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(54) Title: PLANTS HAVING ENHANCED TOLERANCE TO INSECT PESTS AND RELATED CONSTRUCTS AND METHODS INVOLVING INSECT TOLERANCE GENES

(57) Abstract: The disclosure discloses isolated polynucleotides and polypeptides, and recombinant DNA constructs useful for conferring improved tolerance in plants to insect pests; compositions (such as plants or seeds) comprising these recombinant DNA constructs; and methods utilizing these recombinant DNA constructs. The recombinant DNA constructs comprise a polynucleotide operably linked to a promoter that is functional in a plant, wherein said polynucleotides encode insect tolerance polypeptides.

PLANTS HAVING ENHANCED TOLERANCE TO INSECT PESTS AND RELATED CONSTRUCTS AND METHODS INVOLVING INSECT TOLERANCE GENES

FIELD

Thisdisclosure relates to the field of plant breeding and genetics and, in particular, relates to recombinant DNA constructs useful for conferring tolerance to insect pests, and methods for control of insect infestation in plants.

BACKGROUND

Numerous insect species are serious pests to common agricultural crops such as corn, soybean, pea, cotton, rice and similar food and fiber crops. Pests' infestation can cause a huge financial loss annually either in crop loss or in purchasing expensive pesticides to keep check on pests. During the last centuries, the primary method of controlling such pests has been through the application of synthetic chemical insecticidal compounds. However, the widespread use of chemical compounds poses many problems with regard to the environment because of the non-selectivity of the compounds and the development of insect resistance to the chemicals.

Advances in biotechnology in the last decades have presented new opportunities for pest control through genetic engineering. In particular, advances in plant genetics coupled with the identification of insect growth factors and naturally-occurring plant defensive compounds or agents offer the opportunity to create transgenic crop plants capable of producing such defensive agents and thereby protect the plants against insect attack.

Certain species of microorganisms of the genus *Bacillus* are known to possess pesticidal activity against a range of insect pests including Lepidoptera, Diptera, Coleoptera, Hemiptera and others. *Bacillus thuringiensis (Bt)* and *Bacillus popilliae* are among the most successful biocontrol agents discovered to date. Insect pathogenicity has also been attributed to strains of *B. larvae*, *B. lentimorbus*, *B. sphaericus* and *B. cereus*. Microbial insecticides, particularly those obtained from *Bacillus* strains, have played an important role in agriculture as alternatives to chemical pest control.

Transgenic plants that are resistant to specific insect pests have been produced using genes encoding Bacillus thuringiensis (Bt) endotoxins or plant protease inhibitors (PIs). For example, corn and cotton plants have been genetically engineered to produce pesticidal proteins isolated from strains of *Bt*. These genetically engineered crops are now widely used in agriculture and have provided the farmer with an environmentally friendly and commercially attractive alternative to traditional insect control methods. Generally speaking, the use of biopesticides presents a lower risk of pollution and environmental hazards and biopesticides provide greater target specificity than traditional broad spectrum chemical insecticides. In addition, biopesticides often cost less to produce and thus improve economic yield for a wide variety of crops.

While biopesticides have proven to be very successful commercially, these genetically engineered, insect-resistant crop plants provide resistance to only a narrow range of the economically important insect pests. In some cases, insects can develop resistance to different insecticidal compounds, which raises the need to identify alternative biological control agents for pest control. Accordingly, there remains a need for new pesticidal proteins with different ranges of insecticidal activity against insect pests, e.g., insecticidal proteins which are active against a variety of insects in the order Lepidoptera and the order Coleoptera including but not limited to insect pests that have developed resistance to existing insecticides.

SUMMARY

In one aspect, the present disclosure includes an isolated polynucleotide enhancing insect tolerance of a plant through over-expression, comprising: (a) a polynucleotide with nucleotide sequence of at least 85% sequence identity to SEQ ID NO: 7, 10, 13 or 16; (b) a polynucleotide with nucleotide sequence of at least 85% sequence identity to SEQ ID NO: 8, 11, 14 or 17; (c) a polynucleotide encoding a polypeptide with amino acid sequence of at least 90% sequence identity to SEQ ID NO: 9, 12, 15 or 18; or (d) the full complement of the nucleotide sequence of (a), (b) or (c). The nucleotide sequence comprises SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16 or SEQ ID NO: 17. The amino acid sequence of the polypeptide comprises SEQ

ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 15 or SEQ ID NO: 18.

In another aspect, the present disclosure includes a recombinant DNA construct comprising the isolated polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide comprises (a) a polynucleotide with nucleotide sequence of at least 85% sequence identity to SEQ ID NO: 7, 8, 10, 11, 13, 14, 16 or 17; (b) a polynucleotide encoding a polypeptide with amino acid sequence of at least 90% sequence identity to SEQ ID NO: 9, 12, 15 or 18; or (c) the full complement of the nucleotide sequence of (a) or (b); the at least one regulatory sequence is a promoter functional in a plant.

In another aspect, the present disclosure includes a plant or seed comprising a recombinant DNA construct comprising the polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide comprises (a) a polynucleotide with nucleotide sequence of at least 85% sequence identityto SEQ ID NO: 7, 8, 10, 11, 13, 14, 16or 17; (b) a polynucleotide encoding a polypeptide with amino acid sequence of at least 90% sequence identityto SEQ ID NO: 9, 12, 15- or 18; or (c) the full complement of the nucleotide sequence of (a) or (b).

In another aspect, the present disclosure includes a plant comprising in its genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory element, wherein the polynucleotide comprises (a) a polynucleotide with nucleotide sequence of at least 85% sequence identity to SEQ ID NO: 7, 8, 10, 11, 13, 14, 16 or 17; (b) a polynucleotide encoding a polypeptide with amino acid sequence of at least 90% sequence identity to SEQ ID NO: 9, 12, 15 or 18; or (c) the full complement of the nucleotide sequence of (a) or (b); the said plant exhibits increased tolerance to an insect pest when compared to a control plant.

In another aspect, the present disclosure includes any of the plants of the disclosure, wherein the plant is selected from the group consisting of rice, maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, barley, millet, sugar cane and switchgrass.

In another aspect, the present disclosure includes increased insect pest tolerance, wherein the insect toleranceis created or enhanced against any species of the orders selected from the group consistingof orders Coleoptera, Diptera, Hymenoptera,

Lepidoptera, Mallophaga, Homoptera, Hemiptera Orthroptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Lepidoptera and Coleoptera.

In another aspect, methods are provided for increasing tolerance in a plant to an insect pest, comprising: (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity compared to SEQ ID NO: 9, 12, 15 or 18; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; and (c) obtaining a progeny plant derived from the transgenic plant of step (b), wherein the said progeny plant comprises in its genome the recombinant DNA construct and exhibits increased tolerance to an insect pest when compared to a control plant not comprising the recombinant DNA construct.

In another aspect, methods are provided for evaluating tolerance in a plant to an insect pest, comprising: (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity when compared to SEQ ID NO: 9, 12, 15 or 18; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; (c) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (d) evaluating the progeny plant for tolerance to an insect pest compared to a control plant not comprising the recombinant DNA construct.

In another aspect, the present disclosure concerns a recombinant DNA construct comprising any of the isolated polynucleotides of the present disclosure operably linked to at least one regulatory sequence, and a cell, a plant, and a seed comprising the recombinant DNA construct. The cell may be eukaryotic, e.g., a yeast, insect or plant cell, or prokaryotic, e.g., a bacterium.

BRIEF DESCRIPTION OF THE

DRAWINGS AND SEQUENCE LISTINGS

The disclosure can be more fully understood from the following detailed description and the accompanying drawings and Sequence Listing which form a part of this application.

FIG. 1shows the activated expression levels of *OsKUN1* genes in different tissues of line AH67515 plants as revealed by real-time RT-PCR analyses. ZH11 is wild type of Zhonghua 11.The numbers on the top of the columns are the fold-changes compared to Zhonghua 11 leaves.

FIG. 2 shows the relative expression levels of *OsCOA26* transgene in leaves of different transgenic rice lines by real-time PCR analyses. The base expression level in ZH11-TC is set at 1.00, the numbers on the top of the columns are fold-changes compared to ZH11-TC rice. ZH11-TC is tissue cultured Zhonghua 11.

FIG. 3 shows the relative expression levels of *OsROMT17* transgene in leaves of different transgenic rice lines by real-time PCR analyses. The base expression level in ZH11-TC is set at 1.00, the numbers on the top of the columns are fold-changes compared to ZH11-TC rice.

FIG. 4 shows the relative expression levels of *OsITP2* transgene in leaves of different transgenic rice lines by real-time PCR analyses. The base expression level in ZH11-TC is set at 1.00, the numbers on the top of the columns are fold-changes compared to ZH11-TC rice.

FIG. 5 shows the relative expression levels of *OsKUN1* transgene in leaves of different transgenic rice lines by real-time PCR analyses. The base expression level in ZH11-TC is set at 1.00, the numbers on the top of the columns are fold-changes compared to ZH11-TC rice.

Table 1.SEQ ID NOs for nucleotide and amino acid sequences provided in the sequence listing

Table 2.Scoring Scales for Asian corn borer and Oriental armyworm assays

Table 3. Asian corn borer assay of AH68151 seedlings under laboratory screening condition

Table 4. Asian corn borer assay of AH68231 seedlings under laboratory

screeningcondition

Table 5. Asian corn borer assay of AH67515 seedlings under laboratory screening condition

Table 6. Oriental armyworm assay of ATLsseedlings under laboratory screening condition

Table 7. Rice stem borer assay of ATLsseedlings under laboratory screening condition

Table 8. Rice insect tolerance gene names, Gene IDs (from TIGR) and Construct IDs

Table 9. Primers for cloning insect tolerance genes

Table 10. PCR reaction mixture

Table 11. PCR cycle conditions for cloning insect tolerance genes

Table 12. Asian corn borer assay of *OsCOA26*transgenic rice under laboratory screening condition at line level (1st experiment)

Table 13.Asian corn borer assay of *OsCOA26*transgenic rice under laboratory screen condition at line level (2nd experiment)

Table14.Asian corn borer assay of *OsCOA26*transgenic rice under laboratory screen condition at line level (3rd experiment)

Table 15.Armworm assay of *OsCOA26*transgenic rice under laboratory screen condition at line level

Table 16.Rice stem borer assay of *OsCOA26*transgenic rice under greenhouse screen condition at line levelTable 17. Asian corn borer assay of *OsROMT17*transgenicrice under laboratory screening condition at line level (1st experiment)

Table 18. Asian corn borer assay of *OsROMT17*transgenicrice under laboratory screening condition at line level (2nd experiment)

Table 19. Asian corn borer assay of *OsROMT17*transgenicrice under laboratory screening condition at line level (3rd experiment)

Table 20.Armworm assay of *OsROMT17*transgenic rice under laboratory screen condition at line level

Table 21. Rice stem borer assay of *OsROMT17*transgenic rice under greenhouse screen condition at line level

Table 22. Asian corn borer assay of*OsITP2*transgenic rice under laboratory screening condition at line level (1st experiment)

Table 23.Asian corn borer assay of *OsITP2*transgenic rice under laboratory screen condition at line level (2nd experiment)

Table 24.Asian corn borer assay of *OsITP2*transgenic rice under laboratory screen condition at line level (3rd experiment)

Table 25.Armworm assay of *OsITP2*transgenic rice under laboratory screen condition at line level

Table 26.Rice stem borer assay of *OsITP2*transgenic rice under greenhouse screen condition at line level

Table 27. Asian corn borer assay of *OsKUN1* transgenic rice under laboratory screening condition at line level (1st experiment)

Table 28.Asian corn borer assay of *OsKUN1*transgenic rice under laboratory screen condition at line level (2nd experiment)

Table 29.Asian corn borer assay of *OsKUN1*transgenic rice under laboratory screen condition at line level (3rd experiment)

Table 30.Armworm assay of *OsKUN1* transgenic rice under laboratory screen condition at line level (1st experiment)

Table 31.Armworm assay of *OsKUN1* transgenic rice under laboratory screen condition at line level (2nd experiment)

Table 32.Rice stem borer assay of *OsKUN1* transgenic rice plants under laboratory screen condition at line level (1st experiment)

Table 33.Rice stem borer assay of *OsKUN1* transgenic rice plants under laboratory screen condition at line level (2nd experiment)

Table 1. SEQ ID NOs for nucleotide and amino acid sequences provided in the sequence listing

Source species	Clone Designation	SEQ ID NO: (Nucleotide)	SEQ ID NO: (Amino Acid)
Oryza sativa	T-DNA flanking sequence in AH68151 (left LB)	1	n/a
Oryza sativa	T-DNA flanking sequence in AH68151 (right LB)	2	n/a
Oryza sativa	T-DNA flanking sequence in AH68231 (LB)	3	n/a

Oryza sativa	T-DNA flanking sequence in AH67515 (LB)	4	n/a
Oryza sativa	T-DNA flanking sequence in AH67515 (RB)	5	n/a
Artificial sequence	DP0158 vector	6	n/a
Oryza sativa	*OsCOA26*	7, 8	9
Oryza sativa	*OsROMT17*	10, 11	12
Oryza sativa	*OsITP2*	13,14	15
Oryza sativa	*OsKUN1*	16, 17	18
Artificial	Primers	19- 36	n/a

The sequence descriptions and Sequence Listing attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825. The Sequence Listing contains the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IUBMB standards described in *Nucleic Acids Res.* 13:3021-3030 (1985) and in the *Biochemical J.* 219 (2):345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NO: 1 is the nucleotide sequence of flanking sequence of the inserted T-DNA at the left left-border (LB) in AH68151 line.

SEQ ID NO: 2 is the nucleotide sequence of flanking sequence of the inserted T-DNA at the right left-border (RB) in AH68151 line.

SEQ ID NO: 3 is the nucleotide sequence of flanking sequence of the inserted T-DNA at the left border in AH68231 line.

SEQ ID NO: 4 is the nucleotide sequence of flanking sequence of the inserted T-DNA at the left border in AH67515 line.

SEQ ID NO: 5 is the nucleotide sequence of flanking sequence of the inserted T-DNA at the right border in AH67515 line.

SEQ ID NO: 6 is thenucleotide sequence of vector DP0158.

SEQ ID NO: 7is the nucleotide sequence of gDNA of *OsCOA26*gene.

SEQ ID NO: 8is the nucleotide sequence of CDS of *OsCOA26* gene.

SEQ ID NO: 9is the amino acid sequence of OsCOA26.

SEQ ID NO: 10is the nucleotide sequence of cDNA of *OsROMT17*gene.

SEQ ID NO: 11is the nucleotide sequence of CDS of *OsROMT17* gene.

SEQ ID NO: 12is the amino acid sequence of OsROMT17.

SEQ ID NO: 13is the nucleotide sequence of gDNA of *OsITP2*gene.

SEQ ID NO: 14is the nucleotide sequence of CDS of *OsITP2* gene.

SEQ ID NO: 15is the amino acid sequence of OsITP2.

SEQ ID NO: 16is the nucleotide sequence of cDNA of *OsKUN1* gene.

SEQ ID NO: 17is the nucleotide sequence of CDS of *OsKUN1* gene.

SEQ ID NO: 18is the amino acid sequence of OsKUN1.

SEQ ID NO: 19is forward primer for cloning gDNA of*OsCOA26* gene.

SEQ ID NO: 20is reverse primer for cloning gDNA of *OsCOA26* gene.

SEQ ID NO: 21is forward primer for cloning cDNA of *OsROMT17*gene.

SEQ ID NO: 22is reverse primer for cloning cDNA of *OsROMT17* gene.

SEQ ID NO: 23is forward primer for cloning gDNA of*OsITP2*gene.

SEQ ID NO: 24is reverse primer for cloning gDNA of *OsITP2*gene.

SEQ ID NO: 25 is forward primer for cloning cDNA of *OsKUN1* gene.

SEQ ID NO: 26 is reverse primer for cloning cDNA of *OsKUN1* gene.

SEQ ID NO: 27 is forward primer for real-time RT-PCR analysis of *OsKUN1* gene.

SEQ ID NO: 28 is reverse primer forreal-time RT-PCR analysis of *OsKUN1* gene.

SEQ ID NO: 29 is forward primer for real-time RT-PCR analysis of *OsCOA26*gene.

SEQ ID NO: 30 is reverse primer forreal-time RT-PCR analysis of *OsCOA26*gene

SEQ ID NO: 31 is forward primer for real-time RT-PCR analysis of *OsROMT17*gene.

SEQ ID NO: 32 is reverse primer forreal-time RT-PCR analysis of *OsROMT17*gene.

SEQ ID NO: 33 is forward primer for real-time RT-PCR analysis of *OsITP2*gene.

SEQ ID NO: 34 is reverse primer forreal-time RT-PCR analysis of *OsITP2*gene.

SEQ ID NO: 35 is forward primer for real-time RT-PCR analysis of *OsKUN1*gene.

SEQ ID NO: 36 is reverse primer forreal-time RT-PCR analysis of *OsKUN1*gene.

DETAILED DESCRIPTION

The disclosure of each reference set forth herein is hereby incorporated by reference

in its entirety.

As used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to “a plant” includes a plurality of such plants; reference to “a cell” includes one or more cells and equivalents theredisclosureof known to those skilled in the art, and so forth.

As used herein:

The term “OsCOA26” is a Caffeoyl-Coenzyme A 3-O-Methyltransferase(CCOAOMT) and refers to a rice polypeptide that confers increased tolerance to an insect pest and is encoded by the rice gene locus LOC_Os08g38920.1.“*COA26* polypeptide” refers herein to the OsCOA26 polypeptide and its homologs from other organisms.

The OsCOA26 polypeptide (SEQ ID NO: 9) is encoded by the coding sequence (CDS) (SEQ ID NO: 8) or nucleotide sequence (SEQ ID NO: 7) at rice gene locus LOC_Os08g38920.1. This polypeptide is annotated as “caffeoyl-CoA O-methyltransferase, putative, expressed” in TIGR (the internet atplantbiologymsu.edu/index.shtml), and in NCBI (on the worldwebat ncbi.nlm.nih.gov), however does not have any prior assigned function.

The term “OsROMT17(Caffeoyl-CoA 3-O-MethyltransferaseROMT17)” refers to a rice polypeptide that confers increased tolerance to an insect pest and is encoded by the rice gene locus LOC_Os08g38910.2. “ROMT17 polypeptide” refers herein to the OsROMT17 polypeptide and its homologs from other organisms.

The OsROMT17 polypeptide (SEQ ID NO: 12) is encoded by the coding sequence (CDS) (SEQ ID NO: 11) or nucleotide sequence (SEQ ID NO: 10) at rice gene locusLOC_Os08g38910.2. This polypeptide is annotated as “caffeoyl-CoA O-methyltransferase, putative, expressed” in TIGR, however does not have any prior assigned function.

The term “OsITP2 (insect tolerance polypeptide)” refers to a rice polypeptide that confers increased tolerance to an insect pest and is encoded by the rice gene locus LOC_Os01g53940.1. “ITP2 polypeptide” refers herein to the OsITP2 polypeptide and its homologs from other organisms.

The OsITP2 polypeptide (SEQ ID NO: 15) is encoded by the coding sequence (CDS) (SEQ ID NO: 14)or nucleotide sequence (SEQ ID NO: 13) at rice gene locus

LOC_Os01g53940.1. This polypeptide is annotated as "expressed protein" in TIGR, and "hypothetical protein" in NCBI, however no conserved domain detected.

The term "OsKUN1 (Kunitz-type trypsin inhibitor precursor)" refers to a rice polypeptide that confers increased tolerance to an insect pest and is encoded by the rice gene locus LOC_Os04g44470.1. "KUN1 polypeptide" refers herein to the OsKUN1 polypeptide and its homologs from other organisms.

The OsKUN1 polypeptide (SEQ ID NO: 18) is encoded by the coding sequence (CDS) (SEQ ID NO: 17) or nucleotide sequence (SEQ ID NO: 16) at rice gene locus LOC_ Os04g44470.1. This polypeptide is annotated as "KUN1-Kunitz-type trypsin inhibitor precursor, expressed" in TIGR.

The term "insect toleranceprotein" is used herein to refer to a polypeptide that inhibits the growth of, stunts the growth of, and/or killsone or more insect pests, including, but not limited to, members of the Lepidoptera, Diptera, Hemiptera and Coleoptera orders.

The terms "monocot" and "monocotyledonous plant" are used interchangeably herein. A monocot of the currentdisclosure includes the Gramineae.

The terms "dicot" and "dicotyledonous plant" are used interchangeably herein. A dicot of the current disclosure includes the following families: Brassicaceae, Leguminosae, and Solanaceae.

The terms "full complement" and "full-length complement" are used interchangeably herein, and refer to a complement of a given nucleotide sequence, wherein the complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary.

An "Expressed Sequence Tag" ("EST") is a DNA sequence derived from a cDNA library and therefore is a sequence which has been transcribed. An EST is typically obtained by a single sequencing pass of a cDNA insert. The sequence of an entire cDNA insert is termed the "Full-Insert Sequence" ("FIS"). A "Contig" sequence is a sequence assembled from two or more sequences that can be selected from, but not limited to, the group consisting of an EST, FIS and PCR sequence. A sequence encoding an entire or functional protein is termed a "Complete Gene Sequence" ("CGS") and can be derived from an FIS or a contig.

"Transgenic" refers to any cell, cell line, callus, tissue, plant part or plant, the genome

of which has been altered by the presence of a heterologous nucleic acid, such as a recombinant DNA construct, including those initial transgenic events as well as those created by sexual crosses or asexual propagation from the initial transgenic event. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

A "control" or "control plant" or "control plant cell" provides a reference point for measuring changes in phenotype of a subject plant or plant cell which was genetically altered by, such as transformation, and has been affected as to a gene of interest. A subject plant or plant cell may be descended from a plant or cell so altered and will comprise the alteration.

A control plant or plant cell may comprise, for example: (a) a wild-type plant or cell, i.e., of the same genotype as the starting material for the genetic alteration which resulted in the subject plant or cell; (b) a plant or plant cell of the same genotype as the starting material but which has been transformed with a null construct (i.e., with a construct which has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a plant or plant cell which is a non-transformed segregant among progeny of a subject plant or plant cell; (d) a plant or plant cell genetically identical to the subject plant or plant cell but which is not exposed to a condition or stimulus that would induce expression of the gene of interest; or (e) the subject plant or plant cell itself, under conditions in which the gene of interest is not expressed.

In this disclosure, ZH11-TCand empty vector plants indicate control plants. ZH11-TC represents rice plants generated from tissue cultured Zhonghua 11, and empty vector represents plants transformed with empty vector DP0158.

"Genome" as it applies to plant cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components (e.g., mitochondrial, plastid) of the cell.

"Plant" includes reference to whole plants, plant organs, plant tissues, seeds and plant

cells and progeny of same. Plant cells include, without limitation, cells from seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.

"Progeny" comprises any subsequent generation of a plant.

"Transgenic plant" includes reference to a plant which comprises within its genome a heterologous polynucleotide. The heterologous polynucleotide can be stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant DNA construct. A T_0 plant is directly recovered from the transformation and regeneration process. Progeny of T_0 plants are referred to as T_1 (first progeny generation), T_2 (second progeny generation), etc.

"Heterologous" with respect to sequence means a sequence 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.

"Polynucleotide", "nucleic acid sequence", "nucleotide sequence", or "nucleic acid fragment" are used interchangeably and is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deoxycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deoxythymidylate, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

"Polypeptide", "peptide", "amino acid sequence" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The terms "polypeptide", "peptide", "amino acid sequence", and "protein" are also inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation.

"Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell.

"cDNA" refers to a DNA that is complementary to and synthesized from an mRNA template using the enzyme reverse transcriptase. The cDNA can be single-stranded or converted into the double-stranded form using the Klenow fragment of DNA polymerase I.

"Mature" protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or pro-peptides present in the primary translation product hasbeen removed.

"Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and/or pro-peptides still present. Pre- and pro-peptides may be and are not limited to intracellular localization signals.

"Isolated" refers to materials, such as nucleic acid molecules and/or proteins, which are substantially free or otherwise removed from components that normally accompany or interact with the materials in a naturally occurring environment. Isolated polynucleotides may be purified from a host cell in which they naturally occur. Conventional nucleic acid purification methods known to skilled artisans may be used to obtain isolated polynucleotides. The term also embraces recombinant polynucleotides and chemically synthesized polynucleotides.

"Recombinant" refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques. "Recombinant" also includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid or a cell derived from a cell so modified, but does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

"Non-genomic nucleic acid sequence"or "non-genomic nucleic acid molecule" or "non-genomic polynucleotide" refers to a nucleic acid molecule that has one or more change in the nucleic acid sequence compared to a native or genomic nucleic acid sequence. In some embodiments the change to a native or genomic nucleic acid molecule

includes but is not limited to: changes in the nucleic acid sequence due to the degeneracy of the genetic code; codon optimization of the nucleic acid sequence for expression in plants; changes in the nucleic acid sequence to introduce at least one amino acid substitution, insertion, deletion and/or addition compared to the native or genomic sequence; removal of one or more intron associated with the genomic nucleic acid sequence; insertion of one or more heterologous introns; deletion of one or more upstream or downstream regulatory regions associated with the genomic nucleic acid sequence; insertion of one or more heterologous upstream or downstream regulatory regions; deletion of the 5' and/or 3' untranslated region associated with the genomic nucleic acid sequence; insertion of a heterologous 5' and/or 3' untranslated region; and modification of a polyadenylation site. In some embodiments the non-genomic nucleic acid molecule is a cDNA. In some embodiments the non-genomic nucleic acid molecule is a synthetic nucleic acid sequence.

"Recombinant DNA construct" refers to a combination of nucleic acid fragments that are not normally found together in nature. Accordingly, a recombinant DNA construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that normally found in nature.

The terms "entry clone" and "entry vector" are used interchangeably herein.

"Regulatory sequences" and "regulatory elements" are used interchangeably and refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to, promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

"Promoter" refers to a nucleic acid fragment capable of controlling transcription of another nucleic acid fragment.

"Promoter functional in a plant" is a promoter capable of controlling transcription in plant cells whether or not its origin is from a plant cell.

"Tissue-specific promoter" and "tissue-preferred promoter" are used interchangeably

and refer to a promoter that is expressed predominantly but not necessarily exclusively in one tissue or organ, but that may also be expressed in one specific cell.

"Developmentally regulated promoter" refers to a promoter whose activity is determined by developmental events.

"Operably linked" refers to the association of nucleic acid fragments in a single fragment so that the function of one is regulated by the other. For example, a promoter is operably linked with a nucleic acid fragment when it is capable of regulating the transcription of that nucleic acid fragment.

"Expression" refers to the production of a functional product. For example, expression of a nucleic acid fragment may refer to transcription of the nucleic acid fragment (e.g., transcription resulting in mRNA or functional RNA) and/or translation of mRNA into a precursor or mature protein.

"Phenotype" means the detectable characteristics of a cell or organism.

"Introduced" in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct) into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid fragment into a eukaryotic or prokaryotic cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

A "transformed cell" is any cell into which a nucleic acid fragment (e.g., a recombinant DNA construct) has been introduced.

"Transformation" as used herein refers to both stable transformation and transient transformation.

"Stable transformation" refers to the introduction of a nucleic acid fragment into a genome of a host organism resulting in genetically stable inheritance. Once stably transformed, the nucleic acid fragment is stably integrated in the genome of the host organism and any subsequent generation.

"Transient transformation" refers to the introduction of a nucleic acid fragment into the nucleus, or DNA-containing organelle, of a host organism resulting in gene expression without genetically stable inheritance.

"Allele" is one of several alternative forms of a gene occupying a given locus on a chromosome. When the alleles present at a given locus on a pair of homologous chromosomes in a diploid plant are the same that plant is homozygous at that locus. If the alleles present at a given locus on a pair of homologous chromosomes in a diploid plant differ that plant is heterozygous at that locus. If a transgene is present on one of a pair of homologous chromosomes in a diploid plant that plant is hemizygous at that locus.

A "chloroplast transit peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the chloroplast or other plastid types present in the cell in which the protein is made. "Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast transit peptide. A "signal peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the secretory system (Chrispeels (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol. 42*:21-53). If the protein is to be directed to a vacuole, a vacuolar targeting signal (*supra*) can further be added, or if to the endoplasmic reticulum, an endoplasmic reticulum retention signal (*supra*) may be added. If the protein is to be directed to the nucleus, any signal peptide present should be removed and instead a nuclear localization signal included (Raikhel (1992) *Plant Phys. 100*:1627-1632). A "mitochondrial signal peptide" is an amino acid sequence which directs a precursor protein into the mitochondria (Zhang and Glaser (2002) *Trends Plant Sci 7*:14-21).

Sequence alignments and percent identity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the MEGALIGN® program of the LASERGENE® bioinformatics computing suite (DNASTAR® Inc., Madison, WI). Unless stated otherwise, multiple alignment of the sequences provided herein were performed using the Clustal V method of alignment (Higgins and Sharp, *CABIOS.* 5:151-153 (1989)) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal V method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences, using the Clustal V program, it is possible to

obtain “percent identity” and “divergence” values by viewing the “sequence distances” table on the same program; unless stated otherwise, percent identities and divergences provided and claimed herein were calculated in this manner.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter “Sambrook”).

Turning now to the embodiments:

Embodiments include isolated polynucleotides and polypeptides, recombinant DNA constructs useful for conferring insect tolerance, compositions (such as plants or seeds)comprising these recombinant DNA constructs, and methods utilizing these recombinant DNA constructs.

Isolated Polynucleotides and Polypeptides

The present disclosureincludes the following isolated polynucleotides and polypeptides:

In some embodiments, polynucleotides are provided encoding COA26 polypeptides, ROMT17 polypeptides, ITP2 polypeptides or KUN1 polypeptides.

In some embodiments, isolated polynucleotidesare provided comprising: (i) a nucleic acid sequence encoding a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, when compared to SEQ ID NO: 9, 12, 15 or18; or (ii) a full complement of the nucleic acid sequence of (i), wherein the full complement and the nucleic acid sequence of (i) consist of the same number of nucleotides and are 100% complementary. Any of the foregoing isolated polynucleotides may be utilized in any recombinant DNA constructs of the present disclosure.

In some embodiments, isolated polypeptidesare provided having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%,

78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 9, 12, 15 or18. The polypeptidesareinsect tolerance polypeptideCOA26, ROMT17, ITP2 or KUN1.

In some embodiments,isolated polynucleotide are provided comprising (i) a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, when compared to SEQ ID NO: 7, 8, 10, 11, 13, 14, 16 or 17; or (ii) a full complement of the nucleic acid sequence of (i). Any of the foregoing isolated polynucleotides may be utilized in any recombinant DNA constructs of the present disclosure. The isolated polynucleotide preferably encodes an insect tolerance protein. Over-expression of this polypeptide increases planttolerance to an insect pest.

Recombinant DNA Constructs

In one aspect, the present disclosureincludes recombinant DNA constructs.

In one embodiment, a recombinant DNA construct comprises a polynucleotide operably linked to at least one regulatory sequence (e.g., a promoter functional in a plant), wherein the polynucleotide comprises (i) a nucleic acid sequence encoding an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, when compared to SEQ ID NO: 9, 12, 15 or18; or (ii) a full complement of the nucleic acid sequence of (i).

In another embodiment, a recombinant DNA construct comprises a polynucleotide operably linked to at least one regulatory sequence (e.g., a promoter functional in a plant), wherein said polynucleotide comprises (i) a nucleic acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%,

or 100% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO: 7, 8, 10, 11, 13, 14, 16 or17; or (ii) a full complement of the nucleic acid sequence of (i).

In another embodiment, a recombinant DNA construct comprises a polynucleotide operably linked to at least one regulatory sequence (e.g., a promoter functional in a plant), wherein said polynucleotide encodes a COA26, ROMT17, ITP2 or KUN1 protein. This polypeptide provide tolerance to an insect pest activity, and may be from, for example, *Oryza sativa,Oryza australiensis*, *Oryza barthii*, *Oryza glaberrima*(African rice), *Oryza latifolia*, *Oryza longistaminata*, *Oryza meridionalis*, *Oryza officinalis*, *Oryza punctata*, *Oryza rufipogon* (brownbeard or red rice), *Oryza nivara* (Indian wild rice), *Arabidopsis thaliana*, *Zea mays*, *Glycine max*, *Glycine tabacina*, *Glycine soja* or *Glycine tomentella*.

It is understood, as those skilled in the art will appreciate, that the disclosureencompasses more than the specific exemplary sequences. Alterations in a nucleic acid fragment which result in the production of a chemically equivalent amino acid at a given site, but do not affect the functional properties of the encoded polypeptide, are well known in the art. For example, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the polypeptide molecule would also not be expected to alter the activity of the polypeptide. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products.

"Suppression DNA construct" is a recombinant DNA construct which when transformed or stably integrated into the genome of the plant, results in "silencing" of a target gene in the plant. The target gene may be endogenous or transgenic to the plant. "Silencing", as used herein with respect to the target gene, refers generally to the suppression of levels of mRNA or protein/enzyme expressed by the target gene, and/or the level of the enzyme

activity or protein functionality. The terms "suppression", "suppressing" and "silencing", used interchangeably herein, includes lowering, reducing, declining, decreasing, inhibiting, eliminating or preventing. "Silencing" or "gene silencing" does not specify mechanism and is inclusive, and not limited to, anti-sense, cosuppression, viral-suppression, hairpin suppression, stem-loop suppression, RNAi-based approaches, and small RNA-based approaches.

A suppression DNA construct may comprise a region derived from a target gene of interest and may comprise all or part of the nucleic acid sequence of the sense strand (or antisense strand) of the target gene of interest. Depending upon the approach to be utilized, the region may be 100% identical or less than 100% identical (e.g., at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) to all or part of the sense strand (or antisense strand) of the gene of interest.

Suppression DNA constructs are well-known in the art, are readily constructed once the target gene of interest is selected, and include, without limitation, cosuppression constructs, antisense constructs, viral-suppression constructs, hairpin suppression constructs, stem-loop suppression constructs, double-stranded RNA-producing constructs, and more generally, RNAi (RNA interference) constructs and small RNA constructs such as siRNA (short interfering RNA) constructs and miRNA (microRNA) constructs.

"Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target gene or gene product. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target isolated nucleic acid fragment (U.S. Patent No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence.

"Cosuppression" refers to the production of sense RNA transcripts capable of suppressing the expression of the target gene or gene product. "Sense" RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or *in vitro*.

Cosuppression constructs in plants have been previously designed by focusing on over-expression of a nucleic acid sequence having homology to a native mRNA, in the sense orientation, which results in the reduction of all RNA having homology to the over-expressed sequence (see Vaucheret et al., *Plant J.* 16:651-659 (1998); and Gura, *Nature* 404:804-808 (2000)).

Another variation describes the use of plant viral sequences to direct the suppression of proximal mRNA encoding sequences (PCT Publication No. WO 98/36083 published on August 20, 1998).

RNA interference (RNAi) refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire et al., *Nature* 391:806 (1998)). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing (PTGS) or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire et al., *Trends Genet.* 15:358 (1999)).

Small RNAs play an important role in controlling gene expression. Regulation of many developmental processes, including flowering, is controlled by small RNAs. It is now possible to engineer changes in gene expression of plant genes by using transgenic constructs which produce small RNAs in the plant.

Small RNAs appear to function by base-pairing to complementary RNA or DNA target sequences. When bound to RNA, small RNAs trigger either RNA cleavage or translational inhibition of the target sequence. When bound to DNA target sequences, it is thought that small RNAs can mediate DNA methylation of the target sequence. The consequence of these events, regardless of the specific mechanism, is that gene expression is inhibited.

MicroRNAs (miRNAs) are noncoding RNAs of about 19 to about 24 nucleotides (nt) in length that have been identified in both animals and plants (Lagos-Quintana et al., *Science* 294:853-858 (2001), Lagos-Quintana et al., *Curr. Biol.* 12:735-739 (2002); Lau et al., *Science* 294:858-862 (2001); Lee and Ambros, *Science* 294:862-864 (2001); Llave et al., *Plant Cell* 14:1605-1619 (2002); Mourelatos et al., *Genes. Dev.* 16:720-728 (2002); Park et

al., *Curr. Biol.* 12:1484-1495 (2002); Reinhart et al., *Genes. Dev.* 16:1616-1626 (2002)). They are processed from longer precursor transcripts that range in size from approximately 70 to 200 nt, and these precursor transcripts have the ability to form stable hairpin structures.

MicroRNAs (miRNAs) appear to regulate target genes by binding to complementary sequences located in the transcripts produced by these genes. It seems likely that miRNAs can enter at least two pathways of target gene regulation: (1) translational inhibition; and (2) RNA cleavage. MicroRNAs entering the RNA cleavage pathway are analogous to the 21-25 nt short interfering RNAs (siRNAs) generated during RNA interference (RNAi) in animals and posttranscriptional gene silencing (PTGS) in plants, and likely are incorporated into an RNA-induced silencing complex (RISC) that is similar or identical to that seen for RNAi.

Regulatory Sequences:

A recombinant DNA construct of the present disclosuremay comprise at least one regulatory sequence.

A regulatory sequence may be a promoter or enhancer.

A number of promoters can be used in recombinant DNA constructs of the present disclosure. The promoters can be selected based on the desired outcome, and may include constitutive, tissue-specific, inducible, or other promoters for expression in the host organism.

Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters".

High level, constitutive expression of the candidate gene under control of the 35S or UBI promoter may (or may not) have pleiotropic effects, although candidate gene efficacy may be estimated when driven by a constitutive promoter. Use of tissue-specific and/or stress-specific promoters may eliminate undesirable effects, but retain the ability to enhance insect tolerance. This type of effect has been observed in *Arabidopsis* for drought and cold tolerance (Kasuga et al., *Nature Biotechnol.* 17:287-91 (1999)).

Suitable constitutive promoters for use in a plant host cell include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell et al.,

Nature 313:810-812 (1985)); rice actin (McElroy et al., *Plant Cell* 2:163-171 (1990)); ubiquitin (Christensen et al., *Plant Mol. Biol.* 12:619-632 (1989) and Christensen et al., *Plant Mol. Biol.* 18:675-689 (1992)); pEMU (Last et al., *Theor. Appl. Genet.* 81:581-588 (1991)); MAS (Velten et al., *EMBO J.* 3:2723-2730 (1984)); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, those discussed 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; 5,608,142; and 6,177,611.

In choosing a promoter to use in the methods of the disclosure, it may be desirable to use a tissue-specific or developmentally regulated promoter.

A tissue-specific or developmentally regulated promoter is a DNA sequence which regulates the expression of a DNA sequence selectively in the cells/tissues of a plant critical to tassel development, seed set, or both, and limits the expression of such a DNA sequence to the period of tassel development or seed maturation in the plant. Any identifiable promoter may be used in the methods of the present disclosurewhich causes the desired temporal and spatial expression.

Promoters which are seed or embryo-specific and may be useful in the disclosureinclude soybean Kunitz trypsin inhibitor (Kti3, Jofuku and Goldberg, *Plant Cell* 1:1079-1093 (1989)), patatin (potato tubers) (Rocha-Sosa, M., et al., *EMBO J.* 8:23-29 (1989)), convicilin, vicilin, and legumin (pea cotyledons) (Rerie, W.G., et al., *Mol. Gen. Genet.* 259:149-157 (1991); Newbigin, E.J., et al., *Planta* 180:461-470 (1990); Higgins, T.J.V., et al., *Plant. Mol. Biol.* 11:683-695 (1988)), zein (maize endosperm) (Schemthaner, J.P., et al., *EMBO J.* 7:1249-1255 (1988)), phaseolin (bean cotyledon) (Segupta-Gopalan, C., et al., *Proc. Natl. Acad. Sci.* U.S.A. 82:3320-3324 (1995)), phytohemagglutinin (bean cotyledon) (Voelker, T. et al., *EMBO J.* 6:3571-3577 (1987)), B-conglycinin and glycinin (soybean cotyledon) (Chen, Z-L, et al., *EMBO J.* 7:297-302 (1988)), glutelin (rice endosperm), hordein (barley endosperm) (Marris, C., et al., *Plant Mol. Biol.* 10:359-366 (1988)), glutenin and gliadin (wheat endosperm) (Colot, V., et al., *EMBO J.* 6:3559-3564 (1987)), and sporamin (sweet potato tuberous root) (Hattori, T., et al., *Plant Mol. Biol.* 14:595-604 (1990)). Promoters of seed-specific genes operably linked to heterologous coding regions in chimeric gene constructions maintain their temporal and spatial

expression pattern in transgenic plants. Such examples include *Arabidopsis thaliana* 2S seed storage protein gene promoter to express enkephalin peptides in *Arabidopsis* and *Brassica napus* seeds (Vanderkerckhove et al., *Bio/Technology* 7:L929-932 (1989)), bean lectin and bean beta-phaseolin promoters to express luciferase (Riggs et al., *Plant Sci.* 63:47-57 (1989)), and wheat glutenin promoters to express chloramphenicol acetyl transferase (Colot et al., *EMBO J.* 6:3559- 3564 (1987)).

Inducible promoters selectively express an operably linked DNA sequence in response to the presence of an endogenous or exogenous stimulus, for example by chemical compounds (chemical inducers) or in response to environmental, hormonal, chemical, and/or developmental signals. Inducible or regulated promoters include, for example, promoters regulated by light, heat, stress, flooding or drought, phytohormones, wounding, or chemicals such as ethanol, jasmonate, salicylic acid, or safeners.

Promoters for use in the current disclosureinclude the following: 1) the stress-inducible RD29A promoter (Kasuga et al., *Nature Biotechnol.* 17:287-91 (1999)); 2) the barley promoter, B22E; expression of B22E is specific to the pedicel in developing maize kernels ("Primary Structure of a Novel Barley Gene Differentially Expressed in Immature Aleurone Layers", Klemsdal et al., *Mol. Gen. Genet.* 228(1/2):9-16 (1991)); and 3) maize promoter, Zag2 ("Identification and molecular characterization of ZAG1, the maize homolog of the *Arabidopsis* floral homeotic gene AGAMOUS", Schmidt et al., *Plant Cell* 5(7):729-737 (1993); "Structural characterization, chromosomal localization and phylogenetic evaluation of two pairs of *AGAMOUS*-like *MADS-box* genes from maize", Theissen et al., *Gene* 156(2):155-166 (1995); NCBI GenBank Accession No. X80206)). Zag2 transcripts can be detected five days prior to pollination to seven to eight days after pollination ("DAP"), and directs expression in the carpel of developing female inflorescences and Ciml which is specific to the nucleus of developing maize kernels. Ciml transcript is detected four to five days before pollination to six to eight DAP. Other useful promoters include any promoter which can be derived from a gene whose expression is maternally associated with developing female florets.

For the expression of a polynucleotide in developing seed tissue, promoters of particular interest include seed-preferred promoters, particularly early kernel/embryo

promoters and late kernel/embryo promoters. Kernel development post-pollination is divided into approximately three primary phases. The lag phase of kernel growth occurs from about 0 to 10-12 DAP. During this phase the kernel is not growing significantly in mass, but rather important events are being carried out that will determine kernel vitality (e.g., number of cells established). The linear grain fill stage begins at about 10-12 DAP and continues to about 40 DAP. During this stage of kernel development, the kernel attains almost all of its final mass, and various storage products (i.e., starch, protein, oil) are produced. Finally, the maturation phase occurs from about 40 DAP to harvest. During this phase of kernel development the kernel becomes quiescent and begins to dry down in preparation for a long period of dormancy prior to germination. As defined herein "early kernel/embryo promoters" are promoters that drive expression principally in developing seed during the lag phase of development (i.e., from about 0 to about 12 DAP). "Late kernel/embryo promoters", as defined herein, drive expression principally in developing seed from about 12 DAP through maturation. There may be some overlap in the window of expression. The choice of the promoter will depend on the ABA-associated sequence utilized and the phenotype desired.

Early kernel/embryo promoters include, for example, *Cim1* that is active 5 DAP in particular tissues (WO 00/11177), which is herein incorporated by reference. Other early kernel/embryo promoters include the seed-preferred promoters *end1* which is active 7-10 DAP, and *end2*, which is active 9-14 DAP in the whole kernel and active 10 DAP in the endosperm and pericarp(WO 00/12733), herein incorporated by reference. Additional early kernel/embryo promoters that find use in certain methods of the present disclosure include the seed-preferred promoter *ltp2* (U.S. Pat. No. 5,525,716); maize *Zm40* promoter (U.S. Pat.No. 6,403,862); maize *nuc1c*(U.S. Pat.No. 6,407,315); maize *ckx1-2* promoter (U.S. Pat.No. 6,921,815 and US Patent Application Publication Number 2006/0037103); maize *lec1* promoter (U.S. Pat.No. 7,122,658); maize *ESR* promoter (U.S. Pat.No. 7,276,596); maize *ZAP* promoter (U.S. Patent Application Publication Numbers 20040025206 and 20070136891); maize promoter *eep1* (U.S. Patent Application Publication Number 20070169226); and maize promoter *ADF4* (U.S. Patent Application No. 60/963,878, filed 7 Aug. 2007). Additional promoters for regulating the expression of the nucleotide

sequences of the present disclosurein plants are stalk-specific promoters. Such stalk-specific promoters include the alfalfa S2A promoter (GenBank Accession No. EF030816; Abrahams et al., *Plant Mol. Biol.* 27:513-528 (1995)) and S2B promoter (GenBank Accession No. EF030817) and the like, herein incorporated by reference.

Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments.

Promoters for use in the current disclosuremay include: RIP2, mLIP15, ZmCOR1, Rab17, CaMV 35S, RD29A, B22E, Zag2, SAM synthetase, ubiquitin, CaMV 19S, nos, Adh, sucrose synthase, R-allele, the vascular tissue preferred promoters S2A (Genbank accession number EF030816) and S2B (GenBank Accession No. EF030817), and the constitutive promoter GOS2 from *Zea mays*.Other promoters include root preferred promoters, such as the maize NAS2 promoter, the maize Cyclo promoter (US Publication No. 2006/0156439, published July 13, 2006), the maize ROOTMET2 promoter (WO 2005/063998, published July 14, 2005), the CR1BIO promoter (WO 2006/055487, published May 26, 2006), the CRWAQ81 promoter (WO 2005/035770, published April 21, 2005) and the maize ZRP2.47 promoter (NCBI Accession No. U38790; NCBI GI No. 1063664).

Recombinant DNA constructsof the present disclosuremay also include other regulatory sequences including, but not limited to, translation leader sequences, introns, and polyadenylation recognition sequences. In another embodiment of the present disclosure, a recombinant DNA construct of the present disclosurefurther comprises an enhancer or silencer.

An intron sequence can be added to the 5' untranslated region, the protein-coding region or the 3' untranslated region to increase the amount of the mature message that accumulates in the cytosol. Inclusion of a spliceable intron in the transcription unit in both plant and animal expression constructs has been shown to increase gene expression at both the mRNA and protein levels up to 1000-fold (Buchman and Berg, *Mol. Cell Biol.* 8:4395-4405 (1988); Callis et al., *Genes Dev.* 1:1183-1200 (1987)).

An enhancer or enhancer element refers to a cis-acting transcriptional regulatory

element, a.k.a. cis-element, which confers an aspect of the overall expression pattern, but is usually insufficient alone to drive transcription, of an operably linked polynucleotide sequence. An isolated enhancer element may be fused to a promoter to produce a chimeric promotercis-element, which confers an aspect of the overall modulation of gene expression. Enhancers are known in the art and include the SV40 enhancer region, the CaMV 35S enhancer element, and the like. Some enhancers are also known to alter normal regulatory element expression patterns, for example, by causing a regulatory element to be expressed constitutively when without the enhancer, the same regulatory element is expressed only in one specific tissue or a few specific tissues. Duplicating the upstream region of the CaMV35S promoter has been shown to increase expression by approximately tenfold (Kay, R. et al., (1987) Science 236: 1299-1302).

Enhancers for use in the current disclosure may include CaMV 35S (Benfey, et al., (1990) EMBO J. 9:1685-96); 4xB3 P-CaMV.35S Enhancer Domain -- four tandem copies of the B3 domain (208 to 155) as described in U.S. Pat. No. 5,097,025; 4xAS-1 P-CaMV.35S EnhancerDomain- four tandem copies of the "activation sequence" (83 to 62) as described in U.S. Pat. No. 5,097,025; 2xB1-B2 P-CaMV.35S Enhancer Domain -- two tandem copies of the B1-B2 domain (148 to 90) as described in U.S. Pat. No. 5,097,025; 2xA1-B3 P-CaMV.35S Enhancer Domain -- two tandem copies of the A1-B3 domain (208 to 46) as described in U.S. Pat. No. 5,097,025; 2xB1-B5 P-CaMV.35S Enhancer Domain -- two tandem copies of the B1-B5 domain (343 to 90) as described in U.S. Pat. No. 5,097,025; the omega enhancer or the omega prime enhancer (Gallie, et al., (1989) Molecular Biology of RNA ed. Cech (Liss, New York) 237-256 and Gallie, et al., (1987) Gene 60:217-25), the enhancers of U.S. Pat.No. 7,803,992, the sugarcane bacilliform viral (SCBV) enhancer element (WO2013130813).

Any plant can be selected for the identification of regulatory sequences and genes to be used in recombinant DNA constructs of the present disclosure. Examples of suitable plant targets for the isolation of genes and regulatory sequences would include but are not limited to alfalfa, apple, apricot, *Arabidopsis,* artichoke, arugula, asparagus, avocado, banana, barley, beans, beet, blackberry, blueberry, broccoli, brussels sprouts, cabbage, canola, cantaloupe, carrot, cassava, castorbean, cauliflower, celery, cherry, chicory, cilantro,

citrus, clementines, clover, coconut, coffee, corn, cotton, cranberry, cucumber, Douglas fir, eggplant, endive, escarole, eucalyptus, fennel, figs, garlic, gourd, grape, grapefruit, honey dew, jicama, kiwifruit, lettuce, leeks, lemon, lime, Loblolly pine, linseed, maize, mango, melon, mushroom, nectarine, nut, oat, oil palm, oil seed rape, okra, olive, onion, orange, an ornamental plant, palm, papaya, parsley, parsnip, pea, peach, peanut, pear, pepper, persimmon, pine, pineapple, plantain, plum, pomegranate, poplar, potato, pumpkin, quince, radiata pine, radicchio, radish, rapeseed, raspberry, rice, rye, sorghum, Southern pine, soybean, spinach, squash, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, sweetgum, tangerine, tea, tobacco, tomato, triticale, turf, turnip, a vine, watermelon, wheat, yams, and zucchini.

Compositions

A composition of the present disclosureis a plant comprising in its genome any of the recombinant DNA constructsof the present disclosure(such as any of the constructs discussed above). Compositions also include any progeny of the plant, and any seed obtained from the plant or its progeny, wherein the progeny or seed comprises within its genome the recombinant DNA construct. Progeny includes subsequent generations obtained by self-pollination or out-crossing of a plant. Progeny also includes hybrids and inbreds.

In hybrid seed propagated crops, mature transgenic plants can be self-pollinated to produce a homozygous inbred plant. The inbred plant produces seed containing the newly introduced recombinant DNA construct. These seeds can be grown to produce plants that would exhibit an altered agronomic characteristic, or used in a breeding program to produce hybrid seed, which can be grown to produce plants that would exhibit such an altered agronomic characteristic. The seeds may be maize seeds, or rice seeds.

The plant may be a monocotyledonous or dicotyledonous plant, for example, a maize or soybean plant, such as a maize hybrid plant or a maize inbred plant. The plant may also be sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley or millet.

The recombinant DNA construct is stably integrated into the genome of the plant.

Embodiments include but are not limited to the following:

1. A transgenic plant (for example, a rice, maize or soybean plant) comprising in its

genome a recombinant DNA construct comprising a polynucleotide operably linked to at least one heterologous regulatory sequence, wherein said polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, when compared to SEQ ID NO: 9, 12, 15 or 18; and wherein said transgenic plant exhibits increased tolerance to an insect pestwhen compared to a control plant not comprising said recombinant DNA construct.

2. The transgenic plant of embodiment 1, wherein the polynucleotide encodes a COA26, ROMT17, ITP2 or KUN1polypeptide (for example from *Oryza sativa*, *Oryza australiensis*, *Oryza barthii*, *Oryza glaberrima* (African rice), *Oryza latifolia*, *Oryza longistaminata*, *Oryza meridionalis*, *Oryza officinalis*, *Oryza punctata*, *Oryza rufipogon*(brownbeard or red rice), *Oryza nivara* (Indian wild rice), *Arabidopsis thaliana*, *Cicer arietinum*, *Solanum tuberosum*, *Brassica oleracea*,*Zea mays*, *Glycine max*, *Glycine tabacina*, *Glycine soja* or *Glycine tomentella*.

3. The transgenic plant of any one of embodiments 1 to 2, wherein the transgenic plant further comprises at least one polynucleotide encoding an insecticidal polypeptide.

4. The transgenic plant of any one of embodiments 1 to 2, wherein the transgenic plant further comprises at least one recombinant polynucleotide encoding a polypeptide of interest.

5. Any progeny of the above plants in embodiments 1-4, any seeds of the above plants in embodiments 1-4, any seeds of progeny of the above plants in embodiments 1-4, and cells from any of the above plants in embodiments 1-4and progeny thereof.

In any of the foregoing embodiments 1-5or any other embodiments of the present disclosure, the recombinant DNA construct may comprises at least one heterologous promoter functional in a plant as a regulatory sequence.

By “insecticidal protein” is used herein to refer to a polypeptide that has toxic activity against one or more insect pests, including, but not limited to, members of the Lepidoptera, Diptera, Hemiptera and Coleoptera orders or the Nematoda phylum or a protein that has

homology to such a protein. Pesticidal proteins have been isolated from organisms including, for example, *Bacillus* sp., *Pseudomonas* sp., *Photorhabdus* sp., *Xenorhabdus* sp., *Clostridium bifermentans* and *Paenibacillus popilliae.* Pesticidal proteins include but are not limited to: insecticidal proteins from *Pseudomonas* sp. such as PSEEN3174 (Monalysin; (2011) *PLoS Pathogens* 7:1-13); from *Pseudomonasprotegens* strain CHA0 and Pf-5 (previously *fluorescens*) (Pechy-Tarr, (2008) *Environmental Microbiology* 10:2368-2386; GenBank Accession No. EU400157); from *Pseudomonas Taiwanensis* (Liu, *et al.*, (2010) *J. Agric. Food Chem.*, 58:12343-12349) and from *Pseudomonas pseudoalcligenes* (Zhang, *et al.*, (2009) *Annals of Microbiology* 59:45-50 and Li, *et al.*, (2007) *Plant Cell Tiss. Organ Cult.*89:159-168); insecticidal proteins from *Photorhabdus* sp. and *Xenorhabdus* sp. (Hinchliffe, *et al.*, (2010) *The Open Toxicology Journal*, 3:101-118 and Morgan, *et al.*, (2001) *Applied and Envir.Micro.* 67:2062-2069); US Patent Number 6,048,838, and US Patent Number 6,379,946; a PIP-1 polypeptide of US publication number US2014008054; an AfIP-1A and/or AfIP-1B polypeptide of US Serial Number 13/800233; a PHI-4 polypeptide of US Serial Number 13/839702; and δ-endotoxins including, but not limited to, the Cry1, Cry2, Cry3, Cry4, Cry5, Cry6, Cry7, Cry8, Cry9, Cry10, Cry11, Cry12, Cry13, Cry14, Cry15, Cry16, Cry17, Cry18, Cry19, Cry20, Cry21, Cry22, Cry23, Cry24, Cry25, Cry26, Cry27, Cry 28, Cry 29, Cry 30, Cry31, Cry32, Cry33, Cry34, Cry35,Cry36, Cry37, Cry38, Cry39, Cry40, Cry41, Cry42, Cry43, Cry44, Cry45, Cry 46, Cry47, Cry49, Cry 51, Cry55, Cry56, Cry57, Cry58, Cry59, Cry60, Cry61, Cry62, Cry63, Cry64, Cry65, Cry66, Cry67, Cry68, Cry69, Cry70, Cry71 and Cry72 classes of δ-endotoxin genes and the *B. thuringiensis* cytolytic cyt1 and cyt2 genes. Members of these classes of *B. thuringiensis* insecticidal proteins include, but are not limited to Cry1Aa1 (Accession # AAA22353); Cry1Aa2 (Accession # Accession # AAA22552); Cry1Aa3 (Accession # BAA00257); Cry1Aa4 (Accession # CAA31886); Cry1Aa5 (Accession # BAA04468); Cry1Aa6 (Accession # AAA86265); Cry1Aa7 (Accession # AAD46139); Cry1Aa8 (Accession # I26149); Cry1Aa9 (Accession # BAA77213); Cry1Aa10 (Accession # AAD55382); Cry1Aa11 (Accession # CAA70856); Cry1Aa12 (Accession # AAP80146); Cry1Aa13 (Accession # AAM44305); Cry1Aa14 (Accession # AAP40639); Cry1Aa15 (Accession # AAY66993); Cry1Aa16 (Accession # HQ439776); Cry1Aa17 (Accession # HQ439788); Cry1Aa18 (Accession # HQ439790);

Cry1Aa19 (Accession # HQ685121); Cry1Aa20 (Accession # JF340156); Cry1Aa21 (Accession # JN651496); Cry1Aa22 (Accession # KC158223); Cry1Ab1 (Accession # AAA22330); Cry1Ab2 (Accession # AAA22613); Cry1Ab3 (Accession # AAA22561); Cry1Ab4 (Accession # BAA00071); Cry1Ab5 (Accession # CAA28405); Cry1Ab6 (Accession # AAA22420); Cry1Ab7 (Accession # CAA31620); Cry1Ab8 (Accession # AAA22551); Cry1Ab9 (Accession # CAA38701); Cry1Ab10 (Accession # A29125); Cry1Ab11 (Accession # I12419); Cry1Ab12 (Accession # AAC64003); Cry1Ab13 (Accession # AAN76494); Cry1Ab14 (Accession # AAG16877); Cry1Ab15 (Accession # AAO13302); Cry1Ab16 (Accession # AAK55546); Cry1Ab17 (Accession # AAT46415); Cry1Ab18 (Accession # AAQ88259); Cry1Ab19 (Accession # AAW31761); Cry1Ab20 (Accession # ABB72460); Cry1Ab21 (Accession # ABS18384); Cry1Ab22 (Accession # ABW87320); Cry1Ab23 (Accession # HQ439777); Cry1Ab24 (Accession # HQ439778); Cry1Ab25 (Accession # HQ685122); Cry1Ab26 (Accession # HQ847729); Cry1Ab27 (Accession # JN135249); Cry1Ab28 (Accession # JN135250); Cry1Ab29 (Accession # JN135251); Cry1Ab30 (Accession # JN135252); Cry1Ab31 (Accession # JN135253); Cry1Ab32 (Accession # JN135254); Cry1Ab33 (Accession # AAS93798); Cry1Ab34 (Accession # KC156668); Cry1Ab-like (Accession # AAK14336); Cry1Ab-like (Accession # AAK14337); Cry1Ab-like (Accession # AAK14338); Cry1Ab-like (Accession # ABG88858); Cry1Ac1 (Accession # AAA22331); Cry1Ac2 (Accession # AAA22338); Cry1Ac3 (Accession # CAA38098); Cry1Ac4 (Accession # AAA73077); Cry1Ac5 (Accession # AAA22339); Cry1Ac6 (Accession # AAA86266); Cry1Ac7 (Accession # AAB46989); Cry1Ac8 (Accession # AAC44841); Cry1Ac9 (Accession # AAB49768); Cry1Ac10 (Accession # CAA05505); Cry1Ac11 (Accession # CAA10270); Cry1Ac12 (Accession # I12418); Cry1Ac13 (Accession # AAD38701); Cry1Ac14 (Accession # AAQ06607); Cry1Ac15 (Accession # AAN07788); Cry1Ac16 (Accession # AAU87037); Cry1Ac17 (Accession # AAX18704); Cry1Ac18 (Accession # AAY88347); Cry1Ac19 (Accession # ABD37053); Cry1Ac20 (Accession # ABB89046); Cry1Ac21 (Accession # AAY66992); Cry1Ac22 (Accession # ABZ01836); Cry1Ac23 (Accession # CAQ30431); Cry1Ac24 (Accession # ABL01535); Cry1Ac25 (Accession # FJ513324); Cry1Ac26 (Accession # FJ617446); Cry1Ac27 (Accession # FJ617447); Cry1Ac28 (Accession # ACM90319);

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Examples of δ-endotoxins also include but are not limited to Cry1A proteins of US Patent Numbers 5,880,275 and 7,858,849; a DIG-3 or DIG-11 toxin (N-terminal deletion of α-helix 1 and/or α-helix 2 variants of cry proteins such as Cry1A, Cry3A) of US Patent

Numbers 8,304,604, 8.304,605 and 8,476,226; Cry1B of US Patent Application Serial Number 10/525,318; Cry1C of US Patent Number 6,033,874; Cry1F of US Patent Numbers 5,188,960 and 6,218,188; Cry1A/F chimeras of US Patent Numbers 7,070,982; 6,962,705 and 6,713,063); a Cry2 protein such as Cry2Ab protein of US Patent Number 7,064,249); a Cry3A protein including but not limited to an engineered hybrid insecticidal protein (eHIP) created by fusing unique combinations of variable regions and conserved blocks of at least two different Cry proteins (US Patent Application Publication Number 2010/0017914); a Cry4 protein; a Cry5 protein; a Cry6 protein; Cry8 proteins of US Patent Numbers 7,329,736, 7,449,552, 7,803,943, 7,476,781, 7,105,332, 7,378,499 and 7,462,760; a Cry9 protein such as such as members of the Cry9A, Cry9B, Cry9C, Cry9D, Cry9E and Cry9F families; a Cry15 protein of Naimov, *et al.*, (2008) *Applied and Environmental Microbiology*, 74:7145-7151; a Cry22, a Cry34Ab1 protein of US Patent Numbers 6,127,180, 6,624,145 and 6,340,593; a CryET33 and cryET34 protein of US Patent Numbers 6,248,535, 6,326,351, 6,399,330, 6,949,626, 7,385,107 and 7,504,229; a CryET33 and CryET34 homologs of US Patent Publication Number 2006/0191034, 2012/0278954, and PCT Publication Number WO 2012/139004; a Cry35Ab1 protein of US Patent Numbers 6,083,499, 6,548,291 and 6,340,593; a Cry46 protein, a Cry 51 protein, a Cry binary toxin; a TIC901 or related toxin; TIC807 of US Patent Application Publication Number 2008/0295207; ET29, ET37, TIC809, TIC810, TIC812, TIC127, TIC128 of PCT US 2006/033867; AXMI-027, AXMI-036, and AXMI-038 of US Patent Number 8,236,757; AXMI-031, AXMI-039, AXMI-040, AXMI-049 of US Patent Number 7,923,602; AXMI-018, AXMI-020 and AXMI-021 of WO 2006/083891; AXMI-010 of WO 2005/038032; AXMI-003 of WO 2005/021585; AXMI-008 of US Patent Application Publication Number 2004/0250311; AXMI-006 of US Patent Application Publication Number 2004/0216186; AXMI-007 of US Patent Application Publication Number 2004/0210965; AXMI-009 of US Patent Application Number 2004/0210964; AXMI-014 of US Patent Application Publication Number 2004/0197917; AXMI-004 of US Patent Application Publication Number 2004/0197916; AXMI-028 and AXMI-029 of WO 2006/119457; AXMI-007, AXMI-008, AXMI-0080rf2, AXMI-009, AXMI-014 and AXMI-004 of WO 2004/074462; AXMI-150 of US Patent Number 8,084,416; AXMI-205 of US Patent Application Publication Number

2011/0023184; AXMI-011, AXMI-012, AXMI-013, AXMI-015, AXMI-019, AXMI-044, AXMI-037, AXMI-043, AXMI-033, AXMI-034, AXMI-022, AXMI-023, AXMI-041, AXMI-063 and AXMI-064 of US Patent Application Publication Number 2011/0263488; AXMI-R1 and related proteins of US Patent Application Publication Number 2010/0197592; AXMI221Z, AXMI222z, AXMI223z, AXMI224z and AXMI225z of WO 2011/103248; AXMI218, AXMI219, AXMI220, AXMI226, AXMI227, AXMI228, AXMI229, AXMI230 and AXMI231 of WO 2011/103247; AXMI-115, AXMI-113, AXMI-005, AXMI-163 and AXMI-184 of US Patent Number 8,334,431; AXMI-001, AXMI-002, AXMI-030, AXMI-035 and AXMI-045 of US Patent Application Publication Number 2010/0298211; AXMI-066 and AXMI-076 of US Patent Application Publication Number 2009/0144852; AXMI128, AXMI130, AXMI131, AXMI133, AXMI140, AXMI141, AXMI142, AXMI143, AXMI144, AXMI146, AXMI148, AXMI149, AXMI152, AXMI153, AXMI154, AXMI155, AXMI156, AXMI157, AXMI158, AXMI162, AXMI165, AXMI166, AXMI167, AXMI168, AXMI169, AXMI170, AXMI171, AXMI172, AXMI173, AXMI174, AXMI175, AXMI176, AXMI177, AXMI178, AXMI179, AXMI180, AXMI181, AXMI182, AXMI185, AXMI186, AXMI187, AXMI188, AXMI189 of US Patent Number 8,318,900; AXMI079, AXMI080, AXMI081, AXMI082, AXMI091, AXMI092, AXMI096, AXMI097, AXMI098, AXMI099, AXMI100, AXMI101, AXMI102, AXMI103, AXMI104, AXMI107, AXMI108, AXMI109, AXMI110, AXMI111, AXMI112, AXMI114, AXMI116, AXMI117, AXMI118, AXMI119, AXMI120, AXMI121, AXMI122, AXMI123, AXMI124, AXMI1257, AXMI1268, AXMI127, AXMI129, AXMI164, AXMI151, AXMI161, AXMI183, AXMI132, AXMI138, AXMI137 of US Patent Application Publication Number 2010/0005543, AXMI232, AXMI233 and AXMI249 of US Patent Application Publication Number 201400962281; cry proteins such as Cry1A and Cry3A having modified proteolytic sites of US Patent Number 8,319,019; a Cry1Ac, Cry2Aa and Cry1Ca toxin protein from *Bacillus thuringiensis* strain VBTS 2528 of US Patent Application Publication Number 2011/0064710. Other Cry proteins are well known to one skilled in the art (see, Crickmore, *et al.*, "Bacillus thuringiensis toxin nomenclature"(2011), at lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/ which can be accessed on the world-wide web using the "www" prefix). The insecticidal activity of Cry proteins is well known to one skilled in the art (for review, see, van Frannkenhuyzen, (2009) *J. Invert. Path.*101:1-16).

The use of Cry proteins as transgenic plant traits is well known to one skilled in the art and Cry-transgenic plants including but not limited to plants expressing Cry1Ac, Cry1Ac+Cry2Ab, Cry1Ab, Cry1A.105, Cry1F, Cry1Fa2, Cry1F+Cry1Ac, Cry2Ab, Cry3A, mCry3A, Cry3Bb1, Cry34Ab1, Cry35Ab1, Vip3A, Cry9c and CBI-Bt have received regulatory approval (see, Sanahuja, (2011) *Plant Biotech Journal* 9:283-300 and the CERA. (2010) GM Crop Database Center for Environmental Risk Assessment (CERA), ILSI Research Foundation, Washington D.C. at cera-gmc.org/index.php?action=gm_crop_database which can be accessed on the world-wide web using the "www" prefix). More than one pesticidal proteins well known to one skilled in the art can also be expressed in plants such as Vip3Ab & Cry1Fa (US2012/0317682); Cry1BE & Cry1F (US2012/0311746); Cry1CA & Cry1AB (US2012/0311745); Cry1F & CryCa (US2012/0317681); Cry1DA & Cry1BE (US2012/0331590); Cry1DA & Cry1Fa (US2012/0331589); Cry1AB & Cry1BE (US2012/0324606); Cry1Fa & Cry2Aa and Cry1I & Cry1E (US2012/0324605); Cry34Ab/35Ab and Cry6Aa (US20130167269); Cry34Ab/VCry35Ab & Cry3Aa (US20130167268); and Cry3A and Cry1Ab or Vip3Aa (US20130116170). Pesticidal proteins also include insecticidal lipases including lipid acyl hydrolases of US Patent Number 7,491,869, and cholesterol oxidases such as from *Streptomyces* (Purcell et al. (1993) *Biochem Biophys Res Commun* 15:1406-1413). Pesticidal proteins also include VIP (vegetative insecticidal proteins) toxins of US Patent Numbers 5,877,012, 6,107,279 6,137,033, 7,244,820, 7,615,686, and 8,237,020 and the like. Other VIP proteins are well known to one skilled in the art (see, lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html which can be accessed on the world-wide web using the "www" prefix). Pesticidal proteins also include toxin complex (TC) proteins, obtainable from organisms such as *Xenorhabdus, Photorhabdus* and *Paenibacillus* (see, US Patent Numbers 7,491,698 and 8,084,418). Some TC proteins have "stand alone" insecticidal activity and other TC proteins enhance the activity of the stand-alone toxins produced by the same given organism. The toxicity of a "stand-alone" TC protein (from *Photorhabdus, Xenorhabdus* or *Paenibacillus*, for example) can be enhanced by one or more TC protein "potentiators" derived from a source organism of a different genus. There are three main types of TC proteins. As referred to herein, Class

A proteins ("Protein A") are stand-alone toxins. Class B proteins ("Protein B") and Class C proteins ("Protein C") enhance the toxicity of Class A proteins. Examples of Class A proteins are TcbA, TcdA, XptA1 and XptA2. Examples of Class B proteins are TcaC, TcdB, XptB1Xb and XptC1Wi. Examples of Class C proteins are TccC, XptC1Xb and XptB1Wi. Pesticidal proteins also include spider, snake and scorpion venom proteins. Examples of spider venom peptides include but are not limited to lycotoxin-1 peptides and mutants thereof (US Patent Number 8,334,366).

The examples below describe some representative protocols and techniques for simulating plant insect feeding conditions and/or evaluating plants under such conditions.

1. Progeny of a transformed plant which is hemizygous with respect to a recombinant DNA construct, such that the progeny are segregating into plants either comprising or not comprising the recombinant DNA construct: the progeny comprising the recombinant DNA construct would be typically measured relative to the progeny not comprising the recombinant DNA construct(i.e., the progeny not comprising the recombinant DNA constructis the control or reference plant).

2. Introgression of a recombinant DNA construct into an inbred line, such as in maize, or into a variety, such as in soybean: the introgressed line would typically be measured relative to the parent inbred or variety line (i.e., the parent inbred or variety line is the control or reference plant).

3. Two hybrid lines, where the first hybrid line is produced from two parent inbred lines, and the second hybrid line is produced from the same two parent inbred lines except that one of the parent inbred lines contains a recombinant DNA construct: the second hybrid line would typically be measured relative to the first hybrid line (i.e., the first hybrid line is the control or reference plant).

4. A plant comprising a recombinant DNA construct: the plant may be assessed or measured relative to a control plant not comprising the recombinant DNA construct but otherwise having a comparable genetic background to the plant (e.g., sharing at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity of nuclear genetic material compared to the plant comprising the recombinant DNA construct. There are many laboratory-based techniques available for the analysis, comparison and

characterization of plant genetic backgrounds; among these are Isozyme Electrophoresis, Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Arbitrarily Primed Polymerase Chain Reaction (AP-PCR), DNA Amplification Fingerprinting (DAF), Sequence Characterized Amplified Regions (SCARs), Amplified Fragment Length Polymorphisms (AFLP®s), and Simple Sequence Repeats (SSRs) which are also referred to as Microsatellites.

Furthermore, one of ordinary skill in the art would readily recognize that a suitable control or reference plant to be utilized when assessing or measuring an agronomic characteristic or phenotype of a transgenic plant would not include a plant that had been previously selected, via mutagenesis or transformation, for the desired agronomic characteristic or phenotype.

"Pest" includes but is not limited to, insects, fungi, bacteria, nematodes, mites, ticks and the like. Insect pests include insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera Orthroptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Lepidoptera and Coleoptera.

Those skilled in the art will recognize that not all compounds are equally effective against all pests. Compounds of the embodiments display activity against insect pests, which may include economically important agronomic, forest, greenhouse, nursery ornamentals, food and fiber, public and animal health, domestic and commercial structure, household and stored product pests.

Larvae of the order Lepidoptera include, but are not limited to,armyworms, cutworms, loopers and heliothines in the family Noctuidae including *Spodoptera frugiperda* JE Smith (fall armyworm); *S. exigua* Hübner (beet armyworm); *S. litura* Fabricius (tobacco cutworm, cluster caterpillar); *Mamestra configurata* Walker (bertha armyworm); *M. brassicae* Linnaeus (cabbage moth); *Agrotis ipsilon* Hufnagel (black cutworm); *A. orthogonia* Morrison (western cutworm); *A. subterranea* Fabricius (granulate cutworm); *Alabama argillacea* Hübner (cotton leaf worm); *Trichoplusia ni* Hübner (cabbage looper); *Pseudoplusia includens* Walker (soybean looper); *Anticarsia gemmatalis* Hübner (velvetbean caterpillar); *Hypena scabra* Fabricius (green cloverworm); *Heliothis virescens* Fabricius

(*tobacco*budworm); *Pseudaletia unipuncta* Haworth (armyworm); *Athetis mindara* Barnes and Mcdunnough (rough skinned cutworm); *Euxoa messoria* Harris (darksided cutworm); *Earias insulana* Boisduval (spiny bollworm); *E. vittella* Fabricius (spotted bollworm); *Helicoverpa armigera* Hübner (American bollworm); *H. zea* Boddie (corn earworm or cotton bollworm); *Melanchra picta* Harris (zebra caterpillar); *Egira (Xylomyges) curialis* Grote (citrus cutworm); *Mythimna separate* (OrientalArmyworm); borers, casebearers, webworms, coneworms, grass moths from the family Crambidae including*Ostrinia furnacalis* (Asian Corn Borer) and *Ostrinia nubilalis* (European Corn Borer), and skeletonizers from the family Pyralidae *Ostrinia nubilalis* Hübner (European corn borer); *Amyelois transitella* Walker (naval orangeworm); *Anagasta kuehniella* Zeller (Mediterranean flour moth); *Cadra cautella* Walker (almond moth); *Chilo suppressalis* Walker (rice stem borer); *C. partellus*, (sorghum borer);*Corcyra cephalonica* Stainton (rice moth); *Crambus caliginosellus* Clemens (corn root webworm); *C. teterrellus* Zincken (bluegrass webworm); *Cnaphalocrocis medinalis* Guenée (rice leaf roller); *Desmia funeralis* Hübner (grape leaffolder); *Diaphania hyalinata* Linnaeus (melon worm); *D. nitidalis* Stoll (pickleworm); *Diatraea grandiosella* Dyar (southwestern corn borer), *D. saccharalis* Fabricius (surgarcane borer); *Eoreuma loftini* Dyar (Mexican rice borer); *Ephestia elutella* Hübner (tobacco (cacao) moth); *Galleria mellonella* Linnaeus (greater wax moth); *Herpetogramma licarsisalis* Walker (sod webworm); *Homoeosoma electellum* Hulst (sunflower moth); *Elasmopalpus lignosellus* Zeller (lesser cornstalk borer); *Achroia grisella* Fabricius (lesser wax moth); *Loxostege sticticalis* Linnaeus (beet webworm); *Orthaga thyrisalis* Walker (tea tree web moth); *Maruca testulalis* Geyer (bean pod borer); *Plodia interpunctella* Hübner (Indian meal moth); *Scirpophaga incertulas* Walker (yellow stem borer); *Udea rubigalis* Guenée (celery leaftier); and leafrollers, budworms, seed worms and fruit worms in the family Tortricidae *Acleris gloverana* Walsingham (Western blackheaded budworm); *A. variana* Fernald (Eastern blackheaded budworm); *Archips argyrospila* Walker (fruit tree leaf roller); *A. rosana* Linnaeus (European leaf roller); and other *Archips* species, *Adoxophyes orana* Fischer von Rösslerstamm (summer fruit tortrix moth); *Cochylis hospes* Walsingham (banded sunflower moth); *Cydia latiferreana* Walsingham (filbertworm); *C. pomonella* Linnaeus (coding moth); *Platynota flavedana* Clemens (variegated leafroller); *P. stultana* Walsingham (omnivorous

leafroller); *Lobesia botrana* Denis & Schiffermüller (European grape vine moth); *Spilonota ocellana* Denis & Schiffermüller (eyespotted bud moth); *Endopiza viteana* Clemens (grape berry moth); *Eupoecilia ambiguella* Hübner (vine moth); *Bonagota salubricola* Meyrick (Brazilian apple leafroller); *Grapholita molesta* Busck (oriental fruit moth); *Suleima helianthana* Riley (sunflower bud moth); *Argyrotaenia spp.*; *Choristoneura spp.*.

Selected other agronomic pests in the order Lepidoptera include, but are not limited to, *Alsophila pometaria* Harris (fall cankerworm); *Anarsia lineatella* Zeller (peach twig borer); *Anisota senatoria* J.E. Smith (orange striped oakworm); *Antheraea pernyi* Guérin-Méneville (Chinese Oak Tussah Moth); *Bombyx mori* Linnaeus (Silkworm); *Bucculatrix thurberiella* Busck (cotton leaf perforator); *Colias eurytheme* Boisduval (alfalfa caterpillar); *Datana integerrima* Grote & Robinson (walnut caterpillar); *Dendrolimus sibiricus* Tschetwerikov(Siberian silk moth), *Ennomos subsignaria* Hübner (elm spanworm); *Erannis tiliaria* Harris (linden looper); *Euproctis chrysorrhoea* Linnaeus (browntail moth); *Harrisina americana* Guérin-Méneville (grapeleaf skeletonizer); *Hemileuca oliviae* Cockrell (range caterpillar); *Hyphantria cunea* Drury (fall webworm); *Keiferia lycopersicella* Walsingham (tomato pinworm); *Lambdina fiscellaria fiscellaria* Hulst (Eastern hemlock looper); *L. fiscellarialugubrosa* Hulst (Western hemlock looper); *Leucoma salicis* Linnaeus (satin moth); *Lymantria dispar* Linnaeus (gypsy moth);*Manduca quinquemaculata*Haworth (five spotted hawk moth, tomato hornworm); *M. sexta* Haworth (tomato hornworm, tobacco hornworm); *Operophtera brumata* Linnaeus(winter moth); *Paleacrita vernata* Peck (spring cankerworm); *Papilio cresphontes* Cramer (giant swallowtail orange dog); *Phryganidia californica* Packard (California oakworm); *Phyllocnistis citrella* Stainton (citrus leafminer); *Phyllonorycter blancardella* Fabricius (spotted tentiform leafminer); *Pieris brassicae* Linnaeus (large white butterfly); *P. rapae* Linnaeus (small white butterfly); *P. napi* Linnaeus (green veined white butterfly); *Platyptilia carduidactyla* Riley (artichoke plume moth); *Plutella xylostella* Linnaeus (diamondback moth); *Pectinophora gossypiella* Saunders (pink bollworm); *Pontia protodice* Boisduval and Leconte (Southern cabbageworm); *Sabulodes aegrotata* Guenée (omnivorous looper); *Schizura concinna* J.E. Smith (red humped caterpillar); *Sitotroga cerealella* Olivier (Angoumois grain moth); *Thaumetopoea pityocampa* Schiffermuller(pine processionary caterpillar); *Tineola bisselliella* Hummel

(webbing clothesmoth); *Tuta absoluta* Meyrick (tomato leafminer); *Yponomeuta padella* Linnaeus (ermine moth); *Heliothis subflexa* Guenée; *Malacosoma spp.* and *Orgyia spp.*

Of interest are larvae and adults of the order Coleoptera including weevils from the families Anthribidae, Bruchidae and Curculionidae (including, but not limited to: *Anthonomus grandis* Boheman (boll weevil); *Lissorhoptrus oryzophilus* Kuschel (rice water weevil); *Sitophilus granarius* Linnaeus (granary weevil); *S. oryzae* Linnaeus (rice weevil); *Hypera punctata* Fabricius (clover leaf weevil); *Cylindrocopturus adspersus* LeConte (sunflower stem weevil); *Smicronyx fulvus* LeConte (red sunflower seed weevil); *S. sordidus* LeConte (gray sunflower seed weevil); *Sphenophorus maidis* Chittenden (maize billbug)); flea beetles, cucumber beetles, rootworms, leaf beetles, potato beetles and leafminers in the family Chrysomelidae (including, but not limited to: *Leptinotarsa decemlineata* Say (Colorado potato beetle); *Diabrotica virgiferavirgifera* LeConte (western corn rootworm); *D. barberi* Smith and Lawrence (northern corn rootworm); *D. undecimpunctata howardi* Barber (southern corn rootworm); *Chaetocnema pulicaria* Melsheimer (corn flea beetle); *Phyllotreta cruciferae* Goeze (Crucifer flea beetle); *Phyllotreta striolata* (stripped flea beetle); *Colaspis brunnea* Fabricius (grape colaspis); *Oulema melanopus* Linnaeus (cereal leaf beetle); *Zygogramma exclamationis* Fabricius (sunflower beetle)); beetles from the family Coccinellidae (including, but not limited to: *Epilachna varivestis* Mulsant (Mexican bean beetle)); chafers and other beetles from the family Scarabaeidae (including, but not limited to: *Popillia japonica* Newman (Japanese beetle); *Cyclocephala borealis* Arrow (northern masked chafer, white grub);*C. immaculata* Olivier (southern masked chafer, white grub); *Rhizotrogus majalis* Razoumowsky (European chafer); *Phyllophaga crinita* Burmeister (white grub); *Ligyrus gibbosus* De Geer (carrot beetle)); carpet beetles from the family Dermestidae; wireworms from the family Elateridae, *Eleodes* spp., *Melanotus* spp.; *Conoderus* spp.; *Limonius* spp.; *Agriotes* spp.; *Ctenicera* spp.; *Aeolus* spp.; bark beetles from the family Scolytidae and beetles from the family Tenebrionidae.

Adults and immatures of the order Diptera are of interest, including leafminers *Agromyza parvicornis* Loew (corn blotch leafminer); midges (including, but not limited to: *Contarinia sorghicola* Coquillett (sorghum midge); *Mayetiola destructor* Say (Hessian fly);

Sitodiplosis mosellana Géhin (wheat midge); *Neolasioptera murtfeldtiana* Felt, (sunflower seed midge)); fruit flies (Tephritidae), *Oscinella frit* Linnaeus (fruit flies); maggots (including, but not limited to: *Delia platura* Meigen (seedcorn maggot); *D. coarctata* Fallen (wheat bulb fly) and other *Delia* spp., *Meromyza americana* Fitch (wheat stem maggot); *Musca domestica* Linnaeus (house flies); *Fannia canicularis* Linnaeus, *F. femoralis* Stein (lesser house flies); *Stomoxys calcitrans* Linnaeus (stable flies)); face flies, horn flies, blow flies, *Chrysomya* spp.; *Phormia* spp. and other muscoid fly pests, horse flies *Tabanus* spp.; bot flies *Gastrophilus* spp.; *Oestrus* spp.; cattle grubs *Hypoderma* spp.; deer flies *Chrysops* spp.; *Melophagusovinus* Linnaeus (keds) and other *Brachycera*, mosquitoes *Aedes* spp.; *Anopheles* spp.; *Culex* spp.; black flies *Prosimulium* spp.; *Simulium* spp.; biting midges, sand flies, sciarids, and other *Nematocera*.

Included as insects of interest are adults and nymphs of the orders Hemiptera and Homoptera such as, but not limited to, adelgids from the family Adelgidae, plant bugs from the family Miridae, cicadas from the family Cicadidae, leafhoppers, *Empoasca* spp.; from the family Cicadellidae, planthoppers from the families Cixiidae, Flatidae, Fulgoroidea, Issidae and Delphacidae, treehoppers from the family Membracidae, psyllids from the family Psyllidae, whiteflies from the family Aleyrodidae, aphids from the family Aphididae, phylloxera from the family Phylloxeridae, mealybugs from the family Pseudococcidae, scales from the families Asterolecanidae, Coccidae, Dactylopiidae, Diaspididae, Eriococcidae Ortheziidae, Phoenicococcidae and Margarodidae, lace bugs from the family Tingidae, stink bugs from the family Pentatomidae, cinch bugs, *Blissus* spp.; and other seed bugs from the family Lygaeidae, spittlebugs from the family Cercopidae squash bugs from the family Coreidae and red bugs and cotton stainers from the family Pyrrhocoridae.

Agronomically important members from the order Homoptera further include, but are not limited to: *Acyrthisiphon pisum* Harris (pea aphid); *Aphis craccivora* Koch (cowpea aphid); *A. fabae* Scopoli (black bean aphid); *A. gossypii* Glover (cotton aphid, melon aphid); *A. maidiradicis* Forbes (corn root aphid); *A. pomi* De Geer (apple aphid); *A. spiraecola* Patch (spirea aphid); *Aulacorthum solani* Kaltenbach (foxglove aphid); *Chaetosiphon fragaefolii* Cockerell (strawberry aphid); *Diuraphis noxia* Kurdjumov/Mordvilko (Russian wheat aphid); *Dysaphis plantaginea* Paaserini (rosy apple aphid); *Eriosoma lanigerum*

Hausmann (woolly apple aphid); *Brevicoryne brassicae* Linnaeus (cabbage aphid); *Hyalopterus pruni* Geoffroy (mealy plum aphid); *Lipaphis erysimi* Kaltenbach (turnip aphid); *Metopolophium dirrhodum* Walker (cereal aphid); *Macrosiphum euphorbiae* Thomas (potato aphid); *Myzus persicae* Sulzer (peach-potato aphid, green peach aphid); *Nasonovia ribisnigri* Mosley (lettuce aphid); *Pemphigus* spp. (root aphids and gall aphids); *Rhopalosiphum maidis* Fitch (corn leaf aphid); *R. padi* Linnaeus (bird cherry-oat aphid); *Schizaphis graminum* Rondani (greenbug); *Sipha flava* Forbes (yellow sugarcane aphid); *Sitobion avenae* Fabricius (English grain aphid); *Therioaphis maculata* Buckton (spotted alfalfa aphid); *Toxoptera aurantii* Boyer de Fonscolombe (black citrus aphid) and *T. citricida* Kirkaldy (brown citrus aphid); *Adelges* spp. (adelgids); *Phylloxera devastatrix* Pergande (pecan phylloxera); *Bemisia tabaci* Gennadius (tobacco whitefly, sweetpotato whitefly); *B. argentifolii* Bellows & Perring (silverleaf whitefly); *Dialeurodes citri* Ashmead (citrus whitefly); *Trialeurodes abutiloneus* (bandedwinged whitefly) and *T. vaporariorum* Westwood (greenhouse whitefly); *Empoasca fabae* Harris (potato leafhopper); *Laodelphax striatellus* Fallen (smaller brown planthopper); *Macrolestes quadrilineatus* Forbes (aster leafhopper); *Nephotettix cinticeps* Uhler (green leafhopper); *N. nigropictus* Stål (rice leafhopper); *Nilaparvata lugens* Stål (brown planthopper); *Peregrinus maidis* Ashmead (corn planthopper); *Sogatella furcifera* Horvath (white-backed planthopper); *Sogatodes orizicola* Muir (rice delphacid); *Typhlocyba pomaria* McAtee (white apple leafhopper); *Erythroneoura* spp. (grape leafhoppers); *Magicicada septendecim* Linnaeus (periodical cicada); *Icerya purchasi* Maskell (cottony cushion scale); *Quadraspidiotus perniciosus* Comstock (San Jose scale); *Planococcus citri* Risso (citrus mealybug); *Pseudococcus* spp. (other mealybug complex); *Cacopsylla pyricola* Foerster (pear psylla); *Trioza diospyri* Ashmead (persimmon psylla).

Agronomically important species of interest from the order Hemiptera include, but are not limited to: *Acrosternum hilare* Say (green stink bug); *Anasa tristis* De Geer (squash bug); *Blissus leucopterus leucopterus* Say (chinch bug); *Corythuca gossypii* Fabricius (cotton lace bug); *Cyrtopeltis modesta* Distant (tomato bug); *Dysdercus suturellus* Herrich-Schäffer (cotton stainer); *Euschistus servus* Say (brown stink bug); *E. variolarius*Palisot de Beauvois (one-spotted stink bug); Graptostethus spp. (complex of

seed bugs); *Leptoglossus corculus* Say (leaf-footed pine seed bug); *Lygus lineolaris* Palisot de Beauvois (tarnished plant bug); *L. Hesperus* Knight (Western tarnished plant bug); *L. pratensis* Linnaeus (common meadow bug); *L. rugulipennis* Poppius(European tarnished plant bug); *Lygocoris pabulinus* Linnaeus (common green capsid); *Nezara viridula* Linnaeus (southern green stink bug); *Oebalus pugnax* Fabricius (rice stink bug); *Oncopeltus fasciatus* Dallas (large milkweed bug); *Pseudatomoscelis seriatus* Reuter (cotton fleahopper).

Furthermore, embodiments may be effective against Hemiptera such, *Calocoris norvegicus* Gmelin (strawberry bug); *Orthops campestris* Linnaeus; *Plesiocoris rugicollis* Fallen (apple capsid); *Cyrtopeltis modestus* Distant (tomato bug); *Cyrtopeltis notatus* Distant (suckfly); *Spanagonicus albofasciatus* Reuter (whitemarked fleahopper); *Diaphnocoris chlorionis* Say (honeylocust plant bug); *Labopidicola allii* Knight (onion plant bug); *Pseudatomoscelis seriatus* Reuter (cotton fleahopper); *Adelphocoris rapidus* Say (rapid plant bug); *Poecilocapsus lineatus* Fabricius (four-lined plant bug); *Nysius ericae* Schilling(false chinch bug); *Nysius raphanus* Howard (false chinch bug); *Nezara viridula* Linnaeus(Southern green stink bug); *Eurygaster spp.; Coreidae spp.; Pyrrhocoridae spp.; Tinidae spp.; Blostomatidae spp.; Reduviidae spp.* and *Cimicidae spp.*

Also included are adults and larvae of the order Acari (mites) such as *Aceria tosichella* Keifer (wheat curl mite); *Petrobia latens* Müller (brown wheat mite); spider mites and red mites in the family Tetranychidae, *Panonychus ulmi* Koch (European red mite); *Tetranychus urticae* Koch (two spotted spider mite); (*T.mcdanieli* McGregor (McDaniel mite); *T. cinnabarinus* Boisduval (carmine spider mite); *T. turkestani* Ugarov & Nikolski (strawberry spider mite); flat mites in the family Tenuipalpidae, *Brevipalpus lewisi* McGregor (citrus flat mite); rust and bud mites in the family Eriophyidae and other foliar feeding mites and mites important in human and animal health, i.e., dust mites in the family Epidermoptidae, follicle mites in the family Demodicidae, grain mites in the family Glycyphagidae, ticks in the order Ixodidae. *Ixodes scapularis* Say (deer tick); *I. holocyclus* Neumann (Australian paralysis tick); *Dermacentor variabilis* Say (American dog tick); *Amblyomma americanum* Linnaeus (lone star tick) and scab and itch mites in the families' Psoroptidae, Pyemotidae and Sarcoptidae.

Insect pests of the order Thysanura are of interest, such as *Lepisma saccharina* Linnaeus (silverfish); *Thermobia domestica* Packard (firebrat).

Additional arthropod pests covered include: spiders in the order Araneae such as *Loxosceles reclusa* Gertsch and Mulaik (brown recluse spider) and the *Latrodectus mactans* Fabricius (black widow spider) and centipedes in the order Scutigeromorpha such as *Scutigera coleoptrata* Linnaeus (house centipede).

Insect pest of interest include the superfamily of stink bugs and other related insects including but not limited to species belonging to the family *Pentatomidae (Nezara viridula, Halyomorpha halys, Piezodorus guildini, Euschistus servus, Acrosternum hilare, Euschistus heros, Euschistus tristigmus, Acrosternum hilare, Dichelops furcatus, Dichelops melacanthus,* and *Bagrada hilaris (*Bagrada Bug)), the family *Plataspidae (Megacopta cribraria* - Bean plataspid)andthe family *Cydnidae*(*Scaptocoris castanea* - Root stink bug) and Lepidoptera species including but not limited to: diamond-back moth, e.g., *Helicoverpa zea* Boddie; soybean looper, e.g., *Pseudoplusia includens* Walker and velvet bean caterpillar e.g., *Anticarsia gemmatalis* Hübner.

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

Methods for measuring pesticidal activity are well known in the art. See, for example, Czapla and Lang, (1990) *J. Econ. Entomol.*83:2480-2485; Andrews, *et al.*, (1988) *Biochem. J.* 252:199-206; Marrone, *et al.*, (1985) *J. of Economic Entomology* 78:290-293 and US Patent Number 5,743,477, all of which are herein incorporated by reference in their entirety. Generally, the protein is mixed and used in feeding assays. See, for example Marrone, *et al.*, (1985) *J. of Economic Entomology* 78:290-293. Such assays can include contacting plants with one or more pests and determining the plant's ability to survive and/or cause the death of the pests.

As used herein, the term "pesticidal activity" is used to refer to activity of an organism

or a substance (such as, for example, a protein), whether toxic or inhibitory, that can be measured by, but is not limited to, pest mortality, pest weight loss, pest repellency, pest growth stunting, and other behavioral and physical changes of a pest after feeding and exposure for an appropriate length of time. In this manner, pesticidal activity impacts at least one measurable parameter of pest fitness. Similarly, "insecticidal activity" may be used to refer to "pesticidal activity" when the pest is an insect pest. "Stunting" is intended to mean greater than 50% inhibition of growth as determined by weight. General procedures for monitoring insecticidal activity include addition of the experimental compound or organism to the diet source in an enclosed container. Assays for assessing insecticidal activity are well known in the art. See, e.g., U.S. Pat.Nos. 6,570,005 and 6,339,144; herein incorporated by reference in their entirety. The optimal developmental stage for testing for insecticidal activity is larvae or immature forms of an insect of interest. The insects may be reared in total darkness at about 20~ 30°C and about 30% ~ 70% relative humidity. Bioassays may be performed as described in Czapla and Lang (1990) J. Econ. Entomol. 83(6):2480-2485. Methods of rearing insect larvae and performing bioassays are well known to one of ordinary skill in the art.

Toxic and inhibitory effects of insecticidal proteins include, but are not limited to, stunting of larval growth, killing eggs or larvae, reducing either adult or juvenile feeding on transgenic plants relative to that observed on wild-type, and inducing avoidance behavior in an insect as it relates to feeding, nesting, or breeding as described herein, insect resistance can be conferred to an organism by introducing a nucleotide sequence encoding an insecticidal protein or applying an insecticidal substance, which includes, but is not limited to, an insecticidal protein, to an organism (e.g., a plant or plant part thereof).As used herein, "controlling a pest population" or "controls a pest" refers toany effect on a pest that results in limiting the damage that the pest causes. Controlling apest includes, but is not limited to, killing the pest, inhibiting development of the pest, alteringfertility or growth of the pest in such a manner that the pest provides less damage to theplant, decreasing the number of offspring produced, producing less fit pests, producing pestsmore susceptible to predator attack or deterring the pests from eating the plant.

Methods

Methods include but are not limited to methods for increasing tolerance in a plant to an insect pest, methods for evaluating insect resistance, methods for controlling an insect population, methods for killing an insect population, methods for controlling an insect population resistance to an insecticidal polypeptide, and methods for producing seed. The plant may be a monocotyledonous or dicotyledonous plant, for example, a rice, maize, *Arabidopsis*, soybean plant. The plant may also be sunflower, sorghum, canola, wheat, alfalfa, cotton, barley or millet. The seed may be a rice, maize, *Arabidopsis* or soybean seed, for example a maize hybrid seed or maize inbred seed.

Methods include but are not limited to the following:

A method for transforming a cell comprising transforming a cell with any of the isolated polynucleotides of the present disclosure. The cell transformed by this method is also included. In particular embodiments, the cell is eukaryotic cell, e.g., a yeast, insect or plant cell, or prokaryotic, e.g., a bacterium.

A method for producing a transgenic plant comprising transforming a plant cell with any of the isolated polynucleotides or recombinant DNA constructs of the present disclosureand regenerating a transgenic plant from the transformed plant cell. The disclosureis also directed to the transgenic plant produced by this method, and transgenic seed obtained from this transgenic plant.

A method for isolating a polypeptide of the disclosure from a cell or culture medium of the cell, wherein the cell comprises a recombinant DNA construct comprising a polynucleotide of the disclosure operably linked to at least one regulatory sequence, and wherein the transformed host cell is grown under conditions that are suitable for expression of the recombinant DNA construct.

A method of altering the level of expression of a polypeptide of the disclosure in a host cell comprising: (a) transforming a host cell with a recombinant DNA construct of the present disclosure; and (b) growing the transformed host cell under conditions that are suitable for expression of the recombinant DNA construct wherein expression of the recombinant DNA construct results in production of altered levels of the polypeptide of the disclosure in the transformed host cell.

A method of increasing tolerance in a plant to an insect pest comprising: (a) introducing

into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence (for example, a promoter functional in a plant), wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity, when compared to SEQ ID NO: 9, 12, 15 or18; and (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct and exhibits increased tolerance to an insect pest when compared to a control plant not comprising the recombinant DNA construct. The method may further comprise (c) obtaining a progeny plant derived from the transgenic plant, wherein said progeny plant comprises in its genome the recombinant DNA construct and exhibits increased tolerance to an insect pest when compared to a control plant not comprising the recombinant DNA construct.

A method of increasing tolerance in a plant to an insect pest, comprising: (a) introducing into a regenerable plant cell a DNA construct comprising at least one heterologous regulatory element as to operably link the regulatory element to a nucleic acid sequence encoding a COA26, ROMT17, ITP2 or KUN1 polypeptide in the plant genome; and (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the DNA construct, has increased expression of the COA26, ROMT17, ITP2 or KUN1 polypeptide, and exhibits increased tolerance to an insect pest when compared to a control plant not comprising the DNA construct. The method may further comprise (c) obtaining a progeny plant derived from the transgenic plant, wherein said progeny plant comprises in its genome the DNA construct, has increased expression of the COA26, ROMT17, ITP2 or KUN1 polypeptide and exhibits increased tolerance to an insect pest compared to a control plant not comprising the DNA construct.

In some embodiments methods are provided for controlling an insect pest comprising over-expressing in a plant a COA26, ROMT17, ITP2 or KUN1 polypeptide. In some embodiments the method for controlling an insect pest comprises transforming a plant or

plant cell with the DNA constructs of the present disclosure.

In some embodiments methods are provided for killing an insect pest comprising over expressing in a plant a COA26, ROMT17, ITP2 or KUN1 polypeptide. In some embodiments the method for killing an insect pest comprises transforming a plant or plant cell with the DNA constructs of the present disclosure.

A method of evaluating tolerance to an insect pest in a plant, comprising (a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence (for example, a promoter functional in a plant), wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identitywhen compared to SEQ ID NO: 9, 12, 15 or18; (b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; and (c) evaluating the transgenic plant for insect tolerancecompared to a control plant not comprising the recombinant DNA construct. The method may further comprise (d) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and (e) evaluating the progeny plant for insect tolerance compared to a control plant not comprising the recombinant DNA construct.

As used herein, "controlling a pest population" or "controls a pest" refers toany effect on a pest that results in limiting the damage that the pest causes. Controlling apest includes, but is not limited to, killing the pest, inhibiting development of the pest, alteringfertility or growth of the pest in such a manner that the pest provides less damage to theplant, decreasing the number of offspring produced, producing less fit pests, producing pestsmore susceptible to predator attack or deterring the pests from eating the plant.

A method of producing seed comprising any of the preceding methods, and further comprising obtaining seeds from said progeny plant, wherein said seeds comprise in their genome said recombinant DNA construct.

In some embodiments the disclosure provides seeds that comprise in their genome the

recombinant DNA construct of the disclosure.

Seed Treatment

To protect and to enhance yield production and trait technologies, seed treatment options can provide additional crop plan flexibility and cost effective control against insects, weeds and diseases. Seed material can be treated with one or more of the insecticidal proteins or polypeptides disclosed herein. For e.g., such seed treatments can be applied on seeds that contain a transgenic trait including transgenic corn, soy, brassica, cotton or rice. Combinations of one or more of the insecticidal proteins or polypeptides disclosed herein and other conventional seed treatments are contemplated. Seed material can be treated, typically surface treated, with a composition comprising combinations of chemical or biological herbicides, herbicide safeners, insecticides, fungicides, germination inhibitors and enhancers, nutrients, plant growth regulators and activators, bactericides, nematocides, avicides and/or molluscicides. These compounds are typically formulated together with further carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation. The coatings may be applied by impregnating propagation material with a liquid formulation or by coating with a combined wet or dry formulation. Examples of the various types of compounds that may be used as seed treatments are provided in The Pesticide Manual: A World Compendium, C.D.S. Tomlin Ed., and Published by the British Crop Production Council, which is hereby incorporated by reference.

Some seed treatments that may be used on crop seed include, but are not limited to, one or more of abscisic acid, acibenzolar-S-methyl, avermectin, amitrol, azaconazole, azospirillum, azadirachtin, azoxystrobin, Bacillus spp. (including one or more of cereus, firmus, megaterium, pumilis, sphaericus, subtilis and/or thuringiensis species), bradyrhizobium spp. (including one or more of betae, canariense, elkanii, iriomotense, japonicum, liaonigense, pachyrhizi and/or yuanmingense), captan, carboxin, chitosan, clothianidin, copper, cyazypyr, difenoconazole, etidiazole, fipronil, fludioxonil, fluoxastrobin, fluquinconazole, flurazole, fluxofenim, harpin protein, imazalil, imidacloprid, ipconazole, isoflavenoids, lipo-chitooligosaccharide, mancozeb, manganese, maneb, mefenoxam, metalaxyl, metconazole, myclobutanil, PCNB, penflufen, penicillium, penthiopyrad, permethrine, picoxystrobin, prothioconazole, pyraclostrobin, rynaxypyr, S-metolachlor,

saponin, sedaxane, TCMTB, tebuconazole, thiabendazole, thiamethoxam, thiocarb, thiram, tolclofos-methyl, triadimenol, trichoderma, trifloxystrobin, triticonazole and/or zinc. PCNB seed coat refers to EPA Registration Number 00293500419, containing quintozen and terrazole. TCMTB refers to 2-(thiocyanomethylthio) benzothiazole.

Seed varieties and seeds with specific transgenic traits may be tested to determine which seed treatment options and application rates may complement such varieties and transgenic traits in order to enhance yield. For example, a variety with good yield potential but head smut susceptibility may benefit from the use of a seed treatment that provides protection against head smut, a variety with good yield potential but cyst nematode susceptibility may benefit from the use of a seed treatment that provides protection against cyst nematode, and so on. Likewise, a variety encompassing a transgenic trait conferring tolerance to an insect pest may benefit from the second mode of action conferred by the seed treatment, a variety encompassing a transgenic trait conferring herbicide resistance may benefit from a seed treatment with a safener that enhances the plants resistance to that herbicide, etc. Further, the good root establishment and early emergence that results from the proper use of a seed treatment may result in more efficient nitrogen use, a better ability to withstand drought and an overall increase in yield potential of a variety or varieties containing a certain trait when combined with a seed treatment.

In any of the preceding methods or any other embodiments of methods of the present disclosure, the step of determining an alteration of an agronomic characteristic in a transgenic plant, if applicable, may comprise determining whether the transgenic plant exhibits an alteration of at least one agronomic characteristic when compared, under varying environmental conditions, to a control plant not comprising the recombinant DNA construct.

In any of the preceding methods or any other embodiments of methods of the present disclosure, the step of determining an alteration of an agronomic characteristic in a progeny plant, if applicable, may comprise determining whether the progeny plant exhibits an alteration of at least one agronomic characteristic when compared, under varying environmental conditions, to a control plant not comprising the recombinant DNA construct.

In any of the preceding methods or any other embodiments of methods of the present

disclosure, in said introducing step said regenerable plant cell may comprises a callus cell, an embryogenic callus cell, a gametic cell, a meristematic cell, or a cell of an immature embryo. The regenerable plant cells may derive from an inbred maize plant.

In any of the preceding methods or any other embodiments of methods of the present disclosure, said regenerating step may comprise: (i) culturing said transformed plant cells in a media comprising an embryogenic promoting hormone until callus organization is observed; (ii) transferring said transformed plant cells of step (i) to a first media which includes a tissue organization promoting hormone; and (iii) subculturing said transformed plant cells after step (ii) onto a second media, to allow for shoot elongation, root development or both.

In any of the preceding methods or any other embodiments of methods of the present disclosure, alternatives exist for introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence. For example, one may introduce into a regenerable plant cell a regulatory sequence (such as one or more enhancers, optionally as part of a transposable element), and then screen for an event in which the regulatory sequence is operably linked to an endogenous gene encoding a polypeptide of the instant disclosure.

The introduction of recombinant DNA constructs of the present disclosure into plants may be carried out by any suitable technique, including but not limited to direct DNA uptake, chemical treatment, electroporation, microinjection, cell fusion, infection, vector mediated DNA transfer, bombardment, or *Agrobacterium* mediated transformation. Techniques for plant transformation and regeneration have been described in International Patent Publication WO 2009/006276, the contents of which are herein incorporated by reference.

In addition, methods to modify or alter the host endogenous genomic DNA are available. This includes altering the host native DNA sequence or a pre-existing transgenic sequence including regulatory elements, coding and non-coding sequences. These methods are also useful in targeting nucleic acids to pre-engineered target recognition sequences in the genome. As an example, the genetically modified cell or plant described herein, is generated using "custom" engineered endonucleases such as meganucleases produced to modify plant genomes (e.g., WO 2009/114321; Gao et al. (2010) Plant Journal

1:176-187). Another site-directed engineering is through the use of zinc finger domain recognition coupled with the restriction properties of restriction enzyme (e.g., Urnov, et al. (2010) *Nat Rev Genet.* 11(9):636-46; Shukla, et al. (2009) *Nature* 459 (7245):437-41). A transcription activator-like (TAL) effector-DNA modifying enzyme (TALE or TALEN) is also used to engineer changes in plant genome. See e.g., US20110145940, Cermak et al., (2011) Nucleic Acids Res. 39(12) and Boch et al., (2009), Science 326(5959): 1509-12. Site-specific modification of plant genomes can also be performed using the bacterial type II CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system. See e.g., Belhaj et al., (2013), Plant Methods 9: 39; The CRISPR/Cas system allows targeted cleavage of genomic DNA guided by a customizable small noncoding RNA.

The development or regeneration of plants containing the foreign, exogenous isolated nucleic acid fragment that encodes a protein of interest is well known in the art. The regenerated plants are self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present disclosure containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

Stacking of traits in transgenic plant

Transgenic plants may comprise a stack of one or more insecticidal or insect tolerancepolynucleotides disclosed herein with one or more additional polynucleotides resulting in the production or suppression of multiple polypeptide sequences. Transgenic plants comprising stacks of polynucleotide sequences can be obtained by either or both of traditional breeding methods or through genetic engineering methods. These methods include, but are not limited to, breeding individual lines each comprising a polynucleotide of interest, transforming a transgenic plant comprising a gene disclosed herein with a subsequent gene and cotransformation of genes into a single plant cell. As used herein, the term "stacked" includes having the multiple traits present in the same plant (i.e., both traits are incorporated into the nuclear genome, one trait is incorporated into the nuclear genome and one trait is incorporated into the genome of a plastid or both traits are incorporated into

the genome of a plastid). In one non-limiting example, "stacked traits" comprise a molecular stack where the sequences are physically adjacent to each other. A trait, as used herein, refers to the phenotype derived from a particular sequence or groups of sequences. Co-transformation of genes can be carried out using single transformation vectors comprising multiple genes or genes carried separately on multiple vectors. If the sequences are stacked by genetically transforming the plants, the polynucleotide sequences of interest can be combined at any time and in any order. The traits can be introduced simultaneously in a co-transformation protocol with the polynucleotides of interest provided by any combination of transformation cassettes. For example, if two sequences will be introduced, the two sequences can be contained in separate transformation cassettes (trans) or contained on the same transformation cassette (cis). Expression of the sequences can be driven by the same promoter or by different promoters. In certain cases, it may be desirable to introduce a transformation cassette that will suppress the expression of the polynucleotide of interest. This may be combined with any combination of other suppression cassettes or overexpression cassettes to generate the desired combination of traits in the plant. It is further recognized that polynucleotide sequences can be stacked at a desired genomic location using a site-specific recombination system. See, for example, WO 1999/25821,WO 1999/25854, WO 1999/25840, WO 1999/25855 and WO 1999/25853, all of which are herein incorporated by reference.

EXAMPLES

The present disclosureis further illustrated in the following examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these examples, while indicating embodiments of the disclosure, are given by way of illustration only. From the above discussion and these examples, one skilled in the art can ascertain the essential characteristics of this disclosure, and without departing from the spirit and scope thereof, can make various changes and modifications of the disclosureto adapt it to various usages and conditions. Furthermore, various modifications of the disclosure in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are

also intended to fall within the scope of the appended claims.

EXAMPLE 1

Creation of a Rice Population with an Activation-Tagging Construct

A binary construct that contains four multimerized enhancers elements derived from the Cauliflower Mosaic Virus 35S (CaMV 35S) promoter was used, and the rice activation tagging population was developed from Zhonghua11 (*Oryza sativa L.*) which was transformed by *Agrobacteria*-mediated transformation method as described by Lin and Zhang ((2005) *Plant Cell Rep.* 23:540-547).Zhonghua11 was cultivated by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. The first batch of seeds used in this research was provided by Beijing Weiming Kaituo Agriculture Biotech Co.,Ltd.Calli induced from embryos was transformed with *Agrobacteria* with the vector. The transgenic lines generated were developed and the transgenic seeds were harvested to form the rice activation tagging population.

EXAMPLE 2

Seedling Screens to Identify Lines with Enhanced Tolerance to Asian Corn Borer(*Ostrinia furnacalis*) Insect under LaboratoryConditions

Asian corn borer (ACB) (*Ostrinia furnacalis* (Guenée)) is an important insect pest for maize in Asia. This insect is distributed from China to Australia and the Solomon Islands. In northern parts of its range, the moths have one or a few generations per year, but in the tropics, generations are continuous and overlapping. The caterpillars can cause severe yield losses in corn, both by damage to the kernels and by feeding on the tassels, leaves, and stalks. Survival and growth of the caterpillar is highest on the reproductive parts of the plant. Other economic plants attacked include bell pepper, ginger and sorghum. Recently, the Asian corn borer appears to have become an important pest of cotton. A number of wild grasses are also used as hosts(D. M. Nafusa & I. H. Schreinera. 2012. Review of the biology and control of the Asian corn borer, *Ostrinia furnacalis* (Lep: Pyralidae).*Tropical Pest Management.* 37: 41-56).

ACB insect was used to identify rice ATLs which can inhibit larva development. Asian corn borer populations were obtained from the Institute of Plant Protection of Chinese Academy of Agricultural Sciences. This population was reared for more than 10

generations at 25-27 °C, 60-80% relative humidity, under photo-period of 16L: 8D. The larvae were fed with artificial diet (Zhou Darong, Ye Zhihua, Wang Zhenying, 1995),and the eggs were hatched in incubator at 27 °C. The newly hatched larvae were used in assays.

The T_2 seeds which showed red color under green fluorescent light (transgenic seeds) wereused for insect toleranceassays except as otherwise specifically noted. One hundred fiftyseeds of each activation tagged line (ATL)weresterilized by 800 ppm carbendazol for 8 h at 32 °C and washed 3-5 times, then placed on a layer of wet gauze in petri dash (12 x12cm). The germinated seeds were cultured in distilled water at 28 °C for 10 days and the seedlings which were 8-10cm in height were used to feed ACB larvae.

Screening Method:

The 32-well plates (4 x 4 x 2 cm for each well)(Pitman, N.J. USA-609-582-2392)wereused andone-third volumeof 1% agar solution was filled in each well to keep humidity. The 32-well plate could be divided into 8 blocks with each block of 4 wells for one rice ATL seedlings. Twenty rice seedlings without seeds and roots were inserted into the agar, sixACB neonate larvaewere inoculated into the well with a brush, then special lids (Pitman, N.J. USA-609-582-2392)werecovered the well. The tissue cultured ZH11 (ZH11-TC)were used as control, and the control seedlings wererandomly placed in the blocks. The plates were placed in a chamber with temperature at 27.5 °C and 60%relative humidity,and rotated 90 degree each day from the second day. The insect larvae development was measured visually 5 days later, and the tolerant values were calculated.

The threelargest larvae in each well were selected, compared with the larvae in the well with ZH11-TC seedlings, and then a tolerantvalue was obtained according to Table 2. If the larvae in the control well developed to third instar, then the larval development was considered as normal and the tolerantvalue is 0;if the larvae developed to second instar, it was smaller compared to the normal developed larvae and the tolerant value is1;and if the larvaedeveloped to first instar, it is very smaller and the tolerant value is2.

Larvae growth inhibitory ratewas used as a parameter for ACB insect tolerance assay, which is the percentage of the inhibited number over the statistics number of larvae, wherein the inhibited number of larvae is the sum of the tolerant value of 12 test insects from four wells in one repeat and the statistics number of larvae is the sum of the number of

all the observed insects and number of larvae at 1st instar.Then the raw data wereanalyzed by Chi-square, the lines with P<0.01 wereconsidered as ACB tolerance positive lines.

Table 2.Scoring Scales for Asian corn borer and Oriental armyworm assays

Tolerantvalue	Instars of larvae	Size of larvae
0	3rd instar	Normal
1	2nd instar	Smaller
2	1st instar	Severe smaller

The ACB tolerant lines from the primary screenswill be re-screened in two continued screens (2nd and 3rd round of screens) with two repeats to confirm the insect tolerance. The ATLs which passed the 3rd screens were considered as ACB tolerant lines.

<u>Screening results:</u>

1) AH68151 seedlings

After ACB neonate larvaeinoculatingseedlings for 5 days in the screens, the seedlings of ZH11-TC were significantly damaged by ACB insects, while AH68151seedling were less damaged, and the insects fed with AH68151 was smaller thanthat fed withZH11-TC control.As shown in Table 3, 8of the 12 observed larvae with AH68151 seedlings developed to 2nd instar, whereas all of the 12 observed insects with ZH11-TC seedlings grew normally into 3rd instar.Thelarvae growth inhibitory rate of AH68151 was 66.67%, which was significantly greater than that of ZH11-TC seedlings (0.00%). These results show that AH68151 seedlings inhibited the development of ACB larvae. In the second screen, the larvae growth inhibitory rates of AH68151 in two repeats were 83.33% and 33.33%, respectively, whereas the larvae growth inhibitory rates of ZH11-TC controls both were 0.00%.The larvae growth inhibitory rates of AH68151 were significantly greater than ZH11-TC. The two repeats of AH68151 in the 3rd screening displayed the same trend. These results consistently demonstrate that feeding ACB with AH68151 seedlings can preventthe ACB larvae from developing intoadults.

Table 3.Asian corn borer assay of AH68151 seedlings under laboratory screening condition

Line ID	Screening round	Number of larvaeat 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	Pvalue	P≤0.01
AH68151	1st-1	0	8	12	66.67	0.0005	Y
ZH11-TC		0	0	12	0.00		

AH68151	2nd-1	0	10	12	83.33	0.0000	Y
ZH11-TC		0	0	12	0.00		
AH68151	2nd-2	0	4	12	33.33	0.0285	
ZH11-TC		0	0	12	0.00		
AH68151	3rd-1	0	7	12	58.33	0.0017	Y
ZH11-TC		0	0	12	0.00		
AH68151	3rd-2	0	8	9	88.89	0.0000	Y
ZH11-TC		0	0	12	0.00		

2) AH68231 seedlings

After ACB neonate larvae inoculating seedlings for 5 days in the screens, the seedlings of ZH11-TC were significantly damaged by ACB insects, while AH68231 seedling were less damaged, and the insects fed with AH68231 was smaller than that fed with ZH11-TC control. Table 4 shows the three rounds screening results for AH68231 seedlings. In the first screening,eight insects in AH68231 seedlings' wells developed into 2nd instar, while all observed 12 insects fed with ZH11-TC seedlings normally grew into 3rd instar.Thelarvae growth inhibitory rate of AH68231 (66.67%) was significantly greater than that of ZH11-TC seedlings (0.00%). These results indicated AH68231 seedlings inhibited the development of ACB larvae.Therefore, it was further screened. In the second screening, the larvae growth inhibitory rates of AH68231 in two repeats were 66.67% and 44.44%, respectively, which were significantly greater than that of their corresponding ZH11-TC controls. The larvae growth inhibitory rates of AH68231 seedlings were also significantly greater than that of their corresponding ZH11-TC controls in two repeats of 3rd round screening, respectively. These results clearly and consistently demonstrate that AH68231 seedling can inhibit the development of ACB insect and AH68231 was an ACB tolerantline.

Table 4. Asian corn borer assay of AH68231 seedlings under laboratory screening condition

Line ID	Screening round	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	*P*value	*P*≤0.01
AH68231	1st-1	0	8	12	66.67	0.0005	Y
ZH11-TC		0	0	12	0.00		
AH68231	2nd-1	0	8	12	66.67	0.0005	Y
ZH11-TC		0	0	12	0.00		
AH68231	2nd-2	0	4	9	44.44	0.0103	

ZH11-TC		0	0	12	0.00		
AH68231	3rd-1	0	12	12	100.00	0.0000	Y
ZH11-TC		0	0	12	0.00		
AH68231	3rd-2	0	7	9	77.78	0.0002	Y
ZH11-TC		0	0	12	0.00		

3) AH67515 seedlings

After ACB neonate larvae inoculating seedlings for 5 days in the screens, the seedlings of ZH11-TC were significantly damaged by ACB insects, while AH67515 seedling were less damaged, and the insects fed with AH67515 was smaller than that fed with ZH11-TC control. As shown in Table 5, in the first screening, after inoculating ACB neonate larvae on AH67515 seedlings, 9 insects developed to 2nd instar, whereas all observed 12 insects fed by ZH11-TC seedlings normally developed to 3rd instar. The larvae growth inhibitory rate of AH67515 seedling(75%) was significantly greater than that of ZH11-TC seedlings (0.00%). These results indicate thatAH67515 seedlings inhibited the development of ACBlarvae. One repeat was carried out in the second screening;the larvae growth inhibitory rate of AH67515 seedlings was58.33%,which wasalso significantly greater than ZH11-TC control. The two repeats of AH67515 seedlings in the 3rd screening displayed the same trend. These results consistently demonstrate that AH67515 seedling can inhibit the development of ACB insect and AH67515 was an ACB insect tolerance line.

Table 5.Asian corn borer assay of AH67515 seedlings under laboratory screening condition

Line ID	Screening round	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	*P*value	*P*≤0.01
AH67515	1st-1	0	9	12	75.00	0.0001	Y
ZH11-TC		0	0	12	0.00		
AH67515	2nd-1	0	7	12	58.33	0.0017	Y
ZH11-TC		0	0	12	0.00		
AH67515	3rd-1	0	2	6	33.33	0.0339	
ZH11-TC		0	0	12	0.00		
AH67515	3rd-2	0	9	12	75.00	0.0001	Y
ZH11-TC		0	0	12	0.00		

EXAMPLE 3

Cross-validation of ACB Tolerance ATLs with Oriental Armyworm *(Mythimna separata)* under LaboratoryConditions

Oriental armyworm (OAW) was used in cross-validations of insecticidal activity. OAWbelongs to Lepidoptera Noctuidae, and is a polyphagous insect pest.The eggs of OAW wereobtained from the Institute of Plant Protection of Chinese Academy of Agricultural Sciences and hatched in an incubator at 27°C.The neonate larvaewere used in this cross validation assay.

Rice ATL plants were cultured as described in Example 2, and the experiments design was similar as to ACB insect assay described in Example 2. Fivedays later, all the survived larvae were visually measuredand given tolerant values according to Table 2.

Larvae growth inhibitory ratewas used as a parameter for this insect tolerance assay, which is the percentage of the inhibited number over the statistics number of larvae, wherein the inhibited number is the sum of the tolerance value of all observedtest insects from four wells in one repeat and the statistics number of larvae is the sum of the number of all the observed insects and number of larvae at 1stinstar.

The raw data were analyzed by Chi-square, the lines with P<0.01 wereconsidered as OAW tolerantpositive lines.

Screening results:

Table 6 shows the OAW screening results of AH68151, AH68231,and AH67515. For AH68151 seedlings, only 1 larva of all observed 21 larvae in four wells developed to 3rd instar, 15 larvae developed to 2nd instar, and 5 larvae developed to 1st instar; while 18 larvae in the ZH11-TC control wells grew to 3rd instar and 3 larvae grew to 2nd instar. The larvae growth inhibitory rate of AH68151 seedlings was 96.15%, which was significantly greater than that of ZH11-TC control (14.29%). Fourlarvae of 21 observed larvae fed with AH68231 seedling developed to 3rd instar, 14 larvae developed to 2nd instar and 3 larvae developed to 1st instar. The larvae growth inhibitory rate of AH68231 seedlings was 83.33% and was significantly greater than its ZH11-TC control. AH67515 seedlings also exhibited greater larvae growth inhibitory rate (61.90%) than its ZH11-TC control. After OAW neonate larvae inoculating seedlings for 5 days in the screens, the seedlings of ZH11-TC

were significantly damaged by OAW insects, while the seedlings of AH68151, AH68231 and AH67515 were less damaged, and the insects fed with the transgenic seedlings was smaller than that fed with ZH11-TC control. These results demonstrate that all of these three ATLs also inhibit the development of OAW larvae and were OAW insect tolerant positive lines.

Table 6. Oriental armyworm assay of ATLsseedlings under laboratory screening condition

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	*P*value	*P*≤0.01
AH68151	5	15	21	96.15	0.0000	Y
ZH11-TC	0	3	21	14.29		
AH68231	3	14	21	83.33	0.0000	Y
ZH11-TC	0	3	21	14.29		
AH67515	1	11	20	61.90	0.0015	Y
ZH11-TC	0	3	21	14.29		

EXAMPLE 4

Cross-validation of ACB Tolerance Positive ATLswith Rice Stem Bore(*Chilo suppressalis*)underLaboratoryScreening Conditions

Ricestem borer(RCB) belongs to Lepidoptera Pyralidaeand it is a very important rice pest.They infest plants from the seedling stage to maturity. Although worldwide in distribution, rice stem borers are particularly destructive in Asia, the Middle East, and the Mediterranean regions.

The eggs of RSB wereobtained from the Institute of Plant Protection of Chinese Academy of Agricultural Sciences and hatched in an incubator at 27°C.The neonate larvaewere used in this cross validation assay.

ATLs seedlingswere cultured in greenhouse. Two types of lamps wereprovided as light source, i.e. sodium lamp and metal halide lamp, with the ratio of 1:1. Lamps provide the 16 h/8 h period of day/night, and were placed approximately 1.5m above the seedbed. The light intensity 30 cm above the seedbed is measured as 10,000-20,000 lx in sunny day, while 6,000-10,000lx in cloudy day, the relative humidity ranges from 30% to 90%, and the temperature ranges from 20 to 35°C. The tillered seedlings cultured with modified IRRI

nutrient solution for 40-d were used in this assay.

Screening method:

Two main stems of ATLs or ZH11-TC rice plants cultured for 40-d were cut into 7-8 cm, and inserted into agar in an 100 mL triangular flask, and then 10RSB neonate larvaewere inoculatedon the top of main stems with a brush in each triangular flask. The triangular flasks were placed in chamber with temperatureat 27.5 °C and 70%relative humidity. The ZH11-TC main stems were used as control, andsix repeats were designed in the experiments.

Mortality rate and larvae growth inhibitory rate were measured 7 day after inoculation. The mortality rate is the percentage of number of died larvae over the number of inoculated larvae, and the larvae growth inhibitory rate is the percentage of the sum of number of died larvae, number of larvae at 1st instar and number of larvae at 2nd instar over the number of inoculated larvae.

The raw data were analyzed by Chi-square, the lines with P<0.01 are considered as RSB tolerancepositive lines.

Screening results:

1) AH68151 stems

Of all the 60 RSB larvae fed with the AH68151 stems, 21 larvae died, 13 larvae grewinto 1st instar, and 26 larvae grew into 2nd instar; while 8 larvae fed with ZH11-TC controls died, 5 larvae grew into 2nd instar, and 47 larvae grew into 3rd instar.The mortality rate and larvae growth inhibitory rate of AH68151 main stems were 35% and 100%, respectively. The mortality rate and larvae growth inhibitory rate of ZH11-TCcontrolswere 13.33% and 21.67%, respectively.These results clearly show thatAH68151 can significantly inhibit the growth and developmentof RSB larvae.

2) AH68231 stems

ForAH68231 stems fed RSB larvae, 24 larvae died and 4 larvae developed to 2nd instar; whereas15 larvae fed with ZH11-TC controls died, and 2 larvae developed to 2nd instar. The mortality rate and larvae growth inhibitory rate of AH68231 main stems were greater than that of ZH11-TC main stems, indicating that AH68231 seedlings can inhibit the growth of RSB larvae. The inhibitory effect of AH68231 is significantly less than AH68151 and AH67515 (Table 7).

3) AH67515 stems

Two repeats were performed withAH67515 seedlings, 49 of all 60 inoculated RSB larvae died and 5 larvae developed to 2nd instar, the mortality rate and larvae growth inhibitory rate were 81.67% and 90.00%, respectively, in the first repeat. In the second repeat, the mortality rate and the inhibitory rate were 46.67% and 96.67%. The mortality rate and the inhibitory rate were significantly greater than that of their corresponding ZH11-TC controls. These results clearly demonstrate that AH67515 seedlings inhibit the development of RSB larvae, and AH67515 was a RSB insect tolerance positive line.

Table 7. Rice stem borer assay of ATLs seedlings under laboratory screening condition

Line ID	Number of dead larvae	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total larvae	Mortality rate (%)	Larvae growth inhibitory rate (%)	*P* value	*P*≤0.01
AH68151	21	13	26	60	35.00	100.00	0.0000	Y
ZH11-TC	8	0	5	60	13.33	21.67		
AH68231	24	0	4	60	40.00	46.67	0.2246	
ZH11-TC	15	0	2	60	25.00	28.33		
AH67515	49	0	5	60	81.67	90.00	0.0008	Y
ZH11-TC	15	0	2	60	25.00	28.33		
AH67515	28	11	19	60	46.67	96.67	0.0000	Y
ZH11-TC	8	0	5	60	13.33	21.67		

AH68151, AH68231and AH67515seedlings all showed significant inhibitory impact on the growth and development of ACB, OAW and RSB insects, indicating the potential broad spectrum of insecticidal activities.

In light of these results, the gene(s) which contributed to the enhanced insect tolerance of Line AH68151, AH68231,and AH67515, respectively, were isolated.

EXAMPLE 5

Identification of Activation-Tagged Genes

Genes flanking the T-DNA insertion locus in the insect tolerant lineAH68151, AH68231, AH67515were identified using one, or both, of the following two standard procedures: (1) Plasmid Rescue (Friedrich J. Behringer and June I. Medford. (1992), *Plant Molecular Biology Reporter* Vol. 10, 2:190-198); and (2) Inverse PCR (M. J. McPherson and Philip

Quirke. (1991), *PCR: a practical approach*, 137-146). For lines with complex multimerized T-DNA inserts, plasmid rescue and inverse PCR may both prove insufficient to identify candidate genes. In these cases, other procedures, includingTAIL PCR (Liu et al. (1995), *Plant J. 8*:457-463) can be employed.

A successful sequencing result is one where a single DNA fragment contains a T-DNA border sequence and flanking genomic sequence. Once a tag of genomic sequence flanking a T-DNA insert is obtained, candidate genes are identified by alignment to publicly available rice genome sequence. Specifically, the annotated gene nearest the 35S enhancer elements/T-DNA RB are candidates for genes that are activated.

To verify that an identified gene is truly near a T-DNA and to rule out the possibility that the DNA fragment is a chimeric cloning artifact, a diagnostic PCR on genomic DNA is done with one oligo in the T-DNA and one oligo specific for the local genomic DNA. Genomic DNA samples that give a PCR product are interpreted as representing a T-DNA insertion. This analysis also verifies a situation in which more than one insertion event occurs in the same line, e.g., if multiple differing genomic fragments are identified in Plasmid Rescue and/or Inverse-PCR analyses.

Genomic DNA was isolated from leaf tissues of the AH68151, AH68231and AH67515 lines using CTAB method (Murray, M.G. and W.F. Thompson. (1980) *Nucleic Acids Res.*8: 4321-4326).

The flanking sequences of T-DNA insertion locus wereobtainedby molecular technology.

The tandem T-DNAs were inserted between 24620468-24620511bp in chromosome 8 of AH68151 (MSU7.0 http://rice.plantbiology.msu.edu/index.shtml), and there were 75 bp deletionat the left Left-Border (LB) and 344 bp deletionat right LB of the T-DNA. The nucleotide sequences of left LB and right LB flanking sequence of T-DNA in AH68151 were shown as SEQ ID NO: 1 and 2.

For the AH68231 line, the LB of T-DNA was inserted at 31008857 bp in chromosome 1. The nucleotide sequences of LB flanking sequence of T-DNA in AH68231 were shown as SEQ ID NO: 3.

For the AH67515 line, the T-DNA was inserted between 26314055-26314087 bp in

chromosome 4. The nucleotide sequences of LB and RB flanking sequences of T-DNA in AH67515 were shown as SEQ ID NO: 4 and 5.

The expression levels of somegenes in ATL lines of AH68151, AH68231and AH67515 were identified by real-time RT-PCR analyses. Leaf, stem and root samples are collected from ATLs rice plants at 4-leaf-stage, and the total RNA was extracted using RNAiso Plus kit (TaKaRa) according to manufacturer's instruction separately. The cDNA were prepared by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) and from 500 ng total RNA. The real-time RT-PCR (SYBR[R]Premix Ex Taq™, TaKaRa) was conducted using 7,500 Fast real-time RT-PCR equipment and according to the manual (ABI). EF-1α gene is used as an internal control to show that the amplification and loading of samples from the ATL line and ZH-TC plants are similar. Gene expression is normalized based on the EF-1α mRNA levels.

The primers for real-time RT-PCR for the *OsKUN1* gene are listed below:

RP-23-F1:5'-GCATCCGCTTCAACGCC -3' (SEQ ID NO: 27)

RP-23-R1:5'-GTCCTGGCACGAGTCCCTG -3' (SEQ ID NO: 28)

As shown in FIG 1, the *OsKUN1* gene was significantly activated in AH67515 plants (leaf, stem and sheath) compared to the wild-type ZH11plants.

The genes showed in Table 8 were up-regulated compared to that of ZH11-TC or wild-type ZH11 control respectively. So, these genes were cloned and validated as to its functions in insect tolerance and other agronomic trait improvement.

Table 8. Rice insect tolerance gene names, Gene IDs(from TIGR) and Construct IDs

ATLs	Gene name	Gene ID	Construct ID
AH68151	*OsCOA26*	LOC_Os08g38920.1	DP0372
	OsROMT17	LOC_Os08g38910.2	DP0399
AH68231	*OsITP2*	LOC_Os01g53940.1	DP0378
AH67515	*OsKUN1*	LOC_Os04g44470.1	DP1251

EXAMPLE 6

Insect ToleranceGenes Cloning and Over-expression Vector Construction

Based on the sequence information of gene IDsshown in Table 8, primers were designed for cloning rice insect tolerance genes. The primers and the expected-lengths of the amplified genes are shown in Table 9.

For *OsROMT17* (DP0399) and *OsKUN1*(DP1251), cDNA was clonedfrom pooled

cDNA from leaf, stem and root tissues of Zhonghua 11 plant as the template. For *OsCOA26* (DP0372), and *OsITP2* (DP0378), their gDNAs were cloned, and amplified using genomic DNA of Zhonghua 11 as the template. The PCR reaction mixtures and PCR procedures are shown in Table 10 and Table 11.

Table 9. Primers for cloning insect tolerance genes

Primer	Sequence	SEQ ID NO:	Gene name	Length of amplified fragment (bp)
gc-3933	5'-TGCGCTGAGGCTCATGTAAGAGGTCCAGATAGCTAGAGAGG-3'	19	*OsCOA26*	1163
gc-3934	5'-ACGGCTGAGGGTACGACAAGATCAACACAACAG	20		
gc-3928	5'-TGCGCTGAGGCATCCCTCGTGTATATAGAGCTT	21	*OsROMT17*	971
gc-3929	5'-ACGGCTGAGGCCAAATCCAGCCCCACTTCAGTC	22		
gc-3988	5'-TGCGCTGAGGCTAATAGTGGTGAAACAAGGAGAGGAGAGC-3'	23	*OsITP2*	1725
gc-3989	5'-ACGGCTGAGGCATCCTCATGATTCACGGCGTAAAATTG-3'	24		
gc-8653	5'-TGCGCTGAGGCACTCCCCTCGTTTCGTCGTGCA	25	*OsKUN1*	664
gc-8654	5'-ACGGCTGAGGCCTCGTTTACTCTGGTGGGCTTG	26		

Table 10. PCR reaction mixture

Reaction mix	50 μL
Template	1 μL
TOYOBO KOD-FX (1.0 U/μL)	1 μL
2×PCR buffer for KOD-FX	25 μL
2 mM dNTPs (0.4 mM each)	10 μL
Primer-F/R (10 μM)	2 μL each
ddH_2O	9 μL

Table 11. PCR cycle conditions for cloning insect tolerance genes

Temperature	Time	
94 °C	3 min	
98 °C	10 s	×30
58 °C	30 s	
68 °C	(1 Kb/min) min	

68 °C 5 min

The PCR amplified products were extracted after the agarose gel electrophoresis using a column kit and then ligated with TA cloning vectors. The sequences and orientation in these constructs were confirmed by sequencing. These genes were cloned into plant binary construct DP0158 (pCAMBIA1300-DsRed) (SEQ ID NO: 6). Thegenerated over-expression vectors are listed in Table 8. The cloned nucleotide sequence in construct of DP0372 and coding sequence of *OsCOA26* are provided as SEQ ID NO: 7 and 8, the encoded amino acid sequence of OsCOA26 is SEQ ID NO: 9; the cloned nucleotide sequence in construct of DP0399 and coding sequence of *OsROTM17* are provided as SEQ ID NO: 10and 11, the encoded amino acid sequence of OsROMT17 is SEQ ID NO: 12; the cloned nucleotide sequence in construct of DP0378 and coding sequence of *OsITP2* are provided as SEQ ID NO: 13and 14, the encoded amino acid sequence of OsITP2 is SEQ ID NO: 15; and the cloned nucleotide sequence in construct of DP1251and coding sequence of *OsKUN1* are provided as SEQ ID NO: 16and 17, the encoded amino acid sequence of OsKUN1 is SEQ ID NO: 18.

Example 7

Transformation to Get the Transgenic Rice Lines

All of the over-expression vectors and empty vectors (DP0158) were transformed into Zhonghua11 (*Oryza sativa L.*) by *Agrobacteria*-mediated method as described by Lin and Zhang ((2005) *Plant Cell Rep.* 23:540-547). The transgenic seedlings (T_0) generated in transformation laboratory were transplanted in the field to get T_1 seeds. The T_1 and T_2 seedswere stored at cold room (4°C).The over-expression vectors contain DsRED and HYG genes. $T_1$and T_2 seeds which showed red color under green fluorescent light were transgenic seeds and were used inthe following insect tolerant assays.Transgene expression analysis in transgenic rice plants:

Transgene expression levels in the transgenic rice plants are analyzed by a standard real-time RT-PCR procedure, such as the QuantiTect® Reverse Transcription Kit from Qiagen® and Real-Time RT-PCR(SYBR^{R}Premix Ex Taq^{TM}, TaKaRa). EF1α gene is used as an internal control to show that the amplification and loading of samples from the transgenic rice and control plantare similar. The expression level is normalized based on the EF1α

mRNA levels.

OsCOA26 transgene expression levels in the DP0372 rice plants were detected using the following primers. As shown in Fig 2, the expression level in ZH11-TC rice is set at 1.00, the transgene expression level in DP0158 rice is similar to that of ZH11-TC, and Os*COA26* over-expressed in all the ten lines.

DP0372-F1: 5'-CTTCTCCGTGCTACTCAAG-3'(SEQ ID NO: 29)

DP0372-R1: 5'-GAACCCGACCATGTAGTC-3'(SEQ ID NO: 30)

As shown in Fig 3, the expression level of *OsROMT17*genein ZH11-TC rice is set at 1.00, the transgene expression level in DP0158 rice is similar to that of ZH11-TC, and *OsROMT17* over-expressed in all the ten lines.

DP0399-F1: 5'-GGCCTACGACAACACGCTCTGG-3'(SEQ ID NO: 31)

DP0399-R1: 5'-GGATGTCCTGGTCGAACTCCTCC-3'(SEQ ID NO: 32)

As shown in Fig 4. *OsITP2* over-expressed in the tested lines, while the expression levels of *OsITP2* were very low in the both controls of ZH11-TC and DP0158 seedlings.

DP0378-F3: 5'-CAACAAAGTTAGAGAGGCAAAGAG-3'(SEQ ID NO: 33)

DP0378-R4: 5'-GTAATTTGCACAAAGAAGTCATTG-3'(SEQ ID NO: 34)

As shown in Fig 5. *OsKUN1*over-expressed in the tested lines, while the expression levels of *OsKUN1* were not detected in the both controls of ZH11-TC and DP0158 seedlings.

DP1251-F1: 5'- CTACTACGTCCTCCCGGCTAG-3'(SEQ ID NO: 35)

DP1251-R1: 5'- CACCGCCGTACTTCTCCAC-3'(SEQ ID NO: 36)

Example 8

ACB Assay of *OsCOA26*-Transgenic Rice Plants under LaboratoryConditions

In order to investigate whether *OsCOA26*transgenic rice can recapitulate the insect tolerance trait of AH68151 line, the *OsCOA26*transgenic rice wasfirst tested against ACB insect. The ACB insect was reared as described in Example 2.

T_2 plants generated with the construct were tested in the assays for three times with sixor four repeats.The seedlings of ZH11-TC and DP0158 were used as controls. More than tenlines transgenic rice were testedand 450 seeds of each line were water cultured for 10 days as described in Example 2. This recapitulation assay usedrandomized block

design. Seedlings of each line were inserted in two wells of the 32-well-plate, and ZH11-TC and DP0158 seedlings were inserted in sixdifferentwells in the same plate.

Larvae growth inhibitoryrate was used as a parameter for ACB insect tolerance assay, which is the percentage of the inhibited larvae number over the statistics number of larvae, wherein the inhibited larvae number is the sum of the tolerancevalue of test insects from 12 or eightwells and the statistics number of larvaeis the sum of the number of all the observed insects and number of larvae at 1st instar.

Randomized block design was used, and 10-19 transgenic lines from a construct were tested in one experimental unit to evaluate the transgene function by SAS PROC GLIMMIX considering construct, line and environment effects. If the larvae growth inhibitory rates of the transgenic rice plants at both construct and line levels were significantly greater than controls (P<0.05), the gene was considered having ACB tolerant function.

ACB screening results:

1) Results of the first validation experiment

After ACB neonate larvae inoculating seedlings for 5 days in the assays, the seedlings of ZH11-TC and DP0158 were significantly damaged by ACB insects, while the *OsCOA26*transgenic seedlings were less damaged, and the insects fed with the *OsCOA26*transgenic seedlings was smaller than that fed with ZH11-TC and DP0158 controls.

Sixteen*OsCOA26*transgenic lines were placed on two separated plates, and repeated for 6 times. A total of 1152 ACB neonate larvae were inoculated on *OsCOA26*transgenic rice seedlings. Five days after inoculation, 974 larvae were found, 28 larvae developed into 1st instar, and 345 larvae developed to 2nd instar.Only nine larvae of all the observed 373 larvae in ZH11-TC seedlings' wells developed to 1st instar and 82 larvae developed to 2nd instar. Similar results were obtained with DP0158 seedlings, 9 larvae of all observed 387 larvae inoculatedon the DP0158 seedling developed to 1st instar, and 79 larvae developed to 2nd instar. The average larvae growth inhibitoryrates of *OsCOA26*transgenic rice, ZH11-TC and DP0158 were 41.43%, 26.19% and 24.68%, respectively. The average larvae growth inhibitory rate of *OsCOA26*transgenic rice was significantly greater than that of ZH11-TC (Pvalue=0.0000) and DP0158 (Pvalue=0.0000) controls. These results

showthat over-expression of *OsCOA26*in rice significantlyincreased ACB insect tolerance of transgenic rice at construct level.

Further analysis at transgenic line level is displayed in Table 12. The 16 lines of *OsCOA26*transgenic rice were placed on two different plates, and the DP0158 and ZH11-TC seedlings on the same plate were used as their controls. Nine transgenic lines were placed on the first plate, and the other 7 lines were placed on the other plate. Seven of 9lines exhibited greater larvae growth inhibitoryrates than ZH11-TC seedlings and all of the 9 lines exhibited greater larvae growth inhibitory rates than DP0158 seedlings in the first plate. All of the 7lines had greater larvae growth inhibitory rates than ZH11-TC seedlings and 5 of the 7 lines had greater larvae growth inhibitoryrates than DP0158 seedlings in the second plates. These results further indicate OsCOA26plays a role in increasing ACB insect tolerancein rice compared to controls at line level.

Table 12. Asian corn borer assay of *OsCOA26*transgenic riceunder laboratory screening condition at line level (1st experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP0372.01	4	15	68	31.94	0.4543		0.0201	Y
DP0372.05	2	18	64	33.33	0.2961		0.0104	Y
DP0372.08	1	26	65	42.42	0.0197	Y	0.0002	Y
DP0372.10	1	30	59	53.33	0.0003	Y	0.0000	Y
DP0372.17	3	14	57	33.33	0.2945		0.0117	Y
DP0372.21	0	13	56	23.21	0.6664		0.3296	
DP0372.24	5	32	62	62.69	0.0000	Y	0.0000	Y
DP0372.25	4	9	57	27.87	0.9609		0.1227	
DP0372.27	0	16	63	25.40	0.7948		0.2170	
ZH11-TC	6	39	182	27.13				
DP0158	5	28	199	18.63				
DP0372.31	1	19	61	33.87	0.1917		0.6239	
DP0372.36	2	32	61	57.14	0.0000	Y	0.0005	Y
DP0372.37	2	22	68	37.14	0.0643		0.3132	
DP0372.39	1	32	35	94.44	0.0000	Y	0.0000	Y
DP0372.40	1	31	65	50.00	0.0005	Y	0.0063	Y
DP0372.41	0	19	67	28.36	0.6208		0.7381	

DP0372.42	1	17	66	28.36	0.6403	0.7173
ZH11-TC	3	43	191	25.26		
DP0158	4	51	188	30.73		

2) Results of the second validation experiment

Ten *OsCOA26*transgenic lines which showed higher larvae growth inhibitory rates in the first validation experiment were selected and tested in this second experiment. The ten lines were placed on one 32-wellplate, and repeated for 6 times.A total of 720 ACB neonate larvae were inoculated on *OsCOA26*transgenic rice seedlings. Five days after inoculation, 600 larvae were found, 20 larvae developed into 1st instar, and 135 larvae developed to 2nd instar.Only 4 larvae of all the observed 197 larvae in ZH11-TC seedlings' wells developed to 1st instar and 30 larvae developed to 2nd instar. Similar results were obtained with DP0158 seedlings, 3 larvae of all observed 190 larvae inoculated on the DP0158 seedling developed to 1st instar, and35 larvae developed to 2nd instar. The average larvae growth inhibitory rates of *OsCOA26*transgenic rice, ZH11-TC and DP0158 were 28.23%, 18.91% and 21.24%, respectively. The average larvae growth inhibitory rate of *OsCOA26*transgenic rice was significantly greater than that of ZH11-TC (*P* value=0.0139) and greater than that of DP0158 (*P*value=0.0703) controls. These results show that over-expression of *OsCOA26*in riceincreased ACB insect tolerance of transgenic rice at construct level.

Further analysis at transgenic line level is displayed in Table 13. Seven of the ten transgenic lines exhibited greater larvae growth inhibitory rates than ZH11-TC and DP0158 seedlings. The larvae growth inhibitory rate of line DP0372.39 is 65.31%, is greatest. The result was same to that in the first validation experiment. These results further indicate OsCOA26plays a role in increasing ACB insect tolerance in rice compared to controls at line level.

Table 13.Asian corn borer assay of *OsCOA26*transgenic rice under laboratory screen condition at line level (2nd experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP0372.01	1	13	61	24.19	0.3825		0.6446	
DP0372.05	2	13	48	34.00	0.0321	Y	0.0794	
DP0372.08	5	10	64	28.99	0.0869		0.2006	

DP0372.10	3	17	66	33.33	0.0163	Y	0.0481	Y
DP0372.17	3	10	66	23.19	0.4632		0.7579	
DP0372.24	2	9	66	19.12	0.9706		0.7113	
DP0372.36	2	12	57	27.12	0.1888		0.3657	
DP0372.37	0	12	59	20.34	0.8071		0.8830	
DP0372.39	2	28	47	65.31	0.0000	Y	0.0000	Y
DP0372.40	0	11	66	16.67	0.6909		0.4325	
ZH11-TC	4	30	197	18.91				
DP0158	3	35	190	21.24				

3) Results of the third validation experiment

The same ten lines were further tested in this third experiment. The ten lines were placed on one 32-wellplate, and repeated for 4 times. Five days after inoculation, 388 larvae were found, 19 larvae developed into 1st instar, and 123 larvae developed to 2nd instar.Only one larva of all the observed 120 larvae in ZH11-TC seedlings' wells developed to 1st instar and 24 larvae developed to 2nd instar. Five larvae of all observed 121 larvae inoculated on the DP0158 seedling developed to 1st instar, and 27 larvae developed to 2nd instar. The average larvae growth inhibitory rates of *OsCOA26*transgenic rice, ZH11-TC and DP0158 were 39.56%, 21.49% and 29.37%, respectively. The average larvae growth inhibitory rate of *OsCOA26*transgenic rice was significantly greater than that of ZH11-TC (*P* value=0.0010) and greater than that of DP0158 (*P* value=0.0536) controls.

Further analysis at transgenic line level is displayed in Table 14. Nine of ten lines had greater larvae growth inhibitory rates than that of ZH11-TC and DP 0158 seedlings, and six lines had significantly greater larvae growth inhibitory rate than that of ZH11-TC. The larvae growth inhibitory rates of five lines were more than 40%.

The line of DP0372.39 had the greatest larvae growth inhibitory rate in three experiments and the line DP0372.24 show less larvae growth inhibitory rate in two experiments. These results clearly demonstrate that*OsCOA26* transgenic rice inhibited the development of ACB insect, the transgenic rice obtained enhanced ACB insect tolerance at seedling stage, and OsCOA26plays a role in increasing ACB insect tolerance in plants.

Table14.Asian corn borer assay of *OsCOA26*transgenic rice under laboratory screen condition at line level (3rd experiment)

Line ID	Number	Number of	Number	Larvae	CK=ZH11-TC		CK=DP0158	

	of larvae at 1st instar	larvae at 2nd instar	of total observed larvae	growth inhibitory rate (%)	*P* value	*P*≤0.05	*P* value	*P*≤0.05
DP0372.01	3	14	42	44.44	0.0059	Y	0.0737	
DP0372.05	3	8	35	36.84	0.0661		0.3882	
DP0372.08	4	11	43	40.43	0.0179	Y	0.1748	
DP0372.10	2	12	39	39.02	0.0339	Y	0.2558	
DP0372.17	2	16	41	46.51	0.0044	Y	0.0640	
DP0372.24	0	8	39	20.51	0.8976		0.2861	
DP0372.36	0	12	34	35.29	0.1083		0.5095	
DP0372.37	1	16	42	41.86	0.0129	Y	0.1126	
DP0372.39	3	15	31	61.76	0.0000	Y	0.0015	Y
DP0372.40	1	11	42	30.23	0.2551		0.9148	
ZH11-TC	1	24	120	21.49				
DP0158	5	27	121	29.37				

EXAMPLE 9

OAW Assay of *OsCOA26*Transgenic Rice Plants under Laboratory Conditions

OAW assay of *OsCOA26*transgenic rice were performed as described in Example 3. Larvae growth inhibitory ratewas used as a parameter for this insect tolerance assay, which is the percentage of the inhibited number over the statistics number of larvae, wherein the inhibited number is the sum of the tolerance value of all observed test insects fromeight or twelvewells and the statistics number of larvae is the sum of the number of all the observed insects and number of larvae at 1st instar.

OAW screening results:

Ten transgenic lines which were tested in the ACB assay were used in this assay. These ten rice lines were placed in one 32-well plate with four repeats. Five days after larvae inoculation, 11 larvae of 312 larvae found in the *OsCOA26*transgenic rice well developed to 1st instar, and 90 larvae developed to 2nd instar. The OAW larvae inhibitory rate was 34.67%. While, 8 of the 99 larvae in the ZH11-TC wells developed to 1st instar, and 10 larvae developed to 2nd instar. The larvae growth inhibitory rate of ZH11-TC seedlings was 24.30%. 5 of 108 larvae in the DP0158 seedling well developed to 1st instar, and 18 larvae developed to 2nd instar. The larvae growth inhibitory rate was 24.78%. The OAW larvae growth inhibitory rate of *OsCOA26*transgenic rice was greater than ZH11-TC (*P*value

=0.0657) and DP0158 (*P* value=0.0736) controls.

Analysis at line level was displayed in Table 15. Nine of ten lines had greater OAW larvae growth inhibitory rates than that of both ZH11-TC and DP0158 controls. The line DP0372.39 which showed greatest ACB larvae growth inhibitory rate also had greatest OAW larvae growth inhibitory rate in the ten tested lines. These results indicated that *OsCOA26*transgenic rice inhibit the development of OAW larvae and had enhanced OAW insect tolerance at seedling stage.

Table 15.Armworm assay of *OsCOA26*transgenic rice under laboratory screen condition atline level

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP0372.01	0	7	32	21.88	0.7786		0.7363	
DP0372.05	1	7	26	33.33	0.3466		0.3717	
DP0372.08	1	6	31	25.00	0.9358		0.9797	
DP0372.10	0	10	33	30.30	0.4945		0.5284	
DP0372.17	1	11	38	33.33	0.2824		0.3063	
DP0372.24	0	12	33	36.36	0.1825		0.1985	
DP0372.36	3	4	25	35.71	0.2331		0.2518	
DP0372.37	1	8	29	33.33	0.3276		0.3524	
DP0372.39	2	15	30	59.38	0.0008	Y	0.0009	Y
DP0372.40	2	10	35	37.84	0.1225		0.1342	
ZH11-TC	8	10	99	24.30				
DP0158	5	18	108	24.78				

EXAMPLE 10

RSB Assay of *OsCOA26*Transgenic Rice Plants under Greenhouse Conditions

RSB assay was performed to investigate whether OsCOA26has RSB tolerance function. The eggs of RSB wereobtained from the Institute of Plant Protection of Chinese Academy of Agricultural Sciences and hatched in an incubator at 27°C.

Three *OsCOA26*transgenic lines which showed better ACB and OAW insect tolerance were tested, and were cultured in greenhouse. Two types of lamps are provided as light source, i.e. sodium lamp and metal halide lamp, the ratio is 1:1. Lamps provide the 16 h/8 h period of day/night, and are placed approximately 1.5 m above the seedbed. The light

intensity 30 cm above the seedbed is measured as 10,000-20,000 lx in sunny day, while 6,000-10,000 lx in cloudy day, the relative humidity ranges from 30% to 90%, and the temperature ranges from 20 to 35 °C. The tillered seedlings cultured with IRRI nutrient solution for 40-d were used in this assay.

Screening method:

Twelve plants of each line were tested. When cultured for 40-d, the tillers except the main tiller were removed, one neonate RSB larva was inoculated on the new leaf of one rice plant, and then the plate was covered by a yarn net cage to avoid the moth entering in the greenhouse. Each line wasrepeated for four times. After cultured for 40-d at 30~35°C in greenhouse, the withered heart rate and mortality rate were calculated using one way ANOVA. When the *P*value≤ 0.05, the transgenic plants will be considered as RSB tolerant.

Rice plants with withered heart are considered as plants damaged by RSB.

The withered heart rate is percentage of number of damaged plants with withered heart over the number of total plants. The mortality rate is percentage of the number of dead plants over the number of total plants.

Screening results:

DP0372.08, DP0372.10 and DP0372.39 were selected and tested.After fed with RSB for 40-d, 13 DP0372.08 rice plants, nine DP0372.10 rice plants and 15 DP0372.39 rice plants survived, while only three DP0158rice plants survived. As shown in Table 16, the withered heart rate and morality rate of DP0372.39 rice plants were significantly lower than that of DP0158control and the morality rate of DP0372.08 and DP0372.10rice plants significantly lower than that of DP0158 control. These results indicate that *OsCOA26*transgenic rice plants had improved tolerance against RSB insect.

Table 16.Rice stem borer assay of *OsCOA26*transgenic rice under greenhouse screen condition at line level

Line ID	Number of total plant	Number of plant with withered heart	Number of survival plant	Withered heart rate (%)	*P* value	Mortality plants (%)	*P* value
DP0372.08	48	47	13	97.92	0.3559	72.92	0.0036
DP0372.10	48	47	9	97.92	0.3559	81.25	0.1763
DP0372.39	48	43	15	89.58	0.0025	68.75	0.0300
DP0158	48	48	3	100.00		93.75	

In summary, *OsCOA26* transgenic rice plants inhibited the development of ACB and

OAW insect larvae, and obtained ACB and OAW insect tolerance at seedling stage; and *OsCOA26*transgenic rice plants exhibited improved tolerance against RSB insect. These results showed *OsCOA26* transgenic rice had significant inhibitory impact on the growth and development of ACB, OAW and RSB insects, indicating that OsCOA26 plays insecticidalactivities in the potential broad spectrum.

EXAMPLE 11

ACB Assay of *OsROMT17*Transgenic Rice Plants under Laboratory Conditions

In order to investigate whether *OsROMT17*transgenic rice can recapitulate the insect tolerance trait of AH68151 line, the*OsROMT17*transgenic rice was tested against ACB insect. The method is described in Example 8.

ACB screening results:

1) Results of first validation experiment

After ACB neonate larvae inoculating seedlings for 5 days in the assays, the seedlings of ZH11-TC and DP0158 were significantly damaged by ACB insects, while the *OsROMT17*transgenic seedlings were less damaged, and the insects fed with the *OsROMT17*transgenic seedlings was smaller than that fed with ZH11-TC and DP0158 controls.

Ten*OsROMT17*transgenic lines were placed on one 32-well plate with6 repeats. A total of 486 ACB neonate larvae were found in *OsROMT17*transgenic seedlings wells, wherein 12 larvae developed to 1st instar and 198 larvae developed to 2nd instar, the average larvae growth inhibitory rate was 44.58%; while 184 larvae were found in ZH11-TC seedling wells, 4 larvae developed to 1st instar and 35 larvae developed to 2nd instar; and 5 larvae of all observed 200 larvae inoculated on the DP0158 seedling developed to 1st instar, and 30 larvae developed to 2nd instar, the other 165 larvae normally developed to 3rd instar. The average larvae growth inhibitory rates of ZH11-TC seedlings and DP0158seedling were 22.87% and 19.51%, respectively. The average larvae growth inhibitory rate of *OsROMT17*transgenic rice was significantly greater than that of ZH11-TC(*P*value=0.0000) and DP0158 (*P*value=0.0000) controls. These results demonstrate that over-expression of *OsROMT17* increased ACB insect tolerances of transgenic rice at construct level.

Further analysis at transgenic line level is displayed in Table 17. The larvae growth

inhibitory rates of 8 lines were more than 35%, significantly greater than that of ZH11-TC and DP0158seedlings. One line (DP0399.50) had slightly greater larvae growth inhibitory rates compared to ZH11-TC and DP0158 seedlings. These results consistently demonstrate that *OsROMT17*transgenic rice showed inhibitory impact on ACB larval growth and *OsROMT17* plays a role in increasing ACB insect tolerance of transgenic rice seedlings at construct and line levels.

Table 17. Asian corn borer assay of *OsROMT17*transgenicrice under laboratory screening condition at line level (1st experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth Inhibitory rate (%)	CK=ZH11-TC		CK =DP0158	
					*P*value	*P*≤0.05	*P*value	*P*≤0.05
DP0399.01.	1	19	28	72.41	0.0000	Y	0.0000	Y
DP0399.06	0	28	40	70.00	0.0000	Y	0.0000	Y
DP0399.07	1	19	40	51.22	0.0007	Y	0.0001	Y
DP0399.09	1	10	64	18.46	0.4609		0.8521	
DP0399.13	0	25	66	37.88	0.0221	Y	0.0040	Y
DP0399.26	3	18	60	38.10	0.0224	Y	0.0041	Y
DP0399.30	1	22	34	68.57	0.0000	Y	0.0000	Y
DP0399.49	3	23	63	43.94	0.0020	Y	0.0003	Y
DP0399.50	2	15	65	28.36	0.3729		0.1341	
DP0399.51	0	19	26	73.08	0.0000	Y	0.0000	Y
ZH11-TC	4	35	184	22.87				
DP0158	5	30	200	19.51				

2) Results of second validation experiment

The same ten*OsROMT17*transgenic lines were placed on one 32-well plate with6 repeats. A total of 464 ACB neonate larvae were found in *OsROMT17*transgenic seedlings wells, wherein 4 larvae developed to 1st instar and 118 larvae developed to 2nd instar, the average larvae growth inhibitory rate was 26.92%; while 175 larvae were found in ZH11-TC seedling wells, 5 larvae developed to 1st instar and 29 larvae developed to 2nd instar; and 25 larvae of all observed 187 larvae inoculated on the DP0158 seedling developed to 2nd instar. The average larvae growth inhibitory rates of ZH11-TC seedlings and DP0158 seedling were 21.67% and 13.37%, respectively. The average larvae growth inhibitory rate of *OsROMT17*transgenic rice was significantly greater than that of DP0158 (*P*

value=0.0003) and greater than that of ZH11-TC (*P*value=0.1215) controls. These results demonstrate that over-expression of *OsROMT17* increased ACB insect tolerances of transgenic rice seedlings at construct level.

Further analysis at transgenic line level is displayed in Table 18. Eight of ten lines had greater larvae growth inhibitory rates than that of both ZH11-TC and DP0158 controls, five lines had significantly greater larvae growth inhibitory rates than that of DP0158 controls.These results demonstrate that *OsROMT17*transgenic rice showed inhibitory impact on ACB larval growth and *OsROMT17* plays a role in increasing ACB insect tolerance of transgenic rice seedlings at construct and line levels.

Table 18. Asian corn borer assay of *OsROMT17*transgenicrice under laboratory screening condition at line level (2nd experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP0399.01	0	12	25	48.00	0.0081	Y	0.0002	Y
DP0399.06	0	12	48	25.00	0.6156		0.0558	
DP0399.07	0	12	49	24.49	0.6713		0.0651	
DP0399.09	0	10	63	15.87	0.3302		0.6261	
DP0399.13	3	10	41	36.36	0.0483	Y	0.0010	Y
DP0399.26	0	11	42	26.19	0.5218		0.0465	Y
DP0399.30	0	10	38	26.32	0.5212		0.0520	
DP0399.49	0	12	42	28.57	0.3368		0.0209	Y
DP0399.50	0	12	61	19.67	0.7480		0.2375	
DP0399.51	1	17	55	33.93	0.0674		0.0012	Y
ZH11-TC	5	29	175	21.67				
DP0158	0	25	187	13.37				

3) Results of third validation experiment

The same ten lines were tested with three repeats. A total of 278 ACB neonate larvae were found in *OsROMT17*transgenic seedlings wells, wherein 10 larvae developed to 1st instar and 87 larvae developed to 2nd instar, the average larvae growth inhibitory rate was 37.15%; while 94 larvae were found in ZH11-TC seedling wells, 5 larvae developed to 1st instar and 27 larvae developed to 2nd instar; and 3 larvae of all observed 91 larvae

inoculated on the DP0158 seedling developed to 1st instar, and 26 larvae developed to 2nd instar. The average larvae growth inhibitory rates of ZH11-TC seedlings and DP0158 seedling were 37.37% and 34.04%, respectively. The average larvae growth inhibitory rate of *OsROMT17* transgenic rice was greater than that of ZH11-TC (*P* value=0.8525) and DP0158 (*P* value=0.7045) controls.

Further analysis at transgenic line level is displayed in Table 19. Six of ten lines had greater larvae growth inhibitory rates than both of ZH11-TC and DP0158 controls.

In summary, these results demonstrate that *OsROMT17* transgenic rice showed inhibitory impact on ACB larval growth and *OsROMT17* plays a role in increasing ACB insect tolerance of transgenic rice seedlings.

Table 19. Asian corn borer assay of *OsROMT17* transgenic rice under laboratory screening condition at line level (3rd experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP0399.01	1	1	8	33.33	0.8118		0.9660	
DP0399.06	1	7	30	29.03	0.4044		0.6103	
DP0399.07	0	16	34	47.06	0.3285		0.1908	
DP0399.09	1	3	33	14.71	0.0247		0.0471	
DP0399.13	2	11	25	55.56	0.1022		0.0551	
DP0399.26	0	7	26	26.92	0.3309		0.4988	
DP0399.30	2	10	34	38.89	0.8734		0.6089	
DP0399.49	1	11	25	50.00	0.2534		0.1501	
DP0399.50	0	13	34	38.24	0.9293		0.6640	
DP0399.51	2	8	29	38.71	0.8943		0.6405	
ZH11-TC	5	27	94	37.37				
DP0158	3	26	91	34.04				

EXAMPLE 12

OAW Assay of *OsROMT17* Transgenic Rice Plants under Laboratory Conditions

OAW assay of *OsROMT17* transgenic rice was performed as described in Example 9. The screening results as below.

Ten same *OsROMT17* transgenic rice lines tested in ACB assay were tested in OAW assay. These ten lines were placed on the one 32-well plate with four repeats. Five days

after co-culture, 403 larvae were found in the *OsROMT17*transgenic rice wells, wherein 69 OAW larvae developed to 2nd instar, while 15 of the 139 larvae in the ZH11-TC well developed to 2nd instar, and 8 of 139 larvae in the DP0158 well developed to 2nd instar. The average OAW larvae growth inhibitory rates of *OsROMT17*transgenic rice, ZH11-TC and DP0158were 17.12%, 10.79% and 5.76%. The OAW larvae growth inhibitory rate of *OsROMT17*transgenic rice was significantly greater than that of DP0158 control (*P* value =0.007).

Analysis at line level was shown in Table 20. Six lines had significant greater larvae growth inhibitory rates than that of DP0158 control. Two lines DP0399.01 and DP0399.51 had greater inhibitory rates than both controls. These results demonstrate that *OsROMT17*transgenic rice had improved OAW tolerance than ZH11-TC and DP0158 controls at seedling stage.

Table 20.Armworm assay of OsROMT17transgenic rice under laboratory screen condition at line level

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP0399.01	0	10	23	43.48	0.0045	Y	0.0003	Y
DP0399.06	0	3	48	6.25	0.3724		0.8955	
DP0399.07	0	9	45	20.00	0.1258		0.0099	Y
DP0399.09	0	3	44	6.82	0.4296		0.8217	
DP0399.13	0	6	40	15.00	0.5465		0.0888	
DP0399.26	0	8	41	19.51	0.1802		0.0167	Y
DP0399.30	0	3	31	9.68	0.6941		0.5602	
DP0399.49	0	8	45	17.78	0.2353		0.0231	Y
DP0399.50	0	9	48	18.75	0.1522		0.0124	Y
DP0399.51	0	10	38	26.32	0.0331	Y	0.0021	Y
ZH11-TC	0	15	139	10.79				
DP0158	0	8	139	5.76				

EXAMPLE 13

RSB Assay of *OsROMT17*Transgenic Rice Plants under Greenhouse Conditions

RSBassay of *OsROMT17* transgenic rice was performed as described in Example 10. The screening results as below.

Three lines (DP0399.01, DP0399.13 and DP0399.51) shown better ACB and OAW tolerance were tested. After fed RSB for 40-d, six DP0399.01 rice plants, 20 DP0399.13rice plants and six DP0399.51 rice plants survived; while three DP0158 rice plants survived. The withered heart rate and morality rate of DP0399.13 were significantly lower than that of DP0158 rice. These results demonstrated that, *OsROMT17*transgenic rice obtained improved RSB tolerance.

Table 21.Rice stem borer assay of *OsROMT17*transgenic rice under greenhouse screen condition at line level

Lines ID	Number of total plants	Number of plants with withered heart	Number of survival plant	Withered heart rate (%)	*P* value	Mortality rate (%)	*P* value
DP0399.01	48	46	6	95.8	0.5370	87.5	0.3867
DP0399.13	48	38	20	79.2	0.0069	58.3	0.0145
DP0399.51	48	43	6	89.6	0.1135	87.5	0.4772
DP0158	48	47	3	97.9		93.8	

OsROMT17 transgenic rice plantsshowed inhibitory impact on ACB and OAW larval growth and *OsROMT17* plays a role in increasing ACB and OAW insect tolerance of transgenic rice seedlings; and *OsROMT17*transgenic rice plants exhibited improved tolerance against RSB insect. These results showed *OsROMT17* transgenic rice had significant inhibitory impact on the growth and development of ACB, OAW and RSB insects, indicating that OsROMT17 plays insecticidalactivities in the potential broad spectrum.

Example 14

ACB Assay of *OsITP2*Transgenic Rice plants under Laboratory conditions

*OsITP2*transgenic rice was tested against ACB larvae as described in Example 8.

Screening results:

1) Results of first validation experiment

After ACB neonate larvae inoculating seedlings for 5 days in the assays, the seedlings of ZH11-TC and DP0158 were significantly damaged by ACB insects, while the *OsITP2*transgenic seedlings were less damaged, and the insects fed with the *OsITP2*transgenic seedlings was smaller than that fed with ZH11-TC and DP0158 controls.

Sixteen*OsITP2*transgenic lines were tested against ACB and were placed on two different plates.A total of 991 ACB neonate larvae were observed after 5 days

inoculatingwith *OsITP2*transgenic rice plants, 5 larvae grew to 1st instar and 351 larvae grew to 2ndinstar; while 400 larvae were observed in the ZH11-TC wells, 3 larvae grew to 1st instar and 69 larvae grew to 2nd instar; and 409 larvae were observed in DP0158 seedlings' wells, 7 larvae grew to 1st instar, and 62 larvae grew to 2nd instar. The average larvae growth inhibitoryrates of *OsITP2*transgenic rice, ZH11-TC seedlings and DP0158seedling were 36.24%, 18.61% and 18.27%, respectively.The average larvae growth inhibitory rate of *OsITP2*transgenic rice was significantly greater than that of ZH11-TC (*P*value=0.0000) and DP0158 *P*value=0.0000) controls at construct level. These results indicate that *OsITP2*transgenic rice exhibitedenhanced tolerance against ACB insect at construct level.

Further analysis at transgenic line level is displayed in Table 22. The 16 lines of *OsITP2*transgenic rice were placed on two different plates, and the DP0158 and ZH11-TC seedlings on the same plate were used as control, respectively. Tentransgenic lines were placed on the first plate, and the other 6 lines were placed on the second plate. 15 of all 16 lines exhibited greater larvae growth inhibitory rates than that of their responding ZH11-TC and DP0158 controls. 6 lines on the first plate and 3 lines on the second plated had significantly greater inhibitory rates than both controls. These results consistently further demonstrate that over-expression *OsITP2*enhanced tolerance against ACB insect in transgenic rice plantsat line level, and OsITP2 plays a role in increasing ACB insect tolerance.

Table 22. Asian corn borer assay of*OsITP2*transgenic rice under laboratory screening condition at line level (1st experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth Inhibitory rate (%)	CK =ZH11-TC		CK= DP0158	
					*P*value	*P*≤0.05	*P* value	*P*≤0.05
DP0378.05	0	42	62	67.74	0.0000	Y	0.0000	Y
DP0378.07	1	26	60	45.90	0.0005	Y	0.0002	Y
DP0378.09	0	18	63	28.57	0.2754		0.2071	
DP0378.10	0	28	68	41.18	0.0022	Y	0.0012	Y
DP0378.11	0	29	58	50.00	0.0002	Y	0.0000	Y
DP0378.15	0	28	60	46.67	0.0004	Y	0.0002	Y
DP0378.18	0	26	49	53.06	0.0002	Y	0.0000	Y
DP0378.21	0	12	62	19.35	0.7432		0.8619	
DP0378.25	0	19	59	32.20	0.1050		0.0735	

DP0378.27	0	14	64	21.88	0.9643		0.8365	
ZH11-TC	0	43	199	21.61				
DP0158	3	37	205	20.67				
DP0378.28	1	11	64	20.00	0.4605		0.4578	
DP0378.29	0	26	62	41.94	0.0000	Y	0.0000	Y
DP0378.31	1	12	71	19.44	0.4637		0.4609	
DP0378.32	0	12	66	18.18	0.5535		0.5510	
DP0378.35	1	20	63	34.38	0.0018	Y	0.0017	Y
DP0378.40	1	28	60	49.18	0.0000	Y	0.0000	Y
ZH11-TC	3	26	201	15.69				
DP0158	4	25	204	15.87				

2) Results of second validation experiment

Ten*OsITP2*transgenic lines which showed better ACB tolerance in the first experiment were placed on one plate and with 6 repeats. A total of 612 ACB neonate larvae were observed in the wells inserted with *OsITP2*transgenic rice plants 5 days after inoculation. 21larvae grew to 1st instar and 253 larvae grew to 2nd instar, and the average ACB larvae growth inhibitory rate was 46.60%; whereas 3 larvae of all the observed 197 larvae fed with ZH11-TC grew to 1st instar and 51 larvae grew to 2nd instar; and 6 larvae of all observed 205 larvae inoculated with the DP0158 seedling grew to 1st instar, and 49 larvae grew to 2nd instar. The average larvae growth inhibitoryrates of ZH11-TC seedling and DP0158 seedlings were 28.50% and 28.91%, respectively. The *OsITP2*transgenic rice exhibited significantly greater average larvae growth inhibitory rate than ZH11-TC (*P*value=0.0000) and DP0158 (*P*value=0.0000) controls at construct level. These results demonstrate that over-expression of *OsITP2*increased tolerance against ACB insect in transgenic rice seedlings at construct level.

Table 23 shows further analysis at transgenic line level. All of the ten transgenic lines exhibited greater larvae growth inhibitory rates than both of ZH11-TC and DP0158 controls. The larvae growth inhibitory rates of sixlines were significantly greater than that of ZH11-TC and DP0158 controls. These results consistently demonstrate over-expression OsITP2 enhanced tolerance against ACB insect in transgenic rice plants.

Table 23.Asian corn borer assay of *OsITP2*transgenic rice under laboratory screen condition at line level (2nd experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP0378.05	6	21	54	55.00	0.0002	Y	0.0003	Y
DP0378.07	0	32	62	51.61	0.0012	Y	0.0016	Y
DP0378.10	2	24	61	44.44	0.0265	Y	0.0337	Y
DP0378.11	1	29	62	49.21	0.0037	Y	0.0049	Y
DP0378.15	1	23	61	40.32	0.0884		0.1082	
DP0378.18	3	36	64	62.69	0.0000	Y	0.0000	Y
DP0378.25	2	20	65	35.82	0.2631		0.3103	
DP0378.29	1	23	63	39.06	0.1333		0.1614	
DP0378.35	0	20	57	35.09	0.3885		0.4462	
DP0378.40	5	25	63	51.47	0.0008	Y	0.0011	Y
ZH11-TC	3	51	197	28.50				
DP0158	6	49	205	28.91				

3) Results of third validation experiment

Ten transgenic lines were further tested in the third experiment with four repeats. Five days after inoculation, 382 larvae were found in the *OsITP2*transgenic rice wells, wherein 27 larvae grew to 1st instar and 142 larvae grew to 2nd instar. The larvae growth inhibitory rate was 47.92%. While, 4 larvae of all the 112 larvae fed with ZH11-TC seedlings grew to 1st instar, and 27 grew to 2nd instar; 4 larvae of all the 116 larvae fed with DP0158 seedlings grew to 1st instar and 26 larvae grew to 2nd instar. The larvae growth inhibitory rates were 30.17% (*P* value=0.0014) and 28.33% (*P* value=0.0003), which were significantly lower than that of *OsITP2*transgenic rice.

Table 24 shows the analysis at line level. The larvae growth inhibitory rates of eight lines were more than 40%, and five lines had significantly greater inhibitory rates than that of ZH11-TC and DP0158 controls. The results in this experiment demonstrate that *OsITP2*transgenic rice had improved ACB larvae tolerance.

In summary, these three validation experiments consistentlyshow that *OsITP2*transgenic rice exhibited greater ACB larvae growth inhibitory rate than both controls, and the lines DP0378.05 and DP0378.18 exhibited better ACB insect tolerance. These results clearly demonstrate over-expression*OsITP2* enhanced tolerance against

ACB insect and OsITP2 plays a role in increasing ACB insect tolerance.

Table 24.Asian corn borer assay of *OsITP2*rice plants under laboratory screen condition at line level (3rd experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP0378.05	4	14	37	53.66	0.0091	Y	0.0041	Y
DP0378.07	0	15	38	39.47	0.2466		0.1506	
DP0378.10	1	17	36	51.35	0.0259	Y	0.0127	Y
DP0378.11	3	11	34	45.95	0.0869		0.0469	Y
DP0378.15	2	18	41	51.16	0.0180	Y	0.0083	Y
DP0378.18	4	23	38	73.81	0.0000	Y	0.0000	Y
DP0378.25	6	7	37	44.19	0.0836		0.0439	Y
DP0378.29	3	11	37	42.50	0.1992		0.1162	
DP0378.35	4	13	40	47.73	0.0475	Y	0.0234	Y
DP0378.40	0	13	44	29.55	0.9140		0.8553	
ZH11-TC	4	27	112	30.17				
DP0158	4	26	116	28.33				

EXAMPLE 15

OAW Assay of *OsITP2*Transgenic Rice Plants under Laboratory Conditions

OAW assay of *OsITP2*transgenic rice was performed as described in Example 9. The screening results as below.

The same ten lines tested in the ACB assay were used and placed in one 32-well plate with four repeats. Five days later after inoculation of OAW neonate larvae, 409 larvae were found in the *OsITP2*transgenic rice well, one larva grew to 1st instar and 135 larvae grew to 2nd instar. The larvae growth inhibitory rate was 33.41%. Whereas, 25 larvae of 123 larvae in the ZH11-TC seedling wells grew to 2nd instar, and 18 larvae of the 114 larvae in DP0158 seedling wells grew to 2nd instar. The OAW larvae growth inhibitory rate of *OsITP2*transgenic rice was significantly greater than that of ZH11-TC (20.33%, *P* value=0.0097) and DP0158 (15.79%, *P* value =0.0010).These results indicate that *OsITP2*transgenic rice exhibited OAW larvae tolerance at construct level.

Analysis at line level shows that four lines had the larvae growth inhibitory rates more than 40%, which were significantly greater than that of ZH11-TC and DP0158 controls.

These results further confirm that over-expression *OsITP2* enhanced tolerance against OAW insect in transgenic rice plants, and OsITP2 plays a role in increasing OAW insect tolerance.

Table 25.Armworm assay of *OsITP2*transgenic rice under laboratory screen condition at line level

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP0378.05	1	14	37	42.11	0.0113	Y	0.0021	Y
DP0378.07	0	10	45	22.22	0.7899		0.3444	
DP0378.10	0	8	42	19.05	0.8589		0.6309	
DP0378.11	0	16	39	41.03	0.0144	Y	0.0027	Y
DP0378.15	0	20	31	64.52	0.0000	Y	0.0000	Y
DP0378.18	0	9	42	21.43	0.8794		0.4147	
DP0378.25	0	22	42	52.38	0.0004	Y	0.0000	Y
DP0378.29	0	10	46	21.74	0.8409		0.3764	
DP0378.35	0	11	42	26.19	0.4319		0.1490	
DP0378.40	0	15	43	34.88	0.0635		0.0137	Y
ZH11-TC	0	25	123	20.33				
DP0158	0	18	114	15.79				

EXAMPLE 16

RSB Assay of *OsITP2*Transgenic Rice Plants under Greenhouse Conditions

OAW assay of *OsITP2* transgenic rice was performed as described in Example 10. The screening results as below.

Three lines (DP0378.05, DP0378.11 and DP0378.18) shown better ACB and OAW tolerance were tested in this assay. After fed RSB for 40-d, eightDP0378.05 rice plants, tenDP0378.11 rice plants and threeDP0378.18 rice plants survived; while only one DP0158 rice plant survived. The morality rate of DP0378.05 and DP0378.11 were significantly lower than that of DP0158 rice. These results demonstrated that, *OsROMT17*transgenic rice exhibited improved RSB tolerance.

Table 26.Rice stem borer assay of *OsITP2*transgenic rice under greenhouse screen condition at line level

Lines	Number of total plants	Number of plant with withered heart	Number of survival plant	Withered heart rate (%)	*P* value	Mortality rate(%)	*P* value

DP0378.05	36	30	8	83.33	0.8593	77.78	0.0352
DP0378.11	36	28	10	77.78	0.8790	72.22	0.1145
DP0378.18	36	36	3	100.00	0.1836	91.67	0.1161
DP0158	36	29	1	80.56		97.22	

In summary, *OsITP2* transgenic rice plants inhibited the development of ACB and OAW insect larvae, and obtained ACB and OAW insect tolerance at seedling stage; and Os*ITP2*transgenic rice plants exhibited improved tolerance against RSB insect. These results showed Os*ITP2* transgenic rice had significant inhibitory impact on the growth and development of ACB, OAW and RSB insects, indicating that OsITP2 plays insecticidalactivities in the potential broad spectrum.

EXAMPLE 17

ACB Assay of *OsKUN1*Transgenic Rice Plants under Laboratory Conditions

In order to investigate whether*OsKUN1* transgenic rice can recapitulate the insect tolerance trait of AH67515 rice, the *OsKUN1*transgenic rice was tested against ACB insect.The method is described in Example 8.

ACB screening results:

1) Results of the first validation experiment

T_1*OsKUN1*transgenic riceplants were first tested in the assays.

After ACB neonate larvae inoculating seedlings for 5 days, the seedlings of ZH11-TC and DP0158 were significantly damaged by ACB insects, while the *OsKUN1*transgenic seedlings were less damaged, and the insects fed with the *OsKUN1*transgenic seedlings was smaller than that fed with ZH11-TC and DP0158 controls.

Ten*OsKUN1*transgenic lines were placed on one plates, and repeated for three times. A total of 360 ACB neonate larvae were inoculated on *OsKUN1*transgenic rice seedlings. Five days after co-culture, 246 larvae were found, and 94 larvae developed to 2nd instar. 29 larvae of all the observed 91 larvae in ZH11-TC seedlings' wells developed to 2nd instar. One larva of all observed 88 larvae inoculated on the DP0158 seedling developed to 1st instar, and 20 larvae developed to 2nd instar. The average larvae growth inhibitory rates of *OsKUN1*transgenic rice, ZH11-TC and DP0158 were 38.21%, 31.87%and 24.72%, respectively. The average larvae growth inhibitory rate of *OsKUN1*transgenic rice was

greater than ZH11-TC control (*P* value=0.1810) and significantlygreater than DP0158 (*P*value=0.0164) control.

Further analysis at transgenic line level is displayed in Table 27. Eightlines exhibited greater larvae growth inhibitory rates than ZH11-TC seedlings and DP0158 seedlings, and three lines exhibited significantly greater larvae growth inhibitory rates than DP0158 seedlings. These results indicate over-expression of *OsKUN1*in rice increased ACB insect tolerance of transgenic rice, and *OsKUN1* plays a role in increasing ACB insect tolerance in rice compared to controls at construct and line level.

Table 27. Asian corn borer assay of *OsKUN1*transgenic rice under laboratory screening condition at line level (1st experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP1251.12	0	11	22	50.00	0.1248		0.0306	Y
DP1251.15	0	9	26	34.62	0.7937		0.3271	
DP1251.19	0	8	17	47.06	0.2393		0.0767	
DP1251.22	0	8	25	32.00	0.9901		0.4718	
DP1251.23	0	10	23	43.48	0.3048		0.0901	
DP1251.24	0	9	15	60.00	0.0504		0.0136	Y
DP1251.29	0	9	19	47.37	0.2100		0.0619	
DP1251.30	0	15	29	51.72	0.0654		0.0123	Y
DP1251.32	0	9	43	20.93	0.2021		0.6339	
DP1251.37	0	6	27	22.22	0.3454		0.7924	
ZH11-TC	0	29	91	31.87				
DP0158	1	20	88	24.72				

2) Results of the second validation experiment

TwelveT_2*OsKUN1*transgenic lines were tested in this second experiment. These twelve lines were placed on one 32-well plate, and repeated for six times. Five days after inoculation, 666 larvae were found, 10 larvae developed to 1st instar, and 297 larvae developed to 2nd instar. Only one larva of all the observed 96 larvae in ZH11-TC seedlings' wells developed to 1st instar and 29 larvae developed to 2nd instar. Two larvae of all observed 101 larvae inoculated on the DP0158 seedling developed to 1st instar, and 38 larvae developed to 2nd instar. The average larvae growth inhibitory rates of

*OsKUN1*transgenic rice, ZH11-TC seedling and DP0158 seedlings were 46.89%, 31.96%and 40.78%, respectively. The average larvae growth inhibitory rate of *OsKUN1*transgenic rice was significantly greater than ZH11-TC (*P* value=0.0093) and greater than DP0158 (*P* value=0.2650) controls.

Further analysis at transgenic line level is displayed in Table 28. Ten of the twelve transgenic lines exhibited greater larvae growth inhibitory rates than both ZH11-TC and DP0158 seedlings. Fivelinesshowed larvae growth inhibitory ratesmore than 50%, which were significantly greater than ZH11-TC seedlings. These results further indicate over-expression of *OsKUN1*in riceincreased ACB insect tolerance of transgenic rice, andOsKUN1 plays a role in increasing ACB insect tolerance in rice compared to controls at line level.

Table 28.Asian corn borer assay of *OsKUN1*transgenic rice under laboratory screen condition at line level (2nd experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP1251.02	0	20	57	35.09	0.7938		0.4115	
DP1251.03	2	27	54	55.36	0.0068	Y	0.0834	
DP1251.04	0	25	54	46.30	0.1059		0.5647	
DP1251.05	1	18	52	37.74	0.4926		0.7119	
DP1251.07	1	25	62	42.86	0.1848		0.8232	
DP1251.08	1	29	58	52.54	0.0120	Y	0.1327	
DP1251.09	2	21	53	45.45	0.0826		0.4843	
DP1251.10	0	30	57	52.63	0.0125	Y	0.1339	
DP1251.11	0	23	56	41.07	0.2509		0.9358	
DP1251.12	3	28	59	54.84	0.0082	Y	0.1030	
DP1251.14	0	25	54	46.30	0.0872		0.5005	
DP1251.15	0	26	50	52.00	0.0290	Y	0.2302	
ZH11-TC	1	29	96	31.96				
DP0158	2	38	101	40.78				

3) Results of the third validation experiment

Twelve transgenic lines were further tested in the third experiment with six repeats. Five days after inoculation, 697 larvae were found in the *OsKUN1* transgenic rice wells,

wherein three larvae grew to 1st instar and 352 larvae grew to 2nd instar. The larvae growth inhibitory rate was 51.36%. While, 43 larvae of all the 130 larvae fed with ZH11-TC seedlings grew to 2nd instar; 36 larvae of all the 123 larvae fed with DP0158 seedlings grew to 2nd instar. The larvae growth inhibitory rates were 33.08% (*P* value=0.0003) and 29.27% (*P* value=0.0000), which were significantly lower than that of *OsKUN1* transgenic rice.

Table 29 shows the analysis at the line level. The larvae growth inhibitory rates of fivelines were more than 50%, and were significantly greater than ZH11-TC and DP0158control; the larvae growth inhibitory rates of other five lines were more than 45%, and were significantly greater than DP0158 control. The results in this experiment demonstrate that *OsKUN1* transgenic rice had improved ACB larvae tolerance.

In summary, these three validation experiments consistently show that *OsKUN1* transgenic rice exhibited greater ACB larvae growth inhibitory rate than both controls, and the transgenic lines DP1251.03, DP1251. 08 and DP1251.12 exhibited better ACB insect tolerance in two experiments. These results clearly demonstrate over-expression*OsKUN1* enhanced tolerance against ACB insect and *OsKUN1* plays a role in increasing ACB insect tolerance.

Table 29.Asian corn borer assay of *OsKUN1*transgenic rice under laboratory screen condition at line level (3rd experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP1251.02	0	27	57	47.37	0.0629		0.0180	Y
DP1251.03	0	35	55	63.64	0.0003	Y	0.0000	Y
DP1251.04	1	25	50	54.00	0.0152	Y	0.0038	Y
DP1251.05	0	26	55	47.27	0.0746		0.0221	Y
DP1251.07	0	25	58	43.10	0.1993		0.0698	
DP1251.08	0	35	57	61.40	0.0009	Y	0.0002	Y
DP1251.09	1	27	61	47.54	0.0737		0.0208	Y
DP1251.10	0	37	64	57.81	0.0017	Y	0.0003	Y
DP1251.11	1	24	63	41.27	0.3295		0.1259	
DP1251.12.	0	35	58	60.34	0.001	Y	0.0002	Y
DP1251.14	0	26	55	47.27	0.0643		0.0187	Y
DP1251.15	0	30	64	46.88	0.0688		0.0190	Y

ZH11-TC	0	43	130	33.08
DP0158	0	36	123	29.27

EXAMPLE 18

OAW Assay of *OsKUN1*Transgenic Rice Plants under Laboratory Conditions

OAW assay of *OsKUN1* transgenic rice was performed as described in Example 9. The screening results as below.

1) Results of the first validation experiments

Twelve transgenic lines which were tested in the ACB assay were used in this assay. These twelve rice lines were placed in one 32-well plate with four repeats. Five days after larvae inoculation, three larvae of 492 larvae found in the*OsKUN1*transgenic rice well developed to 1st instar, and 211 larvae developed to 2nd instar. The OAW larvae inhibitory rate was 43.84%.While, 18 of the 83 larvae in the ZH11-TC wells developed to 2nd instar,the larvae growth inhibitory rate of ZH11-TC seedlings was 21.69%. 27 of the 74 larvae in the DP0158 seedling well developed to 2nd instar. The larvae growth inhibitory rate was 36.49%. The OAW larvae growth inhibitory rate of *OsKUN1*transgenic rice was significantly greater than ZH11-TC (*P* value =0.0007) control and greater than DP0158 (*P* value=0.2768) control.

Analysis at line level was displayed in Table 30.Ten lines showed greater OAW larvae growth inhibitory rates than both ZH11-TC and DP0158 controls, eight lines showed significantly greater larvae growth inhibitory rates than ZH11-TC, and two lines showed significantly greater larvaegrowth inhibitory rates than DP0158 seedlings. These results indicated that over-expression of *OsKUN1* gene in rice plants had inhibition impact on OAW larval growth, and *OsKUN1*transgenic rice had enhanced OAW tolerance at seedling stage.

Table 30.Armworm assay of *OsKUN1*transgenic rice under laboratory screen condition at line level (1st experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP1251.02	2	25	47	59.18	0.0001	Y	0.0185	Y
DP1251.03	1	19	35	58.33	0.0004	Y	0.0351	Y
DP1251.04	0	16	42	38.10	0.0595		0.8757	
DP1251.05	0	18	45	40.00	0.0352	Y	0.7222	

DP1251.07	0	17	42	40.48	0.0349	Y	0.6944	
DP1251.08	0	17	40	42.50	0.0224	Y	0.5438	
DP1251.09	0	24	43	55.81	0.0005	Y	0.0503	
DP1251.10	0	15	38	39.47	0.0471	Y	0.7527	
DP1251.11	0	16	42	38.10	0.0586		0.8701	
DP1251.12	0	11	39	28.21	0.3941		0.4135	
DP1251.14	0	11	39	28.21	0.4291		0.3768	
DP1251.15	0	22	40	55.00	0.0008	Y	0.0668	
ZH11-TC	0	18	83	21.69				
DP0158	0	27	74	36.49				

2) Results of the secondvalidation experiments

Twelve transgenic lines were tested again. These twelve rice lines were placed in one 32-well plate with six repeats. Five days after larvae inoculation, nine larvae of 767 larvae found in the*OsKUN1*transgenic rice wells developed to 1st instar, and 379 larvae developed to 2nd instar. The OAW larvae inhibitory rate was 51.16%.Whereas, three larvae of the 136 larvae in the ZH11-TC wells developed to 1st instar, and 58 larvae developed to 2nd instar,the larvae growth inhibitory rate of ZH11-TC seedlings was 46.04%. 53 of 127 larvae in the DP0158 seedling well developed to 2nd instar. The larvae growth inhibitory rate was 41.73%. The OAW larvae growth inhibitory rate of *OsKUN1*transgenic rice was greater than ZH11-TC (*P* value =0.2580) control and significantly greater than DP0158 (*P* value=0.0460) control.

Analysis at line level was displayed in Table 31. Ten lines showed greater OAW larvae growth inhibitory rates than both ZH11-TC and DP0158 controls, one line showed significantly greater larvae growth inhibitory rates than ZH11-TC, and three lines showed significantly greater larvae growth inhibitory rates than DP0158 seedlings.Two lines (DP1251.03 and DP1251.09) showed better OAW larvae tolerance in the two experiments. These results indicated that *OsKUN1*transgenic rice had enhanced tolerance against OAW larvae at seedling stage.

Table 31.Armworm assay of *OsKUN1*transgenic rice under laboratory screen condition atline level (2nd experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05

DP1251.02	2	29	63	50.77	0.4777		0.1989	
DP1251.03	0	34	61	55.74	0.2114		0.0727	
DP1251.04	0	26	66	39.39	0.4006		0.8115	
DP1251.05	0	23	64	35.94	0.1851		0.4634	
DP1251.07	0	32	67	47.76	0.7596		0.3692	
DP1251.08	0	33	64	51.56	0.4911		0.207	
DP1251.09	3	37	67	61.43	0.0391	Y	0.0095	Y
DP1251.10	0	36	61	59.02	0.0797		0.0229	Y
DP1251.11	1	38	68	57.97	0.101		0.0286	Y
DP1251.12	0	25	54	46.30	0.8957		0.4932	
DP1251.14	2	32	66	52.94	0.337		0.1246	
DP1251.15	1	34	66	53.73	0.3231		0.1188	
ZH11-TC	3	58	136	46.04				
DP0158	0	53	127	41.73				

EXAMPLE 19

RSB Assay of *OsKUN1*Transgenic Rice Plants under Greenhouse Conditions

1) First validation experiment for testing *OsKUN1* transgenic rice against RSB

To investigate the tolerance against RSB, T_1*OsKUN1* transgenic rice plants which were water-cultured for 14 days were used in the RSB assay.

The screening method is similar to the ACB and OAW screening methods. Two leaves about 4 cm were placed in one well of the 32-well plate, and five RSB larvae were inoculated on the leaves in one well, they were co-cultured for four days. The scoringscale in Table 2 was used.

Screening results:

Nine *OsKUN1* transgenic rice lines were tested, and placed on one 32-well plate with four repeats. After co-cultured for four days, 91 of the 313 RSBlarvae in the *OsKUN1*transgenic seedlings wells developed to 2nd instar, the average larvae growth inhibitory rate was 29.07%; whereas, 14 of the 76 larvae in ZH11-TC seedling wellsdeveloped to 2nd instar; and 15 larvae of all observed 77 larvae inoculated on the DP0158 seedling developed to 2nd instar. The RSB larvae growth inhibitory rates of ZH11-TC seedlings and DP0158 seedling were 18.42% and 19.48%, respectively. The RSBlarvae growth inhibitory rate of *OsKUN1*transgenic rice was greater than that of

ZH11-TC (*P* value=0.1278) and DP0158 (*P* value=0.1788) controls.

Further analysis at transgenic line level is displayed in Table 32. Seven lines exhibited greater RSB larvae growth inhibitory rates than ZH11-TC and DP0158 controls; and the RSB larvae growth inhibitory rates of three lines were more than 35%, significantly greater than that of ZH11-TC and/or DP0158 seedlings. These results demonstrate that *OsKUN1*transgenic rice showed inhibitory impact on RSB larval growth and *OsKUN7* plays a role in increasing RSB insect tolerance of transgenic rice seedlings at construct and line levels.

Table 32.Rice stem borer assay of *OsKUN1* transgenic rice plants under laboratory screen condition at line level (1st experiment)

Line ID	Number of larvae at 1st instar	Number of larvae at 2nd instar	Number of total observed larvae	Larvae growth inhibitory rate (%)	CK=ZH11-TC		CK=DP0158	
					P value	*P*≤0.05	*P* value	*P*≤0.05
DP1251.12	0	20	36	55.56	0.0004	Y	0.0006	Y
DP1251.19	0	8	34	23.53	0.5512		0.6445	
DP1251.22	0	4	26	15.38	0.6635		0.5818	
DP1251.23	0	10	37	27.03	0.2977		0.3652	
DP1251.24	0	13	36	36.11	0.0427	Y	0.0569	
DP1251.29	0	14	36	38.89	0.0242	Y	0.0328	Y
DP1251.30	0	9	36	25.00	0.4040		0.4846	
DP1251.32	0	5	35	14.29	0.5839		0.4994	
DP1251.37	0	8	37	21.62	0.6983		0.8029	
ZH11-TC	0	14	76	18.42				
DP0158	0	15	77	19.48				

2) Second validation experiment for testing *OsKUN1* transgenic rice against RSB

The second OAW assay of *OsKUN1* transgenic rice was performed as described in Example 10. The screening results as below.

Fivelines shown better ACB and OAW tolerance were tested in this assay. After fed RSB for 24-d, the withered heart rate was obtained. As shown in Table 33, DP0158 seedlings exhibited greater withered heart rate than these five *OsKUN1* transgenic rice line. The withered heartratesof DP1251.03 and DP1251.12 were significantly lower than that of DP0158 rice. These results demonstrated that*OsKUN1*transgenic rice exhibited improved RSB tolerance.

Table 33.Rice stem borer assay of *OsKUN1*rice plants at T_2 generation under greenhouse

screen condition at line level (2nd experiment)

Line ID	Number of total plants	Number of plant with withered heart	Withered heart rate (%)	*P* value	*P*≤0.05
DP1251.03	96	27	28.13	0.0444	Y
DP1251.05	96	32	33.33	0.2071	
DP1251.08	96	32	33.33	0.1558	
DP1251.12	96	27	28.13	0.0409	Y
DP1251.15	96	27	28.13	0.0628	
DP0158	96	44	45.83		

Two transgenic lines DP1251.12 and DP1251.24 showed better tolerance against ACB and RSB larvae at T_1 generation. Many *OsKUN1* transgenic lines showed inhibition impact on ACB, OAW and RSB insect larvae at T_2 generation. These results showed *OsKUN1* transgenic rice had significant inhibitory impact on the growth and development of ACB, OAW and RSB insects, indicating that OsKUN1 plays insecticidalactivities in the potential broad spectrum.

CLAIMS

What is claimed is:

1. An isolated polynucleotide comprising: (a) a polynucleotide with nucleotide sequence of at least 85% sequence identity to SEQ ID NO: 7, 10, 13 or 16; (b) a polynucleotide with nucleotide sequence of at least 85% sequence identity to SEQ ID NO: 8, 11, 14 or17; (c) a polynucleotide encoding a polypeptide with amino acid sequence of at least 90% sequence identity to SEQ ID NO: 9, 12, 15 or18; or (d) the full complement of the nucleotide sequence of (a), (b) or (c), wherein over-expression of the polynucleotide in a plant increases tolerance to an insect pest.
2. The isolated polynucleotide of claim1 comprises the nucleotide sequence of SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16 or SEQ ID NO: 17.
3. The isolated polynucleotide of claim 1, wherein the isolated polynucleotide encoded polypeptide comprises the amino acid sequence comprises SEQ ID NO: 9, SEQ ID NO: 12, SEQ ID NO: 15 orSEQ ID NO: 18.
4. The isolated polynucleotide of any one ofclaims 1 to 3, wherein the polynucleotide is from Oryza sativa, *Oryza australiensis*, *Oryza barthii*, *Oryza glaberrima*, *Oryza latifolia*, *Oryza longistaminata*, *Oryza meridionalis*, *Oryza officinalis*, *Oryza punctata*, *Oryza rufipogon*, *Oryza nivara*, *Arabidopsis thaliana*, *Cicer arietinum*, *Solanum tuberosum*, *Brassica oleracea*,*Zea mays*, *Glycine max*, *Glycine tabacina*, *Glycine soja* or *Glycine tomentella*.
5. A recombinant vector comprising the polynucleotide of any one of claims 1 to 4.
6. A recombinant DNA construct comprising the isolated polynucleotide of any one of claims 1 to 4 operably linked to at least one heterologous regulatory sequence.
7. A recombinant DNA construct comprising an isolated polynucleotide, encoding a COA26 polypeptide, ROMT17 polypeptide, ITP2 polypeptideand KUN1 polypeptide, operably linked to at least one heterologous regulatory sequence.
8. A transgenic plant, plant cell or seed comprising a recombinant DNA construct, wherein the recombinant DNA construct comprises the polynucleotide of any one of claims 1 to 4operably linked to at least one heterologous regulatory sequence.
9. A transgenic plant or plant cell comprising in its genome a recombinant DNA

construct comprising polynucleotide of any one of claims 1 to 4 operably linked to at least one heterologous regulatory element, wherein said plant exhibits increased toleranceto an insect pest when compared to a control plant.

10. The transgenic plant or plant cell of claim 9, wherein the insect pest is a Lepidopteran.

11. The transgenic plant or plant cell of claim 10, wherein the insect pest is Asian Corn Borer (*Ostrinia furnacalis)*, Rice Stem Borer (*Chilo suppressalis*), and Oriental Armyworm *(Mythimna separata)*.

12. The plant of claim 7 to 11, wherein said plant is selected from the group consisting of rice, maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, barley, millet, sugar cane and switchgrass.

13. A method of increasing tolerance in a plant to an insect pest comprising overexpressing at least one polynucleotide encoding an insect tolerance polypeptide selected from a COA26 polypeptide, ROMT17 polypeptide, ITP2 polypeptideand KUN1 polypeptide.

14. The method of claim 13, wherein the polynucleotide comprises: (a) a polynucleotide with a nucleotide sequence of at least 85% sequence identity to SEQ ID NO: 7, 10, 13 or 16; (b) a polynucleotide with a nucleotide sequence of at least 85% sequence identity to SEQ ID NO: 8, 11,14 or17; and (c) a polynucleotide encoding a polypeptide with amino acid sequence of at least 90% sequence identity to SEQ ID NO: 9, 12, 15 or 18.

15. The method of claim 13 or 14, wherein the plant comprises the DNA construct of claim 7.

16. A method of increasing tolerance in a plant to an insect pest, comprising:

(a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity compared to SEQ ID NO: 9, 12, 15 or18;

(b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct; and

(c) obtaining a progeny plant derived from the transgenic plant of step (b), wherein said progeny plant comprises in its genome the recombinant DNA construct and exhibits increased tolerance to an insect pest when compared to a control plant not comprising the recombinant DNA construct.

17. A method of evaluating tolerance in a plant to an insect pest, comprising:

(a) introducing into a regenerable plant cell a recombinant DNA construct comprising a polynucleotide operably linked to at least one regulatory sequence, wherein the polynucleotide encodes a polypeptide having an amino acid sequence of at least 50% sequence identity when compared to SEQ ID NO: 9, 12, 15 or18;

(b) regenerating a transgenic plant from the regenerable plant cell after step (a), wherein the transgenic plant comprises in its genome the recombinant DNA construct;

(c) obtaining a progeny plant derived from the transgenic plant, wherein the progeny plant comprises in its genome the recombinant DNA construct; and

(d) evaluating the progeny plant for tolerance to an insect pest compared to a control plant not comprising the recombinant DNA construct.

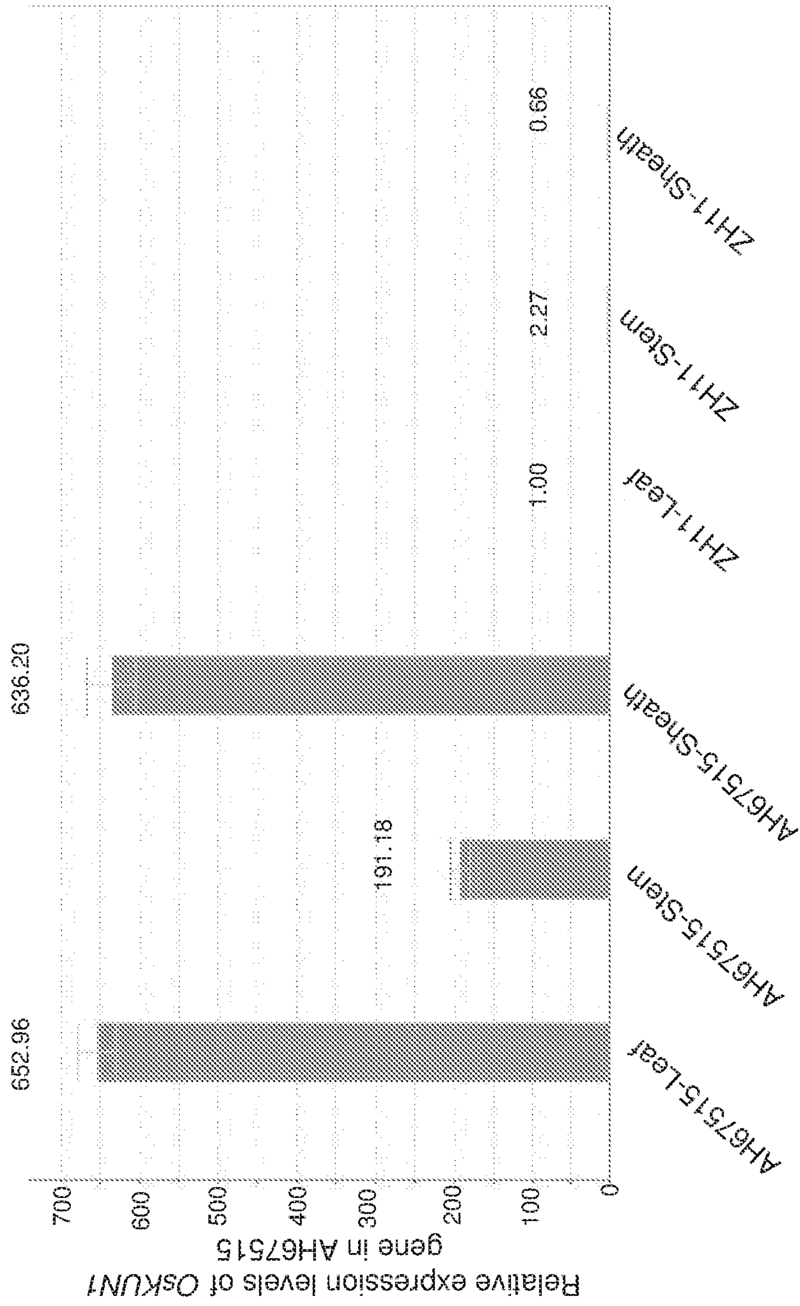

FIG 1.

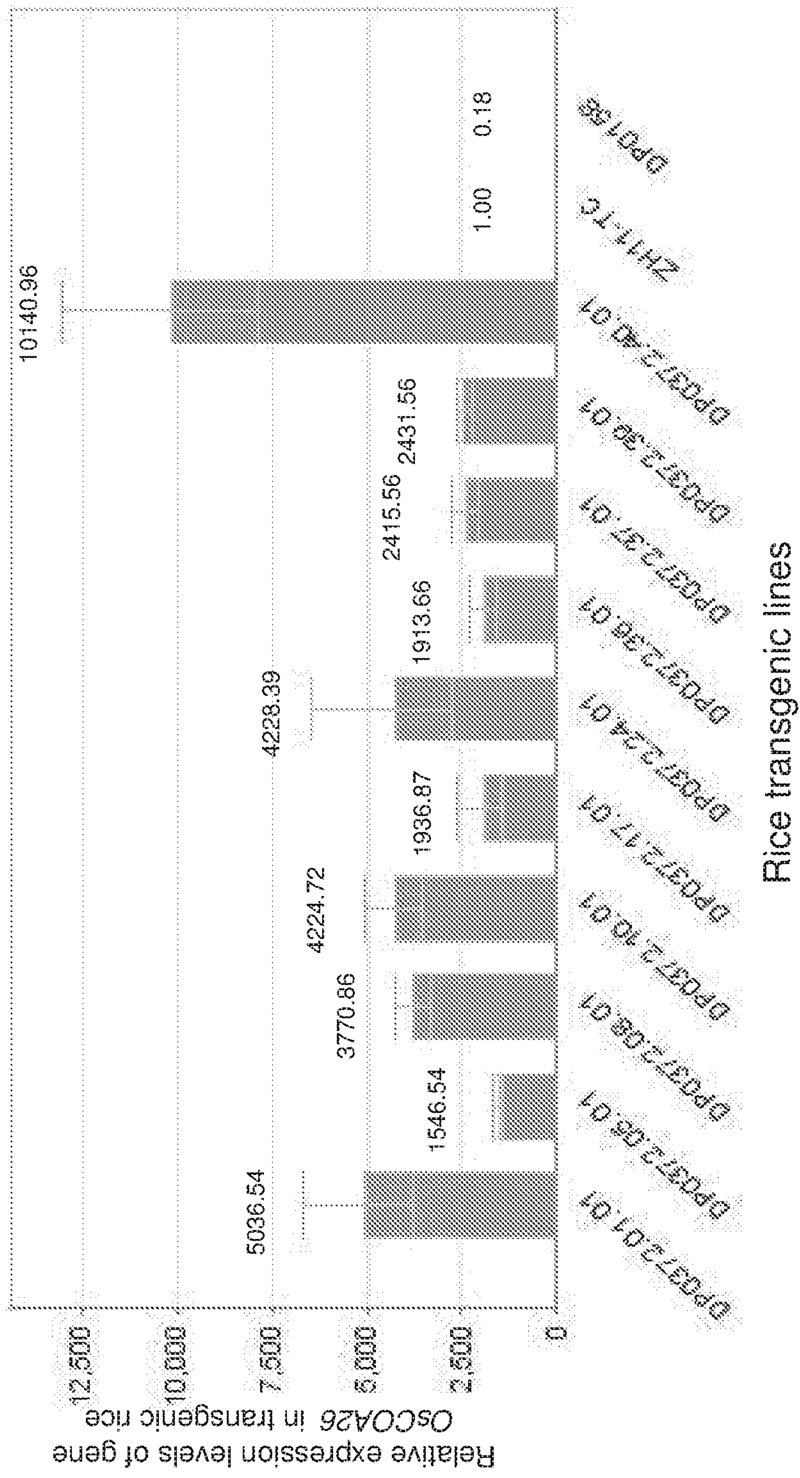

FIG 2.

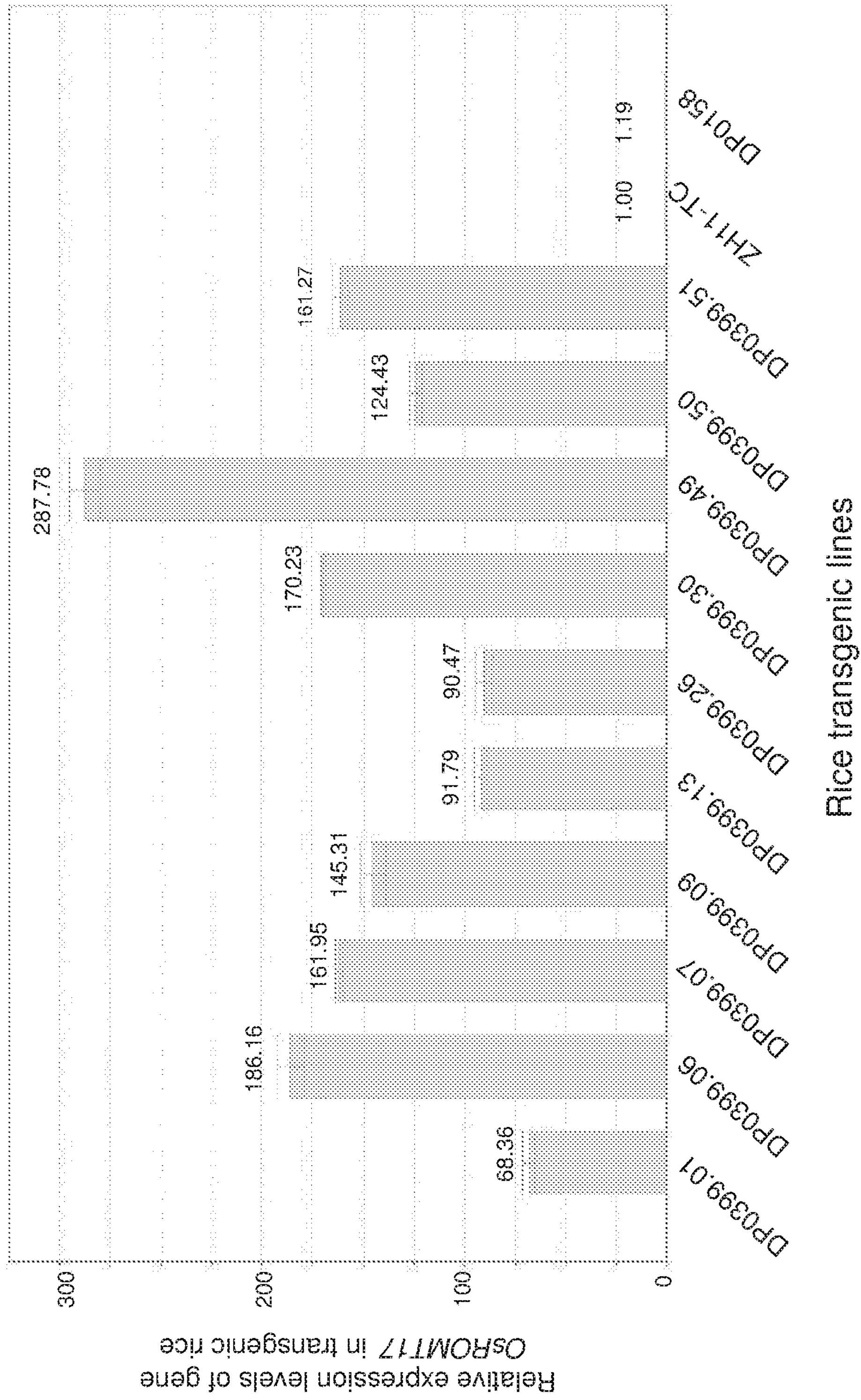

FIG 3.

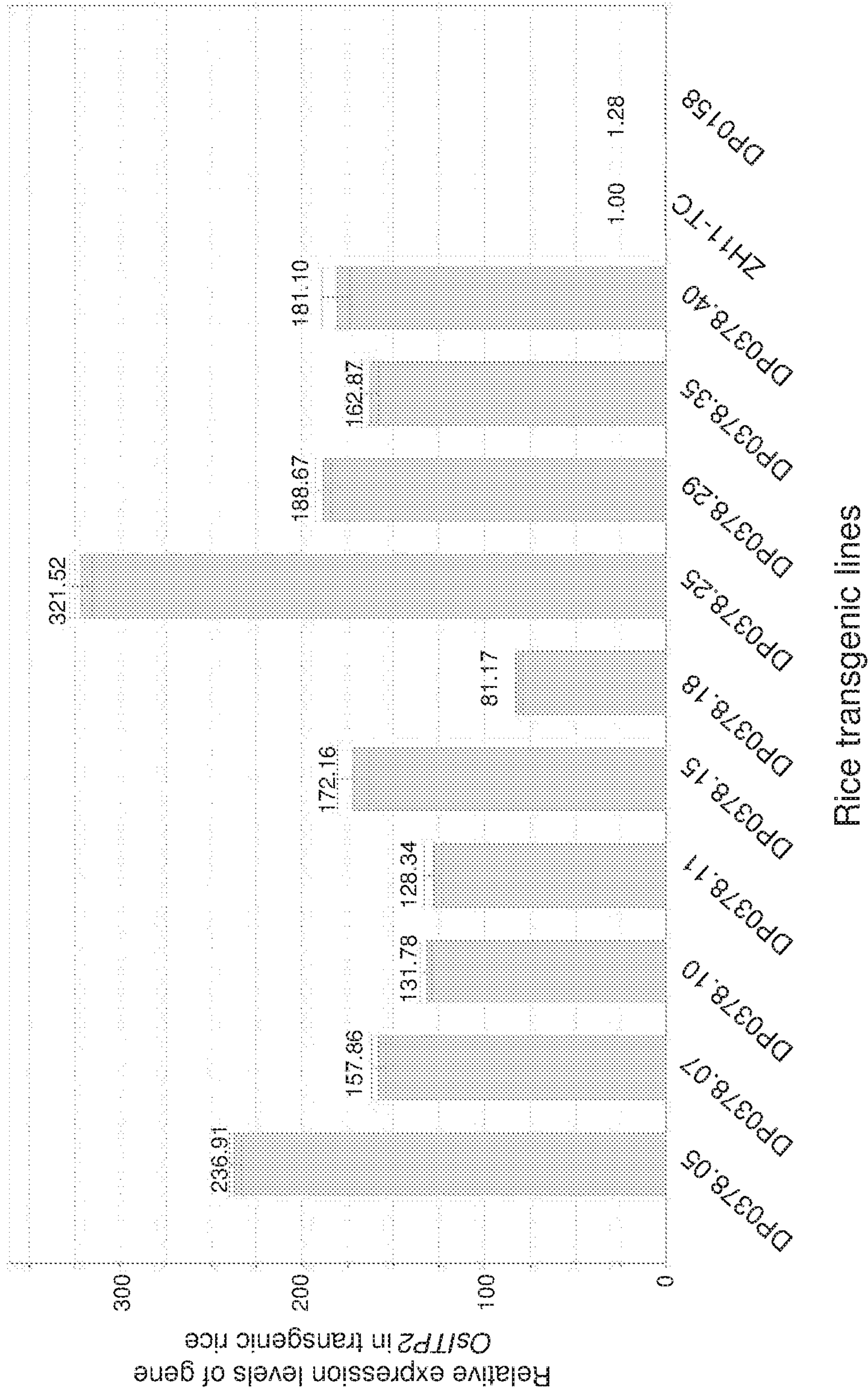

FIG 4.

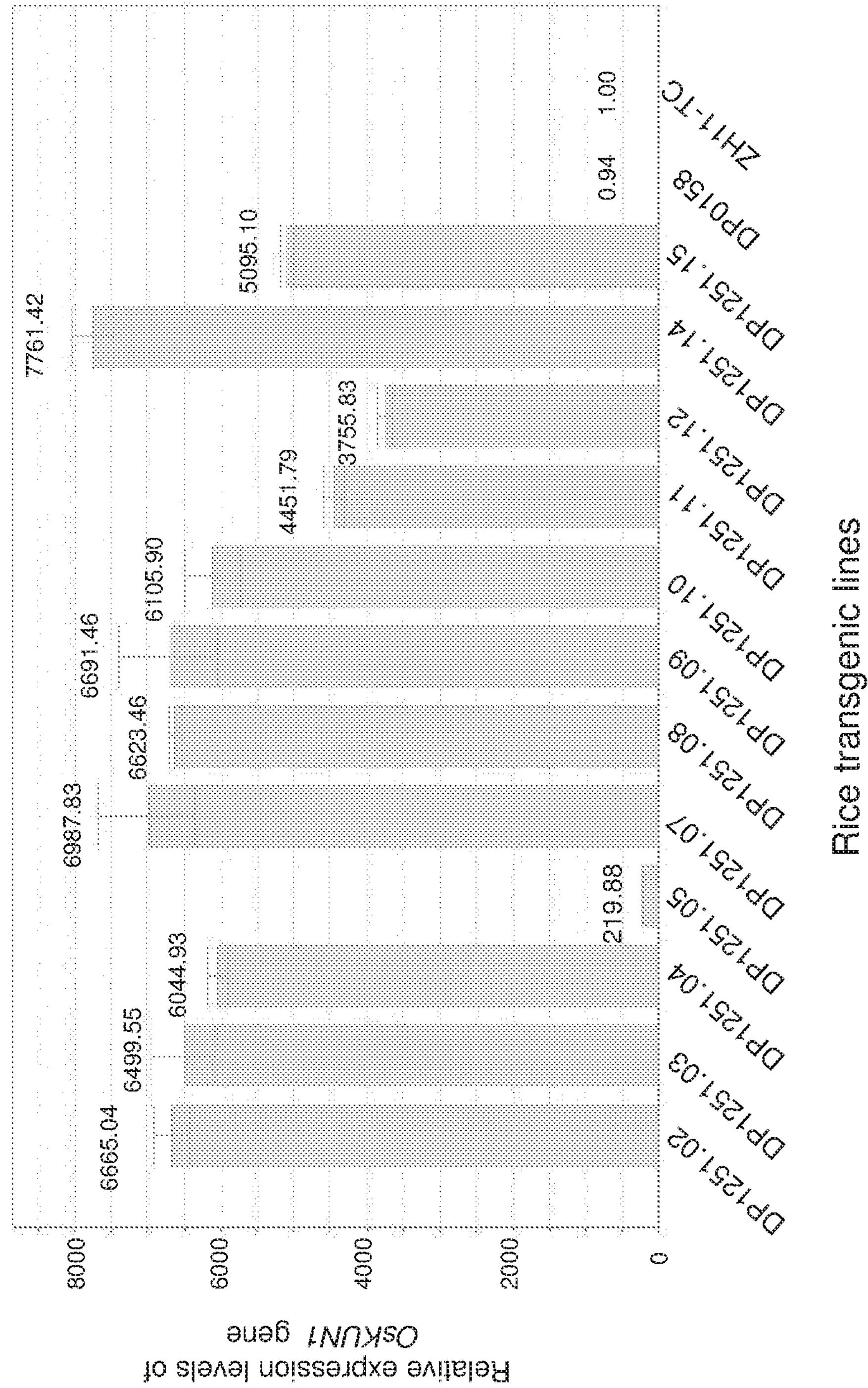

FIG 5.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2015/083237

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/29(2006.01)i； C12N 15/82(2006.01)i； C12N 5/14(2006.01)i； A01H 1/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N; A01H

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

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

CNPAT, WPI, EPODOC, CNKI, NCBI: COA26, caffeoyl CoA 3-O-methyltransferase, resistance, lignin, tolerance, insect, pest, pathogen, transgenic plant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TANAKA， T. et al. ""Oryza sativa Japonica Group Os08g0498600 (Os08g0498600) mRNA， complete cds" retrieved from NCBI Database accession no. NM_001068679.1" *DATABASE GENBANK,* 08 June 2010 (2010-06-08),	1-12
Y	TANAKA， T. et al. ""Oryza sativa Japonica Group Os08g0498600 (Os08g0498600) mRNA， complete cds" retrieved from NCBI Database accession no. NM_001068679.1" *DATABASE GENBANK,* 08 June 2010 (2010-06-08),	13-17
Y	US 6465229 B2 (E. I. DU PONT DE NEMOURS AND COMPANY) 15 October 2002 (2002-10-15) abstract， description column 1 lines 15-42	13-17

☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 September 2015

Date of mailing of the international search report

25 September 2015

Name and mailing address of the ISA/CN

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2015/083237

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

[1] Claims 1-17 direct to isolated polynucleotides for conferring tolerance to insect pests, and methods for control of insect infestation in plants with the said polynucleotides. The said polynucleotides are selected from that encodes COA26 polypeptide (SEQ ID NO: 9), ROMT17 polypeptide (SEQ ID NO: 12), ITP2 polypeptide (SEQ ID NO: 15) or KUN1 polypeptide (SEQ ID NO: 18).

[2] Since the above mentioned 4 polynucleotides are involved, claims 1-17 cover 4 inventions. Each invention relates to one of the 4 polynucleotides. The same or corresponding technical features among the inventions above are as follows: a polynucleotide for increasing tolerance to an insect pest. However, the same or corresponding technical features above are well known in the art.

[3] It follows that the same or corresponding technical features of claims above do not make a contribution over the prior art and can not be considered as special technical features within the meaning of Rule 13.2 PCT. The application, hence does not meet the requirement of unity of invention as defined in Rule 13.1 PCT.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-17 (partial)**

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2015/083237

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
US 6465229 B2	15 October 2002	US 2002081693 A1	27 June 2002