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(54) Title: MAIZE BASAL LAYER ANTIMICROBIAL PROTEIN POLYNUCLEOTIDES AND METHODS OF USE

(57) Abstract: Methods and compositions for modulating development and defense response are provided. Nucleotide sequences encoding maize BTL proteins are provided. The sequence can be used in expression cassettes for antimicrobial resistance, modulating development, developmental pathways, and defense response. Transformed plants, plant cells, tissues, and seed are also provided.



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MAIZE BASAL LAYER ANTIMICROBIAL PROTEIN POLYNUCLEOTIDES AND METHODS OF USE

FIELD OF THE INVENTION

The invention relates to the field of the genetic manipulation of plants, particularly the modulation of gene activity and development in plants and increased disease resistance.

BACKGROUND OF THE INVENTION

5 Disease in plants is caused by biotic and abiotic causes. Biotic causes include fungi, viruses, bacteria, and nematodes. An example of the importance of plant disease is illustrated by phytopathogenic fungi, which cause significant annual crop yield losses as well as devastating epidemics. Plant disease outbreaks have resulted in catastrophic crop
10 failures that have triggered famines and caused major social change. All of the approximately 300,000 species of flowering plants are attacked by pathogenic fungi; however, a single plant species can be host to only a few fungal species, and similarly, most fungi usually have a limited host range. Generally, the best strategy for plant disease control is to use resistant cultivars selected or developed by plant breeders for this purpose.
15 However, the potential for serious crop disease epidemics persists today, as evidenced by outbreaks of the Victoria blight of oats and southern corn leaf blight. Molecular methods of crop protection have the potential to implement novel mechanisms for disease resistance and can also be implemented more quickly than traditional breeding methods. Accordingly, molecular methods are needed to supplement traditional breeding methods to protect plants
20 from pathogen attack.

A host of cellular processes enable plants to defend themselves against disease caused by pathogenic agents. These defense mechanisms are activated by initial pathogen infection in a process known as elicitation. In elicitation, the host plant recognizes a pathogen-derived compound known as an elicitor; the plant then activates disease gene
25 expression to limit further spread of the invading organism. It is generally believed that to overcome these plant defense mechanisms, plant pathogens must find a way to suppress elicitation as well as to overcome more physically-based barriers to infection, such as reinforcement and/or rearrangement of the actin filament networks near the cell's plasma membrane.

Thus, the present invention solves the need for enhancement of the plant's defensive elicitation response via a molecularly-based mechanism which can be quickly incorporated into commercial crops.

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SUMMARY OF THE INVENTION

Plant genes homologous to mammalian Basal Layer Antimicrobial Proteins (BAP's) are provided. Particularly, the nucleotide and amino acid sequences for eight maize Basal Layer Antimicrobial Proteins (BTL's or BETL's) are provided. Hence, the methods and compositions find use in enhancing disease resistance or stress resistance in crop plants.

10

The BTL genes of the present invention may also find use in enhancing the plant pathogen defense system. The methods and compositions can be used to modulate plant development, to promote healing of damaged tissues and to enhance resistance to plant pathogens including fungal pathogens, plant viruses, bacteria, nematodes, microbes, and the like. The method involves stably transforming a plant with a nucleotide sequence coding for a BTL operably linked to a promoter capable of driving expression of a gene in a plant cell. The disease resistance genes of the present invention additionally find use in manipulating these processes in transformed plants and plant cells.

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Transformed plants, plant cells, and seeds, as well as methods for making such plants, plant cells, and seeds are additionally provided. It is recognized that a variety of promoters will be useful in the invention, the choice of which will depend, in part, upon the desired level of expression of the disclosed nucleotide sequences. It is recognized that the levels of expression can be controlled to modulate the levels of expression in the plant cell.

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DETAILED DESCRIPTION OF THE INVENTION

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The present invention provides, inter alia, compositions and methods for modulating the total level of proteins of the present invention and/or altering their ratios in a plant. By "modulation" an increase or decrease in a particular character, quality, substance, or response is intended.

30

The compositions comprise maize nucleotide and amino acid sequences. Particularly, the nucleotide and amino acid sequences for eight maize BTL coding sequences are provided. As discussed in more detail below, the sequences of the invention are involved in basic biochemical pathways that regulate plant growth, development, and pathogen resistance. Methods are provided for the expression of these sequences in a host plant to modulate pathogen responses, defense responses, plant development, and

developmental pathways. The method involves stably transforming a plant with a nucleotide sequence coding for a BTL operably linked with a promoter capable of driving expression of a gene in a plant cell.

BTL's are proteins that have been shown to have antimicrobial activity, and are
5 predominantly expressed in the basal region of the endosperm, (Serna *et al.* (2001) *The Plant Journal* 25:687-698). In maize seeds, the main storage organ is the endosperm, which provides nourishment to the embryo as the seed develops and provides nutrients to the seedling at germination, (Hueros *et al.* (1999) *Plant Physiol.* 121:1143-1152). The uptake of nutrients by the growing endosperm is a critical process during seed development, and is
10 facilitated by specialized cells in the basal region known as Basal Endosperm Transfer Layer (BETL) cells. See Hueros *et al.* (1999) *Plant Physiol.* 121:1143-1152. BETL cells are transfer cells that possess anatomical modifications to enhance solute transport capacity, such as extensive cell wall ingrowths to increase membrane surface area, (Pate and Gunning (1972) *Ann. Rev. Plant. Physiol.* 23:173-196). Thus, the sequences of the invention find use
15 in modulating plant development, promoting healing of damaged tissues, and enhancing resistance to plant pathogens including fungal pathogens, plant viruses, and the like. The method involves stably transforming a plant with a nucleotide sequence capable of modulating the plant pathogen defense system operably linked with a promoter capable of driving expression of a gene in a plant cell.

20 Sequences of the invention, as discussed in more detail below, encompass coding sequences, antisense sequences, and fragments and variants thereof. Transformed plants can be obtained involving alterations in the level, tissue, or timing of expression to achieve enhanced disease or stress resistance. In addition, these methods may involve cross-species expression, meaning expression in a plant species other than the one from which the gene
25 was isolated.

By "antimicrobial activity" the killing or inhibition of growth of a microbe is intended. By "microbe" any organism such as bacteria, fungi, nematodes, and insects that are pathogens or potential pathogens of crop plants is intended. For maize, this would especially include ear mold fungal pathogens, such as *Fusarium moniliforme*.

30 Compositions

Compositions of the invention include the sequences for eight maize nucleotide sequences which have been identified as members of the Basal Layer Antimicrobial Protein (BTL) family in maize that are involved in defense response, development, and

antimicrobial resistance. In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequences shown in SEQ ID NOS:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 28. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described
5 herein, for example those set forth in SEQ ID NOS:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, and 29, and fragments and variants thereof.

The BETL1-2 cDNA (SEQ ID NO:1) is 488 bp long with an open reading frame from nucleotide 66 to 338. It encodes a 90 amino acid residue polypeptide (SEQ ID NO:2) with an approximate molecular weight of 9.97 KDa and a PI of 7.86. BETL1-2 has
10 approximately 74% amino acid sequence identity to the BETL1-1 GAP sequence (Accession No. Z49203).

The BETL1-3 cDNA (SEQ ID NO:5) is 528 bp long with an open reading frame from nucleotide 89 to 358 (SEQ ID NO:7). It encodes an 89 amino acid residue polypeptide (SEQ ID NOS:6 and 8) with an approximate molecular weight of 9.86 KDa and
15 a PI of 7.86. BETL1-3 has approximately 74% amino acid sequence identity to the BETL1-1 GAP sequence (Accession No. Z49203).

The BETL1-4 cDNA (SEQ ID NO:9) is 443 bp long with an open reading frame from nucleotide 54 to 323 (SEQ ID NO:11). It encodes an 89 amino acid residue polypeptide (SEQ ID NOS:10 and 12) with an approximate molecular weight of 10.07 KDa
20 and a PI of 8.24. BETL1-4 has approximately 46% amino acid sequence identity to the BETL1-1 GAP sequence (Accession No. Z49203).

The BETL2-6 cDNA (SEQ ID NO:13) is 402 bp long with an open reading frame from nucleotide 62 to 343 (SEQ ID NO:15). It encodes a 93 amino acid residue polypeptide (SEQ ID NOS:14 and 16) with an approximate molecular weight of 10.41 KDa
25 and a PI of 7.25. BETL2-6 has approximately 93% amino acid sequence identity to the BTL-1b GAP sequence (Accession No. AJ297900). Furthermore, BETL2-6 has approximately 91% amino acid sequence identity to the BTL-1a GAP sequence (Accession No. AJ297901).

The BETL2-7 cDNA (SEQ ID NO:17) is 439 bp long with an open reading frame
30 from nucleotide 64 to 366 (SEQ ID NO:19). It encodes a 100 amino acid residue polypeptide (SEQ ID NOS:18 and 20) with an approximate molecular weight of 10.76 KDa and a PI of 7.93. BETL2-7 has approximately 65% amino acid sequence identity to a *Sorghum bicolor* cDNA GAP sequence (Accession No. BG240586). Furthermore, BETL2-7 has approximately 49% amino acid sequence identity to the BETL2 GAP sequence

(Accession No. AJ133529).

The BETL2-8 cDNA (SEQ ID NO:21) is 466 bp long with an open reading frame from nucleotide 26 to 301 (SEQ ID NO:23). It encodes a 91 amino acid residue polypeptide (SEQ ID NOS:22 and 24) with an approximate molecular weight of 10.06 KDa and a PI of 8.33. BETL2-8 has approximately 65% amino acid sequence identity to the BTL-1b GAP sequence (Accession No. AJ297900). Furthermore, BETL2-6 has approximately 54% amino acid sequence identity to the BTL-1a GAP sequence (Accession No. AJ297901).

The BETL4-2 cDNA (SEQ ID NO:25) is 539 bp long with an open reading frame from nucleotide 54 to 380 (SEQ ID NO:27). It encodes a 108 amino acid residue polypeptide (SEQ ID NOS:26 and 28) with an approximate molecular weight of 12.04 KDa and a PI of 8.33. BETL4-2 has approximately 53% amino acid sequence identity to the BETL4 gene GAP sequence (Accession No. AJ133531).

The BETL4-5 cDNA (SEQ ID NO:29) is 364 bp long and has approximately 45% amino acid sequence identity to the BETL4 gene GAP sequence (Accession No. AJ133531). It encodes a 120 amino acid residue polypeptide (SEQ ID NO:30).

The invention encompasses isolated or substantially purified nucleic acid or protein compositions. An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in the genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Fragments and variants of the disclosed nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By "fragment" a portion of the

nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby is intended. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein and hence have BTL-like activity and thereby affect antimicrobial activity and responses, development, and developmental pathways. Alternatively, fragments of a nucleotide sequence that are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the proteins of the invention.

A fragment of a BTL nucleotide sequence that encodes a biologically active portion of a BTL protein of the invention will encode at least 15, 25, 30, 50, 100, 150, 200, 250, or 300 contiguous amino acids, or up to the total number of amino acids present in a full-length BTL protein of the invention (for example, 90 amino acids for SEQ ID NO:2, 89 amino acids for SEQ ID NOS:6 and 8, 89 amino acids for SEQ ID NO:10 and 12, 93 amino acids for SEQ ID NO:14 and 16, 100 amino acids for SEQ ID NO:18 and 20, 91 amino acids for SEQ ID NO:22 and 24, and 108 amino acids for SEQ ID NO:26 and 28, and 120 amino acids for SEQ ID NO:30). Fragments of a BTL nucleotide sequence that are useful as hybridization probes for PCR primers generally need not encode a biologically active portion of a BTL protein.

Thus, a fragment of a BTL nucleotide sequence may encode a biologically active portion of a BTL protein, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a BTL protein can be prepared by isolating a portion of one of the BTL nucleotide sequences of the invention, expressing the encoded portion of the BTL protein (*e.g.*, by recombinant expression *in vitro*), and assessing the activity of the encoded portion of the BTL protein. Nucleic acid molecules that are fragments of a BTL nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, or 650 nucleotides, or up to the number of nucleotides present in a full-length BTL nucleotide sequence disclosed herein (for example, 488 nucleotides for SEQ ID NO:1, 528 nucleotides for SEQ ID NO:5, 443 nucleotides for SEQ ID NO:9, 402 nucleotides for SEQ ID NO:13, 439 nucleotides for SEQ ID NO:17, 466 nucleotides for SEQ ID NO:21, 539 nucleotides for SEQ ID NO:25, and 364 nucleotides for SEQ ID NO:29).

By "variants" substantially similar sequences is intended. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic

code, encode the amino acid sequence of one of the BTL polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also
5 include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a BTL protein of the invention. Generally, variants of a particular nucleotide sequence of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about
10 98%, 99%, or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

By "variant protein" a protein derived from the native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in
15 the native protein; or substitution of one or more amino acids at one or more sites in the native protein is intended. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, BTL-like activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active
20 variants of a native BTL protein of the invention will have at least about 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99%, or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A
25 biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

Biological activity of the BTL polypeptides can be assayed by any method known in the art. Assays to measure antimicrobial activity and the developmental pathways and
30 defense responses that are influenced by the BTL polypeptides having BTL-like activity are well known in the art. See, WO 99/50427; Serna *et al.* (2001) *The Plant Journal* 25:687-698; Hueros *et al.* (1999) *Plant Physiol.* 121:1143-1152; Hueros *et al.* (1995) *Plant Cell* 7:747-757, all of which are herein incorporated by reference.

The proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Novel proteins having properties of interest may be created by combining elements and fragments of proteins of the present invention, as well as, other proteins. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the BTL proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; US Patent No. 4,873,192; Walker and Gaastra, Eds., (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins, as well as, variations and modified forms thereof. Such variants will continue to possess the desired antimicrobial activity, developmental activity, developmental pathway activity, or defense response activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated by BTL activity assays. See, for example, WO 99/50427; Serna *et al.* (2001) *The Plant Journal* 25:687-698; Hueros *et al.* (1999) *Plant Physiol.* 121:1143-1152; Hueros *et al.* (1995) *Plant Cell* 7:747-757, all of which are herein incorporated by reference. Additionally, differences in the expression of specific genes between uninfected and infected plants can be determined using gene expression profiling. RNA is analyzed using the gene expression

profiling process (GeneCalling®) as described in U.S. Patent No. 5,871,697, herein incorporated by reference.

Variant nucleotide sequences and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different BTL coding sequences can be manipulated to create a new BTL protein possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the BTL gene of the invention and other known BTL genes to obtain a new gene coding for a protein with an improved property of interest, such as an increased K_m in the case of an enzyme. Such shuffling of domains may also be used to assemble novel proteins having novel properties. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Cramer *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-4509; Cramer *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The nucleotide sequences of the invention can be used to isolate corresponding sequences from other organisms, particularly other plants, more particularly other monocots. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire BTL sequences set forth herein or to fragments thereof are encompassed by the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" genes derived from a common ancestral gene and which are found in different species as a result of speciation are intended. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any plant of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning*:

A Laboratory Manual (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, Eds., (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, Eds., (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, Eds., (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (*i.e.*, genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P , or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the BTL sequences of the invention. Methods for the preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, an entire BTL sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to corresponding BTL sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among BTL sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes may be used to amplify corresponding sequences from a chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in an organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By “stringent conditions” or “stringent hybridization conditions” conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (*e.g.*, at least 2-fold over background) are intended. Stringent conditions are

sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides). Duration of hybridization is generally less than about 24 hours, usually about 4 to 12 hours. Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. The T_m (thermal melting point) is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower

than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m).

5 Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the
10 hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, Eds., (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring
15 Harbor Laboratory Press, Plainview, New York).

 Thus, isolated sequences that encode for a BTL polypeptide and which hybridize under stringent conditions to the BTL sequences disclosed herein, or to fragments thereof, are encompassed by the present invention. Such sequences will be at least about 40% to 50% homologous, about 60%, 65%, or 70% homologous, and even at least about 75%,
20 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous with the disclosed sequences. That is, the sequence identity of sequences may range, sharing at least about 40% to 50%, about 60%, 65%, or 70%, and even at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity.

25 The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) “reference sequence”, (b) “comparison window”, (c) “sequence identity”, (d) “percentage of sequence identity”, and (e) “substantial identity”.

 (a) As used herein, “reference sequence” is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a
30 specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

 (b) As used herein, “comparison window” makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) compared to the

reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of
5 gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the
10 algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, and modified as in Karlin and
15 Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT,
20 BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5:151-153; Corpet *et al.* (1988) *Nucleic Acids Res.*
25 16:10881-90; Huang *et al.* (1992) *CABIOS* 8:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403 are based on the
30 algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of

the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.*

25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997)

5 *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (*e.g.*, BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See <http://www.ncbi.nlm.nih.gov>. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to
10 the value obtained using GAP Version 10 using the following parameters: % identity using a GAP Weight of 50 and a Length Weight of 3; % similarity using a GAP Weight of 12 and a Length Weight of 4, or any equivalent program. By “equivalent program” any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence
15 identity when compared to the corresponding alignment generated by the preferred program is intended.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions
20 and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension
25 penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example,
30 the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the

metric maximized in order to align the sequences. Ratio is the Quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value
5 for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences
10 that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (*e.g.*, charge or hydrophobicity) and therefore do not
15 change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a
20 conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, *e.g.*, as implemented in the program PC/GENE (Intelligenetics, Mountain
25 View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (which
30 does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of

positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term “substantial identity” of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term “substantial identity” in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman *et al.* (1970) *J. Mol. Biol.* 48:443. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are “substantially

similar” share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

Diseases and Pests

5 The invention is drawn to compositions and methods for inducing resistance in a plant to plant pests. The anti-pathogenic compositions comprise maize BTL nucleotide and amino acid sequences. Particularly, the maize nucleic acid and amino acid sequences are selected from BETL1-2, BETL1-3, BETL1-4, BETL2-6, BETL2-7, BETL2-8, BETL4-2, and BETL4-5. Accordingly, the compositions and methods are also useful in protecting
10 plants against fungal pathogens, viruses, nematodes, insects, and the like.

 By “disease resistance” it is intended that the plants avoid the disease symptoms that are the outcome of plant-pathogen interactions. That is, pathogens are prevented from causing plant diseases and the associated disease symptoms, or alternatively, the disease symptoms caused by the pathogen are minimized or lessened. The methods of the invention
15 can be utilized to protect plants from disease, particularly those diseases that are caused by plant pathogens.

 By “antipathogenic compositions” it is intended that the compositions of the invention have antipathogenic activity and thus are capable of suppressing, controlling, and/or killing the invading pathogenic organism. An antipathogenic composition of the
20 invention will reduce the disease symptoms resulting from pathogen challenge by at least about 5% to about 50%, at least about 10% to about 60%, at least about 30% to about 70%, at least about 40% to about 80%, or at least about 50% to about 90% or greater. Hence, the methods of the invention can be utilized to protect plants from diseases, particularly those diseases that are caused by plant pathogens.

25 Assays that measure antipathogenic activity are commonly known in the art, as are methods to quantitate disease resistance in plants following pathogen infection. See, for example, U.S. Patent No. 5,614,395, herein incorporated by reference. Such techniques include, measuring over time, the average lesion diameter, the pathogen biomass, and the overall percentage of decayed plant tissues. For example, a plant either expressing an
30 antipathogenic polypeptide or having an antipathogenic composition applied to its surface shows a decrease in tissue necrosis (*i.e.*, lesion diameter) or a decrease in plant death following pathogen challenge when compared to a control plant that was not exposed to the antipathogenic composition. Alternatively, antipathogenic activity can be measured by a decrease in pathogen biomass. For example, a plant expressing an antipathogenic

polypeptide or exposed to an antipathogenic composition is challenged with a pathogen of interest. Over time, tissue samples from the pathogen-inoculated tissues are obtained and RNA is extracted. The percent of a specific pathogen RNA transcript relative to the level of a plant specific transcript allows the level of pathogen biomass to be determined. See, for example, Thomma *et al.* (1998) *Plant Biology* 95:15107-15111, herein incorporated by reference.

Furthermore, *in vitro* antipathogenic assays include, for example, the addition of varying concentrations of the antipathogenic composition to paper disks and placing the disks on agar containing a suspension of the pathogen of interest. Following incubation, clear inhibition zones develop around the discs that contain an effective concentration of the antipathogenic polypeptide (Liu *et al.* (1994) *Plant Biology* 91:1888-1892, herein incorporated by reference). Additionally, microspectrophotometrical analysis can be used to measure the *in vitro* antipathogenic properties of a composition (Hu *et al.* (1997) *Plant Mol. Biol.* 34:949-959 and Cammue *et al.* (1992) *J. Biol. Chem.* 267:2228-2233, both of which are herein incorporated by reference).

In specific embodiments, methods for increasing pathogen resistance in a plant comprise stably transforming a plant with a DNA construct comprising an anti-pathogenic nucleotide sequence of the invention operably linked to promoter that drives expression in a plant. Such methods find use in agriculture particularly in limiting the impact of plant pathogens on crop plants. While the choice of promoter will depend on the desired timing and location of expression of the anti-pathogenic nucleotide sequences, particular promoters include constitutive and pathogen-inducible promoters. Accordingly, transformed plants, plants cells, plant tissues and seeds thereof are provided.

Additionally, the compositions can be used in formulations used for their antimicrobial activities. The proteins of the invention can be formulated with an acceptable carrier into a pesticidal composition(s) for example, a suspension, a solution, an emulsion, a dusting powder, a dispersible granule, a wettable powder, and an emulsifiable concentrate, an aerosol, an impregnated granule, an adjuvant, a coatable paste, and also encapsulations in, for example, polymer substances.

It is understood in the art that plant DNA viruses and fungal pathogens remodel the control of the host replication and gene expression machinery to accomplish their own replication and effective infection. The present invention may be useful in preventing such corruption of the cell.

The methods of the invention can be used with other methods available in the art for enhancing disease resistance in plants. For example, any one of a variety of second nucleotide sequences may be utilized, embodiments of the invention encompass those second nucleotide sequences that, when expressed in a plant, help to increase the resistance of a plant to pathogens. It is recognized that such second nucleotide sequences may be used in either the sense or antisense orientation depending on the desired outcome. Other plant defense proteins include those described in WO 99/43823 and WO 99/43821, both of which are herein incorporated by reference.

Pathogens of the invention include, but are not limited to, viruses or viroids, bacteria, insects, nematodes, fungi, and the like. Viruses include any plant virus, for example, tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, maize dwarf mosaic virus, etc. Specific fungal and viral pathogens for the major crops include, but are not limited to, Soybeans: *Phytophthora megasperma* fsp. *glycinea*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Diaporthe phaseolorum* var. *sojae* (*Phomopsis sojae*), *Diaporthe phaseolorum* var. *caulivora*, *Sclerotium rolsii*, *Cercospora kikuchii*, *Cercospora sojina*, *Peronospora manshurica*, *Colletotrichum dematium* (*Colletotrichum truncatum*), *Corynespora cassicola*, *Septoria glycines*, *Phyllosticta sojicola*, *Alternaria alternata*, *Pseudomonas syringae* p.v. *glycinea*, *Xanthomonas campestris* p.v. *phaseoli*, *Microsphaera diffusa*, *Fusarium semitectum*, *Phialophora gregata*, Soybean mosaic virus, *Glomerella glycines*, Tobacco Ring spot virus, Tobacco Streak virus, *Phakopsora pachyrhizi*, *Pythium aphanidermatum*, *Pythium ultimum*, *Pythium debaryanum*, Tomato spotted wilt virus, *Heterodera glycines*, *Fusarium solani*; Canola: *Albugo candida*, *Alternaria brassicae*, *Leptosphaeria maculans*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Mycosphaerella brassicicola*, *Pythium ultimum*, *Peronospora parasitica*, *Fusarium roseum*, *Alternaria alternata*; Alfalfa: *Clavibacter michiganense* subsp. *insidiosum*, *Pythium ultimum*, *Pythium irregulare*, *Pythium splendens*, *Pythium debaryanum*, *Pythium aphanidermatum*, *Phytophthora megasperma*, *Peronospora trifoliorum*, *Phoma medicaginis* var. *medicaginis*, *Cercospora medicaginis*, *Pseudopeziza medicaginis*, *Leptotrochila medicaginis*, *Fusarium*, *Xanthomonas campestris* p.v. *alfalfae*, *Aphanomyces euteiches*, *Stemphylium herbarum*, *Stemphylium alfalfae*; Wheat: *Pseudomonas syringae* p.v. *atrofaciens*, *Urocystis agropyri*, *Xanthomonas campestris* p.v. *translucens*, *Pseudomonas syringae* p.v. *syringae*, *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium culmorum*, *Ustilago tritici*, *Ascochyta tritici*, *Cephalosporium gramineum*, *Colletotrichum graminicola*,

Erysiphe graminis f.sp. *tritici*, *Puccinia graminis* f.sp. *tritici*, *Puccinia recondita* f.sp. *tritici*,
Puccinia striiformis, *Pyrenophora tritici-repentis*, *Septoria nodorum*, *Septoria tritici*,
Septoria avenae, *Pseudocercospora herpotrichoides*, *Rhizoctonia solani*, *Rhizoctonia*
cerealis, *Gaeumannomyces graminis* var. *tritici*, *Pythium aphanidermatum*, *Pythium*
5 *arrhenomanes*, *Pythium ultimum*, *Bipolaris sorokiniana*, Barley Yellow Dwarf Virus,
Brome Mosaic Virus, Soil Borne Wheat Mosaic Virus, Wheat Streak Mosaic Virus, Wheat
Spindle Streak Virus, American Wheat Striate Virus, *Claviceps purpurea*, *Tilletia tritici*,
Tilletia laevis, *Tilletia indica*, *Rhizoctonia solani*, *Pythium graminicola*, High Plains Virus,
European wheat striate virus; Sunflower: *Plasmophora halstedii*, *Sclerotinia sclerotiorum*,
10 Aster Yellows, *Septoria helianthi*, *Phomopsis helianthi*, *Alternaria helianthi*, *Alternaria*
zinniae, *Botrytis cinerea*, *Phoma macdonaldii*, *Macrophomina phaseolina*, *Erysiphe*
cichoracearum, *Rhizopus oryzae*, *Rhizopus arrhizus*, *Rhizopus stolonifer*, *Puccinia*
helianthi, *Verticillium dahliae*, *Erwinia carotovorum* pv. *carotovora*, *Cephalosporium*
acremonium, *Phytophthora cryptogea*, *Albugo tragopogonis*; Corn: *Fusarium moniliforme*
15 var. *subglutinans*, *Erwinia stewartii*, *Fusarium moniliforme*, *Gibberella zeae* (*Fusarium*
graminearum), *Stenocarpella maydis* (*Diplodia maydis*), *Pythium irregulare*, *Pythium*
debaryanum, *Pythium graminicola*, *Pythium splendens*, *Pythium ultimum*, *Pythium*
aphanidermatum, *Aspergillus flavus*, *Bipolaris maydis* O, T (*Cochliobolus heterostrophus*),
Helminthosporium carbonum I, II & III (*Cochliobolus carbonum*), *Exserohilum turcicum* I,
20 II & III, *Helminthosporium pedicellatum*, *Physoderma maydis*, *Phyllosticta maydis*,
Kabatiella maydis, *Cercospora sorghi*, *Ustilago maydis*, *Puccinia sorghi*, *Puccinia*
polysora, *Macrophomina phaseolina*, *Penicillium oxalicum*, *Nigrospora oryzae*,
Cladosporium herbarum, *Curvularia lunata*, *Curvularia inaequalis*, *Curvularia pallescens*,
Clavibacter michiganense subsp. *nebraskense*, *Trichoderma viride*, Maize Dwarf Mosaic
25 Virus A & B, Wheat Streak Mosaic Virus, Maize Chlorotic Dwarf Virus, *Claviceps sorghi*,
Pseudomonas avenae, *Erwinia chrysanthemi* pv. *zea*, *Erwinia carotovora*, Corn stunt
spiroplasma, *Diplodia macrospora*, *Sclerophthora macrospora*, *Peronosclerospora sorghi*,
Peronosclerospora philippinensis, *Peronosclerospora maydis*, *Peronosclerospora sacchari*,
Sphacelotheca reiliana, *Physopella zeae*, *Cephalosporium maydis*, *Cephalosporium*
30 *acremonium*, Maize Chlorotic Mottle Virus, High Plains Virus, Maize Mosaic Virus, Maize
Rayado Fino Virus, Maize Streak Virus, Maize Stripe Virus, Maize Rough Dwarf Virus;
Sorghum: *Exserohilum turcicum*, *Colletotrichum graminicola* (*Glomerella graminicola*),
Cercospora sorghi, *Gloeocercospora sorghi*, *Ascochyta sorghina*, *Pseudomonas syringae*
p.v. *syringae*, *Xanthomonas campestris* p.v. *holcicola*, *Pseudomonas andropogonis*,

Puccinia purpurea, *Macrophomina phaseolina*, *Perconia circinata*, *Fusarium moniliforme*, *Alternaria alternata*, *Bipolaris sorghicola*, *Helminthosporium sorghicola*, *Curvularia lunata*, *Phoma insidiosa*, *Pseudomonas avenae* (*Pseudomonas alboprecipitans*), *Ramulispora sorghi*, *Ramulispora sorghicola*, *Phyllachara sacchari*, *Sporisorium reilianum* (5 *Sphacelotheca reiliana*), *Sphacelotheca cruenta*, *Sporisorium sorghi*, Sugarcane mosaic H, Maize Dwarf Mosaic Virus A & B, *Claviceps sorghi*, *Rhizoctonia solani*, *Acremonium strictum*, *Sclerophthona macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Sclerospora graminicola*, *Fusarium graminearum*, *Fusarium oxysporum*, *Pythium arrhenomanes*, and *Pythium graminicola*.

10 Nematodes include parasitic nematodes such as root-knot, cyst, and lesion nematodes, including, but not limited to, *Heterodera* and *Globodera* spp; particularly *Globodera rostochiensis* and *Globodera pailida* (potato cyst nematodes); *Heterodera glycines* (soybean cyst nematode); *Heterodera schachtii* (beet cyst nematode); and *Heterodera avenae* (cereal cyst nematode).

15 Insect pests include, but are not limited to, insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera. Insect pests of the invention for the major crops include, but are not limited to, Maize: *Ostrinia nubilalis*, European corn borer; *Agrotis* 20 *ipsilon*, black cutworm; *Helicoverpa zea*, corn earworm; *Spodoptera frugiperda*, fall armyworm; *Diatraea grandiosella*, southwestern corn borer; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Diatraea saccharalis*, sugarcane borer; *Diabrotica virgifera*, western corn rootworm; *Diabrotica longicornis barberi*, northern corn rootworm; *Diabrotica undecimpunctata howardi*, southern corn rootworm; *Melanotus* spp., wireworms; 25 *Cyclocephala borealis*, northern masked chafer (white grub); *Cyclocephala immaculata*, southern masked chafer (white grub); *Popillia japonica*, Japanese beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Anuraphis maidiradicis*, corn root aphid; *Blissus leucopterus leucopterus*, chinch bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus sanguinipes*, 30 migratory grasshopper; *Hylemya platura*, seedcorn maggot; *Agromyza parvicornis*, corn blot leafminer; *Anaphothrips obscurus*, grass thrips; *Solenopsis milesta*, thief ant; *Tetranychus urticae*, twospotted spider mite; Sorghum: *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Feltia subterranea*, granulate cutworm; *Phyllophaga*

- crinita*, white grub; *Eleodes*, *Conoderus*, and *Aeolus spp.*, wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Sipha flava*, yellow sugarcane aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge;
- 5 *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Wheat: *Pseudaletia unipunctata*, army worm; *Spodoptera frugiperda*, fall armyworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis orthogonia*, western cutworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn
- 10 rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella fusca*,
- 15 tobacco thrips; *Cephus cinctus*, wheat stem sawfly; *Aceria tulipae*, wheat curl mite; Sunflower: *Suleima helianthana*, sunflower bud moth; *Homoeosoma electellum*, sunflower moth; *zygogramma exclamationis*, sunflower beetle; *Bothyrus gibbosus*, carrot beetle; *Neolasioptera murtfeldtiana*, sunflower seed midge; Cotton: *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Spodoptera exigua*, beet armyworm;
- 20 *Pectinophora gossypiella*, pink bollworm; *Anthonomus grandis grandis*, boll weevil; *Aphis gossypii*, cotton aphid; *Pseudatomoscelis seriatus*, cotton fleahopper; *Trialeurodes abutilonea*, bandedwinged whitefly; *Lygus lineolaris*, tarnished plant bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Thrips tabaci*, onion thrips; *Frankliniella fusca*, tobacco thrips; *Tetranychus cinnabarinus*,
- 25 carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Rice: *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Colaspis brunnea*, grape colaspis; *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephotettix nigropictus*, rice leafhopper; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; Soybean: *Pseudoplusia*
- 30 *includens*, soybean looper; *Anticarsia gemmatilis*, velvetbean caterpillar; *Plathypena scabra*, green cloverworm; *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Spodoptera exigua*, beet armyworm; *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Epilachna varivestis*, Mexican bean beetle; *Myzus persicae*, green peach aphid; *Empoasca fabae*, potato leafhopper; *Acrosternum hilare*, green

stink bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Hylemya platura*, seedcorn maggot; *Sericothrips variabilis*, soybean thrips; *Thrips tabaci*, onion thrips; *Tetranychus turkestani*, strawberry spider mite; *Tetranychus urticae*, twospotted spider mite; Barley: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Schizaphis graminum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servus*, brown stink bug; *Delia platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Petrobia latens*, brown wheat mite; Oil Seed Rape: *Brevicoryne brassicae*, cabbage aphid; *Phyllotreta cruciferae*, Flea beetle; *Mamestra configurata*, Bertha armyworm; *Plutella xylostella*, Diamond-back moth; *Delia* spp., Root maggots.

Expression of Sequences

The nucleic acid sequences of the present invention can be expressed in a host cell such as bacteria, yeast, insect, mammalian, or preferably plant cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous nucleotide sequence can be from a species different from that from which the nucleotide sequence was derived, or, if from the same species, the promoter is not naturally found operably linked to the nucleotide sequence. A heterologous protein may originate from a foreign species, or, if from the same species, is substantially modified from its original form by deliberate human intervention.

By "host cell" a cell, which comprises a heterologous nucleic acid sequence of the invention is meant. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. Preferably, host cells are monocotyledonous or dicotyledonous plant cells. A particularly preferred monocotyledonous host cell is a maize host cell.

The BTL sequences of the invention are provided in expression cassettes or DNA constructs for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a BTL sequence of the invention. By "operably

linked” a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence is intended. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

Such an expression cassette is provided with a plurality of restriction sites for insertion of the BTL sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a BTL DNA sequence of the invention, and a transcriptional and translational termination region functional in plants. The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By “foreign” it is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of BTL in the host cell (*i.e.*, plant or plant cell). Thus, the phenotype of the host cell (*i.e.*, plant or plant cell) is altered.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See, Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.

Where appropriate, the gene(s) may be optimized for increased expression in the transformed plant. That is, the genes can be synthesized using plant-preferred codons for

improved expression. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels that are average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include, but are not limited to: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *PNAS USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.* (1986) *Virology* 154:9-20); MDMV leader (Maize Dwarf Mosaic Virus); human immunoglobulin heavy-chain binding protein (BiP), (Macejak *et al.* (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, Ed., Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, *e.g.*, transitions and transversions, may be involved.

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance,

such as those encoding neomycin phosphotransferase II (NEO) and hygromycin phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glyphosate, glufosinate, bromoxynil, imidazolinones, and 2,4-

dichlorophenoxyacetate (2,4-D). See generally, Yarranton (1992) *Curr. Opin. Biotech.*

3:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao *et al.* (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley *et al.*

(1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48:555-566; Brown *et al.* (1987)

Cell 49:603-612; Figge *et al.* (1988) *Cell* 52:713-722; Deuschle *et al.* (1989) *Proc. Natl.*

Acad. Sci. USA 86:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-

2553; Deuschle *et al.* (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University

of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow *et al.*

(1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA*

89:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski *et al.*

(1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc.*

Biol. 10:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595;

Kleinschmidt *et al.* (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis,

University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551;

Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913-919; Hlavka *et al.* (1985)

Handbook of Experimental Pharmacology, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.*

(1988) *Nature* 334:721-724 and U.S. Patent Application Serial No. 10/072,307. Such

disclosures are herein incorporated by reference. The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

A number of promoters can be used in the practice of the invention. The promoters

can be selected based on the desired outcome. That is, the nucleic acids can be combined

with constitutive, tissue-preferred, or other promoters for expression in the host cell of

interest. Such constitutive promoters include, for example, the core promoter of the Rsyn7

promoter (WO 99/43838 and U.S. Patent No. 6,072,050); the core CaMV 35S promoter

(Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell*

2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and

Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor.*

Appl. Genet. 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS

promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include,

for example, those disclosed in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

Generally, it will be beneficial to express the gene from an inducible promoter, particularly from a pathogen-inducible promoter. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol.* 89:245-254; Uknes *et al.* (1992) *Plant Cell* 4:645-656; and Van Loon (1985) *Plant Mol. Virol.* 4:111-116. See also, WO 99/43819, which is herein incorporated by reference.

Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol.* 9:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions* 2:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet.* 2:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:2507-2511; Warner *et al.* (1993) *Plant J.* 3:191-201; Siebertz *et al.* (1989) *Plant Cell* 1:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path.* 41:189-200).

Additionally, as pathogens find entry into plants through wounds or insect damage, a wound-inducible promoter may be used in the constructions of the invention. Such wound-inducible promoters include the potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath.* 28:425-449; Duan *et al.* (1996) *Nature Biotechnology* 14:494-498); wun1 and wun2, US Patent No. 5,428,148; win1 and win2 (Stanford *et al.* (1989) *Mol. Gen. Genet.* 215:200-208); systemin (McGurl *et al.* (1992) *Science* 225:1570-1573); WIP1 (Rohrmeier *et al.* (1993) *Plant Mol. Biol.* 22:783-792 and Eckelkamp *et al.* (1993) *FEBS Letters* 323:73-76); MPI gene (Corderok *et al.* (1994) *Plant J.* 6(2):141-150); and the like, herein incorporated by reference.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art

and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners; the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides; and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced BTL expression within a particular plant tissue. Tissue-preferred promoters include those disclosed in Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen. Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

Leaf-specific promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J.* 3:509-18; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

Root-preferred promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and

root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641, which discloses two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa*. The promoters of these genes were linked to a β -glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) (*EMBO J.* 8(2): 343-350) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, which is an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene. The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772); the ZRP2 promoter (U.S. Patent No. 5,633,363); the IFS1 promoter (U.S. Patent Application Serial No. 10/104,706) and the rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson *et al.* (1989) *BioEssays* 10:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); milps (myo-inositol-1-phosphate synthase); and celA (cellulose synthase) (see U.S. Patent No. 6,225,529, herein incorporated by reference). Gama-zein is a preferred endosperm-specific promoter. Glob-1 is a preferred embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β -phaseolin, napin, β -conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, g-zein, waxy, shrunken 1, shrunken 2, globulin 1, etc.

The method of transformation/transfection is not critical to the instant invention. Various methods of transformation or transfection are currently available. As newer

methods are available to transform crops or other host cells they may be directly applied to the present invention. Accordingly, a wide variety of methods have been developed to insert a DNA sequence into the genome of a host cell to obtain the transcription and/or translation of the sequence to effect phenotypic changes in the organism. Thus, any method, which provides for effective transformation/transfection may be employed with the nucleotide sequences of the present invention.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, *i.e.*, monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (U.S. Pat Nos. 5,563,055 and 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, U.S. Patent No. 4,945,050; Tòmes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, Eds., Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926), and Lec1 transformation (WO 00/28058). See also, Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); U.S. Patent Nos. 5,240,855, 5,322,783 and 5,324,646; Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature* (London) 311:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.*

(1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84.

- 5 These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrids having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that constitutive expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure constitutive expression of the desired
- 10 phenotypic characteristic has been achieved.

- The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plants of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus*, *B. rapa*, *B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*),
- 15 rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*,
- 20 *Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*),
- 25 macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

- Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*),
- 30 and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine

(*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and
5 cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.), more preferably corn and soybean plants, yet more preferably corn plants.

Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently
10 are represented by various strains of *E. coli*; however, other microbial strains may also be used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang *et al.* (1977) *Nature* 198:1056),
15 the tryptophan (trp) promoter system (Goeddel *et al.* (1980) *Nucleic Acids Res.* 8:4057), and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake *et al.* (1981) *Nature* 292:128). Examples of selection markers for *E. coli* include, for example, genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction into the appropriate host cell. Bacterial
20 vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using *Bacillus sp.* (Palva *et al.* (1983) *Gene* 22:229-235 and Mosbach *et al.* (1983) *Nature* 302:543-545) and
25 *Salmonella*.

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, a polynucleotide of the present invention can be expressed in these eukaryotic systems. In some embodiments, transformed/transfected plant cells, as discussed *infra*, are employed as
30 expression systems for the production of the proteins of the instant invention.

Synthesis of heterologous nucleotide sequences in yeast is well known. Sherman, F., *et al.* (1982) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, is a well recognized work describing the various methods available to produce a protein in yeast. Two widely utilized yeasts for production of eukaryotic proteins are *Saccharomyces*

cerevisiae and *Pichia pastoris*. Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, an origin of replication, termination sequences and the like, as desired.

A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysate. The monitoring of the purification process can be accomplished by using Western blot techniques, radioimmunoassay or other standard immunoassay techniques.

The sequences of the present invention can also be ligated into various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. Illustrative cell cultures useful for the production of these peptides are mammalian cells. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (e.g. the CMV promoter, a HSV *tk* promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen *et al.* (1986) *Immunol. Rev.* 89:49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for instance, from the American Type Culture Collection.

Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (See, Schneider, *J. Embryol. Exp. Morphol.* 27:353-365 (1987)).

As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, *et al.* (1983) *J. Virol.* 45:773-781). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. See, Saveria-Campo, M., (1985) Bovine Papilloma Virus DNA a Eukaryotic Cloning

Vector in *DNA Cloning Vol. II A Practical Approach*, D.M. Glover, Ed., IRL Press, Arlington, Virginia pp. 213-238.

Animal and lower eukaryotic (e.g., yeast) host cells are competent or rendered competent for transfection by various means. There are several well-known methods of introducing DNA into animal cells. These include, but are not limited to: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing a DNA of interest, treatment of the recipient cells with liposomes containing a DNA of interest, DEAE dextrin, electroporation, biolistics, and micro-injection of a DNA of interest directly into the cells. The transfected cells are cultured by means well known in the art.

See, Kuchler, R.J. (1997) *Biochemical Methods in Cell Culture and Virology*, Dowden, Hutchinson and Ross, Inc.

It is recognized that antisense constructions complementary to at least a portion of the messenger RNA (mRNA) for the BTL sequences of the present invention can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Modifications of the antisense sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%, preferably 80%, more preferably 85% sequence identity to the corresponding antisensed sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene.

Generally, sequences of at least 50 nucleotides, 100 nucleotides, 200 nucleotides, or greater may be used.

The nucleotide sequences of the present invention may also be used in the sense orientation to suppress the expression of endogenous genes in plants. Methods for suppressing gene expression in plants using nucleotide sequences in the sense orientation are known in the art. The methods generally involve transforming plants with a DNA construct comprising a promoter that drives expression in a plant operably linked to at least a portion of a nucleotide sequence that corresponds to the transcript of the endogenous gene. Typically, such a nucleotide sequence has substantial sequence identity to the sequence of the transcript of the endogenous gene, preferably greater than about 65% sequence identity, more preferably greater than about 85% sequence identity, most preferably greater than about 95% sequence identity. See, U.S. Patent Nos. 5,283,184 and 5,034,323; herein incorporated by reference.

In some embodiments, the content and/or composition of polypeptides of the present invention in a plant, may be modulated by altering, *in vivo* or *in vitro*, the promoter of the

nucleotide sequence to up- or down-regulate expression. For instance, an isolated nucleic acid comprising a promoter sequence operably linked to a polynucleotide of the present invention is transfected into a plant cell. Subsequently, a plant cell comprising the promoter operably linked to the polynucleotide of the present invention is selected for by means
5 known to those of skill in the art such as, but not limited to, Southern blot, DNA sequencing, or PCR analysis using primers specific to the promoter and to the polynucleotide of the present invention and detecting amplicons produced therefrom. A plant or plant part altered or modified by the foregoing embodiments is grown under plant forming conditions for a time sufficient to modulate the concentration and/or composition
10 of polypeptides of the present invention in the plant. Plant forming conditions are well known in the art.

In general, the concentration or composition of the polypeptides of the present invention is increased or decreased by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% relative to a native control plant, plant part, or cell lacking the aforementioned
15 recombinant expression cassette. Accordingly, modulation in the present invention may occur during and/or subsequent to growth of the plant to the desired stage of development. Accordingly, modulating nucleic acid expression temporally and/or in particular tissues can be controlled by employing the appropriate promoter operably linked to a polynucleotide of the present invention in, for example, sense or antisense orientation as discussed in greater
20 detail, *supra*. Induction of the expression of a polynucleotide of the present invention can also be controlled by exogenous administration of an effective amount of inducing compound. Inducible promoters and inducing compounds, which activate expression from these promoters, are well known in the art. In preferred embodiments, the polypeptides of the present invention are modulated in monocots, particularly maize.

Molecular Markers

The present invention provides a method of genotyping a plant comprising a polynucleotide of the present invention. Optionally, the plant is a monocot, such as maize or sorghum. Genotyping provides a means of distinguishing homologs of a chromosome
30 pair and can be used to differentiate segregants in a plant population. Molecular marker methods can be used for phylogenetic studies, characterizing genetic relationships among crop varieties, identifying crosses or somatic hybrids, localizing chromosomal segments affecting monogenic traits, map based cloning, and the study of quantitative inheritance. See, e.g., *Plant Molecular Biology: A Laboratory Manual*, Chapter 7, Clark, Ed., Springer-

Verlag, Berlin (1997). For molecular marker methods, see generally, The DNA Revolution by Andrew H. Paterson 1996 (Chapter 2) in: Genome Mapping in plants (Ed., Andrew H. Paterson) by Academic Press/R.G. Lands Company, Austin, Texas, pp. 7-21.

5 The particular method of genotyping in the present invention may employ any number of molecular marker analytic techniques such as, but not limited to, restriction fragment length polymorphisms (RFLPs). RFLPs are the product of allelic differences between DNA restriction fragments resulting from nucleotide sequence variability. As is well known to those of skill in the art, RFLPs are typically detected by extraction of genomic DNA and digestion with a restriction enzyme. Generally, the resulting fragments
0 are separated according to size and hybridized with a probe; single copy probes are preferred. Restriction fragments from homologous chromosomes are revealed. Differences in fragment size among alleles represent an RFLP. Thus, the present invention further provides a means to follow segregation of a gene or nucleic acid of the present invention as well as chromosomal sequences genetically linked to these genes or nucleic acids using
5 such techniques as RFLP analysis. Linked chromosomal sequences are within 50 centiMorgans (cM), often within 40 or 30 cM, preferably within 20 or 10 cM, more preferably within 5, 3, 2, or 1 cM of a gene of the present invention.

In the present invention, the nucleic acid probes employed for molecular marker mapping of plant nuclear genomes hybridize, under selective hybridization conditions, to a
0 gene encoding a polynucleotide of the present invention. In preferred embodiments, the probes are selected from polynucleotides of the present invention. Typically, these probes are cDNA probes or restriction enzyme treated (*e.g.*, *PST I*) genomic clones. The length of the probes is typically at least 15 bases in length, more preferably at least 20, 25, 30, 35, 40, or 50 bases in length. Generally, however, the probes are less than about 1 kilobase in
5 length. Preferably, the probes are single copy probes that hybridize to a unique locus in a haploid chromosome complement. Some exemplary restriction enzymes employed in RFLP mapping are *EcoRI*, *EcoRV*, and *SstI*. As used herein the term "restriction enzyme" includes reference to a composition that recognizes and, alone or in conjunction with another composition, cleaves at a specific nucleotide sequence.

) The method of detecting an RFLP comprises the steps of (a) digesting genomic DNA of a plant with a restriction enzyme; (b) hybridizing a nucleic acid probe, under selective hybridization conditions, to a sequence of a polynucleotide of the present invention of the genomic DNA; (c) detecting therefrom a RFLP. Other methods of differentiating polymorphic (allelic) variants of polynucleotides of the present invention can

be had by utilizing molecular marker techniques well known to those of skill in the art including such techniques as: 1) single stranded conformation analysis (SSCA); 2) denaturing gradient gel electrophoresis (DGGE); 3) RNase protection assays; 4) allele-specific oligonucleotides (ASOs); 5) the use of proteins which recognize nucleotide mismatches, such as the *E. coli* mutS protein; and 6) allele-specific PCR. Other approaches based on the detection of mismatches between the two complementary DNA strands include clamped denaturing gel electrophoresis (CDGE); heteroduplex analysis (HA); and chemical mismatch cleavage (CMC). Thus, the present invention further provides a method of genotyping comprising the steps of contacting, under stringent hybridization conditions, a sample suspected of comprising a polynucleotide of the present invention with a nucleic acid probe. Generally, the sample is a plant sample, preferably, a sample suspected of comprising a maize polynucleotide of the present invention (*e.g.*, gene, mRNA). The nucleic acid probe selectively hybridizes, under stringent conditions, to a subsequence of a polynucleotide of the present invention comprising a polymorphic marker. Selective hybridization of the nucleic acid probe to the polymorphic marker nucleic acid sequence yields a hybridization complex. Detection of the hybridization complex indicates the presence of that polymorphic marker in the sample. In preferred embodiments, the nucleic acid probe comprises a polynucleotide of the present invention.

Formulations

Methods are provided for controlling plant pathogens comprising applying an anti-pathogenic amount of a polypeptide or composition of the invention to the environment of the pathogens. The proteins of the invention can be formulated with an acceptable carrier into a pesticidal composition(s) that is, for example, a suspension, a solution, an emulsion, a dusting powder, a dispersible granule, a wettable powder, an emulsifiable concentrate, an aerosol, an impregnated granule, an adjuvant, a coatable paste, and also encapsulations in, for example, polymer substances.

Such compositions disclosed above may be obtained by the addition of a surface-active agent, an inert carrier, a preservative, a humectant, a feeding stimulant, an attractant, an encapsulating agent, a binder, an emulsifier, a dye, a UV protectant, a buffer, a flow agent, fertilizers, micronutrient donors, or other preparations that influence plant growth. One or more agrochemicals including, but not limited to, herbicides, insecticides, fungicides, bacteriocides, nematocides, molluscicides, acaricides, plant growth regulators, harvest aids, and fertilizers, can be combined with carriers, surfactants, or adjuvants

customarily employed in the art of formulation or other components to facilitate product handling and application for particular target pests. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, *e.g.*, natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, binders, or fertilizers. The active ingredients of the present invention are normally applied in the form of compositions and can be applied to the crop area or plant to be treated, simultaneously or in succession, with other compounds. In some embodiments, methods of applying an active ingredient of the present invention or an agrochemical composition of the present invention (which contains at least one of the proteins of the present invention) are foliar application, seed coating, and soil application. The number of applications and the rate of application depend on the intensity of infestation by the corresponding pest or disease causing organism.

Suitable surface-active agents include, but are not limited to, anionic compounds such as a carboxylate of, for example, a metal; a carboxylate of a long chain fatty acid; an N-acylsarcosinate; mono or di-esters of phosphoric acid with fatty alcohol ethoxylates or salts of such esters; fatty alcohol sulfates such as sodium dodecyl sulfate, sodium octadecyl sulfate, or sodium cetyl sulfate; ethoxylated fatty alcohol sulfates; ethoxylated alkylphenol sulfates; lignin sulfonates; petroleum sulfonates; alkyl aryl sulfonates such as alkyl-benzene sulfonates or lower alkyl-naphthalene sulfonates, *e.g.*, butyl-naphthalene sulfonate; salts of sulfonated naphthalene-formaldehyde condensates; salts of sulfonated phenol-formaldehyde condensates; more complex sulfonates such as the amide sulfonates, *e.g.*, the sulfonated condensation product of oleic acid and N-methyl taurine; or the dialkyl sulfosuccinates, *e.g.*, the sodium sulfonate or dioctyl succinate. Non-ionic agents useful in the present invention include, but are not limited to, condensation products of fatty acid esters, fatty alcohols, fatty acid amides or fatty-alkyl- or -alkenyl-substituted phenols with ethylene oxide, fatty esters of polyhydric alcohol ethers, *e.g.*, sorbitan fatty acid esters, condensation products of such esters with ethylene oxide, *e.g.* polyoxyethylene sorbitar fatty acid esters, block copolymers of ethylene oxide and propylene oxide, acetylenic glycols such as 2, 4, 7, 9-tetraethyl-5-decyn-4, 7-diol, or ethoxylated acetylenic glycols. Examples of a cationic surface-active agent useful in the present invention include, for instance, an aliphatic mono-, di-, or polyamine such as an acetate, naphthenate, or oleate; or oxygen-containing amine such as an amine oxide of polyoxyethylene alkylamine; an amide-linked amine prepared by the condensation of a carboxylic acid with a di- or polyamine; or a quaternary ammonium salt.

Examples of inert materials useful in the present invention include, but are not limited to, inorganic minerals such as kaolin, phyllosilicates, carbonates, sulfates, phosphates, or botanical materials such as cork, powdered corncobs, peanut hulls, rice hulls, and walnut shells.

5 The compositions of the present invention can be in a form suitable for direct application or as a concentrate of a primary composition, which requires dilution with a suitable quantity of water or other diluent before application. The pesticidal concentration will vary depending upon the nature of the particular formulation, specifically, whether it is a concentrate or it is to be used directly. The composition contains 1 to 98% of a solid or
10 liquid inert carrier, and 0 to 50%, preferably 0.1 to 50% of a surfactant. These compositions will be administered at the labeled rate for a commercial product, preferably about 0.01 lb - 5.0 lb per acre when in dry form and at about 0.01 pts - 10 pts per acre when in liquid form.

In a further embodiment, the compositions, as well as the proteins of the present invention can be treated prior to formulation to prolong their activity when applied to the
15 environment of a target pest or disease causing organism as long as the pretreatment is not deleterious to the activity. Such treatment can be by chemical and/or physical means as long as the treatment does not deleteriously affect the properties of the composition(s). Examples of chemical reagents include, but are not limited to, halogenating agents; aldehydes such as formaldehyde and glutaraldehyde; anti-infectives, such as zephiran
20 chloride; alcohols, such as isopropanol and ethanol; and histological fixatives, such as Bouin's fixative and Helly's fixative (see, for example, Humason (1967) *Animal Tissue Techniques* (W.H. Freeman and Co.).

The compositions can be applied to the environment of a pest or disease causing organism by, for example, spraying, atomizing, dusting, scattering, coating or pouring,
5 introducing into or on the soil, introducing into irrigation water, by seed treatment, or dusting at the time when the pest or disease causing organism has begun to appear or before the appearance of pests or disease causing organisms as a protective measure. It is generally important to obtain good control of pests and disease causing organisms in the early stages of plant growth, as this is the time when the plant can be most severely
10 damaged. The compositions of the invention can conveniently contain one or more other insecticides or pesticides if this is thought to be necessary.

In a further embodiment, the BTL's of the invention can be used for coating surfaces to target microbes. In this manner, target microbes include human pathogens or microorganisms. Surfaces that might be coated with the defensive agents of the invention

include carpets and sterile medical facilities. Polymer bound polypeptides of the invention may be used to coat surfaces. Methods for incorporating compositions with anti-microbial properties into polymers are known in the art. See U.S. Patent No.5,847,047 herein incorporated by reference.

- 5 Another embodiment involves the use of the compositions of the invention in the treatment and preservation of textiles. Insect pests devalue and destroy textiles and fabrics including, but not limited to, carpets, draperies, clothing, blankets, and bandages. The compositions of the invention may be applied to finished textile products or may be expressed in plants yielding fibers that are incorporated into fabrics. Insect pests that attack
10 textiles include, but are not limited to, webbing clothes moths and carpet beetles.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

15 Example 1: Transformation and Regeneration of Transgenic Plants

- Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing a BTL nucleotide sequence operably linked to a ubiquitin promoter and the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene* 70:25-37), which confers resistance to the herbicide Bialaphos. Alternatively, the selectable marker gene is provided
20 on a separate plasmid. Transformation is performed as described below. Media recipes follow below.

Preparation of Target Tissue

- The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro
25 detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

30 Preparation of DNA

A plasmid vector comprising the BTL nucleotide sequence operably linked to a ubiquitin promoter is made. This plasmid DNA plus plasmid DNA containing a PAT selectable marker is precipitated onto 1.1 μm (average diameter) tungsten pellets using a CaCl_2 precipitation procedure as follows:

100 µl prepared tungsten particles in water

10 µl (1 µg) DNA in Tris EDTA buffer (1 µg total DNA)

100 µl 2.5 M CaCl₂

5 10 µl 0.1 M spermidine

Each reagent is added sequentially to the tungsten particle suspension, which is maintained on a multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes
10 are centrifuged briefly, liquid removed, washed with 500 ml of 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µls of 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl is spotted onto the center of each macrocarrier and allowed to dry for about 2 minutes before bombardment.

15

Particle Gun Treatment

The sample plates are bombarded at manufacturers recommended levels in a particle gun commercially available from BioRad Laboratories, Hercules, CA. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared
20 particles/DNA.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured
25 every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days
30 until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5 pot) containing potting soil and grown for 1 week in a growth chamber, the plants are subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for altered antimicrobial activity.

Bombardment and Culture Media

- Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416),
 5 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/l
 sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H₂O following
 adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I
 H₂O); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room
 temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416),
 10 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l
 sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H₂O following adjustment to pH
 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H₂O); and 0.85 mg/l
 silver nitrate and 3.0 mg/l Bialaphos (both added after sterilizing the medium and cooling to
 room temperature).
- 15 Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074),
 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCL, 0.10 g/l
 pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I H₂O) (Murashige
 and Skoog (1962) *Physiol. Plant.* 15:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l
 sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H₂O
 20 after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H₂O); and
 1.0 mg/l indole acetic acid and 3.0 mg/l bialaphos (added after sterilizing the medium and
 cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO
 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine
 HCL, 0.10 g/l pyridoxine HCL, and 0.40 g/l glycine brought to volume with polished D-I
 5 H₂O), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H₂O
 after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with
 polished D-I H₂O), sterilized and cooled to 60° C.

Example 2. *Agrobacterium*-mediated Transformation in Maize

- 0 For *Agrobacterium*-mediated transformation of maize with a BTL nucleotide sequence
 of the invention operably linked to a ubiquitin promoter, preferably the method of Zhao is
 employed (U.S. Patent No. 5,981,840 and WO98/32326, the contents of which are hereby
 incorporated by reference). Briefly, immature embryos are isolated from maize and the
 embryos are contacted with a suspension of *Agrobacterium*, where the bacteria are capable
 5 of transferring the DNA construct containing the BTL nucleotide sequence to at least one

cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably, the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional “resting” step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably, the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

Example 3. Soybean Embryo Transformation

Soybean embryos are bombarded with a plasmid containing the BTL nucleotide sequences operably linked to a ubiquitin promoter as follows. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface-sterilized, immature seeds of the soybean cultivar A2872, are cultured in the light or dark at 26°C on an appropriate agar medium for six to ten weeks. Somatic embryos producing secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos that multiplied as early, globular-staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures are maintained in 35 ml liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 ml of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein *et al.* (1987) *Nature* (London) 327:70-73, U.S. Patent No. 4,945,050). A DuPont Biolistic PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene that can be used to facilitate soybean transformation is a transgene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell *et al.* (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz *et al.* (1983) *Gene* 25:179-188), and the 3' region of the
5 nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The expression cassette comprising the BTL nucleotide sequence operably linked to the ubiquitin promoter can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 µl of a 60 mg/ml 1 µm gold particle suspension is added (in order): 5 µl DNA
10 (1 µg/µl), 20 µl spermidine (0.1 M), and 50 µl CaCl₂ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 µl 70% ethanol and resuspended in 40 µl of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five microliters of the DNA-coated gold particles are then
15 loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi, and the chamber is evacuated to
20 a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and again at eleven to twelve days post-bombardment with fresh media containing
25 50 mg/ml hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post-bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent
30 transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

Example 4. Sunflower Meristem Tissue Transformation

Sunflower meristem tissues are transformed with an expression cassette containing the BTL sequence operably linked to a ubiquitin promoter as follows (see also European Patent Number EP 0 486233, herein incorporated by reference, and Malone-Schoneberg *et al.* (1994) *Plant Science* 103:199-207). Mature sunflower seeds (*Helianthus annuus* L.) are dehulled using a single wheat-head thresher. Seeds are surface sterilized for 30 minutes in a 20% Clorox bleach solution with the addition of two drops of Tween 20 per 50 ml of solution. The seeds are rinsed twice with sterile distilled water.

Split embryonic axis explants are prepared by a modification of procedures described by Schrammeijer *et al.* (Schrammeijer *et al.* (1990) *Plant Cell Rep.* 9:55-60). Seeds are imbibed in distilled water for 60 minutes following the surface sterilization procedure. The cotyledons of each seed are then broken off, producing a clean fracture at the plane of the embryonic axis. Following excision of the root tip, the explants are bisected longitudinally between the primordial leaves. The two halves are placed, cut surface up, on GBA medium consisting of Murashige and Skoog mineral elements (Murashige *et al.* (1962) *Physiol. Plant.*, 15:473-497), Shepard's vitamin additions (Shepard (1980) in *Emergent Techniques for the Genetic Improvement of Crops* (University of Minnesota Press, St. Paul, Minnesota), 40 mg/l adenine sulfate, 30 g/l sucrose, 0.5 mg/l 6-benzyl-aminopurine (BTL), 0.25 mg/l indole-3-acetic acid (IAA), 0.1 mg/l gibberellic acid (GA₃), pH 5.6, and 8 g/l Phytagar.

The explants are subjected to microprojectile bombardment prior to *Agrobacterium* treatment (Bidney *et al.* (1992) *Plant Mol. Biol.* 18:301-313). Thirty to forty explants are placed in a circle at the center of a 60X20 mm plate. Approximately 4.7 mg of 1.8 mm tungsten microprojectiles are resuspended in 25 ml of sterile TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and a 1.5 ml aliquot is used per bombardment. Each plate is bombarded twice through a 150 mm nytex screen placed 2 cm above the samples in a PDS 1000[®] particle acceleration device available from BioRad Laboratories, Hercules, CA.

Disarmed *Agrobacterium tumefaciens* strain EHA105 is used in all transformation experiments. A binary plasmid vector comprising the expression cassette that contains the BTL gene operably linked to the ubiquitin promoter is introduced into *Agrobacterium* strain EHA105 via freeze-thawing as described by Holsters *et al.* (1978) *Mol. Gen. Genet.* 163:181-187. This plasmid further comprises a kanamycin selectable marker gene (i.e., *nptII*). Bacteria for plant transformation experiments are grown overnight (28°C and 100 RPM continuous agitation) in liquid YEP medium (10 gm/l yeast extract, 10 gm/l

Bactopeptone, and 5 gm/l NaCl, pH 7.0) with the appropriate antibiotics required for bacterial strain and binary plasmid maintenance. The suspension is used when it reaches an OD₆₀₀ of about 0.4 to 0.8. The *Agrobacterium* cells are pelleted and resuspended at a final OD₆₀₀ of 0.5 in an inoculation medium comprised of 12.5 mM MES pH 5.7, 1 gm/l

5 NH₄Cl, and 0.3 gm/l MgSO₄.

Freshly bombarded explants are placed in an *Agrobacterium* suspension, mixed, and left undisturbed for 30 minutes. The explants are then transferred to GBA medium and co-cultivated, cut surface down, at 26°C and 18-hour days. After three days of co-cultivation, the explants are transferred to 374B (GBA medium lacking growth regulators and a reduced
10 sucrose level of 1%) supplemented with 250 mg/l cefotaxime and 50 mg/l kanamycin sulfate. The explants are cultured for two to five weeks on this selection media and then transferred to fresh 374B medium lacking kanamycin for one to two weeks of continued development. Explants with differentiating, antibiotic-resistant areas of growth that have not produced shoots suitable for excision are transferred to GBA medium containing 250
15 mg/l cefotaxime for a second 3-day phytohormone treatment. Leaf samples from green, kanamycin-resistant shoots are assayed for the presence of NPTII by ELISA and for the presence of transgene expression by assaying for BTL-like activity.

NPTII-positive shoots are grafted to Pioneer[®] hybrid 6440 *in vitro*-grown sunflower seedling rootstock. Surface sterilized seeds are germinated in 48-0 medium (half-strength
20 Murashige and Skoog salts, 0.5% sucrose, 0.3% gelrite, pH 5.6) and grown under conditions described for explant culture. The upper portion of the seedling is removed, a 1 cm vertical slice is made in the hypocotyl, and the transformed shoot inserted into the cut. The entire area is wrapped with parafilm to secure the shoot. Grafted plants can be transferred to soil following one week of *in vitro* culture. Grafts in soil are maintained
25 under high humidity conditions followed by a slow acclimatization to the greenhouse environment. Transformed sectors of T₀ plants (parental generation) maturing in the greenhouse are identified by NPTII ELISA and/or by BTL activity analysis of leaf extracts while transgenic seeds harvested from NPTII-positive T₀ plants are identified by BTL activity analysis of small portions of dry seed cotyledon.

30 An alternative sunflower transformation protocol allows the recovery of transgenic progeny without the use of chemical selection pressure. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, then rinsed three times with distilled water. Sterilized seeds are imbibed in the dark at 26°C for 20 hours on filter paper moistened with

water. The cotyledons and root radical are removed, and the meristem explants are cultured on 374E (GBA medium consisting of MS salts, Shepard vitamins, 40 mg/l adenine sulfate, 3% sucrose, 0.5 mg/l 6-BTL, 0.25 mg/l IAA, 0.1 mg/l GA, and 0.8% Phytagar at pH 5.6) for 24 hours in the dark. The primary leaves are removed to expose the apical meristem, around 40 explants are placed with the apical dome facing upward in a 2 cm circle in the center of 374M (GBA medium with 1.2% Phytagar), and then cultured on the medium for 24 hours in the dark.

Approximately 18.8 mg of 1.8 μ m tungsten particles are resuspended in 150 μ l absolute ethanol. After sonication, 8 μ l of it is dropped on the center of the surface of macrocarrier. Each plate is bombarded twice with 650 psi rupture discs in the first shelf at 26 mm of Hg helium gun vacuum.

The plasmid of interest is introduced into *Agrobacterium tumefaciens* strain EHA105 via freeze thawing as described previously. The pellet of overnight-grown bacteria at 28°C in a liquid YEP medium (10 g/l yeast extract, 10 g/l Bactopeptone, and 5 g/l NaCl, pH 7.0) in the presence of 50 μ g/l kanamycin is resuspended in an inoculation medium (12.5 mM 2-(N-morpholino) ethanesulfonic acid, MES, 1 g/l NH_4Cl and 0.3 g/l MgSO_4 at pH 5.7) to reach a final concentration of 4.0 at OD 600. Particle-bombarded explants are transferred to GBA medium (374E), and a droplet of bacteria suspension is placed directly onto the top of the meristem. The explants are co-cultivated on the medium for 4 days, after which the explants are transferred to 374C medium (GBA with 1% sucrose and no BTL, IAA, GA3 and supplemented with 250 μ g/ml cefotaxime). The plantlets are cultured on the medium for about two weeks under 16-hour day and 26°C incubation conditions.

Explants (around 2 cm long) from two weeks of culture in 374C medium are screened for BTL activity using assays known in the art. After positive (*i.e.*, for BTL expression) explants are identified, those shoots that fail to exhibit BTL activity are discarded, and every positive explant is subdivided into nodal explants. One nodal explant contains at least one potential node. The nodal segments are cultured on GBA medium for three to four days to promote the formation of auxiliary buds from each node. Then they are transferred to 374C medium and allowed to develop for an additional four weeks. Developing buds are separated and cultured for an additional four weeks on 374C medium. Pooled leaf samples from each newly recovered shoot are screened again by the appropriate protein activity assay. At this time, the positive shoots recovered from a single node will

generally have been enriched in the transgenic sector detected in the initial assay prior to nodal culture.

Recovered shoots positive for BTL expression are grafted to Pioneer® hybrid 6440 *in vitro*-grown sunflower seedling rootstock. The rootstocks are prepared in the following manner. Seeds are dehulled and surface-sterilized for 20 minutes in a 20% Clorox bleach solution with the addition of two to three drops of Tween 20 per 100 ml of solution, and are rinsed three times with distilled water. The sterilized seeds are germinated on the filter moistened with water for three days, then they are transferred into 48 medium (half-strength MS salt, 0.5% sucrose, 0.3% gelrite pH 5.0) and grown at 26°C in the dark for three days, then incubated at 16-hour-day culture conditions. The upper portion of selected seedling is removed, a vertical slice is made in each hypocotyl, and a transformed shoot is inserted into a V-cut. The cut area is wrapped with parafilm. After one week of culture on the medium, grafted plants are transferred to soil. In the first two weeks, they are maintained under high humidity conditions to acclimatize to a greenhouse environment.

All publications, patents, and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications, patents, and patent applications are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. An isolated polypeptide selected from the group consisting of:
 - (a) a polypeptide comprising an amino acid sequence set forth in
5 SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, or 30;
 - (b) a polypeptide characterized by at least 60% sequence identity to a sequence of a), wherein said polypeptide retains antimicrobial activity;
 - (c) a polypeptide encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence comprising a sequence set forth in
10 SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29; and,
 - (d) a polypeptide comprising at least 20 consecutive amino acids of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, or 30, wherein said polypeptide has antimicrobial activity.
- 15 2. An isolated nucleotide sequence selected from the group consisting of:
 - (a) a nucleotide sequence comprising a sequence set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29;
 - (b) a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24,
20 26, 28, or 30;
 - (c) a nucleotide sequence comprising at least 100 contiguous nucleotides of a sequence of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity;
 - (d) a nucleotide sequence having at least 60% sequence identity
25 with the nucleotide sequences of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity;
 - (e) a nucleotide sequence comprising an antisense sequence corresponding to a sequence of a) or b); and
 - (f) a nucleotide sequence that hybridizes under stringent
30 conditions to the nucleotide sequences of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity.

3. A DNA construct comprising a nucleotide sequence of claim 2, wherein said nucleotide sequence is operably linked to a promoter that drives expression in a plant cell.
- 5 4. An expression vector comprising the DNA construct of claim 3.
5. A host cell having stably incorporated into its genome at least one nucleotide sequence, wherein said nucleotide sequence is operably linked to a heterologous promoter that drives expression in the host cell, wherein said nucleotide
10 sequence is selected from the group consisting of:
- (a) a nucleotide sequence comprising a sequence set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29;
 - (b) a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24,
15 26, 28, or 30;
 - (c) a nucleotide sequence comprising at least 16 contiguous nucleotides of a sequence of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity;
 - (d) a nucleotide sequence having at least 60% identity with the
20 nucleotide sequences of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity;
 - (e) a nucleotide sequence comprising an antisense sequence corresponding to a sequence of a) or b); and
 - (f) a nucleotide sequence that hybridizes under stringent
25 conditions to the nucleotide sequences of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity.
6. The host cell of claim 5, wherein said cell is a plant cell.
- 30 7. A plant having stably incorporated into its genome at least one nucleotide sequence, said nucleotide sequence operably linked to a heterologous promoter that drives expression in a plant cell, wherein said nucleotide sequence is selected from the group consisting of:

- 51 -

- (a) a nucleotide sequence comprising a sequence set forth in SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29;
- (b) a nucleotide sequence encoding a polypeptide comprising an amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, or 30;
- (c) a nucleotide sequence comprising at least 100 contiguous nucleotides of a sequence of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity;
- (d) a nucleotide sequence having at least 60% sequence identity with the nucleotide sequences of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity;
- (e) a nucleotide sequence comprising an antisense sequence corresponding to a sequence of a) or b); and
- (f) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequences of a), b), or c), wherein said sequence encodes a polypeptide having antimicrobial activity.
8. The plant of claim 7, wherein said promoter is a constitutive promoter.
9. The plant of claim 7, wherein said promoter is a tissue-preferred promoter.
10. The plant of claim 7, wherein said promoter is an inducible promoter.
11. The plant of claim 10, wherein said promoter is a pathogen-inducible promoter.
12. The plant of claim 7, wherein said plant is a monocot.
13. The plant of claim 12, wherein said monocot is selected from the group consisting of maize, wheat, rice, barley, sorghum, and rye.
14. The plant of claim 7, wherein said plant is a dicot.

15. The transformed seed of the plant of claim 7.

16. A method for modulating development in a plant, said method
5 comprising stably incorporating into the genome of said plant at least one nucleotide
sequence operably linked to a promoter wherein said nucleotide sequence is selected
from the group consisting of:

(a) a nucleotide sequence comprising a sequence set forth in SEQ
ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29;

10 (b) a nucleotide sequence encoding a polypeptide comprising an
amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24,
26, 28, or 30;

(c) a nucleotide sequence comprising at least 100 contiguous
nucleotides of a sequence of a) or b), wherein said sequence encodes a polypeptide
15 having antimicrobial activity;

(d) a nucleotide sequence having at least 60% sequence identity
with the nucleotide sequences of a) or b), wherein said sequence encodes a
polypeptide having antimicrobial activity;

(e) a nucleotide sequence comprising an antisense sequence
20 corresponding to a sequence of a) or b); and

(f) a nucleotide sequence that hybridizes under stringent
conditions to the nucleotide sequences of a) or b), wherein said sequence encodes a
polypeptide having antimicrobial activity.

25 17. A method for modulating the defense response in a plant, said method
comprising stably incorporating into the genome of said plant at least one nucleotide
sequence operably linked to a heterologous promoter that drives expression in a plant
cell, wherein said nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence comprising a sequence set forth in SEQ
30 ID NO:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, or 29;

(b) a nucleotide sequence encoding a polypeptide comprising an
amino acid sequence set forth in SEQ ID NO:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24,
26, 28, or 30;

- 53 -

- (c) a nucleotide sequence comprising at least 100 contiguous nucleotides of a sequence of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity;
- (d) a nucleotide sequence having at least 60% sequence identity with the nucleotide sequences of a) or b), wherein said sequence encodes a polypeptide having antimicrobial activity;
- (e) a nucleotide sequence comprising an antisense sequence corresponding to a sequence of a) or b); and
- (f) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequences of a), b), or c), wherein said sequence encodes a polypeptide having antimicrobial activity.
18. The method of claim 17, wherein said promoter is a constitutive promoter.
19. The method of claim 17, wherein said promoter is a pathogen inducible promoter.
20. The method of claim 17, wherein said plant is a dicot.
21. The method of claim 17, wherein said plant is a monocot.

SEQUENCE LISTING

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      35           40           45
Phe Ser Pro Tyr Ser Glu Asp His Cys Asp Arg Gln Asn Cys Arg Phe
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gac cgc cag aac tgc cgc ttc gtc tgc atg tcg cac ggt tac agt gac 304
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ggg ggc tgg tgt gac gag agg gag gtg cgc aaa atg tgc tgc tgc tac 352
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His *
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           35           40           45
Ser Pro Tyr Ser Glu Asp His Cys Asp Arg Gln Asn Cys Arg Phe Val
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Lys Gly Gly Val Val Pro Arg Leu Arg Pro Pro Phe Cys Phe Pro Tyr
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atg gca ctc aac ggc ggt tgg aga aag acc ttc gtg tcc atc ctg acg      48

```

```

Met Ala Leu Asn Gly Gly Trp Arg Lys Thr Phe Val Ser Ile Leu Thr
 1           5           10           15
aca tgc ttt ctt gtg gtg gtt gta att gtt tcc cta tcc tgc gaa gca 96
Thr Cys Phe Leu Val Val Val Val Ile Val Ser Leu Ser Cys Glu Ala
           20           25           30
aaa ggc ggc gtc gtc ccc agg ttg cgg cca cca ttc tgc ttt cca tac 144
Lys Gly Gly Val Val Pro Arg Leu Arg Pro Pro Phe Cys Phe Pro Tyr
           35           40           45
gat cgt gaa tac tgc acg cca ttt cac tgc ggc aaa gtt tgc caa gag 192
Asp Arg Glu Tyr Cys Thr Pro Phe His Cys Gly Lys Val Cys Gln Glu
           50           55           60
tac aac ttc cct gct aag aat ggc ggc tac tgc gat aaa cgt ggt gac 240
Tyr Asn Phe Pro Ala Lys Asn Gly Gly Tyr Cys Asp Lys Arg Gly Asp
           65           70           75           80
cca tgg aag tgc tgc tgt cca tat tga 267
Pro Trp Lys Cys Cys Cys Pro Tyr *
           85

```

<210> 12
 <211> 89
 <212> PRT
 <213> Zea mays

```

<400> 12
Met Met Ala Leu Asn Gly Gly Trp Arg Lys Thr Phe Val Ser Ile Leu
 1           5           10           15
Thr Thr Cys Phe Leu Val Val Val Val Ile Val Ser Leu Ser Cys Glu
           20           25           30
Ala Lys Gly Gly Val Val Pro Arg Leu Arg Pro Pro Phe Cys Phe Pro
           35           40           45
Tyr Asp Arg Glu Tyr Cys Thr Pro Phe His Cys Gly Lys Val Cys Gln
           50           55           60
Glu Tyr Asn Phe Pro Ala Lys Asn Gly Gly Tyr Cys Asp Lys Arg Gly
           65           70           75           80
Asp Pro Trp Lys Cys Cys Cys Pro Tyr
           85

```

<210> 13
 <211> 402
 <212> DNA
 <213> Zea mays

<220>
 <221> CDS
 <222> (62)...(343)
 <223> BTL2-6

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<400> 13
cggacgcgtg ggttcaataa taacacattg ttagtattag accctagcta gttcatcacc 60
c atg gcg aag ttc ttc aac tac acc atc gtc caa gga ctc ttg atg ctt 109
Met Ala Lys Phe Phe Asn Tyr Thr Ile Val Gln Gly Leu Leu Met Leu
 1           5           10           15
tct atg gta ctt ctg gca tca tgc gtc att cat gca cac ata ata agt 157
Ser Met Val Leu Leu Ala Ser Cys Val Ile His Ala His Ile Ile Ser

```

```

                20                25                30
ggg gaa act gaa gag gtt agc aac att ggg agc ccg aca gtg atg gtc 205
Gly Glu Thr Glu Glu Val Ser Asn Ile Gly Ser Pro Thr Val Met Val
          35                40                45

acg atg ggg gca aac cga aag ata att gga gat aat aaa aat tta ttg 253
Thr Met Gly Ala Asn Arg Lys Ile Ile Gly Asp Asn Lys Asn Leu Leu
          50                55                60

tgc tat cta aag gct cta gaa tat tgc tgt gaa agg acc aaa cag tgc 301
Cys Tyr Leu Lys Ala Leu Glu Tyr Cys Cys Glu Arg Thr Lys Gln Cys
          65                70                75                80

tat gat gac ata aag aag tgc ttg gag cac tgc cat ggt tga 343
Tyr Asp Asp Ile Lys Lys Cys Leu Glu His Cys His Gly *
          85                90

aaggtgtaat aaaaggatat gatttctttg gatctaataaa aaaaaaaaaa aaaaaaaaaa 402

<210> 14
<211> 93
<212> PRT
<213> Zea mays

<400> 14
Met Ala Lys Phe Phe Asn Tyr Thr Ile Val Gln Gly Leu Leu Met Leu
1          5          10          15
Ser Met Val Leu Leu Ala Ser Cys Val Ile His Ala His Ile Ile Ser
          20          25          30
Gly Glu Thr Glu Glu Val Ser Asn Ile Gly Ser Pro Thr Val Met Val
          35          40          45
Thr Met Gly Ala Asn Arg Lys Ile Ile Gly Asp Asn Lys Asn Leu Leu
          50          55          60
Cys Tyr Leu Lys Ala Leu Glu Tyr Cys Cys Glu Arg Thr Lys Gln Cys
          65          70          75          80
Tyr Asp Asp Ile Lys Lys Cys Leu Glu His Cys His Gly
          85          90

<210> 15
<211> 282
<212> DNA
<213> Zea mays

<220>
<221> CDS
<222> (1)...(282)

<400> 15
atg gcg aag ttc ttc aac tac acc atc gtc caa gga ctc ttg atg ctt 48
Met Ala Lys Phe Phe Asn Tyr Thr Ile Val Gln Gly Leu Leu Met Leu
1          5          10          15

tct atg gta ctt ctg gca tca tgc gtc att cat gca cac ata ata agt 96
Ser Met Val Leu Leu Ala Ser Cys Val Ile His Ala His Ile Ile Ser
          20          25          30

ggg gaa act gaa gag gtt agc aac att ggg agc ccg aca gtg atg gtc 144
Gly Glu Thr Glu Glu Val Ser Asn Ile Gly Ser Pro Thr Val Met Val
          35          40          45

```

acg atg ggg gca aac cga aag ata att gga gat aat aaa aat tta ttg 192
 Thr Met Gly Ala Asn Arg Lys Ile Ile Gly Asp Asn Lys Asn Leu Leu
 50 55 60

tgc tat cta aag gct cta gaa tat tgc tgt gaa agg acc aaa cag tgc 240
 Cys Tyr Leu Lys Ala Leu Glu Tyr Cys Cys Glu Arg Thr Lys Gln Cys
 65 70 75 80

tat gat gac ata aag aag tgc ttg gag cac tgc cat ggt tga 282
 Tyr Asp Asp Ile Lys Lys Cys Leu Glu His Cys His Gly *
 85 90

<210> 16

<211> 93

<212> PRT

<213> Zea mays

<400> 16

Met Ala Lys Phe Phe Asn Tyr Thr Ile Val Gln Gly Leu Leu Met Leu
 1 5 10 15
 Ser Met Val Leu Leu Ala Ser Cys Val Ile His Ala His Ile Ile Ser
 20 25 30
 Gly Glu Thr Glu Glu Val Ser Asn Ile Gly Ser Pro Thr Val Met Val
 35 40 45
 Thr Met Gly Ala Asn Arg Lys Ile Ile Gly Asp Asn Lys Asn Leu Leu
 50 55 60
 Cys Tyr Leu Lys Ala Leu Glu Tyr Cys Cys Glu Arg Thr Lys Gln Cys
 65 70 75 80
 Tyr Asp Asp Ile Lys Lys Cys Leu Glu His Cys His Gly
 85 90

<210> 17

<211> 439

<212> DNA

<213> Zea mays

<220>

<221> CDS

<222> (64)...(366)

<223> BTL2-7

<400> 17

ggagacactc taaactcaaa caaatacata tattgtcggc attagctctt acccttttca 60
 ccc atg atg acg aag tgc cag aag cgc gcg agc atc caa gga ctt tgg 108
 Met Met Thr Lys Cys Gln Lys Arg Ala Ser Ile Gln Gly Leu Trp
 1 5 10 15
 ctc ctt tcc atg gtt ctt cta gcg tcg tcc tct ctt gtc tgt gca agc 156
 Leu Leu Ser Met Val Leu Leu Ala Ser Ser Ser Leu Val Cys Ala Ser
 20 25 30
 atg gcg gta gat ggg caa acc aaa gag gac atc aac gca acg agt gtg 204
 Met Ala Val Asp Gly Gln Thr Lys Glu Asp Ile Asn Ala Thr Ser Val
 35 40 45
 acg agc atg aac atg acg agg tcg tcg tcg gcg agc tac aac atg act 252
 Thr Ser Met Asn Met Thr Arg Ser Ser Ser Ala Ser Tyr Asn Met Thr
 50 55 60

ggc ggc ggc gga gaa ctt aac aga ggt ccg tgc gtc gtg aga tct gga 300
 Gly Gly Gly Gly Glu Leu Asn Arg Gly Pro Cys Val Val Arg Ser Gly
 65 70 75

ttc tac tgg tgc cag aac att ggc tat ccg acc atg tcg gag tgc ttg 348
 Phe Tyr Trp Cys Gln Asn Ile Gly Tyr Pro Thr Met Ser Glu Cys Leu
 80 85 90 95

aag aac tgc gaa tcg taa agcactggag tatagaaaag gaataaagtg 396
 Lys Asn Cys Glu Ser *
 100

gtctctgcta gcttaatttc tccagtccaa aaaaaaaaaa aaa 439

<210> 18
 <211> 100
 <212> PRT
 <213> Zea mays

<400> 18
 Met Met Thr Lys Cys Gln Lys Arg Ala Ser Ile Gln Gly Leu Trp Leu
 1 5 10 15
 Leu Ser Met Val Leu Leu Ala Ser Ser Ser Leu Val Cys Ala Ser Met
 20 25 30
 Ala Val Asp Gly Gln Thr Lys Glu Asp Ile Asn Ala Thr Ser Val Thr
 35 40 45
 Ser Met Asn Met Thr Arg Ser Ser Ser Ala Ser Tyr Asn Met Thr Gly
 50 55 60
 Gly Gly Gly Glu Leu Asn Arg Gly Pro Cys Val Val Arg Ser Gly Phe
 65 70 75 80
 Tyr Trp Cys Gln Asn Ile Gly Tyr Pro Thr Met Ser Glu Cys Leu Lys
 85 90 95
 Asn Cys Glu Ser
 100

<210> 19
 <211> 303
 <212> DNA
 <213> Zea mays

<220>
 <221> CDS
 <222> (1)...(303)

<400> 19
 atg atg acg aag tgc cag aag cgc gcg agc atc caa gga ctt tgg ctc 48
 Met Met Thr Lys Cys Gln Lys Arg Ala Ser Ile Gln Gly Leu Trp Leu
 1 5 10 15
 ctt tcc atg gtt ctt cta gcg tcg tcc tct ctt gtc tgt gca agc atg 96
 Leu Ser Met Val Leu Leu Ala Ser Ser Ser Leu Val Cys Ala Ser Met
 20 25 30
 gcg gta gat ggg caa acc aaa gag gac atc aac gca acg agt gtg acg 144
 Ala Val Asp Gly Gln Thr Lys Glu Asp Ile Asn Ala Thr Ser Val Thr
 35 40 45
 agc atg aac atg acg agg tcg tcg tcg gcg agc tac aac atg act ggc 192
 Ser Met Asn Met Thr Arg Ser Ser Ser Ala Ser Tyr Asn Met Thr Gly
 50 55 60

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ggc ggc gga gaa ctt aac aga ggt ccg tgc gtc gtg aga tct gga ttc 240
Gly Gly Gly Glu Leu Asn Arg Gly Pro Cys Val Val Arg Ser Gly Phe
65 70 75 80

tac tgg tgc cag aac att ggc tat ccg acc atg tcg gag tgc ttg aag 288
Tyr Trp Cys Gln Asn Ile Gly Tyr Pro Thr Met Ser Glu Cys Leu Lys
85 90 95

aac tgc gaa tcg taa 303
Asn Cys Glu Ser *
100

```

<210> 20
 <211> 100
 <212> PRT
 <213> Zea mays

```

<400> 20
Met Met Thr Lys Cys Gln Lys Arg Ala Ser Ile Gln Gly Leu Trp Leu
1 5 10 15
Leu Ser Met Val Leu Leu Ala Ser Ser Ser Leu Val Cys Ala Ser Met
20 25 30
Ala Val Asp Gly Gln Thr Lys Glu Asp Ile Asn Ala Thr Ser Val Thr
35 40 45
Ser Met Asn Met Thr Arg Ser Ser Ser Ala Ser Tyr Asn Met Thr Gly
50 55 60
Gly Gly Gly Glu Leu Asn Arg Gly Pro Cys Val Val Arg Ser Gly Phe
65 70 75 80
Tyr Trp Cys Gln Asn Ile Gly Tyr Pro Thr Met Ser Glu Cys Leu Lys
85 90 95
Asn Cys Glu Ser
100

```

<210> 21
 <211> 466
 <212> DNA
 <213> Zea mays

<220>
 <221> CDS
 <222> (26)...(301)
 <223> BTL2-8

```

<400> 21
ggttagctct tatcggtcat ccatc atg gcg aga tgc ctc aag tcc tgc agt 52
Met Ala Arg Cys Leu Lys Ser Cys Ser
1 5

gta cat gga ctc tgg ctg ctc tcc atg atc ctt ctt gca tcg tgt gtc 100
Val His Gly Leu Trp Leu Leu Ser Met Ile Leu Leu Ala Ser Cys Val
10 15 20 25

gtt cat gct cac att att aat ggg cgg caa agc aac acc ggg agc ctc 148
Val His Ala His Ile Ile Asn Gly Arg Gln Ser Asn Thr Gly Ser Leu
30 35 40

act atg acc acg acg ggg gaa gca agc atg ata att gga gat gag aaa 196
Thr Met Thr Thr Thr Gly Glu Ala Ser Met Ile Ile Gly Asp Glu Lys
45 50 55

```

gat gca att tgc tat ata aaa gcc gca ttg tat tgc tgc aaa agg act 244
 Asp Ala Ile Cys Tyr Ile Lys Ala Ala Leu Tyr Cys Cys Lys Arg Thr
 60 65 70

ata cag tgc tat cag gac ata gcg caa tgc ttg agg aac tgc cgt aaa 292
 Ile Gln Cys Tyr Gln Asp Ile Ala Gln Cys Leu Arg Asn Cys Arg Lys
 75 80 85

aat gtc taa caagaggcta tgctttctcc aactcgtaga tggagatcat 341
 Asn Val *
 90

aaagatgtta cgttagacct aatcatgcat agaccggagg agaaatataa gctaattagc 401
 ccattccataa ttaataactt cgtattgggt caaaagctat aattgtcatt aaaaaaaaaa 461
 aaaaa 466

<210> 22
 <211> 91
 <212> PRT
 <213> Zea mays

<400> 22
 Met Ala Arg Cys Leu Lys Ser Cys Ser Val His Gly Leu Trp Leu Leu
 1 5 10 15
 Ser Met Ile Leu Leu Ala Ser Cys Val Val His Ala His Ile Ile Asn
 20 25 30
 Gly Arg Gln Ser Asn Thr Gly Ser Leu Thr Met Thr Thr Thr Gly Glu
 35 40 45
 Ala Ser Met Ile Ile Gly Asp Glu Lys Asp Ala Ile Cys Tyr Ile Lys
 50 55 60
 Ala Ala Leu Tyr Cys Cys Lys Arg Thr Ile Gln Cys Tyr Gln Asp Ile
 65 70 75 80
 Ala Gln Cys Leu Arg Asn Cys Arg Lys Asn Val
 85 90

<210> 23
 <211> 276
 <212> DNA
 <213> Zea mays

<220>
 <221> CDS
 <222> (1)...(276)

<400> 23
 atg gcg aga tgc ctc aag tcc tgc agt gta cat gga ctc tgg ctg ctc 48
 Met Ala Arg Cys Leu Lys Ser Cys Ser Val His Gly Leu Trp Leu Leu
 1 5 10 15

tcc atg atc ctt ctt gca tcg tgt gtc gtt cat gct cac att att aat 96
 Ser Met Ile Leu Leu Ala Ser Cys Val Val His Ala His Ile Ile Asn
 20 25 30

ggg cgg caa agc aac acc ggg agc ctc act atg acc acg acg ggg gaa 144
 Gly Arg Gln Ser Asn Thr Gly Ser Leu Thr Met Thr Thr Thr Gly Glu
 35 40 45

gca agc atg ata att gga gat gag aaa gat gca att tgc tat ata aaa 192
 Ala Ser Met Ile Ile Gly Asp Glu Lys Asp Ala Ile Cys Tyr Ile Lys
 50 55 60

gcc gca ttg tat tgc tgc aaa agg act ata cag tgc tat cag gac ata 240
 Ala Ala Leu Tyr Cys Cys Lys Arg Thr Ile Gln Cys Tyr Gln Asp Ile
 65 70 75 80

gcg caa tgc ttg agg aac tgc cgt aaa aat gtc taa 276
 Ala Gln Cys Leu Arg Asn Cys Arg Lys Asn Val *
 85 90

<210> 24
 <211> 91
 <212> PRT
 <213> Zea mays

<400> 24
 Met Ala Arg Cys Leu Lys Ser Cys Ser Val His Gly Leu Trp Leu Leu
 1 5 10 15
 Ser Met Ile Leu Leu Ala Ser Cys Val Val His Ala His Ile Ile Asn
 20 25 30
 Gly Arg Gln Ser Asn Thr Gly Ser Leu Thr Met Thr Thr Thr Gly Glu
 35 40 45
 Ala Ser Met Ile Ile Gly Asp Glu Lys Asp Ala Ile Cys Tyr Ile Lys
 50 55 60
 Ala Ala Leu Tyr Cys Cys Lys Arg Thr Ile Gln Cys Tyr Gln Asp Ile
 65 70 75 80
 Ala Gln Cys Leu Arg Asn Cys Arg Lys Asn Val
 85 90

<210> 25
 <211> 539
 <212> DNA
 <213> Zea mays

<220>
 <221> CDS
 <222> (54) ... (380)
 <223> BTL4-2

<400> 25
 cggacgcgtg ggtccacaag cacctgctat agttgtgtgg tgcaaacacc aaa atg 56
 Met
 1
 ttg cct gcc aaa gtg tca ttt gtt ttc ctg ata gtg tac tgc gca gta 104
 Leu Pro Ala Lys Val Ser Phe Val Phe Leu Ile Val Tyr Cys Ala Val
 5 10 15
 aca ttt tcg cta ggt cag ata gct gtt ggc gag gca tgc aca gtg gat 152
 Thr Phe Ser Leu Gly Gln Ile Ala Val Gly Glu Ala Cys Thr Val Asp
 20 25 30
 cag agg gac aag atc aca acg gac tgc aga gag ttc atc aag ctg aaa 200
 Gln Arg Asp Lys Ile Thr Thr Asp Cys Arg Glu Phe Ile Lys Leu Lys
 35 40 45
 ggc ccc gtc aca gcc ccg tcg tac acc gac gac tgc tgc gtc gcc ata 248
 Gly Pro Val Thr Ala Pro Ser Tyr Thr Asp Asp Cys Cys Val Ala Ile
 50 55 60 65
 aga gcg gtg ccc aat ctc gac atg gag tgc atc att cgc ctg ctc tcc 296
 Arg Ala Val Pro Asn Leu Asp Met Glu Cys Ile Ile Arg Leu Leu Ser

```

              70              75              80
aat aaa cag aag aag aag tac gac gtg gac aag atc cgg cgg ctc ggc 344
Asn Lys Gln Lys Lys Lys Tyr Asp Val Asp Lys Ile Arg Arg Leu Gly
              85              90              95

agc gtc ctc tgc aat cca cat ccg gtg atg acg taa aatgggggtgt 390
Ser Val Leu Cys Asn Pro His Pro Val Met Thr *
              100              105

ggcagtagtt tctgatggag tgcatcgcg tcaacaatatg gagcttgaac tatacaaacc 450
ctactgataa tgtgtcacca tttcatatgt atatgtactc cattcattaa caaaaaaaag 510
tgtcttatat atcaaaaaaa aaaaaaaa 539

```

<210> 26
 <211> 108
 <212> PRT
 <213> Zea mays

```

<400> 26
Met Leu Pro Ala Lys Val Ser Phe Val Phe Leu Ile Val Tyr Cys Ala
 1              5              10              15
Val Thr Phe Ser Leu Gly Gln Ile Ala Val Gly Glu Ala Cys Thr Val
              20              25              30
Asp Gln Arg Asp Lys Ile Thr Thr Asp Cys Arg Glu Phe Ile Lys Leu
              35              40              45
Lys Gly Pro Val Thr Ala Pro Ser Tyr Thr Asp Asp Cys Cys Val Ala
 50              55              60
Ile Arg Ala Val Pro Asn Leu Asp Met Glu Cys Ile Ile Arg Leu Leu
65              70              75              80
Ser Asn Lys Gln Lys Lys Lys Tyr Asp Val Asp Lys Ile Arg Arg Leu
              85              90              95
Gly Ser Val Leu Cys Asn Pro His Pro Val Met Thr
              100              105

```

<210> 27
 <211> 327
 <212> DNA
 <213> Zea mays

<220>
 <221> CDS
 <222> (1)...(327)

```

<400> 27
atg ttg cct gcc aaa gtg tca ttt gtt ttc ctg ata gtg tac tgc gca 48
Met Leu Pro Ala Lys Val Ser Phe Val Phe Leu Ile Val Tyr Cys Ala
 1              5              10              15

gta aca ttt tcg cta ggt cag ata gct gtt ggc gag gca tgc aca gtg 96
Val Thr Phe Ser Leu Gly Gln Ile Ala Val Gly Glu Ala Cys Thr Val
              20              25              30

gat cag agg gac aag atc aca acg gac tgc aga gag ttc atc aag ctg 144
Asp Gln Arg Asp Lys Ile Thr Thr Asp Cys Arg Glu Phe Ile Lys Leu
              35              40              45

aaa ggc ccc gtc aca gcc ccg tcg tac acc gac gac tgc tgc gtc gcc 192
Lys Gly Pro Val Thr Ala Pro Ser Tyr Thr Asp Asp Cys Cys Val Ala
 50              55              60

```

ata aga gcg gtg ccc aat ctc gac atg gag tgc atc att cgc ctg ctc 240
 Ile Arg Ala Val Pro Asn Leu Asp Met Glu Cys Ile Ile Arg Leu Leu
 65 70 75 80

tcc aat aaa cag aag aag aag tac gac gtg gac aag atc cgg cgg ctc 288
 Ser Asn Lys Gln Lys Lys Lys Tyr Asp Val Asp Lys Ile Arg Arg Leu
 85 90 95

ggc agc gtc ctc tgc aat cca cat ccg gtg atg acg taa 327
 Gly Ser Val Leu Cys Asn Pro His Pro Val Met Thr *
 100 105

<210> 28
 <211> 108
 <212> PRT
 <213> Zea mays

<400> 28
 Met Leu Pro Ala Lys Val Ser Phe Val Phe Leu Ile Val Tyr Cys Ala
 1 5 10 15
 Val Thr Phe Ser Leu Gly Gln Ile Ala Val Gly Glu Ala Cys Thr Val
 20 25 30
 Asp Gln Arg Asp Lys Ile Thr Thr Asp Cys Arg Glu Phe Ile Lys Leu
 35 40 45
 Lys Gly Pro Val Thr Ala Pro Ser Tyr Thr Asp Asp Cys Cys Val Ala
 50 55 60
 Ile Arg Ala Val Pro Asn Leu Asp Met Glu Cys Ile Ile Arg Leu Leu
 65 70 75 80
 Ser Asn Lys Gln Lys Lys Tyr Asp Val Asp Lys Ile Arg Arg Leu
 85 90 95
 Gly Ser Val Leu Cys Asn Pro His Pro Val Met Thr
 100 105

<210> 29
 <211> 364
 <212> DNA
 <213> Zea mays

<220>
 <221> misc_feature
 <222> (1)...(364)
 <223> BTL4-5

<221> misc_feature
 <222> (1)...(364)
 <223> n = A,T,C or G

<400> 29
 ctgcactgcg ataataagag atcgatgttt ccgtctaagc aagtatccgn tctcctagtc 60
 cttgtagtggt tttcttctcc cagtcncatc ctccctntga gtggtaagcc atcagtgagc 120
 aaagaacaga angacaagat cctagaggaa tgcgaccggt tcatccgtct cggctacccc 180
 atctatgttg tgtcccggca tagcccctgc tgcgatgctg gtagggcggt tgagaacaga 240
 gacattgact atgttgctct cctgctcaca aangacgaga atgacaagtn cagcncctct 300
 aagatccctgg ngnttcatgg cctctgtgaa cttcctcttg ttctcttcat gacagcagca 360
 aggc 364

<210> 30
 <211> 120
 <212> PRT
 <213> Zea mays

<220>

<221> VARIANT

<222> (1)...(120)

<223> Xaa = Any Amino Acid

<400> 30

Leu	His	Cys	Asp	Asn	Lys	Arg	Ser	Met	Phe	Pro	Ser	Lys	Gln	Val	Ser
1				5					10				15		
Xaa	Leu	Leu	Val	Leu	Val	Val	Val	Ser	Ser	Pro	Ser	Xaa	Ile	Leu	Leu
			20					25				30			
Xaa	Ser	Gly	Lys	Pro	Ser	Val	Ser	Lys	Glu	Gln	Xaa	Asp	Lys	Ile	Leu
		35					40				45				
Glu	Glu	Cys	Asp	Arg	Phe	Ile	Arg	Leu	Gly	Tyr	Pro	Ile	Tyr	Val	Val
	50					55					60				
Ser	Arg	His	Ser	Pro	Cys	Cys	Asp	Ala	Gly	Arg	Ala	Val	Glu	Asn	Arg
65					70					75				80	
Asp	Ile	Asp	Tyr	Val	Val	Leu	Leu	Leu	Thr	Xaa	Asp	Glu	Asn	Asp	Lys
			85						90					95	
Xaa	Ser	Xaa	Ser	Lys	Ile	Leu	Xaa	Xaa	His	Gly	Leu	Cys	Glu	Leu	Pro
			100				105						110		
Leu	Val	Leu	Phe	Met	Thr	Ala	Ala								
		115					120								