Pathogen- or Elicitor-Inducible Transcription Regulatory Element from the Tobacco 5-EPI-Aristolochene Synthase Gene and Plants Transformed Therewith

Joseph Chappell  
*University of Kentucky, chappell@uky.edu*

Shaohui Yin  
*University of Kentucky*

Catherine Cornett  
*University of Kentucky*

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PATHOGEN- OR ELICITOR-INDUCIBLE TRANSCRIPTION REGULATORY ELEMENT FROM THE TOBACCO 5-EPI-ARISTOLOCHENE SYNTHASE GENE AND PLANTS TRANSFORMED THEREWITH

Inventors: Joseph Chappell, Lexington, KY (US); Shaohui Yin, Ardmore, OK (US); Catherine Cornett, Florence, KY (US)

Assignee: University of Kentucky Research Foundation, Lexington, KY (US)

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This patent is subject to a terminal disclaimer.

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Continuation of application No. 08/443,639, filed on May 18, 1995, now Pat. No. 5,981,843.

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ABSTRACT
A tobacco epi-5-aristolochene synthase transcriptional regulatory element functional in plants, plant tissue and in plant cells for pathogen inducible gene expression and a method for increasing the transcriptional expression of downstream genetic information in plants, plant tissue and plant cells are disclosed.

11 Claims, 4 Drawing Sheets
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* cited by examiner
Fig. 1

GUS Activity

(fluorescence units/mg prot·h)

-563 -262 -202 -110 -48 -489 -217 control

EAS-4 EAS-3 35S-CAMV

WITHOUT ELICITOR

WITH ELICITOR

3500
3000
2500
2000
1500
1000
500
0
GUS EXPRESSION
CONTROL
INDUCED

GUS Activity (fluorescence units/mg prot hr)
234
1803

Not Determined
65
23
492
256

Fig. 2A

GUS reporter gene

ATG
+1

+67

-266

EAS4

-567

GUS reporter gene

-212

EAS4

-160

EAS4

GUS reporter gene
Fig. 2B

GUS EXPRESSION
CONTROL

INDUCED

GUS Activity

(ng fluorescence units/mg prot. hr)

-90

35S-CMV

minimal promoter

ATG

+8

10 bp

GUS reporter gene

-187

-256 EAS4

-160 EAS4

-87

-48

GUS reporter gene

-48

10 bp

GUS reporter gene

-12

10 bp
isolation of parA1 gene
-isolation of genomic DNA
-PCR primer design

PCR cloning

BamH1
SstI

parA1

kan^r

pEAS4 600 -GUS-
pBl 101

(pBl 101 a binary transformation vector)
PstI/SstI

BamH1
Cyclase promoter
GUS reporter gene

kan^r

gEAS4 600-parA1-
pBl 101

PstI/SstI

BamH1
Cyclase promoter
parA1

Transformation and regeneration of transgenic tobaccos

Fig. 3
PATHOGEN- OR ELICITOR-INDUCIBLE TRANSCRIPTION REGULATORY ELEMENT FROM THE TOBACCO 5-EPI-ARISTOLOCHENE SYNTHASE GENE AND PLANTS TRANSFORMED THEREWITH

This application is a continuation of Ser. No. 08/443,639, now U.S. Pat. No. 5,981,843, which was filed on May 18, 1995, the disclosure of which is hereby incorporated by reference.

This invention was made, at least in part, with funding from the National Science Foundation and the United States Department of Agriculture. Accordingly, the United States Government may have certain rights in this invention.

THE FIELD OF THE INVENTION

The field of this invention is the area of plant molecular biology, and it relates in particular to transcription regulatory elements: a qualitative regulatory sequence which positively regulates downstream gene expression in plant tissue in response to the stress of an invading microbial pathogen, an elicitor, or other inducing chemical signals and quantitative regulatory sequences which increase the transcriptional expression of associated sequences.

THE BACKGROUND OF THE INVENTION

In plants, disease resistance to fungal, bacterial and viral pathogens is associated with a plant response termed the hypersensitivity response (HR). In the HR, the site in the plant where the potential phytophytopathogen invades undergoes localized cell death, and it is postulated that this localized plant cell death aspect of the HR contains the invading microorganism or virus, thereby protecting the remainder of the plant. Other plant defenses include the production of phytoalexins (antibiotics) and/or lytic enzymes capable of averting pathogen ingress and/or cell wall modifications which strengthen the plant cell wall against physical and/or enzymatic attack.

The HR of plants, including tobacco, can include phytoalexin production as part of the response to invading microorganisms. One class of compounds made by tobacco (Nicotiana tabacum) in response to microbial invaders are the sesquiterpenes.

Cell suspension cultures have provided useful information regarding the regulation of terpene synthesis. Iso-preneoids are ubiquitous in nature, and the early portions of the biosynthetic pathway are shared with the biosynthetic pathway for other isoprenoid compounds such as sterols, carotenoids, dolichol and ubiquinone and growth regulators (e.g., gibberellic acid), which are classified as primary metabolites. Isoprenoid compounds classified as secondary metabolites are not essential for growth, and include mono-, sesquis- and diterpenoids. These secondary metabolite isoprenoids are important mediators of interactions between the plant and its environment.

A variety of compositions can serve as elicitors of plant phytoalexin synthesis. These include, but are not limited to, one or more toxic ions, e.g., mercuric ions, other chemically defined compositions, metabolic inhibitors, cell wall glycans, certain glycoproteins, certain enzymes, fungal spores, chitosans, certain fatty acids and certain oligosaccharides derived from plant cell walls [See, e.g., Sequeira, L. (1983) Annu. Rev. Microbiol. 37:51–79 and references cited therein]. Epi-5-aristolochene synthase (EAS) activity in tobacco plants has been shown to be induced by cell wall fragments of certain Phytophthora species and by Tricho-

derma reesei cellulase but not Aspergillus japonicus pectolyase [Chappell et al. (1991) Plant Physiol. 97:693–698]. Attack by other plant pathogens or an avirulent related strain can also induce phytoalexin synthesis; for example, 

Elicitors are proteins which are produced by plant pathogens and potential plant pathogens, which proteins induce the HR in tobacco plants. Amino acid and nucleotide coding sequences for an elicitor of Phytophthora parasitica have been published [Kamoun et al. (1993) Mol. Plant-Microbe Interactions 6:573–581]. Plant pathogenic viruses including, but not limited to, Tobacco Mosaic Virus (TMV) induce the HR in infected plants. Bacteria which infect plants also can induce HR and thereby disease resistance; representative bacteria eliciting HR include, but are not limited to, Agrobacterium species, Xanthomonas species and Pseudomonas syringae. Plant pathogenic fungi (and certain avirulent strains as well) also induce the HR response, where these include, but are not limited to, Phytophthora parasitica and Peronospora tabaci.

When tobacco cell suspension cultures are treated with an elicitor, squalene synthase is suppressed, thus stopping the flow of common biosynthetic precursors into sterols. The concomitant induction of sesquiterpene cyclase gene expression causes the flow of precursors in sesquiterpenes. The first step in the pathway from farnesyl diphosphate to the sesquiterpene phytoalexin capsidiol in elicitor-induced tobacco tissue is catalyzed by 5-epi-aristolochene synthase (EAS), a sesquiterpene cyclase. The coding sequence and deduced amino acid sequence for one member of the EAS gene family of tobacco have been published [Facchini and Chappell (1992) Proc. Natl. Acad. Sci. USA 89:11088–11092]. The transcriptional expression of one or more members of the EAS gene family is induced in response to elicitors.

There is a long felt need in the art for methods of protecting plants, particularly crop plants, from infection by plant pathogens, including but not limited to, phytopathogenic viruses, fungi and/or bacteria. Especially important from the standpoint of economics and environmental concerns are biological or "natural" methods rather than those which depend on the application of chemicals to crop plants. There is also a long felt need in the art for plant transcriptional regulatory sequences for use in controlling the expression of heterologous DNA sequences in transgenic plants.

SUMMARY OF THE INVENTION

The present invention provides qualitative transcriptional regulatory sequences which regulate downstream gene expression in plant tissue in response to one or more elicitors, other defined inducing compounds or in response to the stress of an invading phytopathogen (the inducible transcription regulatory sequence) and quantitative transcription regulatory sequences which increase the transcription of downstream sequences (the transcription-enhancing sequence). As specifically exemplified herein, these transcriptional regulatory sequences are found in nature upstream and operatively linked to the epi-5-aristolochene synthase gene (EAS4) of tobacco; when operatively linked to a coding sequence (and in the presence of an operatively linked promoter element, from the EAS4 gene or from a heterologous plant-expressible gene) these sequences mediate the inducible transcriptional expression of that coding sequence when the plant or plant tissue is invaded by a
potential phytopathogen (virus, fungus or bacterium) or in response to elicitors such as Trichoderma viride cellulase or plant or fungal cell wall fragments for plants, plant tissue and/or plant suspension culture cells. That potential plant pathogen can be a virus including, but not limited to, tobacco mosaic virus or tobacco vein mottled virus, a bacterium including, but not limited to, Pseudomonas syringae, Xanthomonas campestris or Agrobacterium tumefaciens, or a fungus including, but not limited to, a species of Phytophthora (e.g., P. parasitica) or Peronospora (e.g., P. tabaci). The EAS4 promoter comprising the inducible transcription regulatory element(s) and the transcription-enhancing sequence(s) are disclosed herein as SEQ ID NO:2. In SEQ ID NO:2, the CAAT-homologous sequence of the EAS4 promoter is located at nucleotides 513 to 516, and the TATA-sequence motif is located at nucleotides 540 to 543.

The minimal inducible transcriptional regulatory element within the N. tabacum EAS4 upstream sequence is from nucleotide 458 to nucleotide 475 of SEQ ID NO:2; optionally from 454 to 473; more preferably from nucleotide 413 to 473 in SEQ ID NO:2 provides the inducible transcriptional regulatory element sequences.

Another aspect of the present invention is the transcription-enhancing element derived from the EAS4 promoter and promoter-associated sequences. When operatively linked upstream of a promoter, particularly upstream of a minimal promoter, this element increases the transcriptional expression of the downstream sequences. Transcriptional enhancing activity is mediated by DNA sequence information in the region between nucleotides 371 and 463 in SEQ ID NO:2. Preferably, an EAS4-derived transcription-enhancing sequence comprises a nucleotide sequence as given in SEQ ID NO:2 from nucleotide 371 to nucleotide 463, more preferably from nucleotide 1 to nucleotide 463, and optionally from nucleotide 1 to about nucleotide 1040 of SEQ ID NO:7.

Also provided by the present invention is an expression cassette into which a coding sequence of interest can be cloned, and said coding sequence of interest can be expressed in plant tissue after the introduction of the unit into plant tissue. A preferred coding sequence of interest is that for the ParA1 elicitor protein of Phytophthora parasitica. The coding sequence and deduced amino acid sequence for the ParA1 protein, including the signal peptide, are given in SEQ ID NOs:16 and 17, respectively. The coding sequence and deduced amino acid sequences of the nature ParA1 protein as amplified and described hereinbelow are given in SEQ ID NOs:18 and 19, respectively. Transcription is regulated by the EAS4 promoter sequence and promoter-associated regulatory element or by at least one of the transcription regulatory elements (inducible and/or transcription-enhancing) operably linked to transcription initiation sequences and a heterologous DNA sequence to be expressed.

A further aspect of the present invention are transgenic plant cells, plant tissue and plants which have been genetically engineered to contain and express a nucleotide sequence of interest, preferably a coding sequence, an antisense sequence or other sequence, under the regulatory control of the inducible transcription regulatory element. It is an object of this invention to provide the nucleotide sequences which mediate the induction of the expression of a downstream coding sequence in response to elicitor exposure, potential phytopathogen invasion in a plant, or certain other exogenous inducing signals such as exposure to methyl jasmonate and ethylene. An exemplary elicitor inducible transcription regulatory element is that from the 5′ flanking region of the EAS4 gene of Nicotiana tabacum; as specifically exemplified herein, this sequence is presented in SEQ ID NO:2 from nucleotide 410 to nucleotide 472. Equivalents of the exemplified nucleotide sequence are those nucleotide sequences which similarly direct the induction of the expression of downstream nucleotide sequences. Preferably the inducible transcription regulatory element is associated with EAS4 promoter and promoter-associated sequences (e.g., the combination having the nucleotide sequence as given in SEQ ID NO:2 from nucleotide 410 to nucleotide 573 of SEQ ID NO:2, preferably from nucleotide 361 to 573 of SEQ ID NO:2, more preferably the sequence from 1 to 573 of SEQ ID NO:2).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 presents data for transient expression experiments for GUS regulated by the EAS3 and EAS4 promoter region sequences in comparison to a CaMV 35S-GUS construct (Cauliflower Mosaic Virus 35S promoter—β-glucuronidase reporter gene). These experiments were carried out in tobacco protoplasts into which the DNA constructs had been electroporated. “Uninduced” levels of expression for the EAS-GUS constructs were relatively high because fungal enzymes which digest plant cell walls were used in the preparation of the protoplasts. The y-axis shows units of GUS activity and the 5′ extents of the EAS upstream sequences are given below each bar on the graph. As above, the numbering is relative to the natural transcription start site of EAS4 (or the corresponding base of EAS3); in EAS4 (SEQ ID NO:2) the start site is at nucleotide 573; in EAS3 (SEQ ID NO:1) the potential transcription start site is at nucleotide 489.

FIGS. 2A–2B present information concerning the reporter gene expression directed by the instant inducible transcription regulatory element. FIG. 2A presents a schematic of experiments carried out with the EAS4 promoter region controlling the expression of the GUS reporter gene in stably transformed transgenic plants. FIG. 2B presents the results of “gain of function” assays for the EAS4 promoter-associated sequence-regulating expression of the GUS reporter gene via the CaMV 35S minimal promoter. In both FIGS. 2A and 2B, numbering for the EAS4 upstream region reflect numbering relative to the natural transcription start site of the EAS4 gene (See also SEQ ID NO:2 wherein the transcription start site corresponds to nucleotide 573). In both FIGS. 2A and 2B, the GUS expression is measured in Fluorescence units per milligram protein.

FIG. 3 is a schematic of the genetic manipulation leading to generation of disease-resistant plants. The coding sequence for the ParA1 elicitor is isolated by PCR so as to have BamHI and SstI ends. gEAS4,5′-GUS-pBI101, which directs the expression of the GUS reporter gene under the regulatory control of the EAS promoter, is digested with BamHI and SstI to release the GUS reporter gene. Then the BamHI/SstI-digested parA1 amplifier is ligated to the large fragment produced after digestion of gEAS4,5′-GUS-pBI101 to produce gEAS4,5′-parA1-pBI101, from which the ParA1 elicitor in plant cells or plant tissue is synthesized after induction with a suitable elicitor.

DETAILED DESCRIPTION OF THE INVENTION

5-epi-aristolochene synthase (EAS) is a key enzyme in the synthesis of sesquiterpenoid phytoalexins, for example, in solanaceous plants, including but not limited to Nicotiana species (e.g., tabacum), Capsicum annum and Hyoscyamus
muticus. EAS catalyzes the reaction of farnesyl diphosphate to (+) gemacrene A to eudesmane carbocation to Sepi- aristolochene. Other plants, such as the crucifers, also have sesquiterpene cyclase enzymes.

Treatments which induce host defensive responses in plant tissue or plant cells such as phytoalexin synthesis include cell wall fragments of Phytophthora species and Trichoderma viride cellulture. However, pectolyase from Aspergillus japonicus does not function as an elicitor in tobacco cell culture. Elicitors which induce sesquiterpenoid phytoalexin synthesis have been shown to function at the level of controlling transcription of key biosynthetic enzymes [Vogeli and Chappell (1990) Plant Physiol. 94:1860–1866]. Similar patterns have been observed in other plants, but no transcriptional control sequences which mediate gene induction in response to phytopathogen challenge have been described.

Tobacco (N. tabacum) contains an EAS gene family with some 12–15 members, the coding sequence of EAS4 has been published [Facchini and Chappell (1992) supra]. However, since that time, the present inventors have discovered that the EAS3 does not appear to be expressed in response to the elicitor treatment, and surprisingly, the nucleotide sequences upstream of EAS3 do not appear to mediate the induction of a reporter gene in a chimeric gene construct in elicitor-induced transgenic cell culture. It is noted that the translation start site was incorrectly identified in the 1992 Facchini and Chappell publication. The nucleotide sequence of a genomic clone of EAS4, as it appears in Facchini and Chappell (1992) supra is presented in SEQ ID NO:7, with the deduced amino acid sequence being given in SEQ ID NO:8.

Other aspects of a plant’s defenses against invasion and infection by a phytopathogenic microorganism include the hypersensitive response, which is characterized by necrosis, i.e., programmed cell death in the localized area of attack by the plant pathogen. Elicitor treatments as described above can also induce the necrotic response.

Elicitins are proteins produced by fungal plant pathogens, which proteins elicit a hypersensitive response in an infected plant. Generally, but not necessarily, localized cell death is the result of the elicitin-induced infection in the (infected or challenged) plant tissue. These responses mediate full or partial resistance to destructive infection by the invading, potentially plant pathogenic microorganism. For the purposes of the present invention, a protein of a plant pathogen or potential plant pathogen which induces the hypersensitive response in plant tissue after infection of that plant tissue or after expression of that coding sequence in the plant tissue is considered to fall within a broad definition of an elicitin.


It is understood that it is possible to use in the present invention as it applies to creating transgenic plants with improved disease resistance traits using an elicitin coding sequence expressed under the regulatory control of a pathogen-response transcription regulatory element (and with a minimal promoter functional in those plants) that elicitin protein must be capable of promoting expression of defense genes (including but not limited to those genes governing phytoalexin synthesis, the hypersensitive response and/or localized necrosis) in those plants. Many functional combinations of plant and phytopathogen are known to the art, and the skilled artisan knows how to test the functioning of a particular elicitin in a particular plant tissue (or cells) in the turning on of programmed cell death or phytoalexin synthesis or the like. It is also understood that treatment of plant cells or tissue with compositions such as certain fungal cellulases or certain plant polysaccharide fragments can also induce the host defensive (i.e., hypersensitive) response. Such treatments are used as models for actual plant pathogen attack or invasion.

A non-naturally occurring recombinant nucleic acid molecule, e.g., a recombinant DNA molecule, is one which does not naturally occur in nature, i.e., it is produced either by natural processes using methods known to the art, but is directed by man to produce a desired result or it has been artificially produced from parts derived from heterologous sources, which parts may be naturally occurring or chemically synthesized molecules or portions thereof, and wherein those parts have been joined by ligation or other means known to the art.

A transgenic plant is one which has been genetically modified to contain and express heterologous DNA sequences, either as regulatory RNA molecules or as proteins. As specifically exemplified herein, a transgenic plant is genetically modified to contain and express a heterologous DNA sequence operably linked to and under the regulatory control of transcriptional control sequences by which it is not normally regulated, i.e., under the regulatory control of the inducible transcriptional control sequences of the EAS4 gene of Nicotiana tabacum. As used herein, a transgenic plant also refers to those progeny of the initial transgenic plant which carry and are capable of expressing the heterologous coding sequence under the regulatory control of both qualitative and/or quantitative transcription control sequences described herein. Seeds containing transgenic embryos and/or plantlets are encompassed within this definition.

When production of a heterologous gene or coding sequence of interest is desired under conditions of potential
pathogen invasion or inducer (e.g., elicitor) treatment, that coding sequence is operably linked in the sense orientation to a suitable promoter and under the regulatory control of the inducible regulatory sequences, in the same orientation as the promoter, so that a sense (i.e., functional for translational expression) mRNA is produced. A transcription termination signal functional in a plant cell can be placed downstream of the coding sequence, and a selectable marker which can be expressed in a plant can, be covalently linked to the inducible expression unit so that after this DNA molecule is introduced into a plant cell or tissue, its presence can be selected and plant cells or tissue not so transformed will be killed or prevented from growing. Similarly, a heterologous coding sequence can be expressed under the regulatory control of the inducible transcription regulatory element or the transcription-enhancing element in transgenic plant cell suspension culture, with induction occurring in response to the addition of an elicitor to the cell culture medium.

Where inhibition of gene expression is desired in a plant being invaded by a microbial pathogen, such as a phytopathogenic fungus, then either a portion or all of that coding sequence or cDNA sequence can be operably linked to a promoter functional in plant cells, but with the orientation of the coding sequence opposite to that of the promoter (i.e., in the antisense orientation) so that the transcripted RNA is complementary in sequence to the mRNA, and so that the expression of the antisense molecule is induced in response to pathogen invasion. In addition, there may be a transcriptional termination signal downstream of the nucleotides directing synthesis of the antisense RNA.

The present inventors have isolated a DNA sequence which mediates the inducible expression of a downstream gene in plant cells in response to invasion by a potential plant pathogen and/or treatment with an elicitor or other chemical signals. For example, a combination of ethylene and methyl jasmonate serve to induce downstream gene expression via the qualitative transcription regulatory sequence. It is understood that there may be a multiplicity of sequence motifs within that regulatory sequence, where individual motifs each respond to one or more distinct environmental signal. As specifically exemplified, this transcription-regulating sequence is derived from the EAS4 locus of *N. tabacum*, and it is given in SEQ ID NO:7. The deduced amino acid sequence for the EAS protein is given in SEQ ID NO:8. The open reading frame of the EAS4 gene, which is interrupted by six introns, is provided in SEQ ID NO:7.

A computer search for nucleotide sequence homology in sequences in GenBank to the SEQ ID NO:2 sequence revealed no known nucleotide sequences with significant homology, except for the CAAT and TATA transcriptional control sequences.

Organization of the EAS genes in the *N. tabacum* genome was described in Facchini and Chappell (1992) supra using an EAS probe and Southern hybridization experiments. Under conditions of high stringency, multiple fragments hybridized with analysis indicating that there is a gene family with some 12–16 members in the tobacco genome. In these experiments, however, the probe included the EAS coding sequence rather than the promoter and promoter-associated regulatory sequences.

EAS homologous genes can be identified and isolated from plant species other than *N. tabacum* based on significant degrees of nucleotide sequence homology; i.e., DNA–DNA hybridization under conditions of moderate to high stringency with a tobacco EAS coding sequence probe allows the identification of the corresponding gene from other plant species. A discussion of hybridization conditions can be found for example, in Hames and Higgins (1985) *Nucleic Acid Hybridization*, IRL Press, Oxford, U.K. Generally, sequences which have at least about 70% nucleotide sequence homology can be identified by hybridization under conditions of moderate stringency. Under such conditions, it is generally preferred that a probe of at least 100 bases be used. Most preferably, in the present case, the probe will be derived from the coding portion of the EAS4 coding sequence. Labels for hybridization probes can include, but are not limited to, radioactive groups, fluorescent groups and ligands such as biotin to which specific binding partners (which are in turn labeled) bind. It is the label which allows detection of the hybridization probe to the target nucleic acid molecule. Alternatively, well-known and widely accessible polymerase chain reaction (PCR) technology is advantageously used to amplify sequences with significant nucleotide sequence homology to a target sequence.

It is understood that nucleic acid sequences other than the EAS coding sequence disclosed in SEQ ID NO:7 will function as coding sequences with the amplified EAS4 coding sequence. Nucleic acid sequences are synonymous if the amino acid sequences encoded by those nucleic acid sequences are the same. The degeneracy of the generic code is well-known to the art; i.e., for many amino acids, there is more than one nucleotide triplet which serves as the code for the amino acid. It is also well-known in the biological arts that certain amino acid substitutions can be made in protein sequences without affecting the function of the protein. Generally, conservative amino acid substitutions or substitutions of similar amino acids are tolerated without affecting protein function. Similar amino acids can be those that are similar in size and/or charge properties, for example, aspartate and glutamate and isoleucine and valine are both pairs of similar amino acids. Similarity between amino acid pairs has been assessed in the art in a number of ways. For example, Dayhoff et al. (1978) in *Atlas of Protein Sequence and Structure*, Vol. 5, Suppl. 3, pp. 345–352, which is incorporated by reference herein, provides frequency tables for amino acid substitutions which can be employed as a measure of amino acid similarity. Dayhoff et al.’s frequency tables are based on comparisons of amino acid sequences for proteins having the same function from a variety of evolutionarily different sources.

Terpene cyclase genes can be found in solanaceous plants, including *N. tabacum* and *Hyoscyamus muticus*, as disclosed herein, and in members of the mint family (Labiatae) and the Euphorbiaceae, including but not limited to those which have been demonstrated to contain sequences of significant homology, and in substantially all plants. Preferably, EAS4 homologs will be selected from the Solanaceae. Such sequences can be identified by nucleic acid hybridization experiments or when cloned in expression vectors, by cross reaction to tobacco EAS-specific antibody, or any other means known to the art, including the use of PCR technology carried out using oligonucleotides corresponding to portions of SEQ ID NO:7, preferably in the region encoding EAS. Antibody can be prepared after immunizing an experimental animal with EAS purified as described in Vogeli et al. (1990) *Plant Physiology* 93:182–187 or using a peptide conjugate, where the amino acid sequence of the peptide is taken from a hydrophilic portion of the EAS amino acid sequence (SEQ ID NO:8). Monoclonal and polyclonal antibody production techniques are readily accessible to the art (See, e.g., Campbell (1994) *Monoclonal Antibody Technology*, Laboratory Techniques in Biochemistry and Molecular
Inclusion of additional 5' flanking sequence from the EAS4 gene allows for increased levels of downstream gene expression. Preferred is the use of a sequence including the +266 to +1 region of EAS4 (nucleotides 307 to 573 of SEQ ID NO:2), and more preferred is the sequence including +567 to +1 (nucleotides ▲ to 573 of SEQ ID NO:2).

An alternative to the use of the fusion of the EAS4 transcription regulatory sequence fused to a heterologous minimal promoter is the use of the promoter region of EAS4 in conjunction with the upstream promoter-associated regulatory elements. In such an application the use of nucleotides 307 to 463, or more preferably for greater levels of downstream expression, nucleotides 371 to 463, 311 to 462, and 10 to 573 of SEQ ID NO:2.

In a plant such as N. tabacum, the inducible transcription regulatory element directs the induction of downstream gene expression in response to invading plant pathogens and certain compositions such as some fungal cell walls and certain plant and fungal cell wall fragments. Plant pathogens which can trigger this expression include, but are not limited to, Xanthomonas, Pseudomonas syringae, Phytophthora species including parasitica, and Peronospora species (e.g., tabaci).

Coding sequences suitable for expression in a plant are operably linked downstream of the regulated promoter construct. Transgenic plants can be constructed using the chimeric gene consisting essentially of the regulated promoter, any additional transcription-enhancing sequences, and the desired coding sequence including the necessary sequence signals for its translation. Where disease resistance is to be additionally induced in response to invasion of a transgenic plant tissue by a potential plant pathogen or in response to treatment with an elicitor or other chemical signal which induces EAS4 gene expression, the coding sequence is preferably for an elicitor of a plant pathogenic microorganism, e.g., the parA1 gene product of Phytophthora parasitica (as described in Kamoun et al. (1993) supra). Other elicitor-like proteins have been described in the readily available scientific literature, and include those from Phytophthora species, Peronospora species, and Xanthomonas species, among others.

Alternative coding sequences which can be expressed under the regulatory control of the present inducible transcription regulatory element for improvement of the resistance of a (transgenic) plant or plant tissue exposed to a viral, bacterial or fungal plant pathogen include, but are not limited to, chitinase, TMV coat protein or other plant virus coat protein, Nla virus gene and others.

Additionally, or alternatively, induction of the regulated construct can be induced, for example, by treating the transgenic plant or tissue with an elicitor or with a bacterium, virus or fungus (preferably not pathogenic for the host plant) capable of inducing expression via the inducible transcription regulatory element of a coding sequence not capable of turning on the HR, or disease resistance directly could be achieved. Coding sequences which may be advantageously expressed include an insecticidal protein, such as one of the Bacillus thuringiensis crystal proteins, which when expressed would protect the plant from insect pests.

Phytoalexin synthesis from the native EAS4 gene, or induction of gene expression mediated by the present regulated EAS4 promoter or the inducible transcription regulatory element in combination with at least a heterologous minimal promoter, can be induced by treating the plant tissue or cells with a wide variety of defined chemicals,
crude fungal culture filtrates, fungal cell wall extracts and oligosaccharides from plant or fungal cell walls [Albersheim and Valent (1978) J. Cell. Biol. 78:627–643]. Other compounds capable of inducing the HR include certain cellulases, for example, Trichoderma reesei or Trichoderma viride cellulases, and certain plant or fungal cell wall fragments, among others.


Techniques and agents for introducing and selecting for the presence of heterologous DNA in plant cells and/or tissue are well-known. Genetic markers allowing for the selection of heterologous DNA in plant cells are well-known, e.g., genes carrying resistance to an antibiotic such as kanamycin, hygromycin, gentamicin, or bleomycin. The marker allows for selection of successfully transformed plant cells growing in the medium containing the appropriate antibiotic because they will carry the corresponding resistance gene.

Other techniques for genetically engineering plant cells and/or tissue with an expression cassette comprising an inducible promoter or chimeric promoter fused to a heterologous coding sequence and a transcription termination sequence are to be introduced into the plant cell or tissue by Agrobacterium-mediated transformation, electroporation, microinjection, particle bombardment or other techniques known to the art. The expression cassette advantageously further contains a marker allowing selection of the heterologous DNA in the plant cell, e.g., a gene carrying resistance to an antibiotic such as kanamycin, hygromycin, gentamicin, or bleomycin.

The transcription regulatory sequences, particularly the inducible transcription regulatory element (or the EAS4 promoter with the inducible and preferably the transcription-enhancing element) is useful in controlling gene expression in transgenic plant cells in suspension cell culture as an alternative to expression in transgenic plants. For example, the EAS4 promoter including the transcription initiation signals, the inducible transcription regulatory element and the transcription-enhancing element, can be used to mediate the inducible expression of one or more heterologous coding sequence(s) in transgenic plant cells in suspension cell culture. When desired, expression of the coding sequence of interest is induced by the addition of an inducer or other inducing chemical signal to the culture. Suspension culture cells respond to elicitors readily in comparison to intact plants. The heterologous coding sequence(s) can encode proteins which mediate synthesis of pharmaceutical compounds, poly-β-hydroxybutyrate synthesis or other secondary metabolites, cellulose, starch, sugars, oils, or the heterologous sequences can encode pharmaceutical proteins, insecticidal toxin proteins, antifungal proteins, antiviral proteins such as coat proteins to mediate resistance to virus infection, the N1a protein, chitinases, glucanases, male sterility proteins or sequences, proteins to improve nutritional quality or content, or developmental and/or tissue-specific programs or patterns. It is understood that transgenic plants can be similarly used to express heterologous coding sequences as can transgenic plant cells.

Where transgenic plants are to be induced for phytoalexin synthesis or for the expression of a heterologous coding sequence under the regulatory control of the EAS4 promoter or the inducible transcription regulatory element derived therefrom and/or the transcription-enhancing sequence derived from the EAS4 promoter as well, the elicitor must penetrate the cuticle of the plant to have an inducive effect. Alternatively, the plant tissue can be wounded to facilitate or allow the uptake of the elicitor into the plant tissue. A wide variety of inducing compositions, including elicitors and other chemical signals, such as the combination of ethylene and methyl jasmonate, can be effectively introduced into the transgenic plant suspension cell cultures, where there is significantly less of a barrier to the uptake and/or sensing of the elicitors. Where ethylene is used at a concentration between about 1 and about 50 ppm and the methyl jasmonate is used at a concentration between about 0.1 mM and about 1 mM.


All references cited in the present application are expressly incorporated by reference herein.

The following examples are provided for illustrative purposes and are not intended to limit the scope of the invention as claimed herein. Any variation in the exemplified compositions and methods which occur to the skilled artisan are intended to fall within the scope of the present invention.

EXAMPLES

Example 1

EAS-Specific Antibodies

Monoclonal and polyclonal antibodies specific for tobacco EAS were prepared as described by Vogeli et al. (1990) Plant Physiology 93:182–187. Additional antibody preparations could be made as polyclonal antibodies using purified EAS as antigen or using a peptide sequence conjugated to a carrier protein using well-known techniques. The amino acid sequence of a peptide for antibody production is selected from a particularly hydrophilic region of the protein (For antibody production technique, see, for example, Campbell (1994) Monoclonal Antibody Technology, Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 13, Burdon and Knippenberg, eds, Elsevier, Amsterdam; Harlow and Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Example 2

DNA and Protein Sequence Determination

Sequence determinations of single-stranded and double-stranded DNAs were carried out by the dideoxynucleotide chain termination procedure [Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:8073–8077], with a Sequenase kit from United Biochemical Corp., Cleveland, Ohio) or an automated fluorescence-based system (Applied Biosystems, Foster City, Calif.).

Example 3

Construction of a Full-Length EAS Clone

Nicotiana tabacum L. cv. KY14 cell suspension cultures were treated with Trichoderma viride cellulase (Type RS, Onozuka) at a final concentration of 0.5 µg/ml during rapid growth phase to induce the expression of EAS. Parallel suspension cell cultures which did not receive cellulase served as controls. Cells were collected by gentle vacuum filtration 4 hrs after the addition of the cellulase elicitor to the induced culture.

A cDNA library was prepared in pDNAII (Invitrogen, San Diego, Calif.) from polyA+ RNA extracted from the N. tabacum cells treated for 4 hrs with elicitor. The library was screened by differential hybridization using polyA+ RNA prepared from the induced and control culture. Clones appearing to be positive were further screened by hybrid selection-in vitro translation-immunoprecipitation analysis as described by Alwine et al. (1979) Methods Enzymol. 68:220–242.

A putative positive EAS cDNA clone was used as a hybridization probe for the isolation of additional cDNA and genomic clones. The genomic library thus screened was one constructed in λEMBL3 using MboI partially digested DNA prepared from N. tabacum L cv. NK320 hypocotyl DNA (Clontech, Palo Alto, Calif.). This screening yielded 8 independent clones, each of which appeared to represent a different chromosomal locus. EAS4 and EAS3 genomic clones were described in Facchin and Chappell (1992) supra, but are now known to have been incomplete.

Facchin and Chappell (1992) supra had misidentified the translation start sites of the EAS3 and EAS4 coding sequences in the genomic clone described herein. The correct translation start site for the EAS3 and EAS4 coding sequences have been determined to be methionine codons 165 bp upstream of the ATG codons previously identified as start sites. The corrected start site for EAS4 was mapped using a combination of primer extension assays to identify the transcription start site and additional N-terminal amino acid sequencing data of purified enzyme as noted herein above.

An amplimer of 110 bp was prepared by a polymerase chain reaction to provide a DNA sequence corresponding amino acids 56–92 of the EAS4 protein (see SEQ ID NO:12) and Facchin and Chappell (1992) supra. This amplimer was used as a hybridization probe to screen a clone library in pDNAI (Invitrogen, San Diego, Calif.) prepared from polyA+ RNA from tobacco cell culture cells 4 hr after elicitor treatment (Trichoderma reesei cellulase). This amplimer was made using a sense primer (ATGCGTGTAGCAACCGGAGAG; SEQ ID NO:3) and a reverse primer (TCCAAAATCTCATCAATTC; SEQ ID NO:4), and the genomic EAS4 template in a standard PCR reaction [Salt et al. (1988) Science 239:487–491]. The 110 bp amplimer was isolated after polyacrylamide gel electrophoresis using DE-51 paper (Whatman International, Inc., Clifton, N.J.). The isolated fragment was then radiolabeled with [α-32P]-dCTP using a random priming kit from Stratagene (La Jolla, Calif.) for use as hybridization probe in colony lifts of the cDNA library as previously described [Hahnel and Meselson (1980) Gene 10:63–67]. The longest clone obtained in these experiments appeared to lack 80 bp of 5’ coding sequence.

To obtain a full-length clone, a RT/PCR approach was used. First strand cDNA was prepared from polyA+ RNA prepared from tobacco cells after induction with elicitor as described [Facchin and Chappell (1992) supra] using reverse primer having the sequence AAGATGTCCTTCATATGTA (SEQ ID NO:5). This sequence corresponds to nucleotides 489–477 downstream of the translation start site. The reverse transcriptase reaction was carried out in a 10 µl reaction (1 µg polyA+ RNA, 25 pmol reverse primer, 10 mM DTT, 2.5 mM each dATP, dGTP, dCTP, dTTP, 8 units RNase Block I (Stratagene, La Jolla, Calif.), first strand synthesis buffer used according to the manufacturer’s instructions (Stratagene) for 1 hr at 37° C. This reaction was terminated by treating at 99° C for 5 min. Then 40 µl of master PCR mix was added to the first strand reaction; PCR master mix contains 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% Tween-20, 0.01% (v/v) gelatin, 0.01% NP-40, 2.5 mM each deoxynucleotide triphosphate, 1 unit of Taq polymerase, and 25 pmol forward primer (GGGGATCGAAGTACGTAGCAACCGGAGAG).
The 492 bp reaction product was digested with EcoRI and HindIII and subcloned into similarly cut pBluescript SK (Stratagene). A HindIII/XhoI fragment from another partial cDNA clone was subsequently cloned into the corresponding sites of the S′-terminal sequence clone to generate a full-length cDNA clone named pBSK-TEAS. pBSK-TEAS DNA was transformed into Escherichia coli TB1 using a CaCl₂ protocol [Sambrook et al. (1989) supra]. Determination of the DNA sequence of the insert confirmed that this cDNA had the expected and desired structure (ideoxygenucleotide chain termination procedure, United States Biochemical Corp., Cleveland, Ohio).

Example 4
Identification of EAS Homologous Sequences

Tobacco leaf genomic DNA was isolated as described in Murray and Thompson (1980) Nucleic Acids Research 8:4321–4325. After digestion of aliquots with desired restriction enzymes, the digested DNA samples were electrophoresed on 0.8% agarose gels and the size-separated DNAs were transferred to nylon membranes. DNA blots were hybridized with random primer radiolabeled cEAS1, which is truncated at the 5′ end of the coding region, (prepared as in Sambrook et al. (1989) supra) at 60°C in 0.25 M sodium phosphate buffer, pH 8.0, 0.7% SDS, 1% bovine serum albumin, 1 mM EDTA. The blot was then washed twice at 45°C with 2x SSC, 0.1% SDS and twice with 0.2x SSC, 0.1% SDS (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Relative hybridization levels were estimated from autoradiograms using a video densitometer (MillGen/Biosearch, Ann Arbor, Mich.).

Facchini and Chappell (1992) supra reported that Southern hybridization results indicated that there were 12–16 copies of EAS-homologs in the N. tabacum genome. To address the presence of significantly homologous sequences to tobacco EAS and apparent number of copies per genome of those sequences, Southern hybridization experiments are carried out using DNA isolated from other plant species.

Restriction endonuclease-digested genomic DNAs are separated by agarose gel electrophoresis (0.8% agarose), and then transferred to a Hybond-N* membrane (Amersham Corp., Arlington Heights, Ill.). Radiolabeled probe comprising coding sequences of EAS, and hybridizations are carried out essentially as described in Sambrook et al. (1989) supra. Moderate stringency conditions are used (hybridization in 4x SSC, at 65°C; last wash in 1x SSC, at 65°C).

Alternatively, PCR can be carried out using target DNA as template and primers derived from the EAS4 coding sequence in highly conserved regions (see SEQ ID NO:7) using well-known techniques.

Example 5
Detection of EAS Protein


For detecting the presence of EAS cross-reacting protein material, total protein fractions are prepared from 100 μl aliquots of bacterial culture harvested and concentrated by centrifugation for 2 min in a microfuge. After discarding the culture supernatant, cell pellets are resuspended in 100 μl 50 mM Tris-HCl, pH 6.8, 10 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue, 10% glycerol. For immunological detection 15 μl aliquots are electrophoresed over 11.5% SDS-polyacrylamide gels; for Coomassie blue-staining of the proteins, 35 μl aliquots are similarly electrophoresed. For soluble protein samples, the cells are processed as in the procedure for determination of enzymatic activity (see Back et al. (1995) supra for Facchini and Chappell (1992) supra). For immunological detection 10 μl aliquots are electrophoresed as above; for Coomassie blue-staining, 10–50 μl aliquots were electrophoresed.

After electrophoresis the proteins are stained with Coomassie blue, or the proteins are transferred to nitrocellulose membranes as described [Towbin and Gordon (1984) Jurnal of Immunological Methods 72:313–340] for immunodetection. After incubating for 30 min in 5% low-fat milk in 1x TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl), the nitrocellulose blots were incubated overnight in the same solution containing monoclonal antibody specific for tobacco EAS (1:1000 dilution; Vogeli et al. (1990) Plant Physiology 93:182–187). Goat anti-mouse antibodies linked to alkaline phosphatase and the specific chromogenic dye were then incubated to visualize the binding of the EAS-specific antibody to the proteins immobilized on the nitrocellulose membranes [Leary et al. (1983) Proc. Natl. Acad. Sci. USA 80:4045–4049].

Example 6
Genomic EAS4 Clone

The 5′-truncated cDNA clone cEAS1 described in Facchini and Chappell (1992) supra was used as a hybridization probe for screening a N. tabacum cv. NK326 genomic library in the EMBL3 vector (Clontech, Palo Alto, Calif.). DNA sequences were determined using routine subcloning and DNA sequencing protocols.

The DNA and deduced amino acid sequences of the EAS4 genomic clone are presented in SEQ ID NO: 7–8.

Example 7
Generation of Transgenic Plants

For studies of the function of portions of the upstream untranslated region of the EAS4 gene, HindIII/BamHI-ended fragments of this upstream DNA were cloned into pH101 (Clontech, Palo Alto, Calif.) so that expression of the β-glucuronidase (GUS) reporter gene could be monitored in transformed plant cells. The 5′-flanking sequence of the EAS4 gene is given in SEQ ID NO:1 and the 5′-flanking sequence of the EAS4 gene is given in SEQ ID NO:2. In each of these sequences, the translation start site (ATG) is the last three nucleotides. By primer extension techniques, the EAS4 transcription start site was estimated at nucleotide 573 in SEQ ID NO:2. CAAT and TATA box motifs are identified at nucleotides 429 to 432 and at nucleotides 456 to 459 in SEQ ID NO:1 (EAS3) and at nucleotides 513 to 516 and at nucleotides 540 to 543 in SEQ ID NO:2 (EAS4). The transformed plant cell lines were produced using a modified Agrobacterium tumefaciens transformation protocol. The recombinant plasmids containing the sequences to be introduced into plant tissue were transferred into A. tumefaciens strain GV3850, by triparental mating with E. coli TB1 (pRK23). N. tabacum leaves at a variety of stages of growth were cut into 1 cm² pieces, and dipped in
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a suspension of agrobacterial cells (about \(10^{{10}}\) to \(10^{{15}}\) cells/ml). After 3 to 10 minutes, the leaf segments were then washed in sterile water to remove excess bacterial cells and to reduce problems with excess bacterial growth on the treated leaf segments. After a short drying time (30 to 60 seconds), the treated leaf segments are placed on the surface of Plant Tissue Culture Medium without antibiotics to promote tissue infection and DNA transfer from the bacteria to the plant tissue. Plant Tissue Culture Medium contains per liter: 4.51 g Murashige and Skoog Basal Salts Mixture (Sigma Chemical Company, St. Louis, Mo.), 2.5 mg benzylaminopurine (dissolved in 1 N NaOH), 10 ml of 1 mg/ml indoleacetic acid solution, 30 g sucrose, 2 ml Gamborg’s Vitamin Solution (Sigma Chemical Co., St. Louis, Mo.) and 8 g agar. The pH is adjusted between pH 5.5 and 5.9 with NaOH. After 2 days, the leaf segments were transferred to Plant Tissue Culture Medium containing 300 \(\mu\)g/ml kanamycin, 500 \(\mu\)g/ml mepoxin (Merck, Rahway, N.J.). The kanamycin selects for transformed plant tissue, and the mepoxin selects against the agrobacterial cells.

It is necessary to minimize the exposure of the explant tissue to agrobacterial cells during the transformation procedure in order to limit the possible induction of the regulated parA1 coding sequence during the production of the transgenic plant cells, which would cause a cell death response. Accordingly, the ballistic technique for the introduction of heterologous DNA containing cell suicide genes under the regulatory control of the inducible transcriptional regulatory element is a useful alternative transformation technique because it does not entail the use of agrobacterial cells or fungal cell wall digestive enzymes (as necessary for the generation of protoplasts for electroporation), both of which lead to induction of the coding sequences under the control of that regulatory element.

Transgenic plants were regenerated essentially as described by Horsch et al. (1985) Science 227:1229–1231.

The resulting transgenic plants were tested for the expression of the \(\beta\)-glucuronidase (GUS) reporter gene using 5-bromo-4-chloro-3-indoly-\(\beta\)-D-glucuronic acid as described by Jefferson et al. (1987) EMBO Journal 6:3901–3907, using untreated (control) conditions and inducing conditions. An inducing condition is the intercellular application of \(T.\) viride cellulase to tobacco tissue in the transgenic plants (using a mechanical pipetter to apply 50–100 \(\mu\)l inducing composition to interstitial tissue); controls were mock-applied but not treated with cellulase elicitor. Tobacco tissue was wounded with a scalpel in some experiments to facilitate exposure to the inducing compounds.

**Example 8**

**Deletion Analysis of Promoter and Promoter-Associated Region**

In separate reactions, the EAS4-derived DNA sequence encompassed by –567 to +67 relative to the transcription start site (nucleotides 6 to 642, SEQ ID NO:2; EAS4) was substituted for the Cauliflower Mosaic Virus (CaMV) 3SS promoter [Benfey et al. (1990) EMBO Journal 9:1677–1684] in the GUS-reporter vector pBI221 (Clontech, Palo Alto, Calif.). Deletion mutants in the EAS4 upstream regions were then isolated after restriction endonuclease cutting and Bal31 digestion. Analysis of the eEAS promoter-GUS constructs was carried out in electroporated tobacco cell protoplasts and in stably transformed tobacco lines. Preliminary data for the transient expression demonstrated that SEQ ID NO:1 and SEQ ID NO:2 functioned in regulated gene expression.

The transient expression data obtained with the \(N.\) tabacum protoplasts into which various EAS3 and EAS4 promoter-GUS constructs were introduced are given in FIG. 1. Progressive deletions from the 5’ end of the EAS4 promoter regions reduce the levels of expression, but inducibility is maintained for the –262, –202 and –110 constructs (relative to the transcription start site at nucleotide 573 of SEQ ID NO:2). The data indicate that only very low levels of GUS are expressed via either EAS3 promoter region construct. Similarly, the truncated CaMV 3SS promoter alone is not induced by the elicitor treatment, which provides induction via the inducible transcription regulatory element of EAS4.

The data in FIGS. 2A–2B indicate that deletion of genetic material between –567 and –160 reduce the level of downstream gene expression but does not destroy the inducibility of expression. Therefore, the DNA sequences between –567 and –160 appear to contain transcription-enhancing activity. Most of the transcription-increasing activity appears to reside between –567 and –212, but additional enhancement appears to be mediated by sequence information between –212 and –160 relative to the transcription start site (transcription start site is nucleotide 573; –567 is nucleotide 1, –212 is nucleotide 361, and –160 is nucleotide 413, all in SEQ ID NO:2).

In the Gain of Function assay data in FIG. 2B, the DNA sequence information necessary to mediate induction in response to elicitor treatment is located between –160 and –87 relative to the EAS4 transcription start site (i.e., between nucleotides 413 and 486 of SEQ ID NO:2). In these experiments the EAS-derived sequences were placed in front of a truncated CaMV 35S promoter [Benfey et al. (1990) EMBO J. 9:1677–1684]. This figure also demonstrates that the EAS4-derived transcription regulatory region functions when fused to a heterologous minimal promoter.

In other experiments, either the entire –567 to +67 EAS4 upstream region or 5’ deletions thereof were inserted upstream of the GUS (\(\beta\)-glucuronidase) reporter gene in vector pBI101 (Clontech, Palo Alto, Calif.), and expression levels of the GUS reporter were assayed under inducing and noninducing conditions. 160 bp upstream of the transcription start site of EAS4 were sufficient to direct the regulated expression of the GUS reporter gene, although the presence of additional upstream sequences mediated increased expression.

Constructs containing a minimum of 167 bp upstream of the EAS4 transcription start site gave transient gene expression in electroporated protoplasts and confer elicitor-inducibility of GUS reporter gene expression (minimum of 2.5-fold increase in gene expression). By contrast, the EAS3 upstream region (SEQ ID NO:1) does not appear to support high levels of reporter gene expression in the transient expression system, nor does it appear to confer elicitor-inducibility to the downstream reporter gene.

In part, the elicitor-inducible GUS reporter gene expression was expected in the protoplast system because those protoplasts were generated using fungal cell wall digestive enzymes, and those enzymes have been shown to elicit phytoalexin production and sesquiterpene cyclase gene expression in plants [Chappell et al. (1991) Plant Physiology 97:693–698]. A possible explanation is that the 6 hr time period before the experiment start allows the cells to return to an elicitor-responsive state.

PBI101 is commercially available from Clontech (Palo Alto, Calif.). It contains the CaMV 35S promoter upstream.
of the GUS reporter gene in a pUC19 vector; thus it serves as a vector for transient expression experiments where the recombinant vector is introduced into plant protoplasts. The presence of this plasmid and its derivatives is selected by growth on kanamycin. A "promoter-less" GUS cassette in the Agrobacterium binary plasmid vector pBIN19 (Bevan, M. (1994) Nucl. Acids Res. 12:8711) similarly carries a plant-expressible kanamycin resistance determinant.

Example 9
Identification of Inducible Transcription Regulatory Element

The 5' flanking domains of genomic EAS3 and EAS4 clones were mapped by S1 nuclease protection and primer extension experiments [Sambrook et al. (1989) supra]. Sub-clones comprising up to 1 kb 5' to the translation start site were sequenced and fused to the β-glucuronidase (GUS) reporter gene in PB101 for studies in transgenic plant tissue. The resulting recombinant plasmids were then electroporated into tobacco protoplasts. GUS activity was measured in transient expression assays, and stable transformed tobacco cell lines were also isolated for studies of GUS induction and expression.

 Constructs were prepared containing a minimum of about 200 bp of nucleotide sequence upstream of the EAS4 transcription start site in the modified pBI101 vector, and a β-glucuronidase (GUS) reporter gene were made and analyzed for ability to drive regulated GUS expression. 200 bp of flanking sequence appeared sufficient to drive transient gene expression in electroporated protoplasts and conferred elicitor inducibility to GUS expression (minimum of 2.5 fold induction). Similar experiments with the EAS3 flanking sequence indicated that 200 bp from the EAS3 locus did not support either high levels of GUS expression or elicitor responsiveness in transformed plant cells. Cellulase and elicitors from Phytophthora [Ricci et al. (1989) Eur. J. Biochem. 183:555–563] serve to induce gene expression mediated by the EAS4-derived regulatory sequences.

Further studies related to the identification of sequences important in mediating induced gene expression in response to pathogen invasion, as modeled using cellulase or elicitors, were carried out after oligonucleotide site-directed mutagenesis [Kunkel (1985) Proc. Natl. Acad. Sci. USA 82:488–492] of the putative regulatory region of EAS4. Substitution of GT for the wild-type CA at –233 and –234 relative to the EAS4 transcription start site (nucleotides 334–335 of SEQ ID NO:2) did not appear to alter the expression of the GUS reporter gene as measured after incubation in the presence of elicitor (cellulase) for 20 hours.

Preliminary methylation interference and gel retardation studies carried out essentially as described [Sambrook et al. (1989) supra] indicated that an octamer sequence centered around –233 relative to the translation start site (centered around 334 of SEQ ID NO:2) binds proteins from plant cell nuclei. Methylation interference data suggested that the G at position –233 was preferentially protected against methylation by DMS (dimethyl sulfate) if first allowed to interact with nuclear extracts. The results of gel retardation studies were consistent with those obtained in the methylation protection experiments. When DNA fragments containing the –343 to –140 region (relative to the translation start site) (nucleotides 230 to 433 of SEQ ID NO:2) were examined after reaction to nuclear extracts, mobility in native acrylamide gel electrophoresis appeared retarded. Protein binding was abolished by the GT to CA substitution at positions –234 and –233. Similar results were observed in control and elicitor-induced cell extracts, and reporter gene expression was not changed by this 2 bp mutation. Thus, it is concluded that the region around –233 is not directly involved in the induction of gene expression in response to pathogen invasion or elicitor treatment.

Preliminary experiments indicate that EAS4 DNA sequences between –253 and –48 relative to the EAS4 transcription start site (between nucleotides 320 and 525 of SEQ ID NO:2) have qualitative and quantitative effects on downstream reporter gene expression. Sequences between –110 and –1 of EAS4 relative to the transcription start site of EAS4 (nucleotides 463 to 572 of SEQ ID NO:2) to mediate the inducible response, while sequences between –202 and –110 relative to the EAS4 transcription start site (nucleotides 371 to 463 of SEQ ID NO:2) enhance the levels of both induced and uninduced reporter gene expression.

Example 10
Disease-resistant Transgenic Plants

The parA1 coding sequence was isolated from Phytophora parasitica race O as follows: Genomic DNA was isolated and used as template in PCR with a SIG forward primer (CGTGGATCCCGACTTCGAGAATGAC; SEQ ID NO:9, BamH1 site underlined; nucleotides 25–27 correspond to the translation start site and reverse primer (GGTGAAGCCTCCGACGGAGATGAAC; SEQ ID NO:10, Sstl site underlined) to amplify the coding region of the ParA1 elicitin including the signal peptide coding sequence. To isolate an amplimer corresponding to the coding sequence of the ParA1 elicitin, the MAT forward primer (GGGCGATCTGAGTACTAGTAGTGCACACCCA CGGAGCAACTG, SEQ ID NO:11; BamH1 site, GGAACC, and SpeI site, ACTAG, underlined; transcription start site at nucleotides 12–14 and the reverse primer as before (SEQ ID NO:10) were used with genomic DNA as template. The amplimer sequences of the elicitin-with-signal peptide and the nature elicitin are given in SEQ ID NO:15 and SEQ ID NO:17, respectively.

For subcloning into pBluescript (Stratagene, La Jolla, Calif.) into pEAS4 constructs, the amplimer DNA was digested with BamH1 and Sstl. Where the mature protein’s coding sequence is used, the mature elicitin/pEAS4 construct can be digested with SpeI to insert a plant signal sequence at the 5' end of the open reading frame. The pEAS4-GUS vector is digested with BamH1 and Sstl, with the large fragment of DNA being purified after agarose gel electrophoresis.

FIG. 3 illustrates the molecular manipulations leading to the generation of disease-resistant plants. The coding sequence for the ParA1 elicitin is isolated by PCR so as to have BamH1 and Sstl ends. gEAS4-ΔGUS-pBI101, which directs the expression of the GUS reporter gene under the regulatory control of the EAS promoter, is digested with BamH1 and Sstl to release the GUS reporter gene. Then the BamH1/Sstl-digested ParA1 amplimer is ligated to the large fragment produced after digestion of gEAS4-ΔGUS-pBI101 to produce gEAS4-ΔGUS-ParA1-pBI101, from which the ParA1 elicitin is synthesized after induction with a suitable elicitor once plant cells or tissue have been transformed.

While various embodiments of the present invention have been described in detail, it is apparent that modifications, extensions, adaptations and optimizations may occur in those skilled in the art. It is to be expressly understood that such modifications and adaptations and so on are within the spirit and scope of the present invention, as set forth in the claims.
(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 13

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 512 base pairs
(B) TYPE: nucleic acid
(C) STRANDINESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Nicotiana tabacum

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

CCGCCATTGG AGGATTTTGT ACCTGAGCCT AGCGCGCACC GCCTTTAATT TTAACCGTTC  60
AGAAGAAAC GGGATGCTT GTCAAGCAA CAGAGACGGC CGGACATCAT GCCTGACAC  120
CCGGCGCCTTGAAGCTG ACCCTTGGAA AGAGCTACA GCCTCCTCCA CGCGGATCC  180
GGGATGCGCC CTGACTACAG AAAATGCTTY TCCGGGCACC GGCGCGAGGC CGCGTTTTG  240
AGAAGCTACT TGAACGACCA CAGACCGCG TACGCTATCC GCCTGAGGCT AGAGAGGAT  300
CAGTTTCCTT TTTGCGTATT CGAATGCCTT CAGGCTGGAG CAGGTTAAC  360
GCCGCGTCA ACCTGCGGCC GCGGACGAC TCCCGAGAGA CTTTTACGTG  420
CGCCGATTA ATACATCAAA CTAATCTCCT ACCACTATAAT ATACTTGCTC CTTCTCTCC  480
ATTTAATAG AGTTTCTCTTC TTCTCTCCTT AA  512

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 642 base pairs
(B) TYPE: nucleic acid
(C) STRANDINESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Nicotiana tabacum

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

TAGGCTGACT TGAGGCCTAA TGCTCAAGCA TACTTAAGCC CTCGCCAGTAC ACACCGGCTG  60
GGGAGCTTAC CAAAAACAT CTTGTCTTGT AGAAGGCTCA ATTTTGAACC CCTACAATTT  120
CGACAAAACT TTGCGTTCTG AAAACACTT TGAACAGCTT TGAACATCC AGTCAAAATTA  180
ACTCTAAACT GACCTAAATA ATACATGCT ATACAGCTT TTAAGCTTAC CAAAGTTGACC  240
TCGCGATTAA TAAATCGAAT TTATGCGGCA ACAATACGAT TATGTTTTAT AAATACGATTA  300
ATAGATATT GTAGTGTGCC GGAACACACT TCAAGTACT CCTCTATTTT GTACCTTTTT  360
AAATATATTT ATCAGTGTCA CGAAACAATA AAATAAATAA TTTGGAAAC TCGATCAATA  420
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GACGCCAGAC GCCAACAATG AATCAAAAGG GTCTTAGCTA GTUTAAAGTC TAGTAAGGCA ACTGGGAAAT TAAATGATTA GGTGCTTTTG ATCAATTACA TTAACTAGTC TCTCACCACT ATATATACTT GTCCCTTCTC TTCCATTTAA GTAGAGTTCC TTTCTTTCTT CCTTAAAACT TAAAAGAACA AGTAAAAATA CACTCATCTT TAATTAGCAA

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
   (A) DESCRIPTION: /desc = "Oligonucleotide primer for PCR"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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

ATGCTGTTAG CAACCGGAAG

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 21 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
   (A) DESCRIPTION: /desc = "Oligonucleotide primer for PCR"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

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

ATCCAAAATC TCATCAATTT C

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 18 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
   (A) DESCRIPTION: /desc = "Oligonucleotide primer for PCR"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

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

ATGAGTCCTT ACATGTGA

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 45 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
   (A) DESCRIPTION: /desc = “Oligonucleotide primer for PCR.”

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

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

```
GGAGCTCTGA ATTCCATGCG CTCAAGCAGCA GCAAGTGCA ACTAT
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 4254 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDNESS: double
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: Nicotiana tabacum

(ix) FEATURE:
   (A) NAME/KEY: CDS
   (B) LOCATION: join(1217..1327, 1455..1718, 1806..2182, 2259..2477, 2609..2747, 3262..3558)

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

```
AAGCTTTATG AATTAGATGT AAAAAGACGC AAACTACTTA TATATATTAC CAAAGTAACT TGAAAGTTTA ... AATTACATTA ACTAGTCTCT CACCACTATA TATACTTGTC CCTTCTCTTC CATTTAAGTA GAGTTCCTTT CTTTCTTCCT TAAAACTTAA AAGAACAAGT AAAAATACAC
```

45

60

120

180

240

300

360

420

480

540

600

660

720

780

840

900

980

1002

1080

1140

1200

1280

1340

1400
CTGCAG G TGG TGG AAA GAT TGG GAT TTT GTA ACA ACA TCT CCA TAT GCT
Trp Trp Lys Asp Leu Asp Phe Val Thr Thr Leu Pro Tyr Ala
  225  
  260  
  265  

AGA GAT CCA GTA GTT GAA TGC TAC TTT TGG GCA TTA GUA GTT TAT TTT
Arg Asp Asp Val Val Glu Cys Tyr Phe Trp Ala Leu Gly Val Tyr Phe
  270  
  275  
  280  

GAG CCT CAA TAC TCT CAA GCT CGC GTC ATG CTC GTT AAG ACC ATY TCA
Glu Pro Gin Tyr Ser Ser Ala Arg Val Met Leu Val Lys Thr Ile Ser
  285  
  290  
  295  

ATG ATT TGC ATT GTC GAT GAC ACC TTT GAT GCT TAG GUT ACA GTT AAA
Met Ile Ser Ile Val Asp Asp Thr Phe Asp Ala Tyr Gly Thr Val Lys
  300  
  305  
  310  

GAA CTT GAG GCA TAC ACA GAT GCC ATA CAA AG GTC ATACCT
Glu Leu Glu Ala Tyr Thr Asp Ala Ile Gin Arg
  315  
  320  

CATCACATCA CTTATCCCTT GACGCTGAAT CTCGCTCGA AAGAATTAAG ACGAATT TCT
  2547  

ACTGATTTAC ATGGCTGCTT CTCCCCCAAGT ATGAAATTCG ATCTCAATT GCA
  2607  

GAT TGG GAT ATC AAC GAA ATT CAT CGG CCT CTT GAT TAC ATG AAA ATC
Trp Asp Ile Asp Leu Glu Ile Asp Arg Leu Pro Asp Tyr Met Lys Ile
  325  
  330  
  335  

ATG TAT AAA GCT ATT CTA GAT CTC TAC AAG GAT TAT GTA AAAT AAG TTG
Ser Tyr Lys Ala Leu Asp Tyr Leu Asp Tyr Glu Gly Leu Lys
  340  
  345  
  350  
  355  

TCT ATG GCC GGA AGA TCT ATT GTC TGC CAT GCA ATA GAA AG A
Ser Ser Ala Gly Arg Ser His Ile Val Cys His Ala Ile Gin Arg
  360  
  365  
  370  

GATATGCTG ACAAATTAC TATCGAATA CATTTTCTT CTTACATT TTCTACTTTG
  2807  

GTTAACCTTT TTCTGCTTGT TTAAGTGTAT GAAACTTGAT ACAGTTCAAT CAAATTTTCTT
  2867  

TACAGTTGA ACACATATAT GTTGTATATT CAGC ATG AAA GAA GTA GTA AGA
Met Lys Glu Val Val Arg
  375  

AAAT TAT AAT GTC GAG TCA ACA TGG TTT ATT GAA GUA TAT ATG CCA CCT
Aan Tyr Aan Val Glu Ser Thr Trp Phe Ile Glu Gly Tyr Met Pro Pro
  380  
  385  
  390  

GTT TCT GAA TAC CTA AGC AAT GCA CTA ACA ACT ACC ACA TAT TAC TAC
Val Ser Glu Tyr Leu Ser Asn Ala Leu Ala Thr Thr Tyr Tyr
  395  
  400  
  405  

CTC GCC ACA ACA TCG TAT TGT GCC ATG AAG TCT GCC AGC GAG CAA GAT
Leu Ala Thr Thr Ser Tyr Leu Gly Met Lys Ser Ala Thr Glu Gin Asp
  410  
  415  
  420  

TGT GAG TGG TGG TCA AAG AAT CCA AAA ATT CTT GAA GCA GTT ATT
Phe Glu Trp Leu Ser Lys Asn Pro Lys Ile Leu Glu Ala Ser Val Ile
  425  
  430  
  435  
  440  

ATA TGT CCA GTT ATC GAT GAC ACA GCC AGC TAC GAG GTATATTTG
Ile Cys Arg Val Ile Asp Asp Thr Ala Thr Tyr Glu
  445  
  450  

CATCTCAAGA AATTTATACCA TTATATCGGA TTTGGACAAA CAAAGGCTTG CGAGCAAT
  3218  

TAAGGCAATA TAAAAGCTTAA CCTTTCATT ATCTGTTTTAC TAG GTT GAG AAA AGC
Val Glu Lys Ser
  3273  

AGG GCA AAA ATT GCA ACT GGA ATT GAG TGG TGC ATG AGA GAT TAT GGT
Arg Gly Gin Ile Ala Thr Gly Ile Glu Cys Met Arg Asp Tyr Gly
  460  
  465  
  470  

ATA TCA ACA AAA GAG CCA ATG GCT AAA TTT CAA AAT ATG GCT GAG ACA
Ile Ser Thr Lys Glu Ala Met Ala Lys Phe Gin Asn Met Ala Glu Thr
  475  
  480  
  485  

GCA TGG AAA GAT ATT AAT GAA GGA CTT CTT AGG CCC ACT CCC GTC TCT
  3417  

-continued
Ala Trp Lys Asp Ile Asn Glu Gly Leu Leu Pro Thr Pro Val Ser 490 495 500
ACA GGA TTT TTA ACT CCT ATT CTC ATT CCT GCT GCT ATT GTT GAG GTP 3465
Thr Glu Phe Leu Thr Pro Ile Leu Asn Leu Ala Arg Ile Val Glu Val 505 510 515 520
ACA TAT ATA CAC AAT CTA GAT GGA TAC ACT CAT CCG GAG AAG GTC TTA 3513
Thr Tyr Ile His Asn Leu Asp Gly Tyr Thr His Pro Glu Lys Val Leu 525 530 535
AAA CCT AAC ATT ATT AAC CTA CTT GTG GAC TCC ATC AAA ATT TGA 3558
Lys Pro His Ile Ile Leu Ile Asp Ser Ile Lys Ile 540 545 550
GCT GCC ATT TGT GTC GAT AC TCA GAC GAA AAC TAC TCT TTG TTC TAG 3618
AC TGC TTA CAT GAC GCT TTA AAG AAT GTT GCT TCA AAT TTC AAT 3670
CCG CAT TGT GAG GAA TTC GCT GTA AGT GAA AGT TTA GGA GAT TGT 3738
GTC TCT TGT GAC CTT AAG AAT TCT AAG TCT GAA GGC AAA GAT 3790
AAAT GAC GC TGT TGT GGC TTT GCT GCT TTA ATC ATC TTA AAT GAC GCA 3858
AA AAT GGT GTT GAC CAC TGC TGG TGG GCA GCT GCT GAC TTA TCT GAC TCA 3918
AT AAT TAA GAC TAG AAC TAC TAC AAC AAC AAC TCT TCG TGA TAC TAC TAC 3978
CA GAG AAG TCT ATG TTT TCT GAC GCG TAA CTG AAA GGC GGC AAT TCT GCA 4038
CT AAT GCA GGG AAG GAT TCT GAG GAG GCG TCA GGT TGA TTA AAA GAT 4098
TA TTA GAT AGA CAA TAA AAT TGT GAA GAA TGC TAC TAC TAC TAC TAC 4158
GA AAT GAC ATG GGG TGG AAG CTG TTA GAG GGA ATC AAC GTA TAC GCA 4218
TAT CAAA AAA AAA GAG GTG TCC TCT ACT CCG GCA AGT GGT TAC TTA 4254

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 550 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Met Ala Ser Ala Ala Val Ala Asn Tyr Glu Glu Glu Ile Val Arg Pro
1  5  10  15
Val Ala Asp Phe Ser Ser Pro Ser Leu Trp Gly Asp Gin Phe Leu Ser Phe
20 25 30
Ser Ile Asp Asn Gin Val Ala Glu Tyr Ile Tyr Ala Gin Glu Ile
35 40 45
Glu Ala Leu Lys Glu Gin Thr Arg Ser Met Leu Ala Thr Gly Arg
50 55 60
Lys Leu Ala Asp Thr Leu Asn Leu Ile Asp Ile Glu Arg Leu Gly
65 70 75 80
Ile Ser Tyr His Phe Glu Lys Glu Ile Asp Glu Ile Leu Asp Gin Ile
85 90 95
Tyr Asn Gin Asn Ser Asn Cys Asn Asp Leu Cys Thr Ser Ala Leu Gin
100 105 110
Phe Arg Leu Leu Arg Gin His Gly Phe Asn Ile Ser Pro Glu Ile Phe
115 120 125
Ser Lys Phe Gin Asp Glu Asn Gly Lys Phe Lys Glu Ser Leu Ala Ser
130 135 140
Asp Val Leu Gly Leu Leu Asn Asp Tyr Glu Ala Ser His Val Arg Thr
His Ala Asp Asp Ile Leu Glu Asp Ala Leu Ala Phe Ser Thr Ile His
165 170 175
Leu Glu Ser Ala Ala Pro His Leu Lys Ser Pro Leu Arg Glu Gin Val
180 185 190
Thr His Ala Leu Glu Gin Cys Leu His Lys Gin Val Pro Arg Val Glu
195 200 205
Thr Arg Phe Phe Ile Ser Ser Ile Tyr Asp Lys Glu Gin Ser Lys Asn
210 215 220
Asn Val Leu Leu Arg Phe Ala Lys Leu Asp Phe Asn Leu Leu Gin Met
225 230 235 240
Leu His Lys Gin Glu Leu Ala Gin Val Ser Arg Trp Trp Lys Asp Leu
245 250 255
Asp Phe Val Thr Thr Leu Pro Tyr Ala Arg Asp Arg Val Val Glu Cys
260 265 270
Tyr Phe Trp Ala Leu Gly Val Tyr Phe Glu Pro Gin Tyr Ser Gin Ala
275 280 285
Arg Val Met Leu Val Lys Thr Ser Met Ile Ser Ile Val Asp Asp
290 295 300
Thr Phe Asp Ala Tyr Gly Thr Val Lys Glu Leu Glu Ala Tyr Thr Asp
305 310 315 320
Ala Ile Gin Arg Trp Asp Ile Asn Glu Ile Asp Arg Leu Pro Asp Tyr
325 330 335
Met Lys Ile Ser Tyr Lys Ala Ile Leu Asp Leu Tyr Lys Asp Tyr Glu
340 345 350
Lys Glu Leu Ser Ser Ala Gin Arg Ser His Ile Val Cys His Ala Ile
355 360 365
Glu Arg Met Lys Glu Val Arg Asn Tyr Asn Val Glu Ser Thr Trp
370 375 380
Phe Ile Glu Gly Tyr Met Pro Pro Val Ser Glu Tyr Leu Ser Asn Ala
385 390 395 400
Leu Ala Thr Thr Thr Tyr Tyr Leu Ala Thr Thr Ser Tyr Leu Gly
405 410 415
Met Lys Ser Ala Thr Glu Gin Asp Phe Glu Trp Leu Ser Lys Asn Pro
420 425 430
Lys Ile Leu Glu Ala Ser Val Ile Ile Cys Arg Val Ile Asp Asp Thr
435 440 445
Ala Thr Tyr Glu Val Glu Lys Ser Arg Gin Glu Ile Ala Thr Gly Ile
450 455 460
Glu Cys Cys Met Arg Asp Tyr Gly Ile Ser Thr Lys Glu Ala Met Ala
465 470 475 480
Lys Phe Gin Asn Met Ala Glu Thr Ala Trp Lys Asp Ile Asn Glu Gly
485 490 495
Leu Leu Arg Pro Thr Pro Val Ser Thr Glu Phe Leu Thr Pro Ile Leu
500 505 510
Asn Leu Ala Arg Ile Val Glu Val Thr Tyr Ile His Asn Leu Asp Gly
515 520 525
Tyr Thr His Pro Glu Lys Val Leu Lys Pro His Ile Asn Leu Asn Leu
530 535 540
Val Asp Ser Ile Lys Ile
545 550
(2) INFORMATION FOR SEQ ID NO:9:
  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 30 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "Oligonucleotide primer for PCR."
  (iii) HYPOTHETICAL: NO
  (iv) ANTI-SENSE: NO
  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
  GCTTGATCC CCACTCTAC CGAATGAC  

(2) INFORMATION FOR SEQ ID NO:10:
  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 30 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "Oligonucleotide primer for PCR."
  (iii) HYPOTHETICAL: NO
  (iv) ANTI-SENSE: YES
  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
  GCTGAGCTC CGGAGCCAG AGATCAACC  

(2) INFORMATION FOR SEQ ID NO:11:
  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 42 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: other nucleic acid
      (A) DESCRIPTION: /desc = "Oligonucleotide primer for PCR (reverse)."
  (iii) HYPOTHETICAL: NO
  (iv) ANTI-SENSE: YES
  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
  GCCGAGATCC TATGACTG TGCACCCACA CGACGAAAA TG  

(2) INFORMATION FOR SEQ ID NO:12:
  (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 593 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: double
      (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: DNA (genomic)
  (iii) HYPOTHETICAL: NO
  (iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Phytophthora parasitica

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 207..563

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

GTGACGAAA GCCGAGGTGC TGTGACGATC TTGCTGTGTTG AAATGCTACGC GCCACGUCAA
AACCTACAG GTACACAGC TTCAAATGAA CCTGCAACGC AAGGCGCCAG CCAACTCCAG
CTAATCAAGC CTAGTGGGC CTGCAATGCAT ATTGTACCATTG TCGTCTCAT CCACACCCAC
CCACCTCTC CCCACCTCTA TCGGAA ATG AAC TCC GCC GCT CTG TCC GCC GCC

Met Asn Phe Arg Ala Leu Phe Ala Ala

555 560

ACC GTC GCC GCC CTC GTG GCC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC
Thr Val Ala Ala Val Gly Ser Thr Ser Ala Thr Thr Cys Thr Thr
565 570 575

AGC CAG CAA ACT GCG GCG TAC GTG GCG TCG GTA AGC ATC TCT TCG GAC
Thr Gin Gin Thr Ala Ala Tyr Val Ala Leu Val Ser Ile Leu Ser Asp
580 585 590

AGC TCG TTC AAC CAG TGG TCG ACG GCC TCT TCT TCA ATG ATG ACG
Thr Ser Phe Asn Gin Cys Ser Thr Asp Ser Gly Tyr Ser Met Leu Thr
595 600 605

GCC ACC TCC TTG CCC ACG ACG GAG CAG TAC AAC CTC ATG TCG GCC TCG
Ala Thr Ser Leu Pro Thr Glu Gin Tyr Lys Leu Met Cys Ala Ser
610 615 620

ACG GCC TGC AAG ACG ATG ACG AAG ATC AAC GAC CTG ACG CAC CCC
Thr Ala Cys Lys Thr Met Ile Asn Lys Ile Val Thr Leu Asn Pro
625 630 635 640

GAC TGC GAG TGG ATG CCT AGC ACC GCC CTG GTC GTA AAC GTG TTC
Asp Cys Glu Thr Val Pro Thr Ser Gly Leu Val Leu Val Val Phe
645 650 655

ACG TAC GCG AAC GGG TTC TCG TCT ATG GCC TCG TCA ATG GTA
Thr Tyr Ala Asn Gly Phe Ser Ser Thr Cys Ala Ser Leu
660 665 670

GCCGTTTCA TCTCTGCCTC CAGAATGCAAT

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Met Asn Phe Arg Ala Leu Phe Ala Ala Thr Val Ala Leu Val Gly

1 5 10 15

Ser Thr Ser Ala Thr Thr Thr Thr Cys Thr Thr Thr Gin Gin Thr Ala Ala Tyr

20 25 30

Val Ala Leu Val Ser Ile Leu Ser Asp Thr Ser Phe Asn Gin Cys Ser

35 40 45

Thr Asp Ser Gly Tyr Ser Met Leu Thr Ala Thr Ser Leu Pro Thr Thr

50 55 60

Glu Gin Tyr Lys Leu Met Cys Ala Ser Thr Ala Cys Lys Thr Met Ile

65 70 75 80

Asn Lys Ile Val Thr Leu Asn Pro Pro Asp Cys Glu Leu Thr Val Pro

85 90 95
What is claimed is:

1. A plant or plant cell genetically engineered to contain a chimeric gene comprising, in operable linkage from 5' to 3', (i) a pathogen- or elicitor-inducible transcription regulatory element, wherein said pathogen- or elicitor-inducible transcription regulatory element is selected from the group consisting of (1) a pathogen- or elicitor-inducible transcription regulatory element comprising the nucleotide sequence shown in SEQ ID NO:2 from the nucleotide 458 to nucleotide 473; (2) a pathogen- or elicitor-inducible transcription regulatory element comprising the nucleotide sequence shown in SEQ ID NO:2 from nucleotide 406 to nucleotide 473; and (3) a pathogen- or elicitor-inducible transcription regulatory element comprising the nucleotide sequence shown in SEQ ID NO:2 from nucleotide 371 to nucleotide 473, (ii) a promoter functional in a plant, and (iii) a coding sequence, wherein said promoter and said coding sequence are not naturally associated with said inducible transcription regulatory element, and wherein said coding sequence is expressed under the regulatory control of said inducible transcription regulatory element.

2. The plant or plant cell of claim 1, wherein said pathogen- or elicitor-inducible transcription regulatory element comprises the nucleotide sequence shown in SEQ ID NO:2 from nucleotide 458 to nucleotide 473.

3. The plant or plant cell of claim 1, wherein said pathogen- or elicitor-inducible transcription regulatory element comprises the nucleotide sequence shown in SEQ ID NO:2 from nucleotide 406 to nucleotide 473.

4. The plant or plant cell of claim 1, wherein said pathogen- or elicitor-inducible transcription regulatory element comprises the nucleotide sequence shown in SEQ ID NO:2 from nucleotide 371 to nucleotide 473.

5. The plant or plant cell of claim 1, wherein said promoter functional in a plant is the Cauliflower Mosaic Virus 35S gene minimal promoter.

6. The plant of claim 1, wherein said plant is a dicotyledonous plant.

7. The plant of claim 1, wherein said dicotyledonous plant is a member of the Solanaceae.

8. The plant of claim 1, wherein said plant is Nicotiana tabacum.

9. The plant of claim 1, wherein said plant is a monocotyledonous plant.

10. The plant of claim 1, wherein said plant is a gymnosperm.

11. The plant of claim 1, wherein said plant is a member of the Coniferae.

* * * * *