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**WO 03/093483 A1**

(54) Title: WOUND-INDUCIBLE EXPRESSION IN PLANTS

(57) Abstract: The invention relates to a method for producing plants in which expression of an insecticidal protein is regulated by a wound-induced promoter inducing local expression, preferably the wound-inducible TR2' promoter, to the chimeric genes used in this method and the plants obtained thereby, and to the processes for obtaining resistance to insects feeding on plants by localized expression of an insecticidal protein induced on wounding of plants by insect feeding.

**Wound-inducible expression in plants.**

## FIELD OF THE INVENTION

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The invention relates to a method for producing plants in which expression of an insecticidal protein is regulated by a wound-induced promoter inducing local expression, preferably the wound-inducible TR2' promoter, to the chimeric genes used in this method and the plants obtained thereby, and to the processes for obtaining resistance to insects feeding on plants by localized expression of an insecticidal protein induced on wounding of plants by insect feeding.

## BACKGROUND ART

15 Most successful attempts to make plants that are resistant to insect attack have been obtained by, and are believed to be dependent on, high level expression of insecticidal toxins (Estruch et al., 1997; Witowsky & Siegfried, 1997). Most particularly for the Bt toxins, which as full-length proteins were found to be expressed poorly in plants, efforts have concentrated on increasing expression of these toxins in plants by use of strong constitutive promoters and by  
20 modification of the genes encoding them (Vaeck et al., 1987; Barton et al., 1987). More recently spatial regulation of expression of the insecticidal protein in those tissues susceptible to attack or triggered by feeding of the insect has been suggested as possibly providing advantages for resistance management (Peferoen & Van Rie, 1997). One of the major conditions for obtaining regulatory approval for transgenic insect resistant plants is the  
25 availability of an insect resistance management strategy. The currently favored strategy is the combination of 100% toxicity of the transgenic plants to the target pest(s), obtained by high dose expression of a specific toxin and this during the full life-cycle of the pest, with the use of refuges of non-transgenic plants, which allow the maintenance of the target pest population (De Maagd et al. 1999). In order to be able to comply with such a strategy, the use of strong  
30 constitutive promoters in the engineering of insect-resistant plants has further been encouraged.

Inducible expression of insect resistance has primarily been examined for the potato proteinase-inhibitor genes (*pin1* and *pin2*) which are part of the natural defense system in plants and upon wounding ensure the generation of a systemic signal throughout the plants  
5 (Green and Ryan, 1972; Hilder et al. 1987). Introduction of the *pin2* gene in rice resulted in high level systemic accumulation of the protein in rice plants which showed increased resistance to major pests (Duan et al., 1996). Breitler et al. (2001) describes the use of the C1 region of the maize proteinase inhibitor (MPI) gene to drive wound-inducible expression of the *cry1B* coding sequence in rice and the first transformants were found to effectively protect  
10 rice against stemborer attack, the wound-induced expression was described to be both locally and systemically.

In a small-scale laboratory experiment, transgenic cabbage leaves transformed with the *cry1Ab3* gene placed under the control of the inducible *vspB* promoter from soybean were as toxic to diamondback moth as those transformed with the same gene under control of the 35S  
15 promoter, but wound-inducibility was not demonstrated (Jin et al., 2000).

The TR2' promoter of the mannopine synthase gene of *Agrobacterium tumefaciens*, originally considered to direct constitutive expression (Velten et al. 1984; Vaeck et al. 1987), has been used to direct wound-inducible expression of a native *Cry1Ab* gene in tomato, which led to  
20 relatively low expression and only moderate insect control (Reynaerts & Jansens, 1994). Though a possible broad application for expression of Bt proteins has been suggested (Peferoen,1997), there appear to be discrepancies between the reports on the expression pattern of the TR2' promoter in tobacco and other dicots (Ni et al. 1995). In general, the use of monocot promoters is preferred for optimal expression of genes in monocots (Shimamoto,  
25 1994) and certain promoters have been found to have different cis-acting elements in monocots and dicots (Luan et al. 1992). The contribution of different elements of the TR2' promoter on its activity was investigated in maize protoplasts (Fox et al., 1992), but there have been no reports on the expression pattern of the TR2' promoter in a monocotyledonous plant. Furthermore, the strongest deletion mutant was found to be 20 times less active than the  
30 CaMV 35S promoter (Fox et al., 1992).

Agrobacterium mannopine synthase promoters have been characterized as being constitutive, root-specific, and tissue-specific promoters, e.g., see US patents 6291745, 6320100 and 63133378. In US patent 5641664, it is believed that various constitutive and organ- and tissue-specific promoters that are used to direct expression of genes in transformed dicotyledonous plants will also be suitable for use in transformed monocots. As part of a general list of suitable foreign constitutive promoters for transforming plant cells in this patent are mentioned the TR1' and the TR2' promoters which drive the expression of the 1' and 2' genes, respectively, of the T-DNA of Agrobacterium, and are said to be wound-induced promoters. This patent shows no monocot plant transformed with the TR2' or TR1' promoter, nor is there any suggestion that these promoters are wound-induced promoters in monocot plants or are useful for expression of an insecticidal protein in a monocot plant.

The present invention describes how the TR2' promoter can be used to direct wound-induced expression of an insecticidal protein in monocot plants to obtain insect resistance. Such wound-inducible expression of the TR2' promoter leads to a strong but localized increase of expression of the insecticidal protein. The putative effect on plant vigor and growth, observed with high level expression of some Bt proteins particularly upon repeated inbreeding (such as reported in WO 00/26378 for Cry2Ab) is likely to be reduced as the limited expression of the protein should minimize any burden on functions important for maintaining the agronomic qualities of the engineered crop. This is also an important factor when stacking of different traits (or different Bt proteins) is envisaged. As high-dose expression levels are attained upon contact with the target pest, such plants should comply with current IRM strategies. Additionally, the combination of the specificity of the insect toxin produced with a spatially and temporally limited expression pattern (i.e. in tissues susceptible to wounding, upon wounding) is likely to reduce exposure of non-target organisms, which can be considered advantageous over constitutive production of the toxin by the plants. Thus, an effective system of regulated expression ensuring effective insect resistance for major crops such as corn and rice is of interest from both a regulatory and agricultural point of view.

## SUMMARY OF THE INVENTION

The present invention relates to a method for obtaining wound-induced expression of an insecticidal protein in a monocot plant, which method comprises introducing into the genome of the plant a foreign DNA which is a chimeric gene comprising a DNA sequence encoding the insecticidal protein under control of a promoter region comprising the TR2' promoter. According to a particular embodiment of the invention, the insecticidal protein is a *Bacillus thuringiensis* toxin. In a preferred embodiment of the present invention, the insecticidal protein is an insecticidal protein active against pests of monocot plants, most preferably the insecticidal protein can be a Cry1Ab, Cry1F, Cry2Ae, Cry9C or Cry2Ab protein or an insecticidal fragment or mutant thereof.

Thus, a preferred embodiment of the present invention relates to a method for obtaining insect resistance, preferably high dose insect resistance, in plants, plant cells or plant tissues, more particularly in monocotyledonous, especially gramineae, particularly corn, plants, plant cells or plant tissues, by providing the plants, plant cells or tissue with a foreign DNA comprising a DNA sequence encoding an insecticidal protein under control of a promoter region comprising the TR2' promoter. According to the present invention the TR2' promoter is used to increase expression of the insecticidal protein upon wounding, e.g. by insect feeding. Thus, according to a particular embodiment of the invention, expression of the insecticidal protein in monocotyledonous plants, preferably measured in the greenhouse by an ELISA assay, is low (i.e., below 0.005% total soluble protein (mean value of multiple measurements taken from several, preferably at least 3, particularly at least 5, plants of the same transformation event) in leaves, in the absence of wounding or infestation and is increased, preferably at least doubled, most preferably increased 5 to 100 fold, in the wounded or infested tissues, particularly leaves, within 24 hours.

According to a preferred embodiment of the present invention, a chimeric gene comprising a DNA sequence encoding an insecticidal protein under control of the wound-inducible TR2' promoter is used to confer insect resistance to monocotyledonous plants, especially to gramineae, most particularly to corn, by directing local expression of the insecticidal protein

at the site of insect feeding. Thus, the invention relates to chimeric genes for obtaining wound-inducible expression in monocot plants. In a particular embodiment of the invention, expression (as measured in the greenhouse) is low or not detectable (less than 0.005% of total soluble protein (mean value of multiple measurements taken from several, preferably at least 3, particularly at least 5, plants of the same event)) in leaves of non-wounded, non-infected plants, and, upon infection, increased levels of insecticidal protein (at least double, preferably at least from 5 to 100 fold increase or more) are induced locally in the infected tissues, within 18 hours. According to this aspect of the invention, plants, more particularly monocotyledonous plants are provided that are insect resistant due to the presence in their genome of a foreign DNA comprising a DNA sequence encoding an insecticidal protein, under the control of the TR2' promoter which ensures expression in wounded tissues. According to one embodiment of the invention, expression of an insecticidal protein in the plants is such that, in the absence of wounding of the plant (e.g., when grown in the greenhouse) the insecticidal protein is expressed at low or undetectable levels (i.e. below 0.005% of total soluble protein (mean value of multiple measurements taken from several, preferably at least 3, particularly at least 5, plants of the same event) in leaves, stalk, seed and pollen, preferably at least in leaves and pollen, particularly at least in leaves, and, upon feeding by insects, is increased in the wounded tissue, preferably in the leaves, to a level which is sufficient to kill the feeding pest, preferably to a concentration of at least 0.01% of total soluble protein.

According to a preferred embodiment of the invention the TR2' promoter is used in monocotyledonous plants to confer insect resistance by directing wound-inducible expression of an insecticidal protein which is a Bt toxin. Examples of such DNA sequences encoding Bt toxins are well-known in the art and are described herein.

According to one embodiment of the present invention use of the TR2' promoter in corn to direct wound-inducible expression of a Bt toxin is particularly suited to engineer resistance of corn against the European Corn Borer (ECB), based on the local high-dose expression of the toxin in the plant upon feeding by target insects. The plants or plant parts (cells or tissues) of the present invention, comprising in their genome a foreign DNA comprising a DNA

sequence encoding an insecticidal protein under the control of the TR2' promoter upon wounding produce levels of insecticidal protein that are toxic to ECB larvae, including particularly to ECB larvae of the fourth stage as can be determined by insect efficacy assays described herein. Preferably, mortality rates of ECB fourth instar larvae of at least 97 %, preferably at least 99 %, most preferably of 100%, are obtained.

The present invention further relates to monocotyledonous plants that are resistant to insects while expressing very low basal levels of insecticidal protein in non-wounded leaves of the plant. According to a particular aspect of this invention, monocotyledonous plants, more particularly corn are obtained which combine efficient insect resistance with optimal agronomic characteristics, without penalty on agronomic performances due to expression of the insecticidal protein, as can be ascertained by assessing plant phenotype, segregation, emergence, vigor and agronomic ratings.

According to another aspect of this invention, monocotyledonous plants, particularly corn plants are obtained that are insect resistant and particularly suited for stacking with other traits (e.g. other types of insect resistance, herbicide resistance or agronomic traits).

According to another aspect of the invention, monocotyledonous plants, particularly corn plants are provided, which are resistant to (a) target pest(s), but for which production of the insecticidal protein is low to undetectable, preferably in leaves and pollen, particularly in leaves, in the absence of wounding, limiting exposure of non-target organisms to the insecticidal protein.

According to the present invention, the plants with the characteristics described above are obtained by introduction into the genome of the plant of a DNA sequence encoding an insecticidal protein under control of the TR2' promoter which is demonstrated to function as wound-inducible promoter in monocotyledonous plants, more particularly in corn plants.

## DETAILED DESCRIPTION

The term “gene” as used herein refers to any DNA sequence comprising several operably linked DNA fragments such as a promoter region, a 5' untranslated region (the 5'UTR), a coding region (which may or may not code for a protein), and an untranslated 3' region (3'UTR) comprising a polyadenylation site. Typically in plant cells, the 5'UTR, the coding region and the 3'UTR are transcribed into an RNA of which, in the case of a protein encoding gene, the coding region is translated into a protein. A gene may include additional DNA fragments such as, for example, introns. While a promoter region is required in a gene used for transformation in the current invention, the 3' UTR comprising a polyadenylation site need not be present in the transferred gene itself, but can be found in the upstream plant DNA sequences after insertion of a gene not containing a 3' UTR comprising a polyadenylation site. Similarly, a coding sequence of this invention can be inserted in the plant genome downstream of an existing plant promoter so that expression of the insecticidal protein of the invention occurs from such reconstituted chimeric gene in the plant (e.g., as in promoter tagging experiments).

The term “chimeric” when referring to a gene or DNA sequence refers to a gene or DNA sequence which comprises at least two functionally relevant DNA fragments (such as promoter, 5'UTR, coding region, 3'UTR, intron) that are not naturally associated with each other and/or originate, for example, from different sources. “Foreign” referring to a gene or DNA sequence with respect to a plant species is used to indicate that the gene or DNA sequence is not naturally found in that plant species, or is not naturally found in that genetic locus in that plant species. The term “foreign DNA” will be used herein to refer to a DNA sequence as it has incorporated into the genome of a plant as a result of transformation in that plant or in a plant from which it is a progeny.

A genome of a plant, plant tissue or plant cell, as used herein, refers to any genetic material in the plant, plant tissue or plant cell, and includes both the nuclear and the plastid and mitochondrial genome.

A “fragment” or “truncation” of a DNA molecule or protein sequence as used herein refers to a portion of the original DNA or protein sequence (nucleic acid or amino acid sequence) referred to or a synthetic version thereof (such as a sequence which is adapted for optimal expression in plants), which can vary in length but of which the minimum size is sufficient to ensure the (encoded) protein to be biologically active, the maximum size not being critical. A “variant” or “mutant” of a sequence is used herein to indicate a DNA molecule or protein of which the sequence (nucleic or amino acid) is essentially identical to the sequence to which the term refers.

Sequences which are “essentially identical” means that when two sequences are aligned, the percent sequence identity, i.e. the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the sequences, is higher than 70%, preferably is higher than 85%, more preferably is higher than 90%, especially preferably is higher than 95%, most preferably is between 96 and 100%. The alignment of two nucleotide sequences is performed by the algorithm as described by Wilbur and Lipmann (1983) using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4.

‘Insecticidal’ is used herein to mean toxic to insects which are crop pests. More particularly, in the context of the present invention target insects are the pests of monocotyledonous plants, most particularly of corn, such as, but not limited to major lepidopteran pests, such as *Ostrinia nubilalis* (European corn borer or ECB), *Sesamia nonagrioides* (Mediterranean Stalk borer) and *Helicoverpa zea* (corn earworm), and major coleopteran pests, such as *Diabrotica* spp. insects, particularly *Diabrotica virgifera virgifera* and *Diabrotica undecimpunctata howardi* (Corn rootworm).

An ‘insecticidal protein’ or ‘toxin’ as used herein should be understood as a protein, polypeptide or peptide which is toxic to insects. Examples of such an insecticidal protein are the Bt Cry toxins, mutants or insecticidal fragments thereof (such as those reviewed by Höfte and Whiteley, 1989, in Crickmore et al. (1998) and described in WO 00/26378, WO 97/40162, and US 6,023,013), more particularly the Cry2Ae (WO 02/057664) protein or an

insecticidal fragment thereof, the Cry2Ab protein or an insecticidal fragment thereof, the Cry9C protein or fragments or mutants thereof (e.g., as described in WO94/24264 or WO99/00407), the Cry1F protein or an insecticidal fragment thereof, and the Cry1Ab protein or an insecticidal fragment thereof, as well as the 149B1 combined (about 14 and 44 kilodalton) toxins or insecticidal fragments thereof, and the Cry3Bb protein and insecticidal fragments or mutants thereof as described in US patents 6,548,291, 6,063,597 and 6,501,009. Other insecticidal proteins are for instance the VIPs, particularly VIP3A, more particularly VIP3Aa, VIP3Ab and VIP3Ac proteins or insecticidal fragments thereof (Estruch et al., 1996, WO 96/10083, US patents 6,429,360, 5,877,012), or the proteins encoded by the *mis*, *war* and *sup* sequences (WO98/18932, WO99/57282), and the toxins isolated from *Xenorabdus* and *Photorabdus* ssp. such as those produced by *Photorabdus luminescens* (Forst et al., 1997). Other insecticidal proteins include, but are not limited to, the potato proteinase inhibitor I and II, the cowpea proteinase inhibitor, the cysteine proteinase inhibitor of soybean (Zhao et al., 1996) or the cystatins such as those isolated from rice and corn (Irie et al., 1996), cholesterol oxidases, chitinases, and lectins. An insecticidal protein can be a protoxin (i.e., the primary translation product of a full-length gene encoding an insecticidal protein). Also included are equivalents and variants, derivatives, truncations or hybrids of any of the above proteins which have insecticidal activity. A Bt toxin, or Bt protein, as used herein refers to an insecticidal protein as previously defined which is directly or indirectly derived (e.g., modified so as to improve expression in plants or toxicity to insects) from a protein naturally produced by *Bacillus thuringiensis* and comprises a sequence which is essentially identical to the toxic fragment of a naturally produced Bt toxin, or at least a domain thereof. A Bt toxin or Bt protein, as used herein, can be a crystal protein or an insecticidal part thereof, or can be a non-crystal protein such as a secreted protein or a protein produced mainly during the vegetative phase of a Bt strain, or can be an insecticidal mutant or part thereof. 'A DNA encoding an insecticidal protein' as used herein includes a truncated, modified, synthetic or naturally occurring DNA sequence, encoding an insecticidal protein.

In a particular embodiment of the present invention, the DNA encoding an insecticidal protein is a DNA sequence encoding a Bt toxin, more preferably a DNA sequence modified to increase expression of the insecticidal protein in plants. According to a particular embodiment

of the present invention the DNA sequence encoding an insecticidal protein is a modified cry1Ab DNA sequence which encodes at least part of the Cry1Ab5 protein described by Höfte et al. (1986), preferably a DNA sequence encoding a protein comprising the amino acid sequence from an amino acid position between amino acid positions 1-28 to an amino acid  
5 between amino acid positions 607-725 thereof, most preferably comprising amino acids 1-616. Most preferably the encoded modified Cry1Ab protein has an insertion of an alanine codon (GCT) behind the ATG start codon (AlaAsp2...Asp616).

The expression level of an insecticidal protein in plant material can be determined in a number  
10 of ways described in the art, such as by quantification of the mRNA encoding the insecticidal protein produced in the tissue using specific primers (such as described by Cornelissen & Vandewiele, 1989) or direct specific detection of the amount of insecticidal protein produced, e.g., by immunological detection methods. More particularly, according to the present invention the expression level of insecticidal protein is expressed as the percentage of soluble  
15 insecticidal protein as determined by immunospecific ELISA as described herein related to the total amount of soluble protein (as determined, e.g., by Bradford analysis (Bradford, 1976)). A preferred ELISA in the current invention is a sandwich ELISA (Clark et al., 1986).

A 'wound-inducible' promoter or a promoter directing an expression pattern which is wound-  
20 inducible as used herein means that, at least in the leaves, upon wounding expression of the coding sequence under control of the promoter is significantly increased, i.e. is at least doubled, preferably 5 times increased, most preferably 20 to 100 times increased. 'Wounding' as used herein is intended to mean either mechanical damage or perforation of at least the plant epidermis or outer cell layer by any kind of insect feeding, wounding as used herein can  
25 also be simulated by cutting a leaf with a sharp instrument such as a scalpel. Preferably, according to the present invention, wound-inducible expression of an insecticidal protein, preferably a Bt insecticidal protein, in a plant means that basal expression (i.e., in the absence of wounding, preferably as measured in the greenhouse) of the protein in the leaves of the plant at V4 stage is low, most preferably below 0.005% total soluble protein content (mean  
30 value of measurements taken from several plants of one transformation event), and, upon wounding rises, preferably to a level above 0,01 % of total soluble protein, more preferably to

a level of 0.04% to 0.5% total soluble protein content or higher as measured using a leaf part that was detached after wounding and then incubated in vitro for several hours, e.g., about 12 to about 60 hours, preferably about 18 to about 20 hours. Measurements made using an excised in vitro leaf assay (in such assay an excised leaf or part thereof is incubated in vitro (e.g., in a petri dish on humid filter paper) in a greenhouse or growth chamber at room temperature for several hours, preferably about 12 to 60 hours, more preferably about 18 to 20 hours, after wounding (to better reflect the conditions upon insect feeding) typically will give a higher induction of expression than measurements after wounding in planta without in vitro incubation of a detached leaf or a part thereof. Wound-induced protein levels are typically lower (but still significantly higher than non-induced background levels) when measured around 12 to 60 hours, more preferably around 18 to 20 hours, after wounding of a plant leaf without excising the leaf part for in vitro incubation. For any of the measurements on insecticidal protein concentration in plant tissue, preferably leaves, after wounding, and for measuring the increase in expression as described herein, it is preferred to use the detached tissue, preferably leaf, assay with in vitro incubation, since this likely better reflects the actual concentration induced in the tissue upon insect feeding (control leaves are fresh, unwounded and undergo no in vitro incubation to better reflect the actual concentration in the plant tissues). Basal or background expression levels of a wound-induced promoter, as used herein, are preferably measured from freshly excised, not previously wounded, leaf material taken from greenhouse-grown plants. According to a particular embodiment of the invention, expression of the insecticidal protein at least in the wounded leaves rises to 0.1% total soluble protein content at the site of wounding. It is believed that the percentage of insecticidal protein per total soluble protein in accordance with the invention that is actually ingested by an insect upon insect feeding is higher than the percentage measured in the plant or part thereof, due to the localized expression and the fact that only induced (wounded) tissue will be ingested by the insect. Also, for measurement of protein concentration a certain minimum amount of plant tissue is excised (typically about 2-3 mm around the wound is excised for leaves), to run the insecticidal protein content analysis, hence the presence of unwounded cells is believed to decrease the percentage of insecticidal protein per total soluble protein extracted from the sample.

As typically observed in transformation experiments of this kind, a range of different plant transformation events is obtained after transformation, and a selection procedure is preferred to select the best plants for further development (i.e., for crossing into suitable plant lines adapted to a certain growing region). In such a selection procedure, plants are selected  
5 showing low or undetectable protein levels in plant tissue, preferably in leaves and pollen, most preferably in leaves, which plants upon wound-induction show at least a 5-fold increase in expression in leaves.

A "greenhouse", as used herein, refers to a relatively stable growing environment for plants  
10 which shields plants from normal field conditions, with no or little infestation by insects, rabbits, birds or other animals or external factors (such as wind or storms) which can damage a crop plant in the field. A typical greenhouse is largely made out of transparent materials such as glass or plastic, so as to allow natural day-light to reach the plants, and can have regulated light, growth medium (soil or an artificial medium), water and nutrient supply  
15 and/or temperature control. A greenhouse, as used herein, also includes rooms or boxes with no daylight, as long as a light source and a growth medium is provided so that normal plant growing conditions are established. As such, a greenhouse is the most suitable place to measure basal or background expression levels in leaves for the wound-inducible constructs of this invention (in a non-induced state), for comparative purposes.

20 While similar basal or background expression values of insecticidal protein can be found in leaves of plants in a field, it is expected that infestation by insects or mechanical damage, e.g., by vehicles, rabbits or birds to the plants in a field will increase the expression level by induction of the wound-inducible promoter of this invention, and hence not a real basal level will typically be measured in all plants in a field.

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'High dose' expression, or 'high dose' insect resistance, as used herein when referring to a plant, preferably a monocot plant in accordance with this invention, refers to a concentration of the insecticidal protein in a plant (measured by ELISA as a percentage of the total soluble protein, which total soluble protein is measured after extraction of soluble proteins in an  
30 extraction buffer (e.g., the extraction buffer described in Jansens et al., 1997) using Bradford analysis (Bio-Rad, Richmond, CA; Bradford, 1976)) which kills a developmental stage of the

target insect which is significantly less susceptible, preferably between 25 to 100 times less susceptible to the toxin than the first larval stage of the insect and can thus can be expected to ensure full control of the target insect, most preferably a high dose insect resistance is the obtaining of at least 97 percent, preferably at least 99 percent, most preferably 100 percent, mortality for the fourth larval instar (for insects having 5 larval instars) or the last larval instar (for insects having 4 or less larval instars) of a target insect, as measured 10 to 14 days after insect infestation of such plant in standard insect bioassays, preferably whole plant bioassays, of a plant having high dose expression or high dose insect resistance. The existence of one target insect species (i.e., an insect species which can cause commercially significant damage to a plant species or variety, and which is typically an insect for which a transgenic plant is developed) for which a transformed plant according to this invention provides a high dose insect resistance is sufficient for a plant to be designated as giving "high dose" expression in accordance with this invention. Also included in this invention are also the plant cells or plant tissues, particularly corn plant cells or plant tissues, whether contained in a plant or present in an in vitro culture, transformed with the wound-induced chimeric gene of the invention, that have a high dose insect resistance as described above using insect bio-assays.

High dose when referring to ECB control in corn as used herein refers to the production of insecticidal protein by the plant in mid-whorl stage in an amount which is toxic to ECB larvae of the L4 stage (European Corn Borer. Ecology and Management. 1996. North Central Regional Extension Publication No. 327. Iowa State University, Ames, Iowa) as can be determined by toxicity assays with artificial infestation described herein, wherein mortality of at least 90%, preferably at least 97, more preferably at least 99 %, most preferably 100% of the L4 ECB larvae is obtained in a test 14 days, preferably 10 days, after infestation of the plants with L4 larvae. Surprisingly, a high dose expression of an insecticidal protein is obtained in the corn plants of the invention using the TR2' promoter to drive wound-induced expression, even when expression is low or undetectable by sensitive ELISA protocols in a non-induced state, and expression is only induced upon insect feeding. As is common in plant transformation experiments, some of the plants obtained after transformation will not be suitable for further development, and suitable plant lines with low basal or background expression and high dose wound-induced expression will need to be selected from the

transformation events obtained, and preferably such a plant line contains only one inserted DNA encoding an insecticidal protein. Typically, commercially acceptable plant lines are obtained from over one hundred, preferably from several hundreds, of initial transformed plants.

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According to the present invention 'wound-inducible' expression is furthermore preferably characterized in that the effect of the promoter is local, i.e., is confined essentially to those cells or tissues directly affected by wounding or immediately surrounding the wounded tissue. This as opposed to a systemic effect, which directly or indirectly (through a cascade of reactions) ensures a widespread effect, more particularly wide-spread expression of proteins involved in the natural defense mechanisms of the plant. Preferably, expression of the insecticidal protein in undamaged tissues of the plant is on average not more than 0.01% of the total soluble protein concentration, more preferably not more than 0.005% total soluble protein (mean value of multiple measurements taken from several, preferably at least 3, particularly at least 5, plants from one transformation event), as measured by ELISA (see above) in greenhouse-grown plants. In a preferred embodiment of this invention, wound-inducible promoter activity in leaves, particularly corn leaves, is localized to the region of wounding, and will not be detected (using ELISA assays) in a region of the plant, particularly the leaf, more than 10cm, particularly more than 2 cm, distant of the wounding site (e.g., not in a leaf lower or higher than the wounded leaf in the same plant, nor in the same leaf more than 10 cm, preferably more than 2 cm, away from the wounded site on the leaf). This effectively limits expression in the plant, particularly the leaves, preferably corn leaves, to those places where expression is needed (i.e., where an insect feeds or at least perforates leaf tissue in an attempt to feed) and hence minimizes stress on the plant that could be caused by high-level constitutive expression. Particularly when stacking several genes encoding different proteins (e.g., different insect control proteins) in one plant, wound-induced expression of one or more of the introduced genes is believed to be beneficial.

In another embodiment, the wound-induced expression of an insecticidal protein in accordance with this invention is characterized by a quick induction of expression, rising from basal or background level to a higher protein level (at least twice the amount in the controls,

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preferably at least 5 to 100 times the amount in the controls) at around 18 to 24 hours, preferably at 18, 20 or 24 hours, after wounding using an assay wherein a leaf part is wounded, cut out of the leaf and incubated in vitro for that period of time (the basal or background level is measured in freshly excised and not previously wounded leaf parts, which  
5 are not incubated in vitro).

The “TR2’ promoter” as used herein relates to any promoter comprising the TR2’ (or mannopine synthase, abbreviated as *mas*) functional part of the TR1’-TR2’ dual promoter element from *Agrobacterium* (Velten et al. 1984; Langridge et al. 1989). Thus this can  
10 comprise the TR2’ element either alone or in combination with the divergent TR1’ element (Guevara-Garcia et al., 1998) or other (regulatory) elements, including but not limited to enhancer regions, introns and the like, as long as the wound-induction promoter characteristics in accordance with the present invention are substantially retained. In a preferred embodiment of this invention, transcription is directed from the TR2’ promoter region (and the coding  
15 sequence is hence operably linked to and downstream of the TR2’ promoter sequence), even if the TR1’-TR2’ dual promoter (or any part thereof retaining the TR2’ promoter element) is used. Most particularly, the TR2’ promoter as used herein refers to a promoter region comprising a fragment of SEQ ID NO:1 spanning from a nucleotide position between nucleotide positions 1 and 336 to nucleotide position 483, preferably comprising the sequence  
20 of nucleotides 96 to 483 of SEQ ID NO:1, most preferably comprising SEQ ID NO: 1 or a functional equivalent thereof, i.e., a modification thereof capable of directing wound-induced expression in plants, more particularly in monocotyledonous plants. Such functional equivalents include sequences which are essentially identical to a nucleotide sequence comprising at least nucleotides 328 to 483 (comprising the TR2’ promoter element, Velten et al., 1984) of SEQ ID NO: 1. Such sequences can be isolated from different *Agrobacterium*  
25 strains. Alternatively such functional equivalents correspond to sequences which can be amplified using oligonucleotide primers comprising at least about 25, preferably at least about 50 or up to 100 consecutive nucleotides of nucleotides 328 to 483 of SEQ ID NO:1 in a polymerase chain reaction. Functional equivalents of the TR2’ promoter can also be obtained  
30 by substitution, addition or deletion of nucleotides of the sequence of SEQ ID NO:1 and

includes hybrid promoters comprising the functional TR2' part of SEQ ID NO:1. Such promoter sequences can be partly or completely synthesized.

5 The plants of the present invention are protected against insect pests, by the wound-inducible expression of a controlling amount of insecticidal protein. By controlling is meant a toxic (lethal) or combative (sub-lethal) amount. Preferably, upon induction, a high dose (as hereinbefore defined) is produced. At the same time, the plants should be morphologically normal and may be cultivated in a usual manner for consumption and/or production of products. Furthermore, said plants should substantially obviate the need for chemical or  
10 biological insecticides (to insects targeted by the insecticidal protein).

Different assays can be used to measure the effect of the insecticidal protein expression in the plant. More particularly, the toxicity of the insecticidal protein produced in a corn plant to *Ostrinia nubilalis* or ECB (also referred to herein as ECB efficacy) can be assayed *in vitro* by  
15 testing of protein extracted from the plant in feeding bioassays with ECB larvae or by scoring mortality of larvae distributed on leaf material of transformed plants in a petri dish (both assays described by Jansens et al., 1997). In the field, first brood ECB larvae (ECB1) infestation is evaluated based on leaf damage ratings (Guthrie, 1989) while evaluation of the total number of stalk tunnels per plant and stalk tunnel length are indicative of second brood  
20 ECB (ECB2) stalk feeding damage (see, e.g., Jansens et al., 1997 for stalk tunnel length analysis).

The plants of the present invention optionally also comprise in their genome a gene encoding herbicide resistance. More particularly, the herbicide resistance gene is the *bar* or the *pat*  
25 gene, which confers glufosinate tolerance to the plant, i.e. the plants are tolerant to the herbicide Liberty<sup>TM</sup>. Tolerance to Liberty<sup>TM</sup> can be tested in different ways. For instance, tolerance can be tested by Liberty<sup>TM</sup> spray application. Spray treatments should be made between the plant stages V2 and V6 for best results. Tolerant plants are characterized by the fact that spraying of the plants with at least 200 grams active ingredient/hectare (g.a.i./ha),  
30 preferably 400 g.a.i./ha, and possibly up to 1600 g.a.i./ha (4X the normal field rate), does not kill the plants. A broadcast application should be applied at a rate of 28-34 oz Liberty<sup>TM</sup> +

3lb Ammonium Sulfate per acre. It is best to apply at a volume of 20 gallons of water per acre using a flat fan type nozzle while being careful not to direct spray applications directly into the whorl of the plants to avoid surfactant burn on the leaves. The herbicide effect should appear within 48 hours and be clearly visible within 5-7 days. Examples of other herbicide resistance genes are the genes encoding resistance to phenmedipham (such as the pmph gene, 5 US 5,347,047; US 5,543,306), the genes encoding resistance to glyphosate (such as the EPSPS genes, US 5,510,471), genes encoding bromoxynil resistance (such as described in US 4,810,648) genes encoding resistance to sulfonylurea (such as described in EPA 0 360 750), genes encoding resistance to the herbicide dalapon (such as described in WO 99/27116), and 10 genes encoding resistance to cyanamide (such as described in WO 98/48023 and WO 98/56238) and genes encoding resistance to glutamine synthetase inhibitors, such as PPT (such as described in EP-A-0 242 236, EP-A-0 242 246, EP-A-0 257 542).

According to a preferred embodiment of the invention, the chimeric gene comprising a DNA 15 encoding an insecticidal protein under control of the TR2' promoter can be introduced (simultaneously or sequentially) in combination with other chimeric genes into a plant, so as to obtain different traits in the plant (also referred to as 'stacking'). Similarly, the plant of the invention containing a chimeric gene comprising a DNA encoding an insecticidal protein under control of the TR2' promoter, is particularly suited for combination with other traits. 20 Such other traits include, but are not limited to traits such as those encoded by chimeric genes which confer insect resistance, herbicide resistance, stress or drought tolerance, or traits which modify other agronomic characteristics of the plant. Such a trait can also encompass the synthesis of a product to be recovered from the plant.

25 Introduction of a foreign DNA or a chimeric gene into the genome of a plant, cell or tissue can be achieved in different ways and is not critical to the present invention. Successful genetic transformation of monocots has been obtained by a number of methods including *Agrobacterium-mediated* transformation (as described, for example for corn in US 6,074,877 or US 6,140,553), microprojectile bombardment (as described, for example by Chen et al., 30 1994), direct DNA uptake into protoplasts (as described, for example by Data et al. 1999; Poulsen, 1996) and electroporation (D'Halluin et al., 1992).

The following non-limiting examples describe the construction of chimeric genes comprising a DNA sequence encoding an insecticidal protein under control of the TR2' promoter, for expression in plants and insect resistant plants obtained therewith. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY, in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA and in Volumes I and II of Brown (1998) Molecular Biology LabFax, Second Edition, Academic Press (UK). Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) PCR - Basics: From Background to Bench, First Edition, Springer Verlag, Germany.

Throughout the description and Examples, reference is made to the following sequences represented in the sequence listing:

20

SEQ ID NO:1 : nucleotide sequence of a preferred embodiment of the TR2' promoter  
SEQ ID NO:2 : sequence of pTSVH0212  
SEQ ID NO:3 : sequence of a modified cry1Ab coding sequence

## Examples

**Example 1. Generation of Events with a foreign gene under control of the TR2' promoter.**

5

## a) development of events

For the evaluation of wound-induced expression of a DNA sequence encoding an insecticidal gene, a construct was made comprising a promoter region comprising the TR2' promoter (Velten et al. 1984) directing the expression of a modified cry1Ab protein. The plasmid pTSVH0212 containing the genes of interest placed between the T-DNA borders (also referred to as 'TR2'-Cry1Ab') was used for *Agrobacterium*-mediated transformation (WO 98/37212).

PTSVH0212	3'nos-bar-p35S3><Tr2'-modcry1Ab-3'ocs	(SEQ ID NO:2)
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15 The structure of the PTSVH0212 construct is provided in Table 1. For control plants with constitutive expression of the insecticidal protein, transformations were performed with constructs comprising a DNA sequence encoding the modified Cry1Ab protein under control of either the 35S promoter from Cauliflower Mosaic Virus (Franck et al. 1980)(referred to as 35S-cry1Ab), or the promoter of the GOS2 gene from rice (de Pater et al., 1992) with the  
 20 cab22 leader from Petunia (Harpster et al. 1988)(also referred to as 'Gos/cab-cry1Ab') or the 5' leader sequence of the GOS2 gene from rice, containing the second exon, the first intron and the first exon of the GOS transcript (de Pater et al., 1992)(referred to as 'Gos/gos-cry1Ab'). All constructs included the 35S-bar gene.

Table 1. Nucleotide positions of the genetic elements in pTSVH0212 (TR2'-cry1Ab)

Nt positions	Direction	Description and references
25-1	Counter clockwise	Left Border sequence of TL-DNA of pTiB6S3 (Gielen <i>et al.</i> , 1984).
		Synthetic polylinker sequence
316-56	Counter clockwise	Fragment containing polyadenylation signals from the 3'untranslated region of the nopaline synthase gene from the T-DNA of pTiT37 (Depicker <i>et al.</i> , 1982).
		Synthetic polylinker sequence
887-336	Counter clockwise	<i>bar</i> : the coding sequence of phosphinothricin acetyl transferase of <i>Streptomyces hygrosopicus</i> (Thompson <i>et al.</i> , 1987)
1720-888	Counter clockwise	P35S3: promoter region from the Cauliflower Mosaic Virus 35S transcript (Odell <i>et al.</i> , 1985).
		Synthetic polylinker sequence
2252-1770	Counter clockwise	TR2' Promoter fragment derived from the right T-DNA of octopine type <i>Agrobacterium</i> strain ACH5 (Velten <i>et al.</i> , 1984)
		Synthetic polylinker sequence
2261-4114		<i>Modified cry1Ab</i> : The modified <i>cry1Ab</i> coding sequence encodes part of the Cry1Ab5 protein described by Höfte <i>et al.</i> (1986) and has an insertion of an alanine codon (GCT) 3' of the ATG start codon (SEQ ID NO: 3).
		Synthetic polylinker sequence
4128-4422		Fragment containing polyadenylation signals from the 3'untranslated region of the octopine synthase gene from the TL-DNA of pTiAch5 (De Greve <i>et al.</i> , 1983).
		Synthetic polylinker sequence
4470-4446	Counter clockwise	Right Border sequence of TL-DNA of pTiB6S3 (Gielen <i>et al.</i> , 1984).

Regenerated plantlets were selected based on tolerance to Liberty.

##### 5 b) Evaluation of events

The *Agrobacterium* transformants were checked for presence of vector sequence at the left border of the T-DNA. Southern blot analyses were performed with leaf material of the primary transformants (T0).

**Example 2. Wound-induced expression of an insecticidal protein.**

## i) Basal expression of the insecticidal protein

5 The basal level of expression of the modified Cry1Ab insecticidal protein was determined by a Cry1Ab sandwich ELISA with a polycondensated IgG fraction of a polyclonal rabbit antiserum against Cry1Ab as first antibody and a monoclonal antibody against Cry1Ab as second antibody, after extraction of soluble protein using the extraction method and buffer described in Jansens et al., 1997. Samples of leaves at V3 stage plants, pollen and leaf of R1 stage plants and leaves, stalk and pollen at harvest were taken of plants in the greenhouse (corn stages are as determined in 'How a Corn Plant Develops, Special Report No. 48, Iowa State University of Science and Technology, Cooperative Extension Service, Ames, Iowa, Reprinted June 1993; see also the internet website containing the relevant information at: [http://www.extension.iastate.edu/pages/hancock/agriculture/corn/corn\\_develop/CornGrowthStages.html](http://www.extension.iastate.edu/pages/hancock/agriculture/corn/corn_develop/CornGrowthStages.html)). As a comparison, samples were taken from plants transformed with the Gos/gos-cry1Ab constructs.

Results are provided in Table 2 as percentage of detected Cry1Ab protein per total soluble protein (measured using the Bradford assay (Bio-Rad, Richmond, CA; Bradford, 1976)). The mean value represents the average of 5 samples taken from different plant lines of one transformation event.

Table 2. Expression of Cry1Ab protein in different tissues of plants transformed with the TR2'-cry1Ab and Gos/gos-cry1Ab constructs

Event (construct)		Cry1Ab in % soluble protein / total protein					
		V3 stage	R1 stage		Harvest		
		Leaf	Leaf	Pollen	Leaf	Stalk	Pollen
WI602-0402 (TR2'-cry1Ab)	mean		0	0.000	0.000	0.010	0.000
	st.dev.	0	0	0.000	0.000	0.010	0.010
WI604-1602 (TR2'-cry1Ab)	Mean	0	0	0.000	0.000	0.010	0.000
	st.dev.	0	0	0.000	0.000	0.010	0.000

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WI606-0802 (TR2'-cry1Ab)	Mean	0	0	0.000	0.000	0.020	0.000
	st.dev.	0	0	0.000	0.000	0.040	0.000
WI606-1206 (TR2'-cry1Ab)	Mean	0	0	0.000	0.000	0.010	0.000
	st.dev.	0	0	0.000	0.000	0.010	0.000
WI600-0218 (TR2'-cry1Ab)	Mean	0	0	0.000			
	st.dev.	0	0	0.000			
WI602-0802 (TR2'-cry1Ab)	Mean	0	0	0.000	0.000	0.010	0.010
	st.dev.	0	0	0.000	0.000	0.010	0.010
CE2168-0202 (Gos/gos-cry1Ab)	Mean	0.31	0.26	0.040	0.230	0.800	0.030
	st.dev.	0.04	0.06	0.010	0.060	0.360	0.010
CE2168-1602 (Gos/gos-cry1Ab)	Mean	0.34	0.23	0.050	0.270	0.870	0.030
	st.dev.	0.04	0.05	0.020	0.150	1.450	0.010

For the plants obtained from transformation with the TR2'-cry1Ab construct, mean values for the basal expression of the modified Cry1Ab protein in the greenhouse were found to be below detection limit (0.001% or 0,000 % of total soluble protein) in all leaf samples taken from plants at V3 stage, R1 stage or at harvest. Average basal expression of the insecticidal protein in the stalk was found to be around 0.01% total soluble protein content for most plants at harvest. In plants obtained with the Gos/gos-cry1Ab construct, expression of the Cry1Ab protein was above 0.2% in all leaf samples and up to more than 1% in some of the stalk samples taken at harvest.

10

ii) wound-induced expression of the insecticidal protein

Studies were performed in the greenhouse to determine the expression of the insecticidal Cry1Ab protein upon mechanical damage in non-transformed plants, plants obtained with the TR2'-cry1Ab construct and plants obtained with the Gos/cab-cry1Ab construct. Leaves and roots were damaged by cutting. Wounding was made on fully expanded leaves, each mm a perpendicular cut was made with a scalpel on the leaf without damaging the mid-rib. Leaf samples were taken before and 18h after mechanical damage. Cry1Ab protein levels were measured by ELISA (Table 3) on excised leaf parts (leaf parts were cut out 2 to 3 mm around the wound) that were incubated for 18 hours in a growth chamber at 20° C in a petri dish on a filter paper moistened with Murashige and Skoog medium (MS medium, see Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd

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(UK) and Blackwell Scientific Publications, UK, for composition) – control leaf pieces of similar size were cut from plants and were put immediately on dry ice for protein measurement (without *in vitro* incubation). Means represent averages of 5 plants per transformation event .

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Table 3: expression of insecticidal protein in different plant parts before and after mechanical wounding

Event (construct)		Cry1Ab in % soluble protein / total protein									
		V4 stage		Flowering					Harvest		
		Leaf	Leaf induced	Leaf	Leaf induced	root	Root induced	Pollen	Leaf	Stalk	Kernel
W1600-0218 (TR2'-Cry1Ab)	Mean	0.000	0.020	0.000	0.020	0.023	0.036	0.000	0.000	0.000	0.000
	st.dev.	0.000	0.008	0.000	0.007	0.008	0.011	0.000	0.000	0.000	0.000
W1606-0406 (TR2'-Cry1Ab)	Mean	0.005	0.046	0.000	0.007	0.012	0.008	0.000	\	0.002	0.002
	st.dev.	0.007	0.005	0.000	0.006	0.009	0.009	0.000		0.004	
W1604-1602 (TR2'-Cry1Ab)	Mean	0.002	0.104	0.001	0.020	0.020	0.024	0.000	0.004	0.004	0.001
	st.dev.	0.003	0.012	0.000	0.009	0.008	0.008	0.000	0.002	0.003	0.000
W1606-0802 (TR2'-Cry1Ab)	Mean	0.003	0.064	0.001	0.010	0.027	0.037	0.000	0.002	0.004	0.003
	st.dev.		0.004	0.000	0.009	0.012	0.020	0.000	0.001	0.003	0.001
W1606-1206 (TR2'-Cry1Ab)	Mean	0.001	0.081	0.000	0.022	0.032	0.024	0.000	0.004	0.002	0.001
	st.dev.		0.016	0.000	0.010	0.005	0.011	0.000	0.003	0.001	0.001
CE048-2402 (35S-Cry1Ab)	Mean	0.83	0.614	0.280	0.397	0.196	0.253	0.000	0.376	1.120	0.047
	st.dev.	0.108	0.111	0.027		0.133	0.083	0.000	0.098	0.175	0.014
CE048-2602 (35S-Cry1Ab)	Mean	0.636	0.527	0.007	0.006	0.087	0.045	0.000	0.106	0.040	0.015
	st.dev.	0.16	0.046	0.002	0.002	0.044	0.007	0.000	0.011	0.013	0.004
Control 1 (non-transformed)	Mean	0.000	0.000	0	0	0	0	0	0	0.001	0
	st.dev.	0.000	0.000	0	0	0	0	0	0	0.002	0
Control 2 (non-transformed)	Mean	0.000	0.000	0	0	0	0	0	0	0	0
	st.dev.	0.000	0.000	0	0	0	0	0	0	0	0
CE0104-0202 (Gos/cab-Cry1Ab)	Mean	0.022	0.023	0.008	0.017	0.023	0.016	0.0001	\	\	\
	st.dev.	0.009	0.005	0.005	0.004	0.012	0.005	0.000			
CE1014-0402 (Gos/cab-Cry1Ab)	Mean	0.025	0.017	0.007	0.010	0.028	0.034	0.000	0.010	0.040	0.002
		0.005	0.003	0.001	0.003	0.017	0.013	0.000	0.003	0.020	0.001

10 Expression of the Cry1Ab protein was a gain either absent or around the detection limit in leaves, stalk and kernels of the different TR2'-cry1Ab events tested. No significant expression

was found in leaves and pollen in V4 and flowering plant stage. Constitutive expression of around 0.02-0.03% total soluble protein was seen in the roots for these plants. When leaves are mechanically damaged, expression of the Cry1Ab protein is induced and goes up to 0.05-0.1%. The 35S-cry1Ab events showed constitutive expression of the Cry1Ab protein of around 0.5% in the leaves of V3 plants, irrespective of wounding. During flowering and harvest, expression levels of around 0.1-0.2% were measured before and after wounding.

A similar assay, wherein wounding was in planta (without in vitro incubation of an excised leaf part) on greenhouse-grown plants, resulted in lower expression values, with still a marked increase in expression on wounding shown.

In this assay, second instar ECB larvae were placed in clip-cages on five corn plants of WI602-0402 in VT stage. Of each plant, sixty hours later, samples were taken of the damaged leaf area in the clip-cage, from leaf 2cm or 10cm up and down of the clip-cage, and from a leaf above and beneath the leaf with the clip-cage. Leaf samples were also collected at the start of the experiment. Cry1Ab ELISA was run on the samples, mean (bold) and standard deviation were calculated. Results are shown in Table 4 below.

Table 4. Cry1Ab concentration in TR2'-Cry1Ab plants (wounding by insect feeding).

	Sample	time (h)	Cry1Ab
			%
			damage by L2 ECB feeding
			WI602-0402
undamaged	on plant	0	<b>0,000</b>
			0,000
damaged	on plant, sample injury	60	<b>0,006</b>
			0,001
damaged	on plant, 2cm above injury, dir. Stem	60	<b>0,000</b>
			0,000
damaged	on plant, 2 cm down injury, dir. Leaf tip	60	<b>0,000</b>
			0,000
damaged	on plant, 10cm above injury, dir. Stem	60	<b>0,000</b>
			0,000
damaged	on plant, 10 cm down injury, dir. Leaf tip	60	<b>0,000</b>
			0,000
damaged	on plant, leaf above damaged leaf	60	<b>0,000</b>
			0,000
damaged	on plant, leaf down of damaged leaf	60	<b>0,000</b>
			0,000

In a further assay, five plants in VT stage of events WI604-1602 and WI606-1206 were damaged with a scalpel in the greenhouse. Of each plant, forty hours later, samples were taken of the damaged leaf area (typically 2 to 3 mm around the wound site), from the same leaf 2 cm and 10 cm up and down of the damaged area, and from a leaf higher and lower than the wounded leaf. Leaf samples were also collected at the start of the experiment. Cry1Ab ELISA was run on the samples, mean (bold) and standard deviation were calculated.

Results are shown in Table 5 below.

10

Table 5. Cry1Ab concentration in TR2'-Cry1Ab plant lines (wounding by scalpel).

	sample	time (h)	Cry1Ab	
			%	
			damage with scalpel	
			WI604-1602	WI606-1206
undamaged	on plant	0	<b>0,001</b>	<b>0,000</b>
			0,001	0,000
undamaged	on plant	40	<b>0,001</b>	<b>0,000</b>
			0,001	0,001
damaged	on plant, sample injury	40	<b>0,005</b>	<b>0,006</b>
			0,001	0,002
damaged	on plant, 2cm above injury, dir. stem	40	<b>0,000</b>	<b>0,001</b>
			0,001	0,001
damaged	on plant, 2cm down injury, dir. leaf tip	40	<b>0,001</b>	<b>0,001</b>
			0,001	0,001
damaged	on plant, 10cm above injury, dir. stem	40	<b>0,001</b>	<b>0,001</b>
			0,000	0,001
damaged	on plant, 10cm down injury, dir. leaf tip	40	<b>0,001</b>	<b>0,001</b>
			0,000	0,000
damaged	on plant, leaf above damaged leaf	40	<b>0,001</b>	<b>0,000</b>
			0,001	0,000
damaged	on plant, leaf down of damaged leaf	40	<b>0,000</b>	<b>0,000</b>
			0,001	0,000

15 Measurements in additional field trials confirmed the low basal expression levels of the Cry1Ab protein in plants (using fresh leaf material taken from the plants, not incubated in vitro): in a field trial with 5 inbred plant lines per event comprising the pTR2'-Cry1Ab construct, between 0,001 and 0,003 % of total soluble leaf protein was Cry1Ab protein (mean

of 5 plants per event (standard deviation on average 0,001 to 0,004 %) in the field. Also, an additional field trial showed that more Cry1Ab protein is found in corn plants artificially infested by ECB larvae in the field than in plants not infested with insects. The expression levels for Cry1Ab found in this trial (8 plants) were on average between 0,001 and 0,002 % of total soluble protein in vegetative leaf, between 0,003 and 0,046 % of total soluble protein in R1 leaf, and between 0,009 and 0,017 % of total soluble protein in R5-6 leaf (using fresh leaf material taken from the plants, not incubated *in vitro*). As expected, insect infestation in the field provided for increased Cry1Ab protein concentration in some plants (samples were taken at random from the plants).

### Example 3. Insect resistance of plants with wound-inducible expression.

#### a) Efficacy against controlled infestation with ECB fourth instar larvae

Mid whorl corn plants were placed in a plexi-glass cylinder and infested with 10 or 15 fourth instar European corn borer larvae. After 10 days plants were dissected and percentage survival of larvae was scored per plant. The results are provided in Table 6.

Table 6. Efficacy against fourth instar ECB larvae

Event	Number of plants tested	Averaged percentage survival (s.d.)
WI602-0402	6	0
WI602-0802	6	0
WI604-1602	6	0
WI606-0802	6	0
WI606-1206	6	0
B73 control	9	49.6 (18.8)

The efficacy of the control of fourth instar ECB larvae in controlled infestation was found to be 100% for all TR2'-cry1Ab plants tested. Control plants showed on average a maximum of 50% mortality. As the fourth instar ECB larvae are believed to be between 25 and 100 times less susceptible to the modified Cry1Ab protein than first instar larvae, the level of protein produced in the TR2'-cry1Ab plants can be considered 'high dose', even though expression values as measured in such plants after wound-induction are typically still relatively low.

## b) Efficacy against ECB

Fourteen events were evaluated for ECB efficacy in the greenhouse and in field trials (Table 7). Results are presented as average length of tunnels (with standard deviation value between brackets) over maximum length of tunnels for each plant (average(sd)/max length/pl). In the greenhouse, average length of tunnels were taken of measurements on 10 plants. In the field, ECB efficacy is expressed as the average of 3 values obtained for different groups of 10 plants. Four of the five single-copy events (indicated by an asterisk) gave total ECB2 control, determined as less than 3.5 cm average tunnel length.

Table 7: ECB Efficacy in greenhouse and field trials

Event	ECB efficacy	ECB efficacy	ECB efficacy
	Greenhouse	Field 1	Field 2
	Average(sd)/max length/pl	Average(sd)/max length/pl	Average(sd)/max length/pl
WI602-0402*	0.1(0.31)/1	0.24(0.41)/2	0.21(0.01)/2
WI604-1602*	0.2(0.42)/1	0.05(0.071)/1	
WI606-0406*	51.3(73.5)/195	8.41(1.8)/32	7.45(0.07)/30
WI606-0802*	3.3(2.6)/8	0(0)/0	0.05(0.07)/1
WI606-1206*	0.38(0.74)/2	0.17(0.29)/3	0.1(0.14)/1
WI600-0218	2.0(1.87)/6		
WI600-1402	29.4(45.8)/142	6.55(2.47)/28	
WI602-0202	168.5(26.9)/200	12.6(3.8)/31	
WI604-0604	102.5(83)/251	3.7(2.0)/25	
WI602-0802	0(0)/0	0.33(0.15)/3	
WI602-0204	66(68.2)/160		
WI602-1002	102.4(63.4)/215		
WI606-0602	45.5(52.8)/160		
WI600-0802	0.9(1.44)/4	0.06(0.11)/2	0(0)/0

No penalty on agronomic performance was observed for the plants after second selfing (ear to row) of the different TR2' events in any of the locations tested.

c) Efficacy against *Sesamia nonagrioides*

Five mid whorl corn plants comprising the TR2'-cry1Ab construct were each infested with 2 egg masses. Damage was scored after 14 days and number of larvae were counted. Damage ratings (expressed in plant height and length of tunnels per plant in cm) were averaged over the five plants. Results are given in Table 8.

Table 8.

<u>Event</u>	<u>Number of larvae per plant</u>	<u>Plant height in cm</u>	<u>Cm tunnels per plant</u>
WI600-0802	0.6 (1.3)	198 (8.4)	0
B73 Control	197.2(14.0)	84 (5.5)	70 (9.4)

10

The results indicate that there is also good control of the Mediterranean stalk borer in the TR2'-cry1Ab plants tested.

**Example 4. Comparative analysis.**

15

Analysis of expression using the MPI promoter (described by Breitler et al., 2001 as being a wound-induced promoter) in corn plants with the same Cry1Ab coding sequence as used above is done to compare basal (non-induced) levels and wound-induction in the field. Corn plants expressing the insecticidal Cry1Ab protein portion under control of this MPI promoter show a basal mean expression level of at least about 0.04 % Cry1Ab protein per total soluble protein in leaves in greenhouse tests. Also, in initial greenhouse tests using 2 MPI-Cry1Ab events, some variation in expression levels upon wounding is found, but a wound-induced increase in Cry1Ab protein (mean) expression of at least 5 times that of the basal (mean) level is not observed in these events (after mechanical wounding), when measuring the concentration both in plant material freshly taken from these plants and in detached leaf parts that are incubated in vitro (in both cases, concentration is measured in different samples at 21 hours and 42 hours after wounding). Hence, there is either no or only a weak induction of Cry1Ab protein expression (compared to the basal protein expression level) in these assays with the MPI promoter.

25

Also, in comparative analysis, the performance in the field of the plants of the invention, using the TR2' promoter, is compared to commercially-available corn plants expressing a Cry1Ab protein portion. In these assays, corn plants obtained from events MON810 and Bt11 (see  
5 published USDA petitions of these approved corn events for a detailed description), which use a 35S promoter for high constitutive expression levels, and which are known to provide high dose insect resistance, are used.

In one field trial, the mean tunnel length (in cm per stalk, measured after stalk splitting as described in Jansens et al. (1997)) for artificial infestation with European corn borer is 0, 0,  
10 0.04, 0, and 0,21 cm, respectively, for 5 different Cry1Ab-TR2' corn events, while the mean tunnel length for MON810 corn is 0.3 and for Bt11 corn 0.13 (in non-transformed control corn lines, tunnel lengths from 12.2 to 39.92 cm are found in the same trial).

Hence, comparative analysis of the wound-induced TR2' promoter in corn shows that expression of an insecticidal protein under control of the TR2' promoter in corn provides high  
15 level insect resistance comparable to that obtainable with commercially-available events with constitutive promoters.

In another field trial, the mean tunnel length (in cm per stalk, measured after stalk splitting as described in Jansens et al. (1997)) for artificial infestation with Southwestern corn borer is  
20 0.53, 0.68, 0.3, 0.64, and 0,6 cm, respectively, for the 5 different Cry1Ab-TR2' corn events, while the mean length for MON810 corn is 0.43 and for Bt11 corn 0.15 (in non-transformed control corn lines, tunnel lengths from 32 to 39.6 cm are found in the same trial, some had no plants standing anymore by stalk breakage due to the tunnels).

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## Claims

1. An insect-resistant monocot plant, plant cell or tissue, comprising, stably integrated into its genome, a chimeric gene comprising a DNA sequence encoding an insecticidal protein under control of a promoter region comprising the TR2' promoter.
2. The plant, cell or tissue of claim 1 wherein said plant, cell or tissue expresses a high dose of said insecticidal protein.
3. The plant, cell or tissue of claim 1 or 2, wherein said plant, cell or tissue is a corn plant, cell or tissue and wherein said corn plant, cell or tissue kills at least 97 % of fourth instar European corn borer larvae in a 10 day bio-assay.
4. The plant, cell or tissue of any one of claims 1 to 3, wherein said insecticidal protein is a *Bacillus thuringiensis* toxin.
5. The plant, cell or tissue of any one of claims 1 to 4, wherein said TR2' promoter is the promoter of SEQ ID NO: 1.
6. A method for obtaining wound-induced expression of an insecticidal toxin in a monocot plant, said method comprising introducing into the plant a foreign DNA comprising a chimeric gene comprising:
- a DNA sequence encoding an insecticidal protein, and
  - a plant-expressible promoter region comprising the TR2' promoter.
7. The method of claim 6, wherein said monocot plant expresses a high dose of said insecticidal protein.
8. The method of claim 6 or 7, wherein said monocot plant is corn and wherein said corn plant kills at least 97 % of fourth instar European corn borer larvae in a 10 day bio-assay.

9. The method of any one of claims 6 to 8, wherein said monocot plant is corn, and wherein said wound-induced expression is characterized by a mean basal expression level of the insecticidal protein in leaves of said plant of 0,005 percent of total soluble protein or less in greenhouse-grown plants.

5

10. A method for making an insect resistant monocot plant, said method comprising introducing into the genome of said plant a foreign DNA comprising a chimeric gene comprising:

- a) a DNA sequence encoding an insecticidal protein, and
- 10 b) a plant-expressible promoter region comprising the TR2' promoter.

11. The method of claim 10, wherein said monocot plant expresses a high dose of said insecticidal protein.

15 12. The method of claim 10 or 11, wherein said monocot plant is corn and wherein said corn plant kills at least 97 % of fourth instar European corn borer larvae in a 10-day bio-assay.

13. The method of any one of claims 10 to 12, wherein said monocot plant is corn, and wherein said wound-induced expression is characterized by a mean basal expression level of  
20 the insecticidal protein in leaves of said plant of 0,005 percent of total soluble protein or less in greenhouse-grown plants.

14. A method for obtaining increased expression of an insecticidal protein in a monocot plant upon wounding, wherein the mean expression level in the leaves of said plant in the  
25 greenhouse, in the absence of wounding is 0.005% of total soluble protein or less, and upon wounding said mean expression level increases at least 5-fold, said method comprising introducing into the genome of said plant a foreign DNA comprising a chimeric gene comprising:

- a) a DNA sequence encoding an insecticidal protein, and
- 30 b) a plant-expressible promoter region comprising the TR2' promoter.

15. The method of claim 14, wherein said monocot plant is corn and wherein said corn plant kills at least 97 % of fourth instar European corn borer larvae in a whole plant bio-assay of 10 days.

5

16. The method of claim 14 or 15, wherein said plant is corn and wherein said basal expression of 0,005 % or less is measured in V4 stage leaves.

17. The method of any one of claims 14 to 16, wherein said insecticidal protein is a Bt protein.

10

18. The method of claim 17, wherein said Bt protein is selected from the group of Cry1Ab, Cry1F, Cry2Ae, Cry9C and Cry2Ab and insecticidal fragments thereof.

19. The plant, cell or tissue of claim 1, wherein said protein is the protein encoded by SEQ ID

15 NO: 3.

20. The plant, cell or tissue of claim 4 wherein said Bt protein is selected from the group of Cry1Ab, Cry1F, Cry2Ae, and Cry2A and insecticidal fragments thereof.

20 21. A chimeric gene for use in the method of any one of claims 6 to 18.

winexp wo1.st25  
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INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 03/04699

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 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 7 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)  
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAECK M ET AL: "TRANSGENIC PLANTS PROTECTED FROM INSECT ATTACK" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 328, no. 6125, 1987, pages 33-37, XP002166163 ISSN: 0028-0836 figure 1	21
Y	the whole document	1-20

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

\* Special categories of cited documents:

*A* document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention
*E* earlier document but published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
*O* document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search <b>4 August 2003</b>	Date of mailing of the international search report <b>13/08/2003</b>
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Name and mailing address of the ISA 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	Authorized officer <b>Bucka, A</b>
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## INTERNATIONAL SEARCH REPORT

International Application No

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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	JANSENS STEFAN ET AL: "Phthorimaea operculella (Lepidoptera: Gelechiidae) resistance in potato by expression of the Bacillus thuringiensis CryIA(b) insecticidal crystal protein." JOURNAL OF ECONOMIC ENTOMOLOGY, vol. 88, no. 5, 1995, pages 1469-1476, XP002249951 ISSN: 0022-0493 page 1469, right-hand column	21
Y	the whole document	1-20
Y	BREITLER JEAN CHRISTOPHE ET AL: "The -689/+197 region of the maize protease inhibitor gene directs high level, wound-inducible expression of the cry1B gene which protects transgenic rice plants from stemborer attack." MOLECULAR BREEDING, vol. 7, no. 4, 2001, pages 259-274, XP008020336 ISSN: 1380-3743 cited in the application page 259 -page 260 page 272	1-21
Y	EP 0 400 246 A (PLANT GENETIC SYSTEMS NV) 5 December 1990 (1990-12-05) page 7 -page 9; claim 12	1-21
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A	NI MIN ET AL: "Sequence-specific interactions of wound-inducible nuclear factors with mannopine synthase 2' promoter wound-responsive elements." PLANT MOLECULAR BIOLOGY, vol. 30, no. 1, 1996, pages 77-96, XP002250058 ISSN: 0167-4412 page 81 -page 84	1-21

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PCT/EP 03/04699

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DUAN XIAOLAN ET AL: "Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant." NATURE BIOTECHNOLOGY, vol. 14, no. 4, 1996, pages 494-498, XP008020404 ISSN: 1087-0156 page 494, right-hand column -page 495, right-hand column; table 2</p> <p style="text-align: center;">---</p>	1-21
A	<p>JOUANIN LISE ET AL: "Transgenic plants for insect resistance." PLANT SCIENCE (SHANNON), vol. 131, no. 1, 15 January 1998 (1998-01-15), pages 1-11, XP002249953 ISSN: 0168-9452 page 3, right-hand column page 8, left-hand column</p> <p style="text-align: center;">---</p>	1-21
A	<p>US 5 952 547 A (DOCKX JAN ET AL) 14 September 1999 (1999-09-14) column 12; claims 17,18</p> <p style="text-align: center;">-----</p>	1-21

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Information on patent family members

International Application No

PCT/EP 03/04699

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