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(54) Title: METHODS AND MATERIALS FOR PRODUCING PATHOGEN-RESISTANT PLANTS

(57) Abstract

This invention comprises a mutant plant virus gene which confers resistance on tobacco and tomato plants against tobacco mosaic tobamovirus and tomato mottle geminivirus infections and infection by other related geminiviruses. A gene was initially isolated from the known BC1 gene, between nucleotides 1278 and 2311 of the B component of tomato mottle geminivirus. Upon subcloning of this DNA fragment into an appropriate expression vector and transformation of the gene into tobacco plants, a truncated gene product was produced which confers resistance against viral infection to the recombinant plant in which it is expressed.

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DESCRIPTION

METHODS AND MATERIALS FOR PRODUCING PATHOGEN-RESISTANT PLANTS

Cross Reference to Related Application

This application claims the benefit of U.S. Provisional Application No. 60/015,051, filed April 9, 1996, and U.S. Provisional Application No. 60/002,158, filed August 11, 1995.

Acknowledgement of Government Support

This invention was made with government support under USDA/DSRS CBAG Special Grants Program, grant Nos. 93-34135-8607; 92-34135-7456; and the Florida Tomato Committee Grant No. 90153-C. The government has certain rights in this invention.

Background of the Invention

(i). Field of the Invention

This invention pertains to the field of conferring pathogen resistance to plants. More particularly, the invention is directed to virus-resistant transgenic plants.

(ii). Background of the Invention

Tomato producers suffer significant losses due to tomato mottle geminivirus infection. Currently farmers must purchase chemicals in order to control tomato mottle virus in their tomato fields. Similarly, losses are experienced by farmers producing tobacco as a result of tobacco crop infection by tobacco mosaic tobamoviruses. Accordingly, there is a need for a solution to this problem which is less costly and less damaging to the environment than the chemical controls currently employed.

Production of transgenic plants with enhanced phenotypic characteristics is a relatively recent development in the arsenal available to farmers. Nevertheless, the value of this technology has been demonstrated repeatedly in recent years. However, what is required is the identification of appropriate genes to confer the desired phenotype, in this case, pathogen resistance.

Transformation of plants with portions of viral genomes may result in plants with virus resistance (Beachy, 1993). This phenomenon is known as "pathogen-derived resistance" (Sanford

and Johnson, 1985). The level of resistance obtained is variable. This variability has been attributed to the random nature of the transformation process (Lomonossoff, 1995). Independent lines of plants generated from a single transformation experiment may contain different transgene copy numbers inserted in various chromosomes. Phenotypic differences have been noted among plant lines containing a single copy of the transgene. Some of the variability in transgene expression has also been attributed to tissue culture-induced changes (Phillips *et al.*, 1994). This variability in the phenotype is also observed in subsequent progeny derived from the R_0 plants.

Introduction of a mutation (defective) in one motif of a multimotif protein has been proposed as a strategy for interfering with viral replication. This interference with the function of wild-type genes has been referred to as a dominant negative mutation. Maxwell and his co-workers have constructed transgenic plants expressing a modified tomato mottle geminivirus replication-associated protein (RAP), mutated in a NTP-binding motif, which appears to interfere with viral replication (Hanson, *et al.*, 1991). This dominant negative mutant for the tomato mottle geminivirus RAP gene has been tested for tomato mottle geminivirus resistance in tomatoes. Noris *et al.* (1994, First International Symposium on Geminiviruses, Almeria, Spain) found inhibition of Tomato Yellow Leaf Curl Virus (TYLCV) DNA replication in tobacco protoplasts co-transfected with TYLCV and a construct of a truncated RAP expressed under control of a CaMV 35S promoter.

This control strategy is likely to be very virus specific since the RAP binding sites essential for function have been shown to require a sequence-specific interaction between RAP and the origin of replication (Fontes *et al.*, 1994). This enables the transacting factors of RAP to discriminate between the replication origins of closely related geminiviruses. Because of Geminivirus diversity and adaptability, virus-specific control strategies are of limited value under field conditions.

There have been several reports in recent years relating to the inhibition of infection of certain plants by specific viral pathogens. For example, Von Arnim and Stanley (1992) reported on the inhibition of systemic infection by African Casava Mosaic Virus (ACMV) by a movement protein from the related Geminivirus, Tomato Golden Mosaic Virus (TGMV). This was accomplished by replacing the ACMV coat protein coding sequence with the BL1 or BR1 movement gene sequences from TGMV and then testing the ability of the recombinant ACMV to infect its host, *Nicotiana benthamiana* (which is also the host of TGMV). The authors found that the TGMV gene did not complement the ACMV recombinant, and hypothesized that direct genomic expression of a dominant negative mutant might produce plants resistant against geminiviruses.

Cooper *et al.* (1995) disclosed that transgenic tobacco plants expressing a defective tobacco mosaic virus (TMV) movement protein were resistant to infection by multiple viruses, while

transgenic plants expressing the natural movement protein had increased susceptibility to infection by TMV and other viruses.

Nejidat and Beachy (1990) disclosed that transgenic tobacco plants expressing a TMV coat protein have increased resistance against several of the tobamoviruses. Gilbertson *et al.* (1993) disclosed the reduced pathogenicity of pseudorecombinants of two bipartite geminiviruses, tomato mottle (ToMoV) and TGV-MX1.

Brief Summary of the Invention

We have discovered a mutated plant virus gene which protects tobacco plants against tomato mottle geminivirus and tobacco mosaic tobamovirus infections. This resistance gene has been introduced into tobacco chromosomal DNA by genetic engineering. The transgenic tobacco plants expressing this gene show resistance to tomato mottle geminivirus and tobacco mosaic tobamovirus infections (lack of or reduction of disease symptoms when inoculated with the viruses). The mutated gene can be introduced into chromosomes of desirable tomato and tobacco lines to develop commercially improved tomato and tobacco cultivars/hybrids.

Accordingly, this invention comprises a mutant plant virus gene which confers resistance on tobacco and tomato plants against tobacco mosaic tobamovirus and tomato mottle geminivirus infections, as well as resistance to infections of other related geminiviruses. The known BC1 gene, between nucleotides 1278 and 2311 of the B component of tomato mottle geminivirus, was subcloned into an appropriate expression vector and transformed into tobacco plants. A mutated gene product was produced which confers resistance against viral infection to the recombinant plant in which it is expressed.

One object of this invention is to provide a method for conferring viral resistance on a plant.

Another object of this invention is to provide a mutated BC1 gene and any fragment thereof which confers viral resistance on a plant.

Another object of this invention is to provide novel transgenic plants with enhanced viral resistance.

Other objects and advantages of this invention will become apparent from a review of the complete invention disclosure and the appended claims.

Brief Description of the Figures

Figure 1 is the sequence of the single stranded mutated tomato mottle geminivirus BC1 gene except for positions 1742-1766 which initially were not identified; wild-type nucleotides which are different in the mutant gene are shown in lower case text above the mutant gene sequence.

Figure 2 is the sequence shown in Figure 1 along with its complementary strand; the translational start and stop codons are underlined; the termini are HindIII restriction sites.

Figure 3 is the deduced amino acid sequence of the mutated gene product encoded by the nucleotide sequence of Figure 1, except for positions 151-159 which in initial sequencing efforts were not identified.

Figure 4 shows a comparison of the mutant and wild-type gene products (the mutant protein is the lower sequence).

Figure 5 shows phenotypic comparison of transgenic R_1 tobacco plants expressing BC1 protein of TMoV. Transgenic plants were derived from a R_0 plant which contained two copies of BC1 gene (see Fig. 6) and which did not show any stunting. (A) Plants from left to right: a. transgenic plant (BC1-3-11-5) expressing symptomatic BC1 protein, showing stunting, mottling, and curling on the leaves. Symptoms are more sever than those induced by TMoV infection; b. transgenic plant (BC1-3-11-2) which contains one copy of the non-symptomatic BC1 and the symptomatic BC1 transgene, showing mottling with no stunting; c. transgenic plant (BC1-3-11-6) which contains one copy of non-symptomatic BC1 transgene; and d. non-transgenic tobacco. (B) Plant on the left as in b, Fig. 5A and on the right as in c, Fig. 5A. The plants in A were photographed 45 days after transplanting, and in B 90 days.

Figure 6 shows Southern blot analysis of the R_1 transgenic plant with different phenotypes. Segregation of the BC1 transgene in R_1 generation of transgenic tobacco plants which displayed different phenotypes in Fig. 5 (BC1-3-11-1 and -2, mottling only, -4 and -5, severe stunting and mottling, -6 and -7, no visible symptoms). Blots from BC1-3-16-2 showing stunting and mottling, and BC1-3-6-3 and -4, no visible symptoms are shown for comparative purposes; NT-nontransformed plant; and pKYsBC1, vector construct used for transformation. Genomic DNA of the transgenic plants was extracted and digested with XbaI. Southern blots were subjected to hybridization with ^{32}P-labeled BC1 DNA fragment.

Figure 7 shows Western blot analysis of the P30 fraction of tissue extracts from transgenic R_1 tobacco plants expressing the BC1 gene. Lanes represent extracts from plants described in Fig. 6 except for TMoV-infect. extract from TMoV infected tissue). The subcellular fractions, P1, P30 and S30 were prepared (Pascal *et al.*, 1993) and subjected to SDS-PAGE (Schagger) with some modification and immunoblots using the polyclonal antiserum against expressed BC1 protein. The results of the P1 and S30 fractions are not shown here.

Figure 8 shows Northern blot analysis of transgenic plants which express the BC1 gene, probed with labeled-BC1 DNA. Two BC1 related transcripts were found in the transgenic plants which expressed the full-length BC1 gene, while only one transcript was found in the transgenic

plant which expressed a 3'-truncated form of the BC1 gene (BC1-3-11-6). The samples indicated are as in Fig. 6.

Figures 9A-1 thru 9A-5 and 9B show nucleotide sequences (A) and predicted amino acid sequences (B) of the TMoV BC1 and its transgene mutants. The nucleotide sequence of TMoV BC1 gene from GenBank Accession U14461. The sequence of the PCR amplified BC1 ORF was verified before and after cloning into pGEM-T vector. BC1A sequence determined from an asymptomatic, multicopy transgenic plant which expressed full length BC1 protein. The sequence was analyzed from the PCR product derived from genomic DNA (BC1-3-6-3A). BC1At/r sequence determined from the cDNA, the RT-PCR products, amplified from the total RNA (BC1-3-11-6A). The sequence was also verified by sequencing the PCR product from the genomic DNA and from cloned PCR product. BC1S sequence determined from a symptomatic transgenic plant which expressed full length BC1 protein. The sequence was analyzed after RT-PCR of total RNA (BC1-3-11-5S), after PCR amplification of genomic DNA (BC1-3-11-5S) and after PCR amplification from 3 different lines with a similar phenotype. Note that identical nucleotides and amino acid residues are indicated by (.).

Detailed Description of the Invention

The subject invention concerns a mutated plant virus gene that when expressed in a plant confers on that plant a resistance to infection from plant pathogens. In one embodiment, the mutated virus gene is a BC1 gene of geminivirus. The mutated gene of the present invention can be prepared by inserting the wild-type gene into the genome of a plant and identifying those plants transformed with the gene that exhibit increased resistance to viral infection.

The subject invention also concerns a method for conferring resistance on a plant to infection by plant pathogens. The subject method comprises inserting a wild-type viral movement gene, such as BC1, into the genome of a plant and then identifying those plants that do not exhibit pathogenic symptoms when the inserted gene is expressed but which have enhanced resistance to infection by pathogens.

The subject invention also concerns transgenic plants and plant tissue having a mutated gene of the present invention incorporated into their genome.

The following is a specific example of the subject invention, a method for creating a virus-resistant plant, using the BC1 gene of tomato mottle geminivirus to illustrate the invention. The method is generally and broadly applicable to other plant viruses.

The complete sequence of the BC1 gene of tomato mottle geminivirus is known (Abouzid *et al.*, 1992, herein incorporated by reference). The BC1 gene of tomato mottle geminivirus of the

B component of the genome is isolated in sufficient quantity for subcloning in an expression vector. This may be accomplished by any of several methods well-known in the art. A simple method is to use a pair of specific primers to amplify the desired segment according to the well known polymerase chain reaction (PCR) technique. For this purpose, a useful primer pair such as:

5′-CCCAAGCTTCGAGTTCGAAACTGC-3′ (SEQ ID NO. 1) and

5′-CCCAAGCTTAACGAAGTGTGTTTGAC-3′ (SEQ ID NO. 2)

may be used. All or portions of the BC1 gene may be used for this purpose.

Once sufficient quantities of the gene are obtained, the gene is cloned into a vector for production of a stable source for mass production of the gene. Any vector known in the art can be used for this purpose, and mass quantities of the vector may be cultured, for example, by transformation of competent bacterial cells such as *E. coli* followed by harvesting of the plasmid DNA. Preferably, the gene is inserted into the multiple cloning site of a vector, such as the commercially available pUC vectors or the pGEM vectors, which allow for excision of the gene having restriction termini adapted for insertion into any desirable plant expression or integration vector. For this purpose any vector in which a strong promoter, such as a viral gene promoter, is operatively linked to the coding sequence of the mutant gene of this invention could be used. For example, the powerful 35S promoter of cauliflower mosaic virus could be used for this purpose. In one embodiment of this invention, this promoter is duplicated in a vector known in the art as pKYLX 71:35S^2 (Morgan *et al.*, 1990). However, other plant expression vectors could be used for this purpose.

Once the gene is excised and re-subcloned into a desirable expression vector, the gene is transformed into a bacterium or other vector which is able to introduce the gene into a plant cell. Alternatively, the gene may be introduced into plant cells by a biolistic method (Carrer, 1995). Preferably, competent *Agrobacterium* cells are used for this purpose, and plant sections are exposed to the *Agrobacterium* harboring the BC1 gene. Regeneration of the plant cells in a selective medium to ensure the efficient uptake of the gene is preferred, following which the regenerated plants are grown under optimized conditions for survival.

As a result of this process, it has been discovered that a large proportion of regenerated tobacco plants which were transgenic for the BC1 gene had a spontaneously mutated gene which expressed a mutated gene product. Unexpectedly, the plants harboring the mutated gene had increased resistance to viral infection by both DNA and RNA plant viruses, without any observed deleterious effects resulting from expression of the mutated BC1 gene (in contrast, expression of the wild-type gene produces disease symptoms).

While those skilled in molecular biology are able to clone the known BC1 gene into a plant expression vector to obtain the mutated gene of the present invention, the mutant gene of this invention has also been deposited prior to filing the instant patent application with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 USA. The mutant gene was cloned in a bacterial vector (pGEM-T) and the construct is named TMBC1m. The deposit has been assigned accession number ATCC No. 97244 by the repository.

The subject deposit was deposited under conditions that assure that access to the deposit will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. 122. The deposit will be available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject deposit will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., it will be stored with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the culture. The depositor acknowledges the duty to replace the deposit should the depository be unable to furnish a sample when requested, due to the condition of the deposit. All restrictions on the availability to the public of the subject culture deposit will be irrevocably removed upon the granting of a patent disclosing it.

To use the biological materials deposited, all that is necessary is for the DNA to be solubilized in an appropriate transformation buffer for the cell type into which the gene is to be transformed. For *E. coli*, competent cells are prepared and transformed according to methods well known in the art (see Maniatis *et al.*, 1982), and transformed cells selected in an ampicillin growth medium. The plasmid is then isolated from the *E. coli* and excised from the pGEM-T vector using, for example, HindIII restriction enzyme. The excised gene fragment has a size of about 1100 bp. The HindIII fragment is then cloned into the HindIII site of an appropriate expression vector as described below. In addition to the above, Figure 1 provides the sequence of the mutant gene of this invention, except for a stretch of 25 nucleotides corresponding to positions 1742-1766, which were not identified in initial sequencing efforts. There are several mutations in the polynucleotide of Figure 1. Those mutations are part of the instant invention. Further, Figure 2 provides the complementary strand of the mutant polynucleotide and shows the HindIII termini. Figure 3

provides the deduced amino acid sequence of the mutated gene product, except for amino acids 151-159 which were not identified in the initial sequencing. The differences in amino acid sequence between the wild-type BC1 product and mutant BC1 products are shown in Figure 9B. Figure 4 shows a comparison between the wild-type (upper sequence) and mutant protein (lower sequence) based on initial sequencing efforts.

While this description provides a specific gene and fragments thereof which confer resistance on plants to geminivirus and tobamovirus infection, those skilled in the art will recognize that mutations other than or in addition to the specific mutations shown herein could achieve similar results. In fact, the method taught herein, by which the mutant gene disclosed herein was obtained, is broadly applicable to the obtention of similarly useful mutated movement genes of any virus. Furthermore, it is predictable, based on the instant disclosure, that the instant genes and polynucleotide molecules described herein, as well as likewise-derived genes, can confer resistance on a plant against infection by a wide variety of plant pathogens which depend on movement gene or other gene products for their pathogenesis, including both DNA and RNA viruses.

Example 1 - Development of transgenic tobacco plants

A. Construction of BC1 gene into an expression vector. The BC1 gene (nucleotides between 1278 and 2311 of the B component of tomato mottle geminivirus; Abouzid *et al.*, 1992) was amplified from the extracts of tomato mottle geminivirus infected tomato plants by polymerase chain reaction (PCR) technology. The primers used to amplify viral BC1 were

5′-CCCAAGCTTCGAGTTCGAAACTGC-3′ (SEQ ID NO. 1) and

5′-CCCAAGCTTAACGAAGTGTGTTTGAC-3′ (SEQ ID NO. 2).

The amplified BC1 segment was cloned into a pGEM-T vector and then digested with Hind III. The excised BC1 segment was ligated into the unique Hind III site of the binary pKYLX 71:35 S^2 vector.

B. Agrobacterium transformation. Competent cells of *Agrobacterium tumefaciences* LBA 4404 were prepared as described by An, *et al.*, (1985). The BC1 gene in the pKYLX 71:35 S^2 vector was directly transferred into the *Agrobacterium*. The clone was kept in a -80° C freezer for further use.

C. Plant transformation. The *Agrobacterium* carrying the BC1 gene in the pKYLX 71:35 S^2 vector was used to transform the leaf discs of *Nicotiana tobacum* cv. Xanthi. The *Agrobacterium* cells were cultured in YEP broth containing 50 μg/ml kanamycin and 10 μg/ml tetracycline and 25 μg/ml streptomycin for 24-30 hours. *Agrobacterium* cells were collected and resuspended in YEP

broth. Leaf discs cut from expanded young sterile seedlings were dipped into the *Agrobacterium* suspension and then placed on a selective medium containing 200 μg/ml Mefoxin and 100 μg/ml kanamycin. Regeneration and selection were carried out with the media, and took 6-8 weeks. The kanamycin resistant plants were individually grown in soil under sterile condition for a week, and then transplanted to pots in a growth room and/or greenhouse.

Example 2 - PCR, Southern blot and ELISA analysis

Transformation of the tobacco plants was confirmed by PCR analysis for BC1 gene in chromosomal DNA extracts, by Southern blotting with a BC1 probe, and by ELISA analysis for NPT II (Neomycin Phosphotransferase II). Twenty-three plants were transgenic for BC1.

Example 3 - Western blot analysis

Infected leaves of tomato plants were powdered after freezing in liquid nitrogen and extensively ground with a mortar and pestle in two volumes of ice-cold grinding buffer (GB: 100 mM Tris-HC1, pH 8.0, 10 mM EDTA and 5 ml dithiothreitol)(Deom, *et al.*, 1990). Membrane and cell-wall fractions were prepared as described by Pascal, *et al.*, (1993). The blotting procedure was conducted essentially as described by Towbin, *et al.*, (1979) using a Bio-Rad Mini-Protein Electrophoresis Cell and Bio-Rad Trans-Blot Electrophoretic Transfer Cell. The separator gel for small proteins was prepared with 12.5% polyacrylamide in gel buffer (Laemmli, 1970). The protein gels were transferred to nitrocellulose membrane (Bio-Rad Trans-Blot, 0.4 μm). The detection of expressed BC1 protein in transgenic tobacco plants was conducted with Western-Light~Chemiluminescent Detection System (TROPIX, Inc.). The BC1 protein was detected at a relatively high level and extracts from about 50% of the plants showed a smaller (truncated) BC1 protein (28k Da) than the wild-type (33k Da).

Example 4 - Evaluation of transgenic tobacco plants for symptoms due to the expression of the BC1 gene

The BC1 gene has been implicated as a symptom inducing element of a bipartite geminivirus during infection. Eleven transgenic tobacco plants which expressed the full length BC1 protein showed disease symptoms. Twelve plants expressing the truncated BC1 protein did not show disease symptoms.

Example 5 - Resistance to tomato mottle geminivirus and tobacco mosaic tobamovirus

Transformed tobacco plants (R_1 generation) expressing BC1 were tested for susceptibility to tomato mottle geminivirus infection by natural transmission with the whitefly vector and by mechanical inoculation with extracts from infected plants. The inoculated plants were evaluated for resistance to tomato mottle geminivirus by symptom development, and by enzyme linked immuno-assays (ELISA) using antiserum reactive to tomato mottle geminivirus coat protein. The transgenic plants expressing the truncated BC1 protein were free of symptoms and had very low ELISA readings. Transgenic tobacco plants subjected to mechanical inoculation with tobacco mosaic tobamovirus showed reduced disease symptoms compared to inoculated non-transgenic plants.

Example 6 - Analysis of the BC1 gene expressing truncated protein

The BC1 gene from the tobacco plants expressing the truncated BC1 protein was PCR-amplified and sequenced. This data indicates that the BC1 gene has undergone spontaneous mutation(s) in about 50% of the transgenic BC1 tobacco plants. During the tissue culture phase, plant cells containing the mutated BC1 gene may have a selective advantage over the wild-type BC1 expressing cells.

Example 7 - Production of transgenic tomato plants

The mutated BC1 gene in the pKYLX 71:35 S^2 vector is suitable for the production of tomatoes transgenic for the gene via *Agrobacterium* transformation as described above for tobacco. The mutated BC1 gene provides similar resistance to tomato mottle geminivirus in tomato as seen in transgenic tobacco. The introduction of this mutated BC1 gene into the chromosome of desirable tomato lines leads to tomato mottle geminivirus resistance in commercially acceptable tomato cultivars/hybrids. In addition, it is predictable that this resistance is active against other geminivirus infections. Resistance to tobacco mosaic virus was also detected in the transgenic tobacco expressing the mutated BC1 gene, indicating that resistance to RNA viruses also is possible with the expression of this mutated gene from a DNA plant virus. The mutated gene in tomato offers resistance to tomato mosaic tobamovirus, a virus related to tobacco mosaic tobamovirus.

Example 8 - Production of BC1 gene fragments useful for conferring virus resistance to plants.

Fragments of the mutant BC1 gene which are useful for conferring virus resistance to plants can be produced by use of BAL31 exonuclease for time-controlled limited digestion of the mutant BC1 gene. Methods of using BAL31 exonuclease for this purpose are well known in the art, and have been widely used for over a decade (Wei *et al.*, 1983). By using BAL31 exonuclease, one can

easily remove nucleotides from either or both ends of the mutant BC1 gene to systematically and certainly generate a wide spectrum of DNA fragments which have controlled lengths and are from controlled locations along the entire length of the mutant BC1 gene. Hundreds of such fragments from various points along the entire mutant BC1 gene DNA sequence can be systematically generated in one afternoon. These gene fragments are then cloned into appropriate vectors and ultimately transferred into plant cells according to the methods disclosed above. Plant cells transformed with these fragments are routinely cultured and regenerated into plants, which are then tested for resistance to viruses. In this manner, fragments of the mutant BC1 gene which are sufficient to confer viral resistance are routinely and predictably identified.

Example 9—Production of additional mutants conferring virus resistance to plants.

Tobacco was transformed with the movement protein (pathogenicity) gene (BC1) from tomato mottle geminivirus (TMoV) using *Agrobacterium*-mediated transformation. Different transgenic tobacco lines expressing the BC1 protein had phenotypes ranging from plants with severe stunting and leaf mottling to plants with no visible symptoms. The sequence data for the BC1 transgene for the different phenotypes indicated unexpected mutation(s). A mutated BC1 transgene suppressed the phenotypic expression of the symptomatic BC1 gene in tobacco lines containing both copies of the BC1 gene. The present invention shows spontaneous mutations in the transgene to be common in *Agrobacterium*-mediated transformations, and this phenomenon can be utilized in the creation and selection of pathogen-resistant plants using pathogenicity genes during transformation.

The expression of tomato mottle gemini virus (TMoV) (Abouzid *et al.*, 1992) movement protein gene (BC1) was examined in transgenic tobacco plants for evaluation of function and for possible utilization in pathogen-derived resistance. The BC1 gene has been implicated as a symptom inducing element of a bipartite geminivirus during expression in transgenic plants (Pascal *et al.*, 1993; von Arnim and Stanley, 1992). Transgenic tobacco expressing the BC1 gene was constructed utilizing standard *Agrobacterium*-mediated transformation. Surprisingly, a number of plants expressing TMoV BC1 protein based on Western blot analysis, did not show the expected virus-symptom phenotype. Only 11 of the 19 transgenic R_0 tobacco plants which expressed the BC1 protein showed disease symptoms ranging from mild to severe. The observation that eight plants expressing the BC1 protein did not show symptoms was unexpected.

From further analysis, the three phenotypes were observed in the R_1 generation derived from a R_0 plant which did not show any apparent stunting (Fig. 5). The three observed phenotypes were: 1) Severe stunting and mottling, more severe than the typical symptoms associated with TMoV

infections in tobacco; 2) Mottling with no stunting of growth; and 3) No visible symptoms, plants indistinguishable from nontransformed plants.

These transgenic plants were analyzed by Southern blots to identify gene copy number (Fig. 6). The transgenic plant showing slight mottling with no stunting had two copies of the BC1 gene. Other progeny from this line which had a severe symptom phenotype or a non-symptomatic phenotype only had one copy. Progeny from three other lines examined, one with symptomatic and two with non-symptomatic phenotypes, had 3, 3, and 5 copies of the BC1 gene, respectively.

High levels of the BC1 protein expression were indicated in the young tissues in all transgenic plants by Western blot analysis except for one non-symptomatic line which showed low levels of a truncated BC1 protein. Non-symptomatic plant (BC1-3-6-4; phenotype not shown) had a similar level of BC1 protein as the symptomatic plant (BC1-3-11-5). Extracts from the attenuated-symptom plant (Fig. 5A and B; BC1-3-11-2) showed both full-length and truncated BC1 proteins. The low level of the truncated BC1 protein detection may be due to the loss of epitopes since 121 amino acid residues were lost at the carboxy end (see below). BC1 proteins (full-length or truncated form) from the non-symptomatic, transgenic plants were not detected in older tissue, unlike that seen for the transgenic plants expressing the severe symptom type BC1 protein. This indicated that certain mutations in the BC1 protein may affect its stability *in planta*.

Northern blots indicated a high transcript number for all the transgenic lines (Fig. 8). The non-symptomatic plant shown in Fig. 5A had a smaller than expected transcript. This apparent deletion in the transcript is consistent with the truncated BC1 protein seen in Western blots (Fig. 7). The transcript level for the plants expressing the truncated BC1 protein was high and therefore the low level of truncated BC1 protein detected in Western blots (Fig. 7) is not due to transcript activity. The larger than expected transcript is the result of a readthrough of BC1 termination signals into the vector *rbcS* termination sequences of the pKYLX vector.

The BC1 gene from the transgenic tobacco plants showing the different phenotypes was amplified by polymerase chain reaction (PCR) and sequenced. The sequence data revealed mutations (amino acid residue 215 G-S, 219 S-L, and 247 E-G) near the carboxyl terminus of the BC1 protein (Fig. 9) for the severe stunting phenotype (Fig. 5A). Two mutants were associated with non-symptomatic, transgenic tobacco. One mutant (resolved from BC1-3-6-3) showed several changes near the amino terminus (amino acid residue 6 V-F, 7 N-S, and 35 F-L) while the other (resolved from BC1-3-11-6, Fig. 5A) showed a change in amino acid residue 12 F-C, a deletion of amino acid residues 174-293, and an unidentified fusion sequence of 26 amino acid residues starting after amino acid residue 173 (Fig. 9). This was consistent with the detection of a truncated BC1 protein (~10 kDa smaller in size compared to the wild type) in Western blots from extracts from these transgenic

plants. The transcript for the truncated, BC1 protein was sequenced after reverse transcription of extracted total RNA using oligo dT primer followed by PCR amplification using a BC1 specific primer.

The non-symptomatic, transgenic R_0 tobacco plants revealed segregation in the R_1 generation as indicated by the appearance of several symptomatic plants in this generation. Some lines with symptom attenuation (Fig. 5B) continued to segregate in the R_2 generation but the non-symptomatic plants did not. Southern blot analysis (Fig. 6) indicated multiple copies of the BC1 gene in the R_0 tobacco. Apparently some of the R_0 tobacco lines contained copies of both the symptomatic and non-symptomatic forms of BC1. This was confirmed by Southern blot and Western blot analyses of selected R_1 tobacco plants which were associated with the different phenotypes (Fig. 5). The mottling phenotype with no stunting described above (Fig. 5) had one copy each of the symptomatic and non-symptomatic forms of BC1. Transgenic tobacco containing copies of both symptomatic and non-symptomatic forms of the BC1 gene (Fig. 5B) resulted in a nonstunting with mild mottling phenotype. This indicated that the non-symptomatic BC1 gene suppressed (trans-dominant negative interference) the symptom inducing element(s) of symptomatic BC1 gene in transgenic plants containing both forms. Transgene silencing (Meins Jr. and Kunz, 1995) was not evident in these plants since both proteins were detected in Western blots (Fig. 7, BC1-3-11-2). Furthermore, the expression of the symptomatic phenotype in subsequent generations indicated that the symptomatic BC1 gene was not in an inactive form in the phenotype suppressed R_0 tobacco. The symptom suppression was also effective against virus infection since tobacco plants with the mutated, non-symptomatic BC1 transgene remained free of TMoV symptoms under high disease pressure from viruliferous whiteflies over a 3-month period.

All of the BC1 transgenic plants that were analyzed revealed spontaneous/unexpected mutations in the BC1 gene. Point mutations were found in all transgenes analyzed, and one transgene showed a major deletion at the 3′ end and with a fusion of an unidentified sequence of ~250 nucleotides (no close relationship with sequences in GenBank using BLAST). This may have occurred by a chromosomal cross-over event during plant cell division after the BC1 gene was integrated into the tobacco chromosome. In the latter case, a truncated BC1 protein (~10 kDa smaller in size compared to the wild type) was detected in Western blots and a smaller transcript was detected in Northern blots. This indicated that a deletion in the transgene as well as point mutations (described previously) are sources of variation in transgene expression.

Other studies with the expression of foreign genes in transgenic plants show varying levels of expressivity in the different generated lines or in siblings in a transgenic line (Hull, 1994). Varying levels of resistance in different transgenic plant lines transformed with the same gene appear

to be the norm in pathogen-derived resistance studies. These variations are not adequately explained by positional effects due to the random integration in the plant chromosome during transformation. Silencing of genes in transgenic plants is considered a general phenomenon when multiple copies of transgenes are introduced into plant cells (Meins Jr. and Kunz, 1995). All of our R_0 transgenic plants analyzed contained multiple copies of the BC1 transgene with no apparent suppression of transgene expression.

Because the teachings herein used the classical methods of *Agrobacterium*-mediated transformation commonly used by others in the art, some of the variation in the expected phenotype reported in the literature can be explained by spontaneous mutations occurring during *Agrobacterium*-mediated transformation and during chromosomal rearrangements as reported here for TMoV BC1. Thus it is shown that spontaneous point mutations in the transgene during *Agrobacterium*-mediated transformation and other modifications in the transgene by chromosomal rearrangements affect gene function and regulation with transgenes. The subject invention also concerns the polynucleotide molecules shown in Figure 9A and the polypeptides encoded thereby shown in Figure 9B, as well as other mutated polynucleotides conferring viral resistance that can be produced using the teachings of the present invention.

The spontaneous mutations that can be produced in viral movement genes using the methods and materials of the present invention during *Agrobacterium*-mediated transformation provide a simple way to develop pathogen-resistant plants. For geminiviruses, the introduction of the pathogenicity gene (BC1 for the bipartite geminiviruses, AC4 for the monopartite like tomato yellow leaf curl virus) into plant cells by *Agrobacterium*-mediated transformation will result in selection since transformed cells which express the non-mutated pathogenicity genes will not grow as well as those cells which express the mutated pathogenicity gene. After transformation, visual evaluation for the non-symptomatic phenotype and Western blot analysis for pathogenicity gene protein expression is all that is needed to select geminivirus-resistant plants for further screening and evaluation. All transgenic tobacco with a non-symptomatic phenotype and with TMoV BC1 protein expression showed virus resistance. Similarly, certain pathogenicity genes from bacterial or fungal plant pathogens can be introduced into transgenic plants according to these teachings and the selection pressure will result in pathogen-resistant plants.

The amino acid sequences disclosed herein are based on standard single letter abbreviations for amino acid residues.

While the foregoing description and examples provide details regarding the methods of making and using the invention, including its best mode, it is to be understood that obvious

variations and functional equivalents thereof are to be considered part of this invention and therefore fall within the scope of the claims which follow.

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Claims

1. A mutated plant virus BC1 gene which confers enhanced viral resistance to plants harboring said mutated gene.

2. The gene of claim 1 wherein the viral resistance is against a virus selected from the group consisting of tobamovirus and geminivirus.

3. The mutated gene of claim 1 prepared by the process of isolating the natural gene, inserting the natural gene into the genome of a plant, and identifying plants that have increased resistance to viral infection.

4. The mutated gene of claim 1 which encodes a gene product of about 28 kDa.

5. A method for conferring on a plant enhanced resistance against plant viral infection which comprises insertion of a viral movement gene into said plant and identifying a plant spontaneously expressing a mutant of said gene which confers said enhanced resistance against plant viral infection on said plant while at the same time not inducing pathogenic symptoms in the plant.

6. The method of claim 5 in which the viral movement gene is a plant virus BC1 gene.

7. A transgenic plant having increased resistance to viral infection, said plant being transgenic for a mutated plant virus BC1 gene.

8. The plant of claim 7 which is a transgenic tomato or tobacco plant.

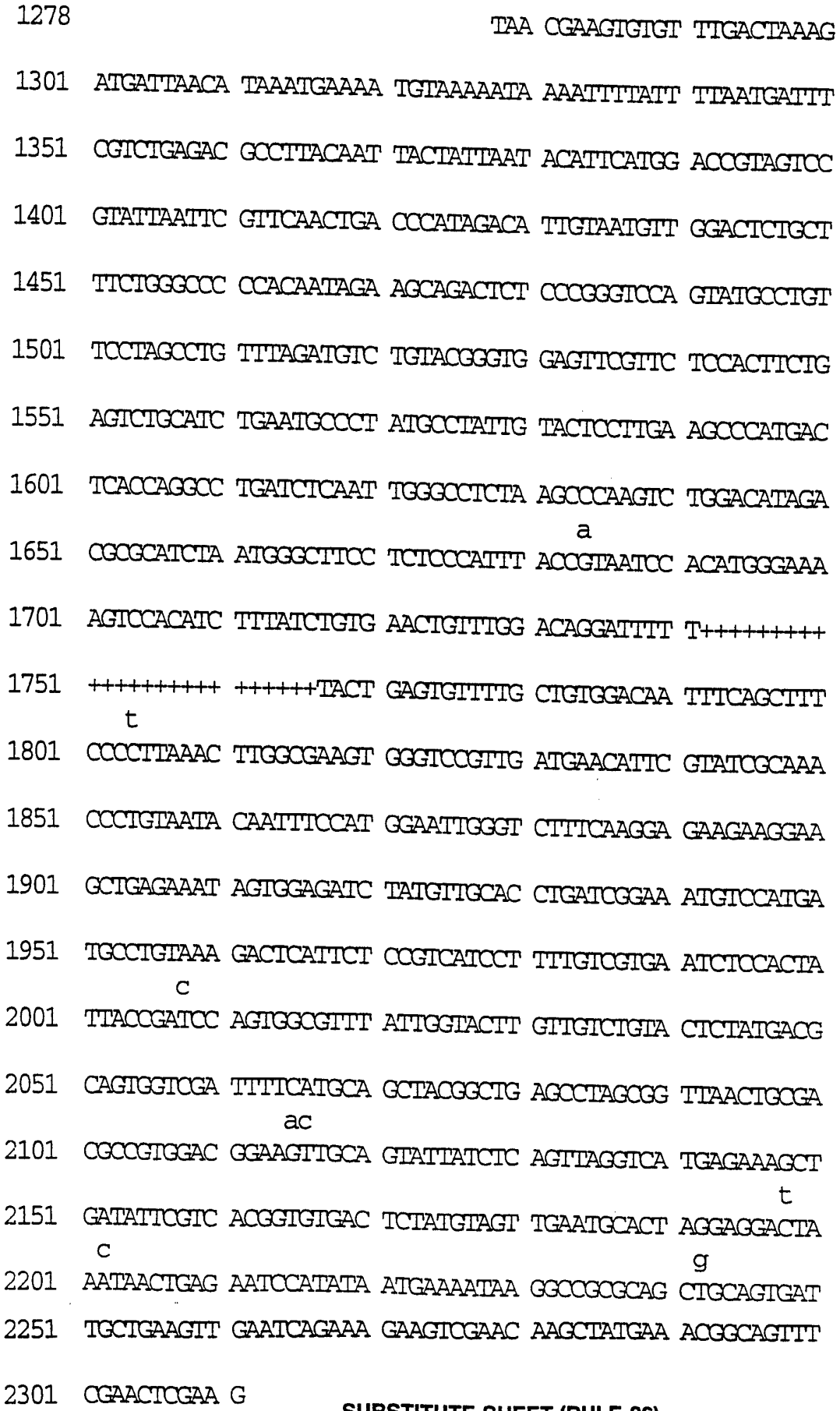
9. The plant of claim 8 in which the plant has enhanced resistance against infection by tomato mottle geminivirus or tobacco mosaic tobamovirus.

10. The mutated gene of claim 1 which comprises any or all of the mutations as compared to the wild-type gene, shown in Figure 1 or Figure 9A.

11. The mutated gene of claim 1 comprising all or a portion of the sequence shown in Figure 1.

12. A mutant BC1 protein comprising any or all of the wild-type to mutant amino acid substitutions shown in Figure 4 or Figure 9B.

FIG. 1A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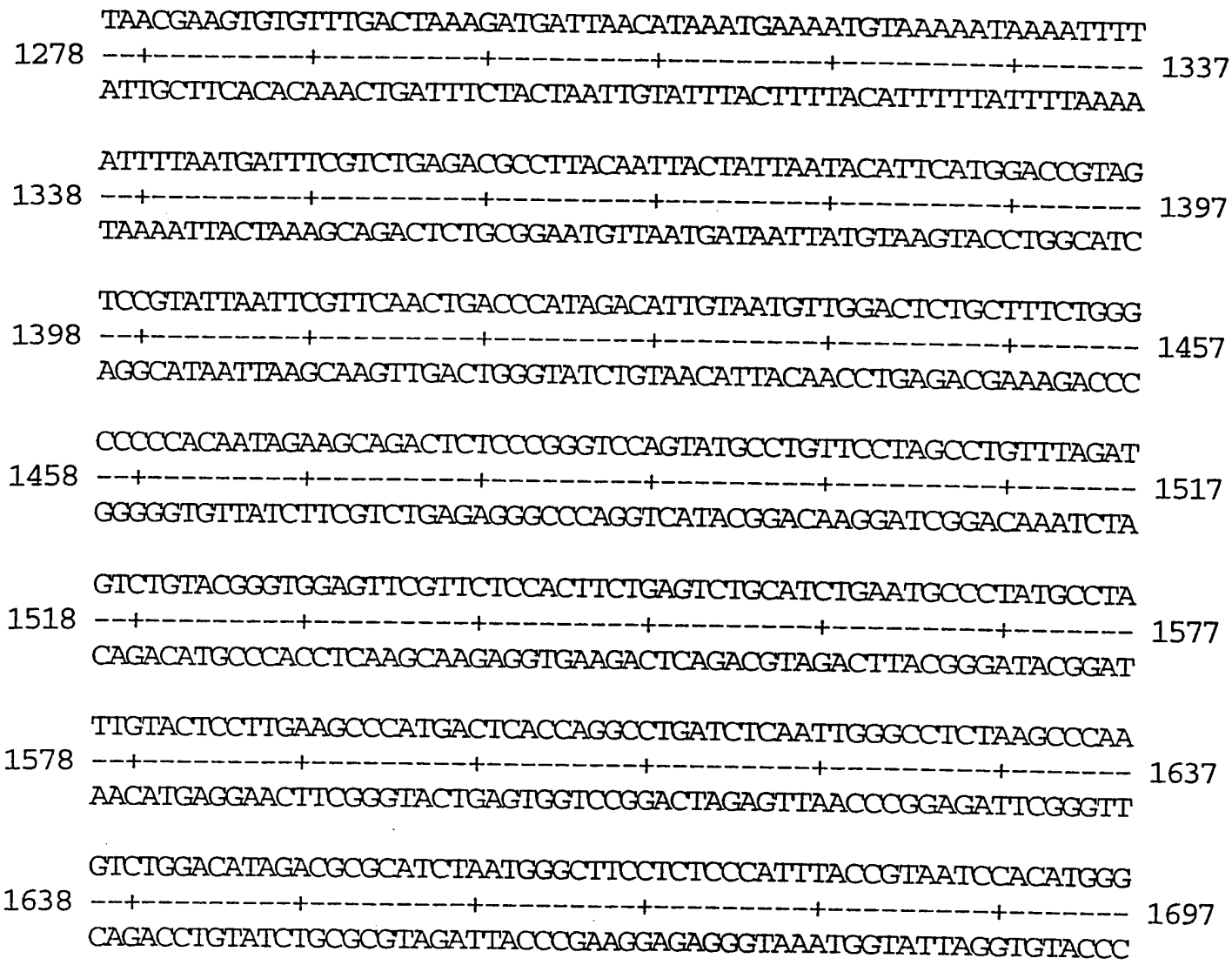
1301 ATGATTAACA TAAATGAAAA TGTAAAAATA AAATTTTATT TTAATGATTT
1351 CGTCTGAGAC GCCTTACAAT TACTATTAAT ACATTCATGG ACCGTAGTCC
1401 GTATTAATTC GTTCAACTGA CCCATAGACA TTGTAATGTT GGACTCTGCT
1451 TTCTGGGCCC CCACAATAGA AGCAGACTCT CCCGGGTCCA GTATGCCTGT
1501 TCCTAGCCTG TTTAGATGTC TGTACGGGTG GAGTTCGTTC TCCACTTCTG
1551 AGTCTGCATC TGAATGCCCT ATGCCTATTG TACTCCTTGA AGCCCATGAC
1601 TCACCAGGCC TGATCTCAAT TGGGCCTCTA AGCCCAAGTC TGGACATAGA
a
1651 CGCGCATCTA ATGGGCTTCC TCTCCCATTT ACCGTAATCC ACATGGGAAA
1701 AGTCCACATC TTTATCTGTG AACTGTTTGG ACAGGATTTT T+++++++++
1751 ++++++++++ ++++++TACT GAGTGTTTTG CTGTGGACAA TTTCAGCTTT
t
1801 CCCCTTAAAC TTGGCGAAGT GGGTCCGTTG ATGAACATTC GTATCGCAAA
1851 CCCTGTAATA CAATTTCCAT GGAATTGGGT CTTTCAAGGA GAAGAAGGAA
1901 GCTGAGAAAT AGTGGAGATC TATGTTGCAC CTGATCGGAA ATGTCCATGA
1951 TGCCTGTAAA GACTCATTCT CCGTCATCCT TTTGTCGTGA ATCTCCACTA
c
2001 TTACCGATCC AGTGGCGTTT ATTGGTACTT GTTGTCTGTA CTCTATGACG
2051 CAGTGGTCGA TTTTCATGCA GCTACGGCTG AGCCTAGCGG TTAACTGCGA
ac
2101 CGCCGTGGAC GGAAGTTGCA GTATTATCTC AGTTAGGTCA TGAGAAAGCT
t
2151 GATATTCGTC ACGGTGTGAC TCTATGTAGT TGAATGCACT AGGAGGACTA
c
g
2201 AATAACTGAG AATCCATATA ATGAAAATAA GGCCGCGCAG CTGCAGTGAT
2251 TGCTGAAGTT GAATCAGAAA GAAGTCGAAC AAGCTATGAA ACGGCAGTTT
2301 CGAACTCGAA G

FIG. 2A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1278 --+---------+---------+---------+---------+---------+---------+------- 1337
ATTGCTTCACACAAACTGATTTCTACTAATTGTATTTACTTTTACATTTTTATTTTAAAA
ATTTTAATGATTTCGTCTGAGACGCCTTACAATTACTATTAATACATTCATGGACCGTAG
1338 --+---------+---------+---------+---------+---------+---------+------- 1397
TAAAATTACTAAAGCAGACTCTGCGGAATGTTAATGATAATTATGTAAGTACCTGGCATC
TCCGTATTAATTCGTTCAACTGACCCATAGACATTGTAATGTTGGACTCTGCTTTCTGGG
1398 --+---------+---------+---------+---------+---------+---------+------- 1457
AGGCATAATTAAGCAAGTTGACTGGGTATCTGTAACATTACAACCTGAGACGAAAGACCC
CCCCCACAATAGAAGCAGACTCTCCCGGGTCCAGTATGCCTGTTCCTAGCCTGTTTAGAT
1458 --+---------+---------+---------+---------+---------+---------+------- 1517
GGGGGTGTTATCTTCGTCTGAGAGGGCCCAGGTCATACGGACAAGGATCGGACAAATCTA
GTCTGTACGGGTGGAGTTCGTTCTCCACTTCTGAGTCTGCATCTGAATGCCCTATGCCTA
1518 --+---------+---------+---------+---------+---------+---------+------- 1577
CAGACATGCCCACCTCAAGCAAGAGGTGAAGACTCAGACGTAGACTTACGGGATACGGAT
TTGTACTCCTTGAAGCCCATGACTCACCAGGCCTGATCTCAATTGGGCCTCTAAGCCCAA
1578 --+---------+---------+---------+---------+---------+---------+------- 1637
AACATGAGGAACTTCGGGTACTGAGTGGTCCGGACTAGAGTTAACCCGGAGATTCGGGTT
GTCTGGACATAGACGCGCATCTAATGGGCTTCCTCTCCCATTTACCGTAATCCACATGGG
1638 --+---------+---------+---------+---------+---------+---------+------- 1697
CAGACCTGTATCTGCGCGTAGATTACCCGAAGGAGAGGGTAAATGGTATTAGGTGTACCC

FIG. 2B

```
      AAAAGTCCACATCTTTATCTGTGAACTGTTTGGACAGGATTTTT++++++++++++++++
1698  --+---------+---------+---------+---------+---------+---------+------- 1757
      TTTTCAGGTGTAGAAATAGACACTTGACAAACCTGTCCTAAAAA++++++++++++++++

      ++++++++++TACTGAGTGTTTTGCTGTGGACAATTTCAGCTTCCCCCTAAACTTGGCGA
1758  --+---------+---------+---------+---------+---------+---------+------- 1817
      ++++++++++ATGACTCACAAAACGACACCTGTTAAAGTCGAAGGGGGATTTGAACCGCT

      AGTGGGTCCGTTGATGAACATTCGTATCGCAAACCCTGTAATACAATTTCCATGGAATTG
1818  --+---------+---------+---------+---------+---------+---------+------- 1877
      TCACCCAGGCAACTACTTGTAAGCATAGCGTTTGGGACATTATGTTAAAGGTACCTTAAC

      GGTCTTTCAAGGAGAAGAAGGAAGCTGAGAAATAGTGGAGATCTATGTTGCACCTGATCG
1878  --+---------+---------+---------+---------+---------+---------+------- 1937
      CCAGAAAGTTCCTCTTCTTCCTTCGACTCTTTATCACCTCTAGATACAACGTGGACTAGC

      GAAATGTCCATGATGCCTGTAAAGACTCATTCTCCGTCATCCTTTTGTCGTGAATCTCCA
1938  --+---------+---------+---------+---------+---------+---------+------- 1997
      CTTTACAGGTACTACGGACATTTCTGAGTAAGAGGCAGTAGGAAAACAGCACTTAGAGGT

      CTATTACCGATCCAGTGGCGTTTATTGGTACTTGTTGTCTGTACTCTATGACGCAGTGGT
1998  --+---------+---------+---------+---------+---------+---------+------- 2057
      GATAATGGCTAGGTCACCGCAAATAACCATGAACAACAGACATGAGATACTGCGTCACCA
```

FIG. 2C

```
     CGATTTTCATGCAGCTACGGCTGAGCCTAGCGGTTAACTGCGACGCCGTGGACGGAAGTT
2058 --+---------+---------+---------+---------+---------+------- 2117
     GCTAAAAGTACGTCGATGCCGACTCGGATCGCCAATTGACGCTGCGGCACCTGCCTTCAA

     GCAGTATTATCTCAGTTAGGTCATGAGAAAGCTGATATTCGTCACGGTGTGACTCTATGT
2118 --+---------+---------+---------+---------+---------+------- 2177
     CGTCATAATAGAGTCAATCCAGTACTCTTTCGACTATAAGCAGTGCCACACTGAGATACA

     AGTTGAATGCACTAGGAGGACTAAATAACTGAGAATCCATATAATGAAAATAAGGCCGCG
2178 --+---------+---------+---------+---------+---------+------- 2237
     TCAACTTACGTGATCCTCCTGATTTATTGACTCTTAGGTATATTACTTTTATTCCGGCGC

     CAGCTGCAGTGATTGCTGAAGTTGAATCAGAAAGAAGTCGAACAAGCTATGAAACGGCAG
2238 --+---------+---------+---------+---------+---------+------- 2297
     GTCGACGTCACTAACGACTTCAACTTAGTCTTTCTTCAGCTTGTTCGATACTTTGCCGTC

     TTTCGAACTCGAAG
2298 --+----------+- 2311
     AAAGCTTGAGCTTC
```

FIG. 3

```
  1  MDSQLFSPPS AFNYIESHRD EYQLSHDLIE IILQLPSIAS QLIARLSRSC
 51  MKIDHCVIEY RQQVPINAIG SVIVEIHDKR MIENESLQAS WIFPIRCNID
101  LHYFSASFFS LKDPIPWKLY YRVCDINVHQ RIHFAKFRGK LKLSIAKHSV
151  +++++++++K ILSKQFIDKD VDFSHVDYGK WERKPIRCAS MSRLGLRGPI
201  EIRPGESWAS RSIIGIGHSD ADSEVENELH PYRHLNRLGI GILDPGESAS
251  IVGAQKAESN IIMSMGQLNE LIRITVHECI NSNCKASQIK SLK*
```

FIGURE 4

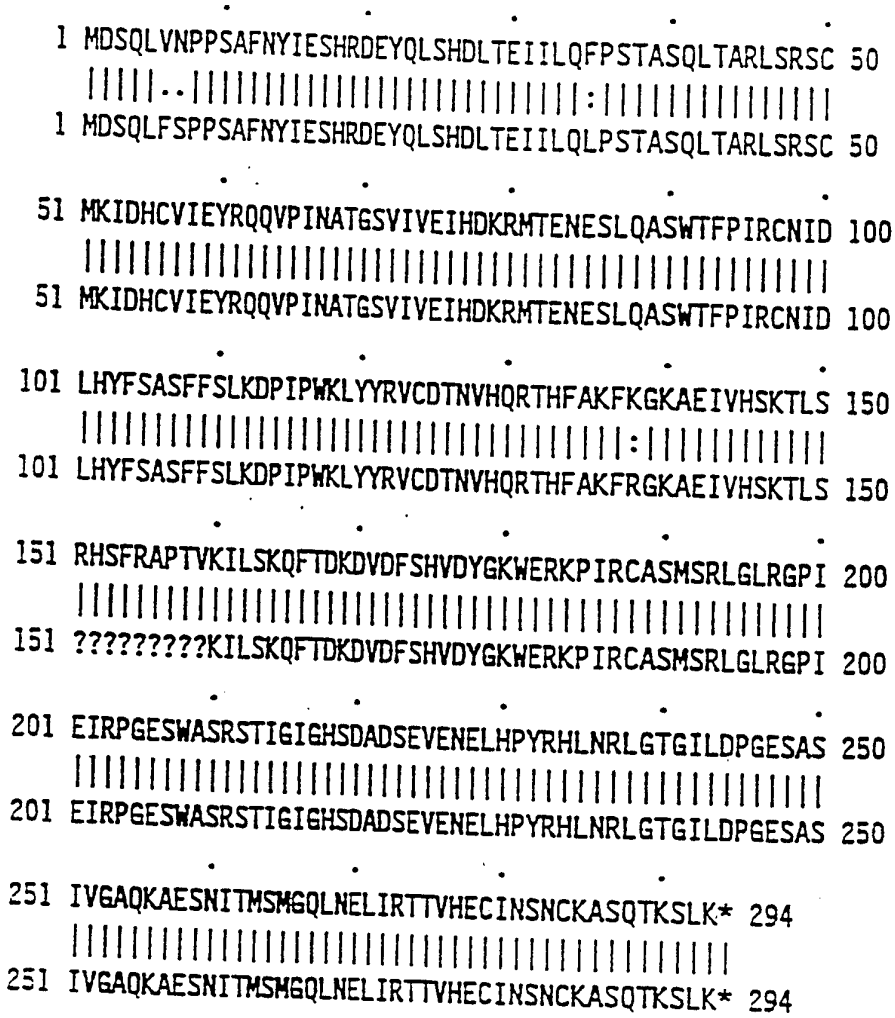
1 MDSQLVNPPSAFNYIESHRDEYQLSHDLTEIILQFPSTASQLTARLSRSC 50
1 MDSQLFSPPSAFNYIESHRDEYQLSHDLTEIILQLPSTASQLTARLSRSC 50
51 MKIDHCVIEYRQQVPINATGSVIVEIHDKRMTENESLQASWTFPIRCNID 100
51 MKIDHCVIEYRQQVPINATGSVIVEIHDKRMTENESLQASWTFPIRCNID 100
101 LHYFSASFFSLKDPIPWKLYYRVCDTNVHQRTHFAKFKGKAEIVHSKTLS 150
101 LHYFSASFFSLKDPIPWKLYYRVCDTNVHQRTHFAKFRGKAEIVHSKTLS 150
151 RHSFRAPTVKILSKQFTDKDVDFSHVDYGKWERKPIRCASMSRLGLRGPI 200
151 ?????????KILSKQFTDKDVDFSHVDYGKWERKPIRCASMSRLGLRGPI 200
201 EIRPGESWASRSTIGIGHSDADSEVENELHPYRHLNRLGTGILDPGESAS 250
201 EIRPGESWASRSTIGIGHSDADSEVENELHPYRHLNRLGTGILDPGESAS 250
251 IVGAQKAESNITMSMGQLNELIRTTVHECINSNCKASQTKSLK* 294
251 IVGAQKAESNITMSMGQLNELIRTTVHECINSNCKASQTKSLK* 294

FIG. 5B

FIG. 5A

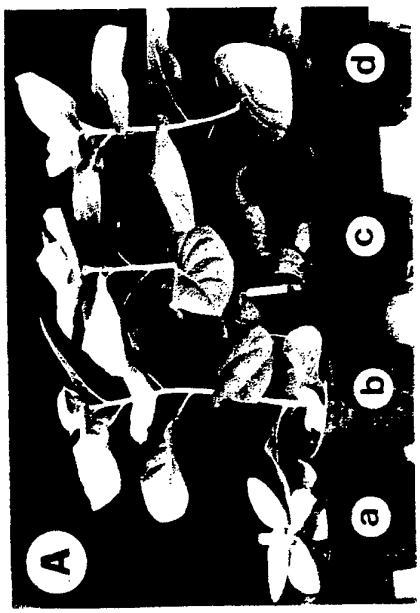

FIG. 6

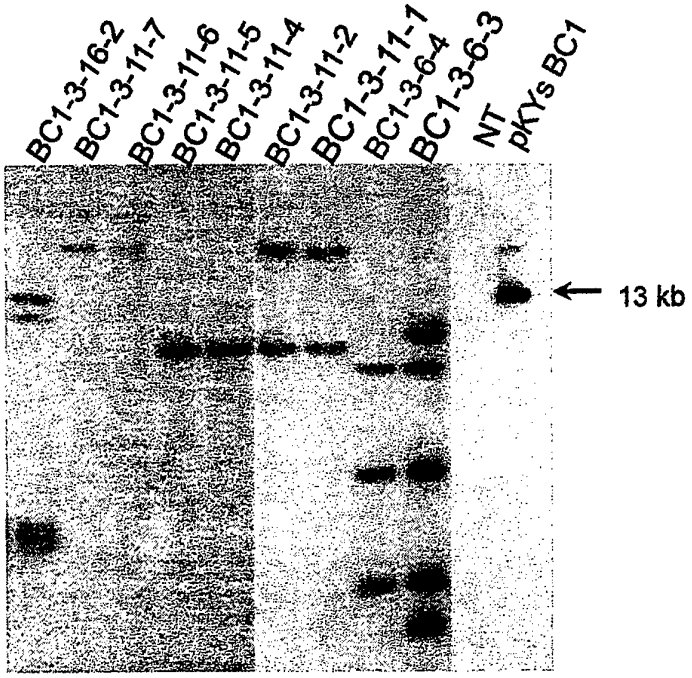

FIG. 7

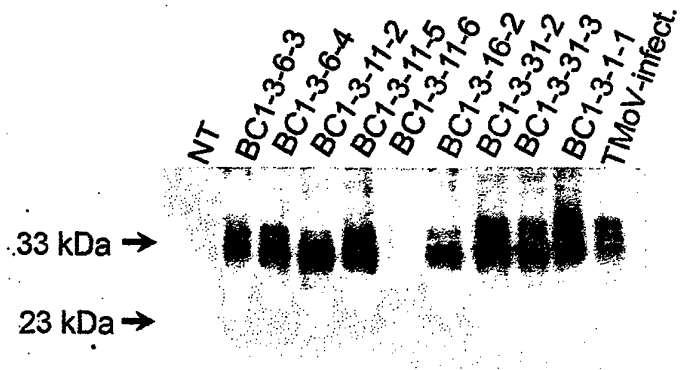

FIG. 8

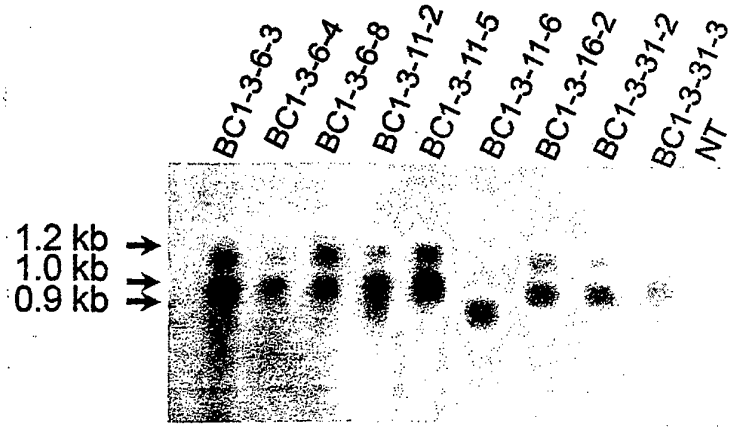

FIG. 9A-1

```
                1                                                    50
BC1wt            CGAGTTCGAA ACTGCCGTTT CATAGCTTGT TCGACTTCTT TCTGATTCAA
BC1S             .......... .......... .......... .......... ..........
BC1A             .......... .......... .......... .......... ..........
BC1scDNA         .......... .......... .......... .......... ..........
BC1At/rCDNA      .......... .......... .......... .......... ..........

               51                                                   100
               CTTCAGCAAT CACTGCCGCT GCGCGGCCTT ATTTTCATTA TATGGATTCT
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........

               101                                                  150
               CAGTTAGTTA ATCCTCCTAG TGCATTCAAC TACATAGAGT CACACCGTGA
               .......... .......... .......... .......... ..........
               ......T... G......... .......... .......... ..........
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........

               151                                                  200
               CGAATATCAG CTTTCTCATG ACCTAACTGA GATAATACTG CAGTTTCCGT
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ......A...
               .......... .......... .......... .......... ..........
               .......... .......... .......... .......... ..........
```

FIG. 9A-2

```
201                                                          250
CCACGGCGTC  GCAGTTAACC  GCTAGGCTCA  GCCGTAGCTG  CATGAAAATC
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........

251                                                          300
GACCACTGCG  TCATAGAGTA  CAGACAACAA  GTACCAATAA  ACGCCACTGG
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........

301                                                          350
GTCGGTAATA  GTGGAGATTC  ACGACAAAAG  GATGACGGAG  AATGAGTCTT
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........

351                                                          400
TACAGGCATC  ATGGACATTT  CCGATCAGGT  GCAACATAGA  TCTCCACTAT
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
..........  ..........  ..........  ..........  ..........
```

FIG. 9A-3

```
401                                                        450
TTCTCAGCTT CCTTCTTCTC CTTGAAAGAC CCAATTCCAT GGAAATTGTA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
451                                                        500
TTACAGGGTT TGCGATACGA ATGTTCATCA ACGGACCCAC TTCGCCAAGT
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
501                                                        550
TTAAGGGGAA GCTGAAATTG TCCACAGCAA AACACTCAGT AGACATTCCC
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
551                                                        600
TTCCGGGCAC CAACAGTAAA AATCCTGTCC AAACAGTTCA CAGATAAAGA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
```

FIG. 9A-4

```
601                                                        650
TGTGGACTTT TCCCATGTGG ATTACGGTAA ATGGGAGAGG AAGCCCATTA
.......... .......... ....T..... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... ....T..... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... ACCATTGAAA CTGAGCCAAA AATGCATGTT

651                                                        700
GATGCGCGTC TATGTCCAGA CTTGGGCTTA GAGGCCCAAT TGAGATCAGG
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
TTGGGGGAAA ATTCCAAATA GTGCAACTTT TGAACAAACA TACGATGGCT

701                                                        750
CCTGGTGAGT CATGGGCTTC AAGGAGTACA ATAGGCATAG GGCATTCAGA
.......... .......... .......... ....A..... .....T....
.......... .......... .......... .......... ..........
.......... .......... .......... ....A..... .....T....
AAAGTAGTAA CAAGGCAAAG GACGTAAACC TAAAATGATG TCCAAAAGCG

751                                                        800
TGCAGACTCA GAAGTGGAGA ACGAACTCCA CCCGTACAGA CATCTAAACA
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
.......... .......... .......... .......... ..........
TAATTATTCA CTGTAGATGT TTGTTTTACC AACCAAAGAA AAACTAAATT
```

FIG. 9A-5

```
801                                                            850
GGCTAGGAAC  AGGCATACTG  GACCCGGGAG  AGTCTGCTTC  TATTGTGGGG
.........  .........  .........  G........  .........
.........  .........  .........  .........  .........
.........  .........  .........  G........  .........
GTGTATCCTA  ATCAACAAAC  TATCAGTAAG  TTTCCAAATT  ATTTCTAAAA

851                                                            900
GCCCAGAAAG  CAGAGTCCAA  CATTACAATG  TCTATGGGTC  AGTTGAACGA
.........  .........  .........  .........  .........
.........  .........  .........  .........  .........
.........  .........  .........  .........  .........
AAAAAAAAAA .........  .........  .........  .........

901                                                            950
ATTAATACGG  ACTACGGTCC  ATGAATGTAT  TAATAGTAAT  TGTAAGGCGT
.........  .........  .........  .........  .........
.........  .........  .........  .........  .........
.........  .........  .........  .........  .........

951                                                           1000
CTCAGACGAA  ATCATTAAAA  TAAAATTTTA  TTTTTACATT  TTCATTTATG
.........  .........  .........  .........  .........
.........  .........  .........  .........  .........
.........  .........  .........  .........  .........

1001                                  1031
TTAATCATCT  TTAGTCAAAC  ACACTTCGTT  A
.........  .........  .........  .
.........  .........  .........  .
AAAAAAAAAA  AAAAA.....  .........  .
```

FIG. 9B

```
          1                                                  50
BC1wt     MDSQLVNPPS AFNYIESHRD EYQLSHDLTE IILQFPSTAS QLTARLSRSC
BC1A      .....FS... .......... .......... .....L.... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... .......... .......... ..........

         51                                                 100
BC1wt     MKIDHCVIEY RQQVPINATG SVIVEIHDKR MTENESLQAS WTFPIRCNID
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... .......... .......... ..........

        101                                                 150
BC1wt     LHYFSASFFS LKDPIPWKLY YRVCDTNVHQ RTHFAKFKGK LKLSTAKHSV
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... .......... .......... ..........

         151                                                200
BC1wt     DIPFRAPTVK ILSKQFTDKD VDFSHVDYGK WERKPIRCAS MSRLGLRGPI
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... .......... .......... .......... ..........
BC1At/r   .......... .......... ....PLKLSQ KCMFWGKIPN SATFEQTYDG*

         201                                                250
BC1wt     EIRPGESWAS RSTIGIGHSD ADSEVENELH PYRHLNRLGT GILDPGESAS
BC1A      .......... .......... .......... .......... ..........
BC1S      .......... ....S...L. .......... .......... ......G...

         251                                          294
BC1wt     IVGAQKAESN ITMSMGQLNE LIRTTVHECI NSNCKASQTK SLK*
BC1A      .......... .......... .......... .......... ...*
BC1S      .......... .......... .......... .......... ...*
```

INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 96/13097

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/34 C12N15/82 A01H5/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, August 1990, WASHINGTON US, pages 6291-6295, XP002024195 STANLEY, J., ET AL.: "Defective viral DNA ameliorates symptoms of geminivirus infection in plants" see the whole document ---	1-4,7,8
X	PROCEEDINGS OF THE SYMPOSIUM MOL. BIOL. TOMATO, 1993, pages 227-238, XP000564182 DE KOUCHKOVSKY F., ET AL: "MOLECULAR BIOLOGY OF TOMATO YELLOW LEAF CURL VIRUS (TYLCV) AND POTENTIAL WAYS TO CONTROL THE DISEASE" see page 230 - page 233 --- -/--	5,6

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 1997	11.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VIROLOGY, vol. 187, 1992, pages 555-564, XP002024283 VON ARNIM, A., ET AL.: "INHIBITION OF AFRICAN CASSAVA MOSAIC VIRUS SYSTEMIC INFECTION BY A MOVEMENT PROTEIN FROM THE RELATED GEMINIVIRUS TOMATO GOLDEN MOSIAC VIRUS" cited in the application see the whole document	1,2,4
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Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, April 1993, WASHINGTON US, pages 3134-3141, XP002024284 WILSON, T.M.A.: "Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms" see page 3138, column 1	7-9
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	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, October 1994, WASHINGTON US, pages 10310-10314, XP002024287 BECK, D.L., ET AL.: "Disruption of virus movement confers broad-spectrum resistance against systemic infection by plant viruses with triple gene block" see the whole document	1-9
3	---	
	-/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE PLANT CELL, vol. 5, July 1993, pages 795-807, XP002024288 PASCAL, E., ET AL.: "Transgenic tobacco plants expressing the geminivirus BL1 protein exhibit symptoms of viral disease" see the whole document	1-12
A	VIROLOGY, vol. 207, 20 February 1995, pages 191-204, XP002024289 INGHAM, D.J., ET AL.: "Both bipartite geminivirus movement proteins define viral host range, but only BL1 determines viral pathogenicity" cited in the application see the whole document	1-12
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P,A	ANNUAL MEETING OF THE AMERICAN PHYTOPATHOLOGICAL ASSOCIATION, PITTSBURGH, PENNSYLVANIA, USA, AUGUST 12-16, 1995. PHYTOPATHOLOGY 85 (10). 1995. 1142. , XP000617062 BROGLIO E P ET AL: "A virus-based vector system for evaluating the usefulness of geminivirus gene constructs in conferring pathogen-derived resistance." see abstract 220	1-12
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