds #31 plants were tested, and compared to one hundred and ninety eight plants in total that did not variegate for necrosis. Plants were inoculated with C. fulvum (5 x 10^5 spores/ml) when they were four weeks old and carried 2 expanded leaves. A similar result was obtained when variegated Cf-9*Ds #50 plants and non-variegated plants were inoculated with C. fulvum. On 18 variegated GAR+ #50 plants 1-3 pustules per leaf formed, whereas on 42 non-variegated GAR− #50 plants over 35 pustules per leaf developed by 14 days after inoculation.

Sensitivity to the pathogen was measured by counting the number of sporulating pustules that were visible on each genotype 14 days and 21 days after inoculation. Samples were also taken for microscopic analysis. The results of the assay after 14 days are shown in Figure 2, and typical infections on each genotype after 21 days are shown in Figure 12.

Figure 2 shows a histogram in which the sensitivity of different individual tomato plants is expressed on the y axis as the number of sporulating pustules per leaf. The Ds carried a GUS gene. M20, M23, M30 and M31 show C. fulvum growth on plants resulting from crosses between Cf-9*Ds and sAc, and derive from Cf-9*Ds #20, Cf-9*Ds #23, Cf-9*Ds #30 and
Cf-9*Ds #31, respectively. These individuals segregate from the Cf-9*Ds and for sAc. Cf0 carries no R genes and M20, M23, M30 and M31 GUS- plants have lost by segregation both Cf-9*Ds and sAc and are thus disease sensitive sibs, providing a good control for disease symptoms in sensitive individuals. If plants receive Ds without sAc they may be GUS+ without expressing the variegation for necrosis which requires both Cf-9*Ds and sAc. As can be seen, the necrotic individuals (which all carry the 35S:Avr9 gene) show distinctly fewer pustules per leaf than their disease sensitive sibs.

Figure 2 shows that in these experiments, Cf0 plants (lacking the Cf-9 gene) exhibited about 38 pustules per leaf and non-variegating individuals derived from Cf-9*Ds #20, Cf-9*Ds #23 or Cf-9*Ds #31 also showed about 38 pustules per leaf. The non-variegated individuals that carried Cf-9*Ds #30 showed about 17 pustules per leaf indicating some residual action of the tagged Cf-9 allele. However, variegated individuals that carried Cf-9*Ds #20, Cf-9*Ds #23, Cf-9*Ds #30 or Cf-9*Ds #31 showed 1-3 pustules per leaf. In total seventy variegated individuals were assessed. These results demonstrate a very significant level of disease control by this method.

Figure 12 shows three leaves. Leaf 1 and Leaf 2 are infected with C. fulvum which confers the white
fluffy appearance. Leaf 1 is CfO and Leaf 2 is a
disease sensitive sib from Cf-9*Ds #23. Leaf 3 showing
minimal sporulation is a necrotic individual (small
sectors of necrosis are discernible) that carried Cf-
9*Ds #23, sAc and 35S:Avr9. Leaf 3 is therefore
expressing GAR.

It is important to recognize that in this example
regions of variegating plants that resist the C. fulvum
pathogen do not contain a functional Cf-9 gene. Indeed
all the cells that do carry a functional Cf-9 gene
(whose function was restored somatically by transposon
excision) are killed as they turn on the defence
response after recognition of the endogenously
expressed Avr9 peptide. Thus, non-resistant cells are
being induced to resistance by necrosis being
manifested in adjacent cells.

EXAMPLE 2

Pathogen resistance of variegated plants employing Cf-9

In addition to demonstrating that variegated
plants produced in Example 1 have enhanced resistance
to C. fulvum, we have established that the plants are
also more resistant to three unrelated fungal
pathogens, Phytophthora infestans (the causal agent of
late blight disease of tomato and potato) and Oidium
lycopersici (a powdery mildew) and Colletotrichum
largenarium (which causes leaf and fruit spot).
For the *P. infestans* experiments, sibling backcross progeny from the mutant *Cf-9* *Ds* lines M31 and M50 that were either variegating for necrosis or not and control plants lacking a *Cf*-gene (*Cf0*) were challenged by a spray application of sporangiospores (10,000 or 100 spores/ml) of the highly virulent isolate DSSI (*Al* mating type). After inoculation, the plants were kept in diffuse light conditions at a constant 100% RH and 16°C and a 12h photoperiod.

Seven days after application of the high spore dose the leaves of the unvariegated plants and those of the *Cf0* plants were completely destroyed by the spread of *P. infestans* lesions which had abundant sporangiospores at their margins. In contrast, the variegated plants were infected with *P. infestans* but the lesions were 3-5 mm in diameter and non-sporulating (Figure 3 A,B). An additional 5-6 days were required before the entire green leaf tissue of the variegated plants was destroyed and fungal sporulation commenced.

At the lower spore dose, by 7 days after inoculation, an average of 8-10 large sporulating lesions were present on each leaf of the unvariegated and *Cf0* plants whereas on the plants variegating for necrosis there were 1-2 small non-sporulating lesions per 10 leaves (Figure 4 A,B). A minimum of 18 plants were used for each genotype/spore.

For the *Oidium lycopersici* experiments the
identical plant genotypes were used. Each leaf was inoculated by brushing with an artist paintbrush the spores from a single 14 day old sporulating pustule over an entire upper surface. The inoculated plants were then kept under diffuse light conditions at 20°C during the 16 h photoperiod and at 18°C during the dark period. The RH was maintained at 70%.

By day 10 post inoculation 8-10 chlorotic lesions were evident on the leaves of the unvariegated and Cf0 plants and in 1-2 of these sporulation had commenced. By contrast on the variegated plants 1-2 smaller chlorotic non-sporulating lesions were present on each leaf (Figure 5). By day 14 post inoculation more than 20 sporulating lesions per leaf were present on the unvariegated plants and these were accompanied by severe chlorotic symptoms on the remainder of the leaf. On the variegated plants 2-4 small sporulating lesions were present per leaf (Figure 5A). An additional 7-10 days were required before a similar level of sporulation and chlorosis formed on the variegated leaves to that found on the unvariegated and Cf0 leaves at day 14 post-inoculation. (16 plants each).

EXAMPLE 3

Variegation in fruit

Dark green sectors formed on green tomato fruits of GAR plants, 5 weeks after flower pollination (Figure
6). These sectors were not visible once the tomato fruit had turned red, which is encouraging for potential commercial exploitation. When mature red fruit taken from GAR⁺ and GAR⁻ plants were injected with 100μl of spores of Colletotrichum lagenarium \(10^4\) spores/ml only the GAR⁻ fruit exhibited the typical soft rot disease symptoms seven days later. Repeated inoculations of the GAR⁺ fruit failed to cause disease.

Collectively, the above results attest to a very significant level of disease control that can be achieved in the plants variegating for restoration of Cf-9 gene function whilst constitutively expressing the Avr9 gene. The data also indicate that the disease control achievable by this method is potentially broad spectrum because the four fungal pathogens controlled have very dissimilar modes of parasitism: C. fulvum is a biotroph that does not form haustoria and grows exclusively in the extracellular spaces of the leaf mesophyll layers; O. Lycopersici is also a biotroph but colonises only the upper leaf epidermis and forms complex intracellular haustoria; P. infestans and C.largenarium are hemibiotroph that initially forms simple haustoria but later on kills host cells in both the epidermal and mesophyll layers.

Homozygous Cf-9*Ds, 35S:SPAvr9 lines have been established for the tomato lines M31 and M50. The F₁
backcross progeny derived from crosses to a homozygous
sAC source, may be assessed for their resistance to
various pathogens, including:

Potato virus X, *Pseudomonas syringae* pv. *tomato*,

Necrotrophic fungi - *Botrytis* spp, *Colletotrichum* spp,
Nematodes - *Meloidogyne* incognata, Aphids - Green Peach
Aphid, and fruit, pod, root or tuber attacking
pathogens. Also, the effect of GAR on the
establishedment of mycorrhizal associations may be
tested.

The enhanced resistance exhibited in the plants
variegating for necrosis has been termed Genetic
Acquired Resistance (GAR). It is distinct from SAR
because it is a heritable trait and is active
throughout the entire plants life.

When the expression of several defence-related
genes were compared in the GAR⁻ and GAR⁺ plants,
significantly higher levels of expression of each gene
were found in the GAR⁺ plants. Examples of this are
shown in Figure 7 for *Cf-9*Ds lines from M23, M31 and
M50 pedigrees using a basic tomato β-1,3 glucanase
probe and a tomato anionic peroxidase probe (pTAP 4.5).

The effectiveness of GAR in suppressing plant
disease appears to be inversely related to sector size.
The two independent *Cf-9*Ds pedigrees that have the
highest frequency of small necrotic sectors (lines M31
and M50) give the best GAR. This indicates that by carefully manipulating the frequency of somatic restoration of Cf-9 function even higher levels of plant protection be developed.

Currently, there are two possible hypotheses to explain GAR. Either the initially activated host cells generate local and systemic signals whilst still alive, and the necrotic lesions are a by-product of the Cf-9-Avr9 mediated responses. Alternatively, the actual death and necrotic reactions, the final response of the activated host cells, generates specific local and systemic signals in a manner analogous to SAR. Exactly how GAR works does not need to be known for the present invention to be operated. Provided the required genetic components are present, GAR plants have enhanced pathogen resistance compared with wild-type.

EXAMPLE 4

Expression of Cf-9 in Heterologous Plants Species and Induction of Cell Necrosis

We have shown that following the transfer of different genomic clones containing the Cf-9 gene into tobacco and potato, these sequences render the transgenic plants responsive to Avr9 elicitor (Figure 8).

Also when transgenic tobacco expression Cf-9 is crossed to transgenic tobacco plants engineered to
express Avr9 peptide constitutively, the F1 seedlings
die within 2 days of seed germination (Figure 9).

When transgenic Arabidopsis expressing Cf-9 is
crossed to Avr9 expressing transgenic Arabidopsis the
F1 seedlings die 10 days after seed germination (Figure
10).

Thus we have shown that in a variety of species,
genesis required for activation of plant defence,
mediated by the Cf-9 protein, are present and
functional.

EXAMPLE 5

Genetic Acquired Resistance Using Cf-9 in Potato

To apply GAR to potato plants a single T-DNA
construct systems is used.

The system is based around a single T-DNA
construct (Figure 11) containing, a Cf-9 gene sequence
under the control of its own promoter which has been
inactivated by an autonomous Ac element that is only
capable of a low level of excision (the Ac (Cla)
element (Keller et al. 1993), and the 35S:SP:Avr9
transgene). The Ac element is inserted at various
positions in the Cf-9 sequence and in both orientations
in order to determine the best configuration to produce
a high frequency of small somatic sectors where Cf-9
function has been restored.

Placing the Cf-9 sequence or other R gene
sequence under the control of a cell-type specific promoter may enhance the GAR phenotype. Potential target cellular sites include the epidermis and the vascular parenchyma cells.

EXAMPLE 6
Expression of Cf-4 in transgenic plants and demonstration of increased pathogen resistance

The Cf-4 gene has been tested in transgenic plants in a number of ways: firstly by inoculation with a race of C. fulvum containing the corresponding avirulence gene Avr4 to test if that race gives an incompatible response on the transgenic plant; secondly by injecting leaves of a transformed plant with intercellular fluid isolated from a compatible interaction containing AVR4; thirdly, by delivering AVR4 in the form of recombinant potato virus X as described previously in studies of the Cf-9/AVR9 interaction (Hammond-Kosack et al., 1995).

The DNA sequence of the C. fulvum gene encoding AVR4 has been reported and the amino acid sequence of the mature processed polypeptide (Joosten et al., 1994). We amplified by PCR the Avr4 gene from C. fulvum race 2,5 using primers to the published sequence and fused a sequence encoding the proposed mature polypeptide to a DNA sequence encoding the N-terminal
signal peptide of the tobacco PR1a protein. This would facilitate targeting of AVR4 to the intercellular space in transgenic plants where it is expressed. This chimeric gene (SPAvr4) was inserted into a cDNA copy of potato virus X, as a ClaI/SalI DNA fragment (SEQ ID NO. 13) as described previously (Hammond-Kosack et al., 1995) to generate PVX:SPAvr4. Infectious transcripts of the recombinant virus were generated by in vitro transcription. All nucleic acid manipulations were performed using standard techniques well known to those skilled in the art.

**Tomato**

Experiments were designed to test the recombinant virus in 3 week old tomato seedlings. In Cf-4 containing plants inoculated cotyledons appeared desiccated and eventually abscised at 3 days post-inoculation (d.p.i.), in contrast to Cf0 controls which only showed signs of slight mechanical damage at the site of virus inoculation. Cf0 plants developed visible symptoms of virus infection at 7-10 d.p.i. comparable to symptoms observed with the wild type virus i.e. chlorotic mosaic symptoms. At 4-5 d.p.i. in plants containing Cf-4 necrotic lesions were observed in the younger leaves, presumably due to systemic spread of the virus as described previously in similar experiments with PVX containing Avr9 on Cf-9 containing
plants (Hammond-Kosack et al., 1995). Other features included necrotic sectors on petioles and the stem. The necrotic phenotype was seen to spread systemically and at 14 d.p.i. the majority of Cf-4 containing seedlings had died. Cf0 control plants did not die but did show symptoms of chlorosis and vein-clearing.

These results confirm that Cf-4 is functional in transgenic tomato plants, resulting in a necrotic defence response in the presence of elicitor AVR4.

Tobacco

Using binary vector cosmids comprising Cf-4, transgenic tobacco plants have also been produced (Fillatti et al., 1987; Horsch et al., 1985) using techniques well known to those skilled in the art.

Transgenic tobacco containing cosmids comprising Cf-4 were inoculated with PVX:SPAvr4. In most transformants necrotic lesions were observed at the site of virus inoculation 3-4 d.p.i. similar in appearance to lesions which appear in response to virus inoculation in some virus resistant varieties. In these individuals the necrosis was not strictly confined to local lesions which eventually coalesced and at 7-10 d.p.i. leaf necrosis was apparent over the entire region of virus inoculation. In several transformants the reaction to PVX:SPAvr4 was more acute and the necrotic leaf sectors could be observed at 3-4
d.p.i. Neither of these phenotypes were observed in transgenic tobacco containing cosmids lacking Cf-4 or in non-transformed control plants challenged with PVX:SPAvr4.

Functional expression of Cf-4 in transgenic tobacco has thus also been shown, with activation of a necrotic defence response in the presence of elicitor AVR4.

Pathogen Resistance

Transgenic plants were propagated by cuttings so that Cf-4 activity could be detected by inoculation with PVX:SPAvr4 on 12 tomato transformants. Transgenic tomato plants containing Cf-4 exhibited leaf necrosis on inoculated leaves 3-4 d.p.i. This necrosis eventually spread systemically as previously observed in Cf-4 containing plants in the experiments described above. Transgenic plants exhibiting necrotic leaf sectors eventually died.

Cuttings of a number of transgenic plants obtained in the first round of transformation experiments were further assayed for Cf-4 function by inoculation with C. fulvum race 5. In 5 transgenic plants tested, a positive correlation was observed between plants exhibiting PVX:SPAvr4 dependent necrosis and resistance to the pathogen. In this experiment pathogen growth was observed on compatible control
plants (Cf0) but not on incompatible control plants (Cf2).

All documents mentioned in the text are incorporated herein by reference.

REFERENCES:

Van Den Ackerveken, et al. (1992) Plant Journal 2, 359-


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GAA GGT CAT ATA CCG GCA TCA TTT CAA AAT TTA CTA GTA CTG GAA GCT
Glu Gly His Ile Pro Ala Ser Phe Gln Asn Leu Ser Val Leu Glu Ser
685 690 695

TTG GAT CTC TCA TCT AAT AAA ATC AGC GGA ATT CGG CAG CAG CTT
Leu Asp Leu Ser Ser Asn Lys Ile Ser Gly Glu Ile Pro Gln Gln Glu Leu
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GCA TCC CTC ACA TCC CTT GAA GTC TTA AAT CTC TCT CAC AAT CAT CTT
Ala Ser Leu Thr Phe Leu Glu Val Leu Asn Leu Ser His Asn His Leu
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Val Gly Cys Ile Pro Lys Gly Lys Gln Phe Asp Ser Phe Gly Asn Thr
730 735 740 745

TCG TAC CAA GGG AAT GAT GGG TTA CGC GGA TTT CCA TCT TCA AAA CTT
Ser Tyr Gln Gly Asn Gsp Gly Leu Arg Gsp Phe Pro Ser Lys Leu
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Cys Gly Gly Glu Asp Glu Val Thr Thr Pro Ala Glu Leu Asp Glu Glu
770 775

GAG GAG GAA GAA GAT TCA CCA ATG ATC AGT TGT CAG GGG GTT CTC GTG
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780 785 790

GGT TAC GGT GCT GAA CTT GGT ATT GGA CTG TCC GTA ATA TAC ATA ATG
Gly Tyr Gly Cys Gly Leu Val Ile Gly Leu Ser Val Ile Tyr Ile Met
795 800 805

TGG TCA ACT CAA TAT CCA GCA TGG TTT TCG AGG ATG GAT TTA AAG TTG
Trp Ser Thr Gln Tyr Pro Ala Trp Phe Ser Arg Met Asp Leu Lys Leu
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SEQ ID NO. 2:

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 Phe Gln Leu Ser Asn Leu Lys Arg Leu Asp Leu Ser Phe Asn Asn Phe
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 Thr Gly Ser Leu Ile Ser Pro Lys Phe Gly Glu Phe Ser Asn Leu Thr
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Leu Glu Cys Leu Tyr Leu Ser Ser Asn His Leu Asn Gly Ser Ile Pro
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Ser Trp Ile Phe Ser Leu Pro Ser Leu Val Glu Leu Asp Leu Ser Asn
380 385 390
Asn Thr Phe Ser Gly Lys Ile Gln Glu Phe Lys Ser Lys Thr Leu Ser
395 400 405
Ala Val Thr Leu Lys Gln Asn Leu Lys Gly Arg Ile Pro Asn Ser
410 415 420 425
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445 450 455
Leu Leu Asp Leu Gly Ser Asn Leu Glu Gly Thr Ile Pro Gln Cys
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Val Val Glu Arg Asn Glu Tyr Leu Ser His Leu Asp Leu Ser Lys Asn
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Arg Leu Ser Gly Thr Ile Asn Thr Thr Phe Ser Val Gly Asn Ile Leu
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Ser Met Ile Asn Cys Lys Tyr Leu Thr Leu Leu Asp Leu Gly Asn Asn
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Lys Ile Leu Ser Leu Arg Ser Asn Lys Leu His Gly Pro Ile Lys Ser
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SEQ ID NO. 3:

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SEQ ID NO. 4:

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48

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4951 TCATGCAAAG GAGAAAGAAG CAGCGAGTGC AAAGAATAA CAGAAAGAAGA
5001 AATATAACACT TCTAGACAAAG TTAACCATAC AGGAAGATTT GATTTTCGAAA
5051 CTTCAGGTAT TCAATCTGAAG CTCTAACCCT TATCTTCTTT AGTTTATTCT
5101 AACACTAAT ATATGCTTTTT TTTTATCCA CAAATACTTA ATAAGCCTTG
5151 ATACAAATTTG CTATAATCACT TTGGAAAGCTG TGATATATAA CAAGGCTTAA
5201 AAATTTTATAG TTTGCTGACT CACTTTCTTA TTTTTCAGAT TTTTACAGGAC
5251 CAAGAAATAGG AAGACGCGCTG TGTAAGGATG TTGCTTCTTC CTAAGTTCGA
5301 GCTTAAAGTTT GTTTGAGATTG ATTTTTAGTT TTATAGGTG TTCTTTCAAGT
5351 GGAGAAAGTTT AATATTGTGA ATTTTGATGA TATATATAAA ATTTTGTTGTA
5401 TGGAATGATGT TGTATGCATT TCTCGAGTCA ATAAAATCTCA CATTCAAGAA
5451 TCATAAAGAGG TTACACCGACG ATAGAAAGATA GAACATACAA AGAAGAATAC
5501 ATTACAACCT TGCGCTTTGGT TATCTTACAC CCGAAAGCTT GTTTTATTAGG
5551 AAGGGAAGGCA CAAGTTTATAT TTTTAGATAT GGGGAGCCTT GGCGTGCTGG
5601 TAAAGTTTTGA GTTTGATAAAGG TAACTTTCTCC TGTTAATGAA TTTATGATTC
5651 ATAGCAAGAGG TGTTTTAAAA ATTTCTGTGG TTTAGTGGT TAATTATTGG
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5751 TCCTTAATAGT CAACTCTCAA GATTCTAAAA TATAAGGACT CTAAGAATAG
5801 CATAGAAAAA AACTGCGATTA TACTAAGGCCG TTGTTGGATC CGGAAGGAAA
5851 TGCTGTGATA GGGCTGTAAA AACATACGTT TAATAGGGTG GGGGTAGAAG
5901 GTACCCAGTGG AAAAATTCTGA GGTGTGGATAG GGTGTGCTCTG CAACAACAAC
5951 TTATATTAACAA AATCTACACAC AACTAACAGAC ATGAGAGTAA AAAATTTTAAAT
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6051 ACATTGTGTA TACCTTTAGG ACAACCATTT ATCACTCAA AAGATCAAG
6101 GATGTGATGCA TTATCTGTTT CTGGAACAA AATATATGAC ATAAATCTTA
6151 CAGGAATCTAG GTTTTGTGGTG GTTGAAACTT CCATAAGGAC TAGTCCAGGA
6201 TACGTGAGATC AAAAGTTTCT TATTGTCAGCC AATCTCTTCTT CGAGTTATCT
6251 GATCCCCGGAA CTGGACAGGAC GAAAGCAGAA CAACAATAATG TACATGAGCG
6301 AGTTACTTGA ATCAAAGAGG ATGAAAAGAG GCACCTCATA CTAAAATGAT
6351 AACACTTAC TAATATGATA CAATATTTA CAGGAAGAAA AAGAAGAATAG
6401 GAAGACGAAAC CGCAACATAC TTTATCTATT AACGAGCAGT GCACTCAAGA
6451 TAATAGTGAT TTTTGGCTCGA G
SEQ ID NO. 6:

1 MMWVSRKVS SLQFRTLFLYL FTVAFASTEAL ATATLKWKAT FKNNQINSFRA

51 SWIPSSNACK DWGYGVCFPNG RWNTLNITNA SVIGTLYAFP FSSLPSLENL

101 DLHSDKNYGT IPEIGNLTN LVYDLNNNNQ ISGTPQQIG LLAKLQIIRI

151 FHNQLNGFIP KEIGYRLSLL KLSLGINFLS GSIPASVGNL NNLSPFLYLYN

201 NQLSISPEEE ISYLRSLETLL DLSQDNALGKS IPASLGNMNLL LSPLQFLYNQN

251 LSGSIPEEEIC YLRLSTYLDL SENALNQSIP ASLGNLNRNL FLFLYQNQLS

301 GSIPEEIGYL RSLNVLGLSE NALNQIPAS LGNLKKNLRL NLYNNQLS

351 IPASLGNLIAN LSNLYLYNNQQ LSGLSIPASSL LNGNNLMSLMYLN YNNQLSIP

401 ASLGNLNLNL RLYLYNNQQL ASIPSEEEIG YSSLTYLSSL GNSNLGLLQIP ASPAMNRNQL

451 FNQMNLALFL FLYENQLASS VPEEIGYRIS LNLVLDSNLA LNSIPASFG

501 NNLNLRLNQL VNNQLSIPSE BEIGYRLSIL NLMDALNLG SGPASFQNL

551 NNLSRLNLVL NQLSISPEEE IC YRLSRNLDGL GLSEALNGS IPASLGNLNL

601 LSNLYLYNNQQ LSGLSIEEIC YLRLSTYLDL GNSNLGKQPL ASLNNLRNQL

651 ALIINDNLNL GIIPSVQCNL TLQSLYLYMPR NLKLGKVPQC LNGISNLQVL

701 SSMSNSFSGE LPSSISNLTS LQIILDFGRN LN LEAGIPQQQG MNNLLEFMD

751 QNNKLSGTLP TNFSIGCSLI SMLLHGNELE DEIPRLDLCN KKLQVLDDLGD

801 NQLNDTFPMW LGTLPRLRLV RLTSDKHLQG IRSSRAEIMF PDLRLRIDLRS

851 NAFSQDLPFTS LFPEHLKGMRT VDKTMEEPSY ESYYDSVVV VTKGELEIV

901 RLILYTVID LSSNKFESHI PSLGDLQAI RILNVSHNAL QGYIPSSILGS

951 LSILESDLS FNQLSGEIPQ QLSLTFLEF LNLNHNLYLG CFPQQFQFRT

1001 FESNSYEGND GLRGYPVSKG CGKDPVEKGN YTVAASLEDQE SNSFFNDFW

1051 KAILMGGYSG LCIGISMIYI LSTGNLRWLL ARIIEKLEH K IIMQQRRKQR

1101 GQRNYRRRRN HF*

SEQ ID NO. 7:

1 MMWVSRKVS SLQFRTLFLYL FTVAFASTEAL ATATLKWKAT FKNNQINSFRA

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151 FHNQLNGFIP KEIGYRLSLL KLSLGINFLS GSIPASVGNL NNLSPFLYLYN

201 NQLSISPEEE ISYLRSLETLL DLSQDNALGKS IPASLGNMNLL LSPLQFLYNQN

251 LSGSIPEEEIC YLRLSTYLDL SENALNQSIP ASLGNLNLNL FLFLYQNQLS
301 GSIEPEEGYL RNIAVLGLSE NALNGSIPAS LGNLKNLSDL NLVNNQLSGS
351 IPASLGLNN LSMLYLYNQ LSGSIPASLG NLNLSMLYL YNNQLGSISIP
401 ASLGNLNLNS RLKYLYNLQLS GSIPEEEYGL SSITYLDSLNS NSINGFIPAS
451 FGNMLNLFNL PYLENSCLASS VPEEIGYLRNS LNVLDSLENSA LNGSSIPASFG
501 NLNLSRNLNL VNQLSGSIPIE IGYRLSLNLD GLSENALNS IPASLGLNAN
551 LSLMLYLYNQ LSGSIEEEIG YLSSLYLQLS GNNSLNLGLP ASFPNNMLNQ
601 ALILNDNNNL GEIPSSVCNL TSLEVLYMPR NLNLGKVQPC LGNISNLQLV
701 SMSSNSFSGE LPSSISNLTS LQILDFGRNN LEGAIPQCQFG NISSLEVFD
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901 RILSLLVTVL LSNKDFEGHJ PSVLGDLCIA RILNVSANH QYGIPSSGLS
951 LSILSLLLS FNQLSGLIEQP QLASLLFLEF LNLSSHNLQG CIPQPGQFRT
1001 FESNYEYNGD GLRGYPVSKG CGKDPVSEK YTVPASLQSE SNSFNFNDW
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1101 GQRRYRRRN FN*

SEQ ID NO. 8:

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51 TCTTTACACT TGCACTTGGCT TCGACTGAGG AGGCACTGCG CTCCTTGAAA
101 TGGAGACCA GTTTCCAAGAA CCAGAATAAT TCCTTTTGAG CTTCCATGAT
151 TCCAGTTCT AATGCACTGCA AGGACTGTTGA TGGATGTTGA TGTTTTAATG
201 GTGCTGGTTAA CACGGTAGAT ATTACAAATG CTAGTGCTAT TGGTAACCTC
251 TATGGCTTTC CATTTCATC CTCCTCCTCT CTTGAAATTC TTGATTCTAG
301 CCAGAACAT AATCTATGGTA CCATCCACC TGAGATGTGT AATCTACAAA
351 ATCTTGCTCA TCTGGACTTG AACAACAATC AGATTTCAAG ACAAATACCA
401 CCCACAAATCG GTTTACTAGC CAAGCTCAGCA ATATCAACGC TATTTCAAC
451 TCAATTAAT AAGTATATC CTAAGAAAT AGTTACCTA AGGTCTTATA
501 CTAACACATC TTTGCTTATC AACCTTTCTTA GTGGCTTCCAT TCTGCTCTCA
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601 CCCCTTGCTCT ATCCCTGGAAG AAAAATAGTA CCTAGATCCT CTTACGAGAC
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SEQ ID NO. 9:

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2601 CAGTTGATAA AACAAATGGG GAAACAAGTG TATGAAGCTA TTAGCATGAC
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3551 AAAAAAAAAA AAAAAAAAAA AAA
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SRTIAPELIS AIREARISIV IFSKNYASST WCLNELVEIH KCPNDLGOMV
IPVFYDVDS EVRQVFGTFEGPF KVFKECTEVS KDKQPDFQKQ RWQVALTDIA
NTAGEDLLNG PNEAMHKVEI SMDVSNKLIT RSKCPFDFFVG IBAINAIAK
VLCLEKESVAR MVQWQSSGI KSTQGRAFL SQLSSQHRR AFLTYKSTFG
SDVSQMKLSW QKELLSEILG QKDIKIEHG FVEQRLNHKK VLLILLDDVND
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KMIDIQAYPK DSPDPDFKEL APEVAELVQS LPLGLSVLGS SLKGRDKDEW
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ROFLTFNEDF REVLEKTEGT ELTLGIRLPH PYLITRSFIL IDESKFQMR
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LRMNYNSLKER LWDQTPQQLS LKMDLYNSY KLKEIPDLSL AINLEELNEL
ECFSELELPS SIONAIKLRE LNCWGGILLID LKSLGMCNLY EYLSVPWSW
90

701  RECTQGIVYF PRKLKSVLWT NCPKLRLPSN FKAELYVELI MEYSELEKLW
751  DGTSQLSGSLK EMNLRYSNNL KEIPDLSLAI NLEELDLFPGC VSLVTLPSSI
801  QNATKLIYLD MSECNLESF PTVPNFLKSLY YLDITGCPCNLF RNPFAIKMGC
851  AWTRLSRTRL PFEGRNEIVY EDCFWKNNLPL AGLYLDCLM RMCPCEFSE
901  QLTFLNVSGL KLEKLIWEGQ SLSLSEMDL SESENLKELP DLKATNLKL
951  LCLSGCKSLV TLPSCHLGNQ NLRLYMNRC TGLEDLPDTV NLSSLETLDL
1001 SGCSSLRTTP LISTNIVCLV LENTAIEEIP DLKATKLES NLDINCKSLV
1051 TLPSCHLGNQ NLRLYMNRC TGLEDLPDTV NLSSLETLDL SGCSSLRTTP
1101 LSTRIBCLY LENTAIEEVP CCIEDPRTLTL VLRMMCCQRL KNISNIFRL
1151 TSLTLADPTD CRGVIKALSD ATVVTMEHDM VSCVPLSEN LETYCERFWDA
1201 CSYYSDDFE VNRNPRLST MTNVDVEFKF CCSITIKECG VRLLYVYQFT
1251 ENHQQITRRSK KRMRVSSLPP

SEQ ID No.11:

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51 GAAATTTAGC TCGAGGTGGC GCACATAGTG AGGTAAGCTG TACATACGTTG
101 TTATTGCGTG ATATTGTCG ATATAATACCT CATCTAAATT ATGAAATAGA
151 CACACAAGGC AAACATCTCT TAATATGTTT TGATATTTTT TAGTGCAAGAA
201 ATGGGTGGTG TAAAAGCTTG GTTTGACAGT CTATATGTCT TTCTCTTCTCA
251 ACTGCTGTTCC TGCTCATGCT GACCTGAAGT TTGCCCGGAA GAACAGGCTC
301 TTGCTCTTTCT AGAATTTCAAG AAACAGTTTA CGTTTAATCC TAAGCTTCT
351 GATTATGTTG ACGAGAAGAG AACTCTTCTCT TGGAAAACAA GCACAGGTTTG
401 CTGTCATAGG GTGGGTGCTAT ATTTGACAGA AACGACAGGA CAAGTTGATAG
451 AGCTTGAACCT CGTTGAGCAG CAATTCAAG GCAAGTTCCA TTCCAATAGT
501 AGCTTTCCTTC AACTTCCCAA TCTCAAAGG AGTTATTGTG CTATTAAATGA
551 TTTCACCTGG TCGGCGATTG CACCTCACCT TTGTTAGGTTGC TCAAGATTGTA
601 CGCATCTCGA TTGGTGCCGAT TCAAGTTTGA GGGGTGTAAT CCCCCTCGAA
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1651 AAGTCCCAGG ATCTTACATC AATGGCAAGT ATTTGACACT ACTTGATCTA
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1801 AACTCTCAGG GAATAAACAC TGTTTATAGG GTCTCTCAAT TCTTGCATCT
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2151 ATACCGGCACT ATTTCAAAA TTTATCGATA CTGGAATCACT TGGATCTTTC
2201 ATCTTAATTTA ATCAGCGGGG AAATTCGCGCA GCAACCTGCA TCCCTCACAT
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SEQ ID No. 12:

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101  SLFQLSNLKR LDDSYNDFTG SPISPKEGFS EDLTHLDLSE SSFRGVPSE
151  ISLHLSLYVL RISNLNLTGQ PHNFELLKKN LTQLKVLDDL SINESSITPL
201  NPSSHTNLW LYPETELGIL PERVFHLSLDL EFLDLSSNPQ LTVRFPPTK
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301  LTNIVFLDLN NNHLEGPIPS NVSGLRNLQI LMLSSSHNNG SIPSIFSLPL
351  SLIGLDSLNN TFSGKIQEFP SKTLSTVTLL QNKLKGIPPH SLNNQKQLQF
401  LLLSHNNISG HISSAICNKL TLILDLGSSN NLEGTFQCV VERNYLHSL
451  DLSNNRLSGT INTTFSGVNI LRVSLHNGNK LTGKVPRSMI NCKYLTLLDL
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601  QQQYDSVRL DSNMIINLSE NRFEGHIPII IGDIVGLRNL MLHSNVLGEH
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751  MISOQGVLVG YGCGLVIGLS VIYIMWSTQY PAWFSRMILK LEHIITKMK
801  KHKKRY
CLAIMS:

1. A method of providing increased pathogen resistance in a plant, or a part or propagule of a plant, by induction of variegation in which a gene is expressed or suppressed in cells resulting in the activation of a plant defence response, which comprises:

   (i) inactivating a nucleotide sequence which contributes to a plant defence response or inactivating one or more nucleotide sequences forming a part of a combination of nucleotide sequences which contributes to a plant defence response;

   (ii) introducing said nucleotide sequence or sequences into the genome of a plant; and

   (iii) restoring said nucleotide sequence or sequences to a functional form in cells of the plant or a descendant thereof, or a part or propagule of the plant or descendant, to result in increased pathogen resistance.

2. A method of providing increased pathogen resistance in a plant, or a part or propagule thereof, by induction of variegation in which a gene is expressed or suppressed resulting in necrosis, which comprises:

   (i) inactivating a nucleotide sequence which contributes to necrosis or inactivating one or more nucleotide sequences forming part of a combination of
nucleotide sequences which contributes to necrosis;
(ii) introducing said nucleotide sequence or
sequences into the genome of a plant; and
(iii) restoring said inactivated nucleotide sequence or
sequences to a functional form in cells of the plant or
a descendant thereof, or a part or propagule of the
plant or descendant, to result in necrosis.

3. A method according to claim 1 or claim 2 wherein
said nucleotide sequence encodes or sequences encode a
substance or a combination of substances which result
in increased pathogen resistance.

4. A method according to any one of the preceding
claims wherein said nucleotide sequence or sequences
comprises a gene and activation of the plant defence
response and/or necrosis due to the expression of said
nucleotide sequence or sequences is not dependent on
the expression of any other gene comprised in said
nucleotide sequence or sequences.

5. A method according to any one of claims 1 to 3
wherein said nucleotide sequence or combination of
nucleotide sequences comprises one or more genes and
wherein activation of the plant defence response and/or
necrosis due to the expression of said nucleotide
sequence or sequences is conditional on the expression
of one or more interacting genes.
6. A method according to claim 5 wherein said nucleotide sequences encodes or nucleotide sequences encode one or more substances which are or together are capable of inducing the plant defence response and/or necrosis, and at least one of said nucleotide sequences is inactivated in step (i).

7. A method according to claim 6 wherein said nucleotide sequence comprises a plant pathogen resistance gene (R) or a mutant, variant or derivative thereof, or a pathogen avirulence gene (Avr) or a mutant, variant or derivative thereof, or another R gene elicitor (E), or both (i) an R gene or a mutant, variant, or derivative thereof and (ii) a corresponding Avr gene, or a mutant, variant or derivative thereof, or another R gene elicitor (E).

8. A method according to claim 7 wherein said plant pathogen resistance gene (R) is a tomato Cf-9 gene or a mutant, variant, derivative or homologue thereof and the avirulence gene is a Cladosporium fulvum Avr-9 gene or a mutant, variant, derivative or homologue thereof, or encodes another Cf-9 elicitor.

9. A method according to claim 7 wherein said plant pathogen resistance gene (R) is a tomato Cf-2 gene or a mutant, variant, derivative or homologue thereof and the avirulence gene is a Cladosporium fulvum Avr-2 gene
or a mutant, variant, derivative or homologue thereof, or encodes another Cf-2 elictor; or wherein said plant pathogene resistance gene (R) is a tomato Cf-4 gene or a mutant, variant, derivative or homologue thereof and the avirulence gene is a *Cladosporium fulvum* Avr-4 gene or a mutant, variant, derivative or homologue thereof, or encodes another Cf-4 elictor; or wherein said plant pathogen resistance gene (R) is the tobacco N' gene or a mutant, variant, derivative or homologue thereof, and the avirulence gene is a suitable Tobacco Mosaic Virus coat protein, or a mutant, variant, derivative or homologue thereof or encodes another N' elictor; or wherein said plant pathogen resistance gene (R) is the potato Rx gene or a mutant, variant, derivative or homologue thereof and the avirulence gene is a suitable PVX coat protein or a mutant, variant, derivative or homologue thereof or another Rx elictor; or wherein said plant pathogen resistance gene is another viral resistance gene and the avirulence gene encodes a corresponding viral coat protein or other elictor of the viral resistance gene.

10. A method according to claim 5 wherein said nucleotide sequence encodes a Cauliflower Mosaic Virus gene VI protein, a bacterial harpin gene protein, an *Arabidopsis* RPP5 gene protein, a ubiquitin conjugating enzyme, an RNase such as Barnase, a mutant, variant, derivative or homologue of any of these, or other toxic
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polypeptide or peptide such as diphtheria toxin or a mutant, variant, derivative or homologue thereof.

11. A method according to claim 4 in which the plant defence response or necrosis is dependent on the expression from a nucleotide sequence leading to the reduction of expression of a gene that negatively regulates the plant defence response, resulting in the plant defence response and/or necrosis.

12. A method according to claim 4 in which the plant defence response or necrosis is dependent on the expression of an allele of a gene from a nucleotide sequence which activates the plant defence response in the absence of a ligand that is capable of interacting with the product of said gene, resulting in the plant defence response and/or necrosis.

13. A method according to claim 5 in which the plant defence response or necrosis is dependent on the expression of a mutant allele of a gene from a nucleotide sequence which is capable of activating the plant defence response and the expression of an enfeebled negative regulator of the defence response, leading to the plant defence response and/or necrosis.

14. A method according to any of the preceding claims wherein the inactivation of said nucleotide sequence or
of one or more of said nucleotide sequences is effected by the insertion therein of a transposable genetic element.

15. A method according to claim 14 wherein said transposable genetic element is a transposon or a nucleotide sequence bordered by specific nucleotide sequences that can be recognised by a site specific recombination system.

16. A method according to any of the preceding claims wherein said plant genome comprises at least one nucleotide sequence encoding a substance capable of restoring said inactivated nucleotide sequence or sequences to a functional form to result in increased pathogen resistance.

17. A method according to claim 16 which comprises restoring said inactivated nucleotide sequence or sequences to a functional form by excision or rearrangement of said transposable genetic element.

18. A method according to claim 17 wherein when said transposable element is a transposon, said plant genome comprises at least one nucleotide sequence coding for a corresponding transposon activation system to effect somatic excision of said transposon.
19. A method according to claim 18 wherein the genes encoding the transposon and transposase are derived from the Activator/Dissociation transposable element family (Ac/Ds) or from the Enhancer/Suppressor mutator transposon family (En/Spm).

20. A method according to claim 17 wherein when said inactive form of said nucleotide sequence or sequences is flanked by recombinase recognition sequences, said recombinase recognition sequences are acted on by a site specific recombination system which comprises a specific recombinase to result in recombination.

21. A transgenic plant, or descendant thereof, or part or propagule of the plant or descendant, obtainable using a method of any of the preceding claims with increased pathogen resistance compared with wild-type.

22. A plant, or a descendant thereof, or a part or propagule of the plant or descendant, or a derivative of any of these, which is phenotypically variegated, comprising a cell or clone expressing a first phenotype and other cells expressing a second phenotype comprising increased pathogen resistance compared with wild-type.

23. A plant, descendant, derivative, part or
propagule according to claim 22 wherein the first phenotype is necrosis and/or a plant defence response phenotype.

24. A plant, descendant, derivative, part or propagule according to claim 22 or claim 23 wherein the phenotypic variegation results from expression in cells with the first phenotype from a nucleotide sequence or sequences which contribute to such phenotype, said expression from said nucleotide sequence or sequences being inactivated in cells not having said first phenotype.

25. A plant, descendant, derivative, part or propagule according to claim 24 wherein said expression results from reactivation of a previously inactivated gene.

26. A plant, descendant, derivative, part or propagule according to claim 24 or claim 25 wherein said inactivation results from insertion of a transposable genetic element into said nucleotide sequence or one or more of said nucleotide sequences.

27. A plant, descendant, derivative, part or propagule according to any one of claims 24 to 26, wherein said nucleotide sequence or sequences comprises: a gene (R) which is a plant pathogen
resistance gene or a mutant, variant or derivative thereof; or a gene (L) which is a pathogen avirulence

gene (Avr) or a mutant, variant or derivative thereof, or another elicitor or ligand gene the product of which

can interact with the product of a R-gene; or both an R
gene and an L gene.

28. A plant, descendant, derivative, part or
propagule according to claim 27 wherein the R gene is a
tomato Cf-9 gene or a mutant, variant, derivative or
homologue thereof and the L gene is a Cladosporium
fulvum Avr-9 gene or a mutant, variant, derivative or
homologue thereof, or encodes another Cf-9 elicitor.

29. A plant, descendant, derivative, part or
propagule according to claim 27 wherein said R gene is:

(i) a pathogen resistance gene from tomato;
(ii) a pathogen resistance gene from tobacco;
(iii) a pathogen resistance gene from potato;
(iv) a pathogen resistance gene from Arabidopsis;
(v) a pathogen resistance gene from flax;

(vi) a nucleotide sequence encoding a CaMV gene VI
protein;
(vii) a nucleotide sequence encoding a bacterial
harpin gene protein;
(viii) a nucleotide sequence encoding a ubiquitin
conjugating enzyme;
(ix) a nucleotide sequence encoding an RNase;
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(x) a nucleotide sequence encoding a toxic peptide;

(xi) a mutant, variant, derivative or homologue of any of (i) to (x);

30. A plant, descendant, derivative, part or propagule according to claim 29 wherein said pathogen resistance gene from tomato is selected from Cladosporium fulvum resistance genes including Cf-2, Cf-4, Cf-5 and Cf-9; said pathogen resistance gene from tobacco is N'; said pathogen resistance gene from potato is Nx; said pathogen resistance gene from Arabidopsis is RPP5 or RP52; said pathogen resistance gene from flax is L6; said RNase is Barnase; or said toxic peptide is diphtheria toxin.

31. A plant, descendant, derivative, part or propagule according to claim 27 wherein said L gene is:

(i) a Cladosporium fulvum avirulence gene or another elicitor of a resistance gene for a Cladosporium fulvum avirulence gene;

(ii) a suitable TMV coat protein or another N' elicitor;

(iii) a suitable PVX coat protein or another Rx elicitor; or

(iv) a mutant, variant, derivative or homologue of any of (i) to (iii).

32. A plant, descendant, derivative, part or
propagule according to claim 31 wherein said 
*Cladosporium fulvum* avirulence gene is *Avr2, Avr4, Avr5* 
or *Avr9.*

33. A cell containing (i) nucleic acid encoding one 
or more than one nucleotide sequence which causes or 
contributes to the plant defence response and/or cell 
necrosis, at least one said nucleotide sequence being 
reversibly inactivated and (ii) nucleic acid encoding a 
molecule or molecules able to reverse the inactivation.

34. A cell according to claim 33 wherein the 
inactivation results from insertion of a transposable 
 genetic element into one or more of said nucleotide 
sequences.

35. A cell according to claim 34 wherein said 
transposable genetic element is a transposon and said 
molecule or molecules provide a corresponding 
transposon activation system to effect excision of said 
transposon.

36. A cell according to any one of claims 33 to 35 
wherein said nucleotide sequence or sequences 
comprises: a gene (R) which is a plant pathogen 
resistance gene or a mutant, variant or derivative 
thereof; or a gene (L) which is a pathogen avirulence 
gene (*Avr*) or a mutant, variant or derivative thereof,
or another elicitor or ligand gene the product of which can interact with the product of a R-gene; or both an R gene and an L gene.

37. A cell according to claim 36 wherein the R gene is a tomato Cf-9 gene or a mutant, variant, derivative or homologue thereof and the L gene is a Cladosporium fulvum Avr-9 gene or a mutant, variant, derivative or homologue thereof, or encodes another Cf-9 elicitor.

38. A cell according to claim 37 wherein said R gene is:

(i) a pathogen resistance gene from tomato;
(ii) a pathogen resistance gene from tobacco;
(iii) a pathogen resistance gene from potato;
(iv) a pathogen resistance gene from Arabidopsis;
(v) a pathogen resistance gene from flax;
(vi) a nucleotide sequence encoding a CaMV gene VI protein;
(vii) a nucleotide sequence encoding a bacterial harpin gene protein;
(viii) a nucleotide sequence encoding a ubiquitin conjugating enzyme;
(ix) a nucleotide sequence encoding an RNase;
(x) a nucleotide sequence encoding a toxic peptide;
(xi) a mutant, variant, derivative or homologue of any of (i) to (x);
39. A cell according to claim 38 wherein said pathogen resistance gene from tomato is selected from *Cladosporium fulvum* resistance genes including *Cf-2, Cf-4, Cf-5* and *Cf-9;* said pathogen resistance gene from tobacco is *N*; said pathogen resistance gene from potato is *Nx;* said pathogen resistance gene from *Arabidopsis* is *RPP5* or *RP52;* said pathogen resistance gene from flax is *L6;* said RNase is Barnase; or said toxic peptide is diphtheria toxin.

40. A cell according to claim 36 wherein said L gene is:

(i) a *Cladosporium fulvum* avirulence gene or another elicitor of a resistance gene for a *Cladosporium fulvum* avirulence gene;

(ii) a suitable TMV coat protein or another *N* elicitor;

(iii) a suitable PVX coat protein or another Rx elicitor; or

(iv) a mutant, variant, derivative or homologue of any of (i) to (iii).

41. A cell according to claim 40 wherein said *Cladosporium fulvum* avirulence gene is *Avr2, Avr4, Avr5* or *Avr9.*

42. A cell according to any one of claims 33 to 41 which is a microbial cell.
43. A cell according to any one of claims 33 to 41 which is a plant cell.

44. A plant or any part or propagule or derivative thereof comprising a cell according to claim 43.

45. A plant, part, propagule or derivative according to claim 44 which is variegated for cells wherein said nucleotide sequence is inactivated or activated.

46. A method of producing a cell according to any one of claims 33 to 45 comprising introduction of nucleic acid (i) and/or (ii) into the cell or an ancestor thereof.

47. A composition of matter comprising any of the following combinations of nucleotide sequences:

(i) a nucleotide sequence comprising R, a nucleotide sequence comprising I and a nucleotide sequence comprising A;

(ii) a nucleotide sequence comprising R, and a nucleotide sequence comprising I and A;

(iii) a nucleotide sequence comprising I, and a nucleotide sequence comprising A and R;

(iv) a nucleotide sequence comprising A, and a nucleotide sequence comprising R and I; and

(v) a nucleotide sequence comprising R, I and A;

wherein R encodes a substance whose presence in a plant
results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and A encodes a substance able to reactivate R inactivated by I.

48. A composition of matter comprising any of the following combinations of nucleotide sequences:
   (i) a nucleotide sequence comprising R, a nucleotide sequence comprising L, a nucleotide sequence comprising I, and a nucleotide sequence comprising A;
   (ii) a nucleotide sequence comprising R, a nucleotide sequence comprising L and I, and a nucleotide sequence comprising (A);
   (iii) a nucleotide sequence comprising R, a nucleotide sequence comprising L and A, and a nucleotide sequence comprising I;
   (iv) a nucleotide sequence comprising R, a nucleotide sequence comprising I and A, and a nucleotide sequence comprising L;
   (v) a nucleotide sequence comprising L, a nucleotide sequence comprising I and R, and a nucleotide sequence comprising A;
   (vi) a nucleotide sequence comprising L, a nucleotide sequence comprising A and R, and a nucleotide sequence comprising I;
   (vii) a nucleotide sequence comprising I, a nucleotide sequence comprising L and R, and a nucleotide sequence comprising A;
(viii) a nucleotide sequence comprising R, and a nucleotide sequence comprising L, I and A;
(ix) a nucleotide sequence comprising L, and a nucleotide sequence comprising I, A and R;
(x) a nucleotide sequence comprising I, and a nucleotide sequence comprising A, R and L;
(xi) a nucleotide sequence comprising A and a nucleotide sequence comprising A, R and I; and
(xii) a nucleotide sequence comprising R, L, I and A;
wherein R and L encode substances whose presence together in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and/or L and A encodes a substance able to reactivate R and/or L inactivated by I.

49. A composition of matter according to claim 47 or 48 which is one or more nucleic acid vectors.

50. A composition of matter according to any one of claims 47 to 49 wherein a cell contains any of said combinations of nucleotide sequences.

51. A plant, or a part, propagule, derivative or descendant thereof, comprising a cell according to the composition of claim 50.

52. A method of producing a plant, or a part,
propagule, derivative or descendant thereof, containing nucleic acid comprising a nucleotide sequence or nucleotide sequences encoding R, I and A, wherein R encodes a substance whose presence in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and A encodes a substance able to reactivate R inactivated by I, comprising crossing plant lines whose genomes comprise any of R, I, A and combinations thereof, to produce the plant or an ancestor thereof.

53. A method according to claim 52 wherein one or more of said plant lines contains nucleic acid comprising any of R, I, A and combinations thereof as a result of transformation of cells of the plant or an ancestor thereof.

54. A method of producing a plant, or a part, propagule, derivative or descendant thereof, containing nucleic acid comprising a nucleotide sequence or nucleotide sequences encoding R, L, I and A, wherein R and L encode substances whose presence together in a plant results in a plant defence response, necrosis and/or increased pathogen resistance, I is a genetic insert able to inactivate R and/or L and A encodes a substance able to reactivate R and/or L inactivated by I, comprising crossing plant lines whose genomes
comprise any of R, L, I, A and combinations thereof, to produce the plant or an ancestor thereof.

55. A method according to claim 54 wherein one or more of said plant lines contains nucleic acid comprising any of R, L, I, A and combinations thereof as a result of transformation of cells of the plant or an ancestor thereof.

56. A plant, or a part, propagule, derivative or descendant thereof, obtainable using a method according to any one of claims 52 to 55.
FIGURE 2
<table>
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<th>Probe</th>
<th>Cf0</th>
<th>Mutant 18</th>
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<td>GAR-</td>
<td>GAR+</td>
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<td>GUS-</td>
<td>GUS+</td>
<td>GUS+</td>
<td>GUS+ N</td>
</tr>
</tbody>
</table>

Basic
β-1,3 glucanase

Anionic peroxidase
pTap 4.5
\[
\begin{array}{c}
\text{Ds} \\
\downarrow \\
+ \\
\text{Cf-9} \\
+ \\
\text{Avr9} \\
+ \\
\text{sAc} \\
X \\
\begin{array}{c}
\text{sAc} \\
+ \\
\text{Cf-9} \\
+ \\
\text{Avr9} \\
+ \\
\text{GAR}^+ \\
\text{sAc} \\
+ \\
\text{Cf-9} \\
+ \\
\text{Avr9} \\
+ \\
\text{Somatic necrotic sectors} \\
\text{GUS}^+ \\
\text{sAc} \\
+ \\
\text{Cf-9} \\
+ \\
\text{Avr9} \\
+ \\
\text{GUS}^- \\
\end{array}
\end{array}
\]
FIGURE 14

A. \[ T_1 \times T_2 \quad T_3 \times T_4 \]
   \[ \downarrow \quad \downarrow \quad \downarrow \]
   \[ P_{1,2} \quad x \quad P_{3,4} \]
   \[ \downarrow \quad \downarrow \]
   \[ P_{1,2,3,4} \]

B. \[ T_{1,2} \times T_3 \]
   \[ \downarrow \quad \downarrow \]
   \[ P_{1,2,3} \quad x \quad T_4 \]
   \[ \downarrow \quad \downarrow \]
   \[ P_{1,2,3,4} \]

C. \[ T_{1,2} \times T_{3,4} \quad T_{1,2,3} \times T_4 \]
   \[ \downarrow \quad \downarrow \]
   \[ P_{1,2,3,4} \quad P_{1,2,3,4} \]

D. \[ T_{1,2,3,4} \]
   \[ \downarrow \quad \text{X} \]
   \[ P_{1,2,3,4} \]