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(71) Applicant: UNIVERSITY OF FLORIDA RESEARCH
FOUNDATION, INC. [US/US]; 223 Grinter Hall,
Gainesville, FL 32611 (US).

(72) Inventors: MOU, Zhonglin; 223 Grinter Hall, Gainesville,
FL 32611 (US). DING, Yezhang; 223 Grinter Hall,
Gainesville, FL 32611 (US). ZHANG, Xudong; 223
Grinter Hall, Gainesville, FL 32611 (US). FOLTA, Kevin,
M.; 223 Grinter Hall, Gainesville, FL 32611 (US).

(74) Agent: HANSON, Robert, E.; Dentons US LLP, P. O.
Box 061080, Wacker Drive Station, Willis Tower, Chica-
go, IL 60606-1080 (US).

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(54) Title: USE OF ELONGATOR GENES TO IMPROVE PLANT DISEASE RESISTANCE

(57) Abstract: The invention provides methods and compositions for producing plants displaying enhanced disease resistance by transgenic over-expression of Elongator Complex subunit *ELP3* or *ELP4* genes. Methods and compositions for production of plants with altered growth habit (e.g. runner development) are also provided.



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USE OF ELONGATOR GENES TO IMPROVE PLANT DISEASE RESISTANCE

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims benefit of United States provisional application No. 62/193,001, filed July 15, 2015, herein incorporated by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

10 This invention was made with U.S. Government support under NSF Grant Number IOS-0842716 awarded by the National Science Foundation. The Government has certain rights in the invention.

INCORPORATION OF SEQUENCE LISTING

15 The Sequence Listing, which is a part of the present disclosure, includes a computer readable form 144,561 bytes file (as measured in MS Word) entitled "UFFL070WO_ST25.txt" created on July 5, 2016 comprising nucleotide and/or amino acid sequences of the present invention submitted via EFS-Web. The subject matter of the Sequence Listing is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

1. Field of the Invention

20 The present invention relates generally to the field of agriculture and plant genetics. More particularly, it concerns genetically modified plants comprising enhanced agronomic properties including improved disease resistance.

2. Description of the Related Art

25 Plants are capable of activating an array of defense mechanisms in response to pathogen attack, some of which are preexisting and others are inducible. However, given the potential susceptibility of plants, new methods and compositions are needed for enhancing the resistance

of plants to disease and other biotic and abiotic stresses. Genetic modification of plants has, in combination with conventional breeding programs, led to significant improvements in plant growth, yield, and disease resistance. This may occur for instance by enhancing the ability of plants to recognize the presence of a pathogen and to improve expression of defense mechanisms.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a transgenic plant comprising a heterologous nucleic acid sequence that encodes ELP3 or ELP4 (also called ELO1), wherein ELP3 or ELP4 is over-expressed in the transgenic plant, and exhibits resistance to a disease-causing plant pathogen as a result of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence. In some embodiments the heterologous nucleic acid sequence comprises at least 70%, 80%, 85%, 90%, 95%, 98%, 99% or more sequence identity to a sequence selected from the group consisting of SEQ ID NOs:1-24

In some embodiments the plant pathogen is a bacterial plant pathogen or a fungal plant pathogen. In certain embodiments the nucleic acid sequence encoding ELP3 or ELP4 is operably linked to a heterologous promoter which is a developmentally-regulated, organelle-specific, inducible, tissue-specific, constitutive, cell-specific, or seed specific promoter. In particular embodiments the promoter is a constitutive or inducible promoter.

In certain embodiments the plant is a member of the Brassicaceae, Cucurbitaceae, Fabaceae, Poaceae, Rosaceae, Rutaceae, Malvaceae, or Solanaceae. In some embodiments the plant is a Brassica. In particular embodiments the plant is a *Fragaria sp.*, a tomato plant, or a citrus plant. The plant may further be defined as an R₀ transgenic plant. In other embodiments the plant is further defined as a progeny plant of any generation of an R₀ transgenic plant, wherein the transgenic plant has inherited the selected DNA from the R₀ transgenic plant.

In some embodiments the plant displays an additional altered agronomic property relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence,

wherein the additional altered agronomic property is selected from the group consisting of: enhanced yield; altered leaf area; altered leaf shape, and altered production of runners.

Another aspect of the invention provides a seed that produces the plant comprising a heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-
5 expressed in the transgenic plant, and exhibits resistance to a disease-causing plant pathogen as a result of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence. A part of the plant is also provided. The plant part may further be defined, in some embodiments of the invention, as a protoplast, cell, meristem, root, leaf, pistil, anther, flower, seed, fruit, embryo, stalk, or petiole.

10 Another aspect of the invention provides a recombinant nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of: (a) a nucleic acid sequence that hybridizes to a nucleic acid sequence complementary to the sequence of any of SEQ ID NOs: 1-24, under wash conditions of 1X SSC and 65°C; (b) a nucleic acid comprising the sequence complementary to SEQ ID NOs: 1-24 or a fragment thereof; and c) a nucleic acid sequence
15 exhibiting at least 80% sequence identity to any of SEQ ID NOs: 1-24; wherein the nucleic acid sequence is operably linked to a heterologous promoter sequence and wherein expression of the nucleic acid molecule in a plant cell results in over-expression of ELP3 or ELP4 relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence. In some embodiments the DNA molecule comprises a nucleic acid sequence exhibiting at least
20 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a complement of any of SEQ ID NOs: 1-24. Further, the heterologous promoter sequence may be developmentally-regulated, organelle-specific, inducible, tissue-specific, constitutive, cell-specific, or seed specific promoter.

Another aspect of the invention relates to a transgenic plant cell comprising the nucleic
25 acid molecule comprising a nucleic acid sequence selected from the group consisting of: (a) a nucleic acid sequence that hybridizes to a nucleic acid sequence complementary to the sequence of any of SEQ ID NOs: 1-24, under wash conditions of 1X SSC and 65°C; (b) a nucleic acid comprising the sequence complementary to SEQ ID NOs: 1-24 or a fragment thereof; and c) a nucleic acid sequence exhibiting at least 80% sequence identity to any of SEQ ID NOs: 1-24;

wherein the nucleic acid sequence is operably linked to a heterologous promoter sequence and wherein expression of the nucleic acid molecule in a plant cell results in over-expression of ELP3 or ELP4 relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence. A transgenic plant or plant part, such as a leaf, seed, flower, ovary, pollen, or fruit comprising such a nucleic acid molecule is also contemplated.

Another aspect of the invention provides a method of conferring disease resistance to a plant comprising: over-expressing a heterologous nucleic acid sequence that encodes ELP3 or ELP4 in a transgenic plant; wherein the transgenic plant exhibits disease resistance, as a result of the over-expression of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence. In certain embodiments the disease is a bacterial plant disease or a fungal plant disease. In some embodiments of the method the disease is caused by a fungus. In particular embodiments the disease is Strawberry Anthracnose or Strawberry Powdery Mildew. In other embodiments the disease is caused by a bacterium. In particular embodiments the disease is caused by a *Pseudomonad*, a *Xanthomonad*, or an *Erwinia sp.* In certain embodiments the disease is Strawberry Angular Leaf Spot, or Bacterial Speck of Tomato.

The invention further relates to a method of plant breeding comprising: (a) crossing a first plant, which is over-expressing a heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the first plant, and wherein the first plant exhibits disease resistance as a result of the heterologous nucleic acid sequence relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence, with a second plant; (b) obtaining one or more progeny plants, and (c) selecting a progeny plant which is over-expressing the heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the progeny plant, and wherein the progeny plant exhibits disease resistance as a result of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein:

FIG. 1. (a): Bacterial multiplication ($\log_{10}$ CFU/leaf disc) of *A. thaliana* ecotype Landsberg *erecta* ("Ler") and *elo1-1* lines challenged with the bacterial pathogen strain *Pseudomonas syringae* pv. *maculicola* ("Psm") ES4326. The asterisk indicates that *elo1-1* is significantly more susceptible than *Ler* to *Psm* ES4326 ($p < 0.05$, Student's t test). (b): Bacterial multiplication ($\log_{10}$ CFU/leaf disc) of *A. thaliana*, ecotype Columbia ("Col-0") and the T-DNA insertion mutant SALK_079193, challenged with *Psm* ES4326. The asterisk indicates that SALK_079193 is significantly more susceptible than Col-0 to *Psm* ES4326 ($p < 0.05$, Student's t test). **FIG. 1(c):** Leaf morphology of Col-0 and the T-DNA insertion mutant line SALK_079193. "CFU": colony-forming unit.

FIG. 2. (a): Germination and development of *Arabidopsis* seedlings on agar plates containing 0.5 mM added salicylic acid ("SA"). (b): Levels of free and total SA measured in control and mutant *Arabidopsis* lines, untreated or challenged with *Psm* ES4326. Different letter above the bars indicate significant difference ($p < 0.05$, one-way ANOVA). The statistical comparisons were performed separately among *Ler2-2*, *npr1-L*, and *elo1 npr1-L* for each treatment.

FIG. 3. (a-b): free SA and total SA (SA+SAG) levels in control and selected *Arabidopsis* mutant lines treated with or without *Psm* ES4326. (c-d): Bacterial multiplication ($\log_{10}$ CFU/leaf disc) in control and selected mutant *Arabidopsis* lines, 0 and 3 days after challenge with *Psm* ES4326. Different letter above the bars indicate significant difference ($p < 0.05$, one-way ANOVA). The comparisons were performed separately for each treatment or time point.

FIG. 4. (a): Expression levels of the six genes (*AtELP1-AtELP6*) encoding Elongator subunits at different developmental stages of *Arabidopsis*. (b): Leaf morphology of *Arabidopsis* control line *Ler*, an *elo1-1* mutant, and complemented transgenic *35S::AtELP4-GFP elo1* and

35S::*AtELP4 elo1* lines. (c): Bacterial multiplication ($\log_{10}$ CFU/leaf disc) of *Psm* ES4326 in control, indicated mutant *Arabidopsis* lines, and complemented transgenic 35S::*AtELP4-GFP elo1* and 35S::*AtELP4 elo1* lines, 0 and 3 days after challenge with ES4326. Different letter above the bars indicate significant difference ($p < 0.05$, one-way ANOVA). The comparisons were performed separately for each time point. (d): Bacterial multiplication ($\log_{10}$ CFU/leaf disc) of *Psm* ES4326 in control (*Ler2-2*) and *AtELP4*-overexpressing lines. Different letter above the bars indicate significant difference ($p < 0.05$, one-way ANOVA).

FIG. 5. Free SA and total SA (SA+SAG) levels ($\mu\text{g/g}$ FW) in *Arabidopsis* control, *elo1-1* mutant, and 35S::*AtELP4* transgenic lines. Different letter above the bars indicate significant difference ($p < 0.05$, one-way ANOVA).

FIG. 6. Time course of induction of selected defense genes in *Arabidopsis* *Ler* and a 35S::*AtELP4* transgenic line following challenge with *Psm* ES4326. Asterisks indicate significant difference between the induction of defense genes in *Ler2-2* and 35S::*AtELP4* plants at the indicated time points compared to time 0 ($p < 0.05$, two-way ANOVA). HPI: hour post-inoculation.

FIG. 7. Developmental phenotypes of transgenic strawberry plants expressing *AtELP3* or *AtELP4*. (a to b): qPCR analyses of the expression levels of *AtELP3* and *AtELP4* in independent *AtELP3*- and *AtELP4*-transgenic lines, respectively. Almost no expression was observed in the wild type strawberry Hawaii-4 (cont.). The order of the transgenic lines is presented in order of increasing expression levels of the transgenes. The *EF1 α* gene was used as an internal control. Gene expression data represent the average of three biological replicates with standard deviation ("SD"). (c): Presence (+) or absence (-) of runners, flowers, and fruits on the transgenic plants. The order of the transgenic lines is the same as in Fig. 7(a), (b). (d): Plant (top) and fruit (bottom) morphology of two *AtELP3*-expressing (E3/66 and E3/72) and two *AtELP4*-expressing (E4/06 and E4/01) independent transgenic lines, and the control Hawaii-4 (Cont.). (e): Plant height (cm) and (f): Fruit weight of one-year-old control and transgenic plants overexpressing *AtELP3* or *AtELP4*. Fruit weight data represent the average of 20 replicates with SD. Asterisks indicate significant difference between the transgenic lines and the control ($p < 0.05$, Student's t test).

FIG. 8. Resistance of *AtELP*- and *AtELP4*-transgenic strawberry plants to anthracnose crown rot. (a): Disease symptoms caused by *Colletotrichum gloeosporioides* on the control (Cont.) and the transgenic lines E3/65 and E4/01. Photos were taken 20 days after infection ("dai"). (b): Disease Incidence ("DI") and (c): Area under the disease progress curve ("AUDPC") for the control (Cont.) and *AtELP3*- and *AtELP4*-transgenic lines infected with *C. gloeosporioides*. Data represent the average of collapsed plants in three independent experiments containing 5 plants each, with SD. DI allowed the calculation of AUDPC. Asterisks indicate significant difference between the transgenic lines and the control ($p < 0.05$, Student's t test).

FIG. 9. Resistance of *AtELP3* and *AtELP4* transgenic strawberry plants to powdery mildew. (a): Phenotype of disease reaction scores ("DS") for leaves inoculated with *P. aphanis*. Scores 0 = resistant, Score 1 = intermediate susceptibility, and Score 2 = susceptible. (b): Disease scores (DS). Data represent the average of five biological replicates with SD. (c): *P. aphanis* spores count score for the control (Cont.) and *AtELP3*- and *AtELP4*-transgenic lines. Spores were counted 10 days after inoculation. FW: fresh weight. Data represent the average of 15 biological replicates with SD. Asterisks indicate significant difference between the transgenic lines and the control ($p < 0.05$, Student's t test).

FIG. 10. Resistance of *AtELP3* and *AtELP4* transgenic strawberry plants to angular leaf spot. (a): Phenotype of disease reaction scores (DS) on leaves of transgenic and wild type strawberry Hawaii-4 (Cont.) plants inoculated with *Xanthomonas fragariae*. Score 0: transient water-soaking from inoculation no longer evident; Score 1: transient water-soaking evident; Score 2: slight chlorosis or necrosis in the center of the inoculation site; Score 3: water-soaking expanding beyond inoculation site with bacterial exudate often evident; and score 4: necrosis spreading beyond the inoculation site and/or secondary infection evident. (b): Disease Reaction scores (DS) of angular leaf spot, and (c): Colony-forming unit (CFU) of *X. fragariae* in the control (Cont.) and *AtELP3* and *AtELP4* transgenic lines. Data represent the mean of 15 independent samples (inoculation sites) with SD. Asterisks indicate significant difference between the transgenic lines and the control ($p < 0.05$, Student's t test).

FIG. 11. Free SA levels in *AtELP3* and *AtELP4* transgenic strawberry plants before and after pathogen inoculation. (a): Free SA in the *AtELP3* and *AtELP4* transgenic plants and the control (Cont.). *AtELP3* and *AtELP4* expression did not alter the basal level of free SA in

transgenic plants. (b): Free SA levels 24h after *C. gloeosporioides* inoculation. SA levels were measured by HPLC. Data represent the average of three biological replicates with SD. Asterisks indicate significant difference between the transgenic line and the control ($p < 0.05$, Student's t test).

- 5 **FIG. 12.** Growth of bacteria and symptomatology of Bacterial Speck on tomato plants. (a): growth curve days, post-inoculation, of *P. syringae* pv. tomato DC3000 in tomato cv. Moneymaker and representative transgenic lines; (b): symptom expression on inoculated control and transgenic plants, six days post inoculation.

BRIEF DESCRIPTION OF THE SEQUENCES

- 10 **SEQ ID NOs:1-12** ELP3 nucleotide sequences from *Arabidopsis thaliana*, *Brassica napus*, *Gossypium arboreum*, *Glycine max*, *Citrus sinensis*, *Solanum lycopersicum*, *Medicago truncatula*, *Fragaria vesca*, *Cucumis melo*, *Zea mays*, *Oryza sativa*, and *Eucalyptis grandis*, respectively.

- 15 **SEQ ID NOs:13-24** ELP4 nucleotide sequences from *Arabidopsis thaliana*, *Brassica napus*, *Gossypium arboreum*, *Glycine max*, *Citrus sinensis*, *Solanum lycopersicum*, *Medicago truncatula*, *Fragaria vesca*, *Cucumis melo*, *Zea mays*, *Oryza sativa*, and *Eucalyptis grandis* respectively.

SEQ ID NOs:25-36 predicted ELP3 polypeptide sequences from *Arabidopsis thaliana*, *Brassica napus*, *Gossypium arboreum*, *Glycine max*, *Citrus sinensis*, *Solanum lycopersicum*, *Medicago truncatula*, *Fragaria vesca*, *Cucumis melo*, *Zea mays*, *Oryza sativa*, and *Eucalyptis grandis* respectively.

5 SEQ ID NOs:37-48 predicted ELP4 polypeptide sequences from *Arabidopsis thaliana*, *Brassica napus*, *Gossypium arboreum*, *Glycine max*, *Citrus sinensis*, *Solanum lycopersicum*, *Medicago truncatula*, *Fragaria vesca*, *Cucumis melo*, *Zea mays*, *Oryza sativa*, and *Eucalyptis grandis* respectively.

DETAILED DESCRIPTION OF THE INVENTION

10 The invention relates, in one aspect, to the surprising discovery that plants such as Brassicas, Citrus, tomatoes, and strawberries, among others, may be engineered to over express certain subunits of the Elongator Complex, including ELP3 and ELP4, in order to exhibit enhanced resistance to bacterial and fungal plant pathogens. This discovery is unexpected because the effect of such over expression was not known or predictable with respect to the level
15 of plant disease resistance as well as effects on morphology, growth, flowering, and yield. Thus, the invention overcomes limitations in the art by providing plants engineered to be resistant to multiple plant pathogens, and also provides methods for producing plants with enhanced resistance to bacterial and fungal plant pathogens. Plants that are provided include transgenic dicotyledonous and monocotyledonous plants comprising heterologous DNA sequences that lead
20 to over-expression of Elongator Complex Subunit 3 ("ELP3") or Elongator Complex Subunit 4 ("ELP4").

Elongator is a multifunctional protein complex involved in many cellular processes, such as transcriptional regulation, histone acetylation and gene silencing (DeFraia and Mou, *Plant Signal. Behavior* 6:19-22, 2011), operating in association with RNA polymerase II (RNAPII)
25 during transcription (Otero *et al.*, *Mol. Cell* 3:109-118, 1999). Composed of six protein subunits (ELP1, ELP2, ELP3, ELP4, ELP5 and ELP6), this complex controls many cellular processes such as histone modification/acetylation, exocytosis, α -tubulin acetylation, response to DNA damage, transcriptional silencing, zygotic paternal genomic demethylation, and tRNA nucleoside modification (Creppe and Buschbeck *J. Biomed. Biotechnol.* 2011:924898, 2011; Esberg *et al.*,

Mol. Cell 24:139-148, 2006; Huang *et al.*, *RNA* 11:424-436, 2005; Li *et al.*, *PLoS Genetics* 5:e1000684, 2009; Okada *et al.*, *Nature* 463:554-558, 2010; Rahl *et al.*, *Mol. Cell* 17:841-853, 2005; Wittschleben *et al.*, *Mol. Cell* 4:123-128, 1999). *ELP1-ELP3* encode the core complex, whereas *ELP4-ELP6* encode components of a subcomplex that forms a hetero-hexameric ring-like structure which is essential for the binding of anticodon stem-loop of substrate tRNAs (Glatt *et al.*, *Nature Struct. Mol. Biol.* 19:314-320, 2012). The Elongator Complex is highly conserved in eukaryotes; however, the effects of its over-expression in higher plant are not well understood. The present disclosure demonstrates that over-expression of *ELP3* and *ELP4* confers enhanced resistance to multiple diseases and provides an alternative for producing plants resistant to fungal and bacterial pathogens.

Plants have developed various defense mechanisms in response to infection. Plants defend themselves against pathogens by recognizing them, and then rapidly and efficiently reprogramming their transcriptional profiles to mount a very complex and accurate precise defense strategy. However, unlike animals, they lack mobile defense cells and a somatic adaptive immune system, and so rely on their innate immunity. This defense leads to the recognition of pathogen-associated molecular patterns (PAMPs), resulting in PAMP-triggered immunity (PTI) that prevents pathogen colonization, along with induction of effector-triggered immunity ("ETI"), that triggers the activation of resistance ("R") proteins. These signals lead to the generation of a systemic acquired resistance ("SAR") response, which confers long-lasting protection against a broad spectrum of microbial pathogens. Salicylic acid ("SA") is a signal molecule essential for activation of SAR and also associated with accumulation of the pathogenesis-related ("PR") proteins that contribute to resistance. Effective SA-mediated resistance requires the transcriptional co-activator NPR1, which regulates the activity of several transcription factors that modulate defense gene expression (Cao *et al.*, *Cell* 88:57-67, 1997; Durrant and Dong, *Ann. Rev. Phytopathol.* 42:185-209, 2004). Mutations in the *NPR1* gene significantly suppress SA-mediated transcriptional reprogramming, and lead to compromised basal immunity, ETI, and SAR (Durrant and Dong, 2004, *ibid*). In addition, these mutations also result in SA hyperaccumulation during pathogen infection (Shah *et al.*, *Mol. Plant Microbe Interact* 10:69-78, 1997; Wildermuth *et al.*, *Nature* 414:562-565, 2001) and failure of seedling

development on Murashige and Skoog (MS) medium containing high concentrations of SA (Cao *et al.*, 1997, *ibid*).

Independently of SAR, components of the Elongator Complex (EC) may also play a role in immunity (Chen *et al.* *Mol. Cell Biol.* 26:6902-6912, 2006; DeFraia *et al.* *Plant J.* 64:511-523, 2010; DeFraia and Mou, *Plant Signal. Behavior* 6:19-22, 2011; Wang *et al.*, *Plant Cell* 25:762-776, 2013; Zhou *et al.*, *Plant J.* 60:79-90, 2009). Plants lacking certain Elongator subunits are more susceptible to plant pathogens. ELP3 contains both histone acetyltransferase (HAT), which connects chromatin structure deformation with elevated transcription activity mediated by RNAPII, and a radical S-AdoMet (SAM) domain, linked to the protein by an iron-sulphur cluster (Paraskevopoulou *et al.*, *Mol. Microbiol.* 59:795-806, 2006). In *Arabidopsis*, AtELP3 plays a role in regulating transcriptional changes induced during pathogen infection and are accompanied by changes in DNA methylation, a hallmark of transcriptional suppression and histone acetylation, which is generally associated with transcriptional activation (DeFraia *et al.* 2010, *ibid*; Kass *et al.*, *Trends Genet.* 13:444-449, 1997; Workman and Kingston, *Ann. Rev. Biochem.* 67:545-579, 1998). In *Arabidopsis*, AtELP4 is required for rapid defense gene induction and establishment of full basal and effector-triggered plant immunity.

In general, mutations of Elongator subunits in *Arabidopsis* result in pleiotropic effect including: abscisic acid (ABA) hypersensitivity; tolerance to drought or oxidative stress; severely aberrant auxin phenotypes like narrow leaves and reduced root growth; accumulation of anthocyanin; and reduced apical dominance (Chen *et al.* 2006, *ibid*; DeFraia *et al.* 2013, *BMC Plant Biol.* 13:102 ; DeFraia *et al.* 2010, *ibid*; Huang *et al.*, *RNA* 11:424-436, 2005; Nelissen *et al.*, *Plant Cell* 15:639-654, 2003; Nelissen *et al.*, *PNAS* 107:1678-1683, 2010; Wang *et al.* 2013, *ibid*; Yan *et al.*, *Plant Physiol. And tRNA Modification* 13:1640-1650, 2014; Zhou *et al.*, *Plant J.* 60:79-90, 2009). However, the effect of ELP3 or ELP4 over-expression was unclear.

In one embodiment, a plant provided by the invention is more resistant to bacterial or fungal diseases than an otherwise similar plant not comprising a recombinant DNA construct that allows for an increase in the level of expression of ELP3 or ELP4. A first plant can be considered more resistant to plant disease than a second plant when the first plant exhibits less disease, such as symptomatology and pathogen multiplication, or the disease progresses more slowly in the first plant than in the second plant. The amount of disease infecting a plant can be

measured by any means known in the art. For instance, "area under the disease progress curve ("AUDPC"; Shaner & Finney, *Phytopathol.* 67:1051-1056, 1977) may be measured. Loss of foliage, necrosis, wilting, or other symptoms can also be measured, as well as levels of pathogen population or multiplication.

5 Plant pathogens contemplated herein for control via enhanced plant resistance include bacterial and fungal plant pathogens, for instance biotrophic or hemibiotrophic pathogens. In certain embodiments the contemplated plant pathogen is a gram-negative plant pathogenic bacterium such as a *Xanthomonas* or a *Pseudomonas*, or an Ascomycete such as a member of the Erysiphaceae or the Glomerellaceae. Levels of resistance may be positively correlated with
10 the levels of transgene expression in transgenic lines.

As an example, ectopic expression of *AtELP3* and *AtELP4* in strawberry increased resistance to *C. gloeosporioides*, causal agent of anthracnose crown rot. This pathogen aggressively invaded the plant tissue and control plants collapsed within 20 days. However, plants over-expressing ELP3 or ELP4 demonstrated a reduction in disease symptoms of at least
15 25% to 50%, and higher levels of transgene expression promoted increased resistance to, for instance, *Podosphaeria aphanis*, with a tested transgenic line E4/01 not presenting any symptoms of disease. Spores counted in leaves of these plants may actually have remained from inoculation, as this fungus can survive on leaves and remain on the plants for a long period of time.

20 *AtELP3* and *AtELP4* plants were also more resistant to a bacterial pathogen. In this instance, inoculation sites did not develop coalescing and necrotic lesions 20 days after infection ("dai") with *Xanthomonas fragariae*; and progression of symptoms and bacterial growth were decreased in most lines, with transgenic lines E4/01 and E4/06 displaying the most resistance. These results showed that *AtELP3* and *AtELP4* expression promoted increased disease resistance
25 in strawberry plants. When compared to strawberry plants overexpressing *AtNPR1* and challenged with the same pathogens (Silva *et al.*, *Transgenic Res.* 1-12, doi:10.1007/s11248-015-9869-5, 2015), ELP3- and ELP4-over-expressing plants showed higher levels of resistance, allowing for improvement in development of strawberry cultivars resistant to diseases.

Contemplated plants displaying enhanced disease resistance and/or altered growth
30 include any plant that is a host of a bacterial or fungal plant pathogen, and which could thus be

made more disease resistant using the constructs and methods as described herein. Exemplary plants include *Arabidopsis thaliana*, *Brassica napus*, *Gossypium sp.*, *Glycine max*, *Citrus sinensis*, *Solanum lycopersicum*, *Medicago truncatula*, *Fragaria vesca*, *Cucumis melo*, *Zea mays*, *Oryza sativa*, and *Eucalyptis grandis* among others. Included are plants grown for food, feed, fuel, timber, forage, or fiber, as well as ornamental plants. In some embodiments, the plant is a member of the Brassicaceae, the Rosaceae, the Fabaceae, the Poaceae, the Solanaceae, or the Rutaceae. In specific embodiments, the plant is a strawberry plant, a soybean plant, a corn plant, a Citrus plant, a tomato plant, a cotton plant, or a canola plant. Methods to produce transgenic plants comprising a heterologous sequence encoding a functional *ELP3* or *ELP4* gene, to over-express such gene products, are also provided.

I. Application of the Invention

As indicated above, one application of the invention is to provide methods to increase the resistance of a plant to bacterial and fungal plant pathogens. Modulation of the phenotype of a plant or plant tissue may be obtained in accordance with the invention by introduction of recombinant nucleic acid sequences that result in increased, *e.g.* constitutive, synthesis of *ELP3* or *ELP4*. Such sequences may be identical to or display, for example, at least 75%, 80%, 85%, 90%, 95%, 98%, or 99% sequence identity with, for instance, SEQ ID NOs:1-24. Transgenic plants comprising a heterologous nucleic acid sequence encoding a functional polypeptide of SEQ ID NOs:25-48, or functional polypeptides displaying at least 75%, 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to any of SEQ ID NOs:25-48 are also contemplated. As used herein, “hybridization” or “hybridizes” is understood to mean the forming of a double or triple stranded molecule or a molecule with partial double or triple stranded nature. As used herein “stringent condition(s)” or “high stringency” are those conditions that allow hybridization between or within one or more nucleic acid strand(s) containing complementary sequence(s), but precludes hybridization of random sequences.

Stringent conditions tolerate little mismatch between a nucleic acid and a target strand. Such conditions are well known to those of ordinary skill in the art, and are preferred for applications requiring high selectivity. Medium stringent conditions may comprise relatively low salt and/or relatively high temperature conditions, such as provided by about 1X SSC, and 65°C. High stringency may be defined as 0.02M to 0.10M NaCl and 50°C to 70°C. Specific

examples of such conditions include 0.02M NaCl and 50°C; 0.02M NaCl and 60°C; and 0.02M NaCl and 70°C.

Alterations of the native amino acid sequence to produce variant polypeptides can be prepared by a variety of means known to those ordinarily skilled in the art. For instance, amino acid substitutions can be conveniently introduced into the polypeptides by changing the sequence of the nucleic acid molecule at the time of synthesis. Site-specific mutations can also be introduced by ligating into an expression vector a synthesized oligonucleotide comprising the modified sequence. Alternately, oligonucleotide-directed, site-specific mutagenesis procedures can be used, such as disclosed in Walder *et al*, (*Gene* 42:133, 1986); and U.S. Patents 4,518,584 and 4,737,462.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (*e.g.* Kyte and Doolittle, *J. Mol. Biol.* 157:105-132, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid may be assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics. These are, for instance: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate/glutamine/aspartate/asparagine (-3.5); lysine (-3.9); and arginine (-4.5). It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biologically functional protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those within ± 1 are more preferred, and those within ± 0.5 are most preferred.

It is also understood in the art that the substitution of like amino acids may be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101 states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids,

correlates with a biological property of the protein. The following hydrophilicity values have been assigned to amino acids: arginine/lysine (+3.0); aspartate/glutamate (+3.0 $\pm$ 1); serine (+0.3); asparagine/glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 $\pm$ 1); alanine/histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine/isoleucine (-1.8);
5 tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4).

It is understood that an amino acid may be substituted by another amino acid having a similar hydrophilicity score and still result in a protein with similar biological activity, *i.e.*, still obtain a biologically functional protein. In making such changes, the substitution of amino acids whose hydropathic indices are within $\pm$ 2 is preferred, those within $\pm$ 1 are more preferred, and
10 those within $\pm$ 0.5 are most preferred.

As outlined above, amino acid substitutions are therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those skilled in the art and include: arginine
15 and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine, and isoleucine.

It is understood that the temperature and ionic strength of a desired stringency are determined in part by the length of the particular nucleic acid(s), the length and nucleobase content of the target sequence(s), the charge composition of the nucleic acid(s), and to the
20 presence or concentration of formamide, tetramethylammonium chloride or other solvent(s) in a hybridization mixture. It is also understood that compositions and conditions for hybridization are mentioned by way of non-limiting examples only, and that the desired stringency for a particular hybridization reaction in a plant cell is often determined empirically by comparison to one or more positive or negative controls. Depending on the application envisioned it is
25 preferred to employ varying conditions of hybridization to achieve varying degrees of selectivity of a nucleic acid towards a target sequence. Thus, recombinant DNA constructs comprising nucleotide sequences identical to, or displaying 75%, 80%, 85%, 90%, 95%, 98%, 99%, or greater identity, over the complete length of their coding regions to any of SEQ ID NOs:1-24 and that encode a functional ELP3 or ELP4 polypeptide may be utilized in the present invention.

The coding sequences of such genes may be operably linked to and under transcriptional control of a constitutive, root-preferred, and/or inducible promoter as outlined below.

II. Plant Transformation Constructs

Certain embodiments of the current invention concern plant transformation constructs.

5 For example, one aspect of the current invention is a plant transformation vector comprising a nucleotide sequence that encodes a polypeptide which functions as an Elongator Complex subunit ELP3 or ELP4. Such coding sequences may be present in one or more plant expression cassettes and/or transformation vectors for introduction to a plant cell.

10 In certain embodiments of the invention, coding sequences are provided operably linked to a heterologous promoter, which exhibits a constitutive or inducible pattern of gene expression. Expression constructs are also provided comprising these sequences, as are plants and plant cells transformed with the sequences.

15 The construction of vectors which may be employed in conjunction with plant transformation techniques using these or other sequences according to the invention will be known to those of skill of the art in light of the present disclosure (see, for example, Sambrook *et al.*, *In.: Molecular Cloning-A Laboratory Manual* (second edition), Cold Spring Harbour Laboratory Press, 1989; Gelvin *et al.*, *Pl. Mol. Biol. Manual* 1990). The techniques of the current invention are thus not limited to any particular nucleic acid sequences.

20 One important use of the sequences provided by the invention will be in the alteration of a plant disease resistance phenotype by genetic transformation with an ELP3- or ELP4-coding gene. Alternatively, plant growth habit (*e.g.* runner formation) may be altered. The ELP3 or ELP4-coding gene that allows for an altered phenotype, such as enhanced plant disease resistance, may be provided along with other sequences, for instance, sequences that function as selectable or screenable markers. Where an expressible coding region that is not necessarily a
25 marker coding region is employed in combination with a marker coding region, one may employ the separate coding regions on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize co-transformation.

DNA segments used for transforming plant cells will, of course, generally comprise the cDNA, gene or genes which one desires to introduce into and have expressed in the host cells. These DNA segments can further include structures such as promoters, enhancers, polylinkers, or even regulatory genes as desired. The DNA segment or gene chosen for cellular introduction will often encode a protein which will be expressed in the resultant recombinant cells resulting in a screenable or selectable trait and/or which will impart an improved phenotype to the resulting transgenic plant. However, this may not always be the case, and the present invention also encompasses transgenic plants incorporating non-expressed transgenes. Preferred components likely to be included with vectors used in the current invention are as follows.

A. Regulatory Elements

Exemplary promoters for expression of a nucleic acid sequence include plant promoter such as the CaMV 35S promoter (Odell *et al.*, *Nature* 313:810-812, 1985), or others such as CaMV 19S (Lawton *et al.*, *Plant Mol. Biol.* 9:315-324, 1987), *nos* (Ebert *et al.*, *PNAS* 84:5745-5749, 1987), *ocs* (Herrera-Estrella *et al.*, *Nature* 303:209-213, 1983), *Adh* (Walker *et al.*, *PNAS* 84:6624-6628, 1987), sucrose synthase (Yang and Russell, *PNAS* 87:4144-4148, 1990), α -tubulin, actin (Wang *et al.*, *Mol. Cell Biol.* 12:3399-3406, 1992), *cab* (Sullivan *et al.*, *Mol. Gen. Genet.* 215:431-440, 1989), PEPCase (Hudspeth and Grula, *Plant Mol. Biol.* 12:579-589, 1989). Tissue specific promoters such as root cell promoters (Conkling *et al.*, *Pl. Physiol.* 93:1203-1211, 1990) and tissue specific enhancers (Fromm *et al.*, *Nature* 319:791-793, 1986) are also contemplated to be useful, as are inducible promoters such as ABA- and turgor-inducible promoters. The choice of such promoter sequences will be known to those of skill in the art in light of the present disclosure.

The DNA sequence between the transcription initiation site and the start of the coding sequence, *i.e.*, the untranslated leader sequence, can also influence gene expression. One may thus wish to employ a particular leader sequence with a transformation construct of the invention. Preferred leader sequences are contemplated to include those which comprise sequences predicted to direct optimum expression of the attached gene, *i.e.*, to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to

those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants will typically be preferred.

It is specifically envisioned that ELP3 or ELP4 coding sequences may be introduced under the control of novel promoters or enhancers, *etc.*, or homologous or tissue specific promoters or control elements. Vectors for use in tissue-specific targeting of genes in transgenic plants will typically include tissue-specific promoters and may also include other tissue-specific control elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure. These include, for example, the *rbcS* promoter, specific for green tissue; the *ocs*, *nos* and *mas* promoters which have higher activity in roots or wounded leaf tissue; a truncated (-90 to +8) 35S promoter which directs enhanced expression in roots and elsewhere, and an α -tubulin gene that also directs expression in roots.

B. Terminators

Transformation constructs prepared in accordance with the invention will typically include a 3' end DNA sequence that acts as a signal to terminate transcription and allow for the poly-adenylation of the mRNA produced by coding sequences operably linked to, for instance, a ELP3 or ELP4 encoding gene. In one embodiment of the invention, the native terminator of a FS or IFS gene is used. Alternatively, a heterologous 3' end may enhance the expression of the ELP3 or ELP4 gene(s). Terminators which are deemed to be particularly useful in this context include those from the nopaline synthase gene of *Agrobacterium tumefaciens* (nos 3' end) (Bevan *et al.*, *NAR* 11:369-385, 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as an Adh intron (Callis *et al.*, *Genes Devel.* 1:1183-1200, 1987), sucrose synthase intron (Vasil *et al.*, *Pl. Physiol.* 91:1575-1579, 1989) or TMV omega element (Gallie *et al.*, *Plant Cell* 1:301-311, 1989), may further be included where desired.

C. Transit or Signal Peptides

Sequences that are joined to the coding sequence of an expressed gene, which are removed post-translationally from the initial translation product and which facilitate the transport

of the protein into or through intracellular or extracellular membranes, are termed transit (usually into vacuoles, vesicles, plastids and other intracellular organelles) and signal sequences (usually to the endoplasmic reticulum, golgi apparatus and outside of the cellular membrane). By facilitating the transport of the protein into compartments inside and outside the cell, these sequences may increase the accumulation of gene product protecting them from proteolytic degradation. These sequences also allow for additional mRNA sequences from highly expressed genes to be attached to the coding sequence of the genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA in front of the gene may increase the overall stability of the mRNA transcript from the gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide. It further is contemplated that targeting of certain proteins may be desirable in order to enhance the stability of the protein (U.S. Patent No. 5,545,818, incorporated herein by reference in its entirety).

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This generally will be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed.

D. Marker Genes

By employing a selectable or screenable marker protein, one can provide or enhance the ability to identify transformants. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker protein and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can "select" for by chemical means, *i.e.*, through the use of a selective agent (*e.g.*, a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, *i.e.*, by

“screening” (*e.g.*, the green fluorescent protein). Of course, many examples of suitable marker proteins are known to the art and can be employed in the practice of the invention.

Included within the terms “selectable” or “screenable markers” also are genes which encode a “secretable marker” whose secretion can be detected as a means of identifying or
5 selecting for transformed cells. Examples include markers which are secretable antigens that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, *e.g.*, by ELISA; small active enzymes detectable in extracellular solution (*e.g.*, α -amylase, β -lactamase, phosphinothricin acetyltransferase); and proteins that are inserted
10 or trapped in the cell wall (*e.g.*, proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an
15 epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

Many selectable marker coding regions are known and could be used with the present
20 invention including, but not limited to, *neo* (Potrykus *et al.*, *Mol. Gen. Genet.* 199:183-188, 1985), which provides kanamycin resistance and can be selected for using kanamycin, G418, paromomycin, *etc.*; *bar*, which confers bialaphos or phosphinothricin resistance; a mutant EPSP synthase protein (Hinchee *et al.*, *Bio/Technol* 6:915-922, 1988) conferring glyphosate resistance; a nitrilase such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker
25 *et al.*, *Science* 242:419-422, 1988); a mutant acetolactate synthase (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate resistant DHFR (Thillet *et al.*, *J. Biol. Chem.* 263:12500-12508, 1988), a dalapon dehalogenase that confers resistance to the herbicide dalapon; or a mutated anthranilate synthase that confers resistance to 5-methyl tryptophan.

An illustrative embodiment of selectable marker capable of being used in systems to select transformants are those that encode the enzyme phosphinothricin acetyltransferase, such as the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *Streptomyces viridochromogenes*. The enzyme phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami *et al.*, *Mol. Gen. Genet.* 205:42-50, 1986; Twell *et al.*, *Pl. Physiol.* 91:1270-1274, 1989) causing rapid accumulation of ammonia and cell death.

Screenable markers that may be employed include a β -glucuronidase (GUS) or *uidA* gene which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.*, *In: Chromosome Structure and Function: Impact of New Concepts, 18th Stadler Genetics Symposium*, 11:263-282, 1988); a β -lactamase gene (Sutcliffe, *PNAS* 75:3737-3741, 1978), which encodes an enzyme for which various chromogenic substrates are known (*e.g.*, PADAC, a chromogenic cephalosporin); a *xyIE* gene (Zukowsky *et al.*, *PNAS* 80:1101-1105, 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an α -amylase gene (Ikuta *et al.*, *Bio/technol* 8:241-242, 1990); a tyrosinase gene (Katz *et al.*, *J. Gen. Microbiol.* 129:2703-2714, 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily-detectable compound melanin; a β -galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (*lux*) gene (Ow *et al.*, *Science* 234:856-859, 1986), which allows for bioluminescence detection; an aequorin gene (Prasher *et al.*, *BBRC* 126:1259-1268, 1985) which may be employed in calcium-sensitive bioluminescence detection; or a gene encoding for green fluorescent protein (Sheen *et al.*, *Plant J.* 8:777-784, 1995; Haseloff *et al.*, *PNAS* 94:2122-2127, 1997; Reichel *et al.*, *PNAS* 93:5888-5893, 1996; Tian *et al.*, *Pl. Cell Rep.* 16:267-271, 1997; WO 97/41228).

Another screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the *lux* gene. The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It also is

envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening.

III. Tissue Cultures

Tissue cultures may be used in certain transformation techniques for the preparation of
5 cells for transformation and for the regeneration of plants therefrom. Maintenance of tissue
cultures requires use of media and controlled environments. "Media" refers to the numerous
nutrient mixtures that are used to grow cells *in vitro*, that is, outside of the intact living organism.
The medium usually is a suspension of various categories of ingredients (salts, amino acids,
growth regulators, sugars, buffers) that are required for growth of most cell types. However,
10 each specific cell type requires a specific range of ingredient proportions for growth, and an even
more specific range of formulas for optimum growth. Rate of cell growth also will vary among
cultures initiated with the array of media that permit growth of that cell type.

Nutrient media is prepared as a liquid, but this may be solidified by adding the liquid to
materials capable of providing a solid support. Agar is most commonly used for this purpose.
15 Bacto™ agar (Difco-BD, Franklin Lakes, NJ), Hazleton agar (Hazleton, Lenexa, KS, USA),
Gelrite® (Sigma, St. Louis, MO), PHYTAGEL (Sigma-Aldrich, St. Louis, MO), and GELGRO
(ICN-MP Biochemicals, Irvine, CA, USA) are specific types of solid support that are suitable for
growth of plant cells in tissue culture.

Some cell types will grow and divide either in liquid suspension or on solid media. As
20 disclosed herein, plant cells will grow in suspension or on solid medium, but regeneration of
plants from suspension cultures typically requires transfer from liquid to solid media at some
point in development. The type and extent of differentiation of cells in culture will be affected
not only by the type of media used and by the environment, for example, pH, but also by whether
media is solid or liquid.

25 Tissue that can be grown in a culture includes meristem cells, callus, immature embryos,
hairy root cultures, and gametic cells such as microspores, pollen, sperm and egg cells. Callus
may be initiated from tissue sources including, but not limited to, immature embryos, seedling
apical meristems, root, leaf, microspores and the like. Those cells which are capable of
proliferating as callus also are candidate recipient cells for genetic transformation.

Somatic cells are of various types. Embryogenic cells are one example of somatic cells which may be induced to regenerate a plant through embryo formation. Non-embryogenic cells are those which typically will not respond in such a fashion. Certain techniques may be used that enrich recipient cells within a cell population, for example by manual selection and culture of friable, embryogenic tissue. Manual selection techniques which can be employed to select target cells may include, *e.g.*, assessing cell morphology and differentiation, or may use various physical or biological means. Cryopreservation also is a possible method of selecting for recipient cells.

Where employed, cultured cells may be grown either on solid supports or in the form of liquid suspensions. In either instance, nutrients may be provided to the cells in the form of media, and environmental conditions controlled. There are many types of tissue culture media comprised of various amino acids, salts, sugars, growth regulators and vitamins. Most of the media employed in the practice of the invention will have some similar components, but may differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending on the growth media. In some media, cells survive but do not divide. Various types of media suitable for culture of plant cells previously have been described. Examples of these media include, but are not limited to, the N6 medium described by Chu *et al.*, *Scientia Sinica* 18:659-668, 1975) and MS media (Murashige and Skoog, *Physiol. Plant.* 15:473-497, 1962).

IV. Methods for Genetic Transformation

Suitable methods for transformation of plant or other cells for use with the current invention are believed to include virtually any method by which DNA can be introduced into a cell, such as by direct delivery of DNA such as by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, *Plant Mol. Biol.* 21:415-428, 1993), by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.*, *ibid*, 1985), by electroporation (U.S. Patent No. 5,384,253, specifically incorporated herein by reference in its entirety), by agitation with silicon carbide fibers (Kaeppeler *et al.*, *TAG* 84:560-566, 1990; U.S. Patent No. 5,302,523, specifically incorporated herein by reference in its entirety; and U.S. Patent No. 5,464,765, specifically incorporated herein by reference in its entirety), by *Agrobacterium*-mediated transformation (U.S. Patent No.

5,591,616 and U.S. Patent No. 5,563,055; both specifically incorporated herein by reference) and by acceleration of DNA coated particles (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,877; and U.S. Patent No. 5,538,880; each specifically incorporated herein by reference in its entirety), *etc.* Through the application of techniques such as these, the cells of virtually any plant species may be stably transformed, and these cells developed into transgenic plants.

A. *Agrobacterium*-mediated Transformation

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described by Fraley *et al.*, *Bio/Technol.* 3:629-635, 1985), Rogers *et al.*, (*Methods Enzymol.* 153:253-277, 1987) and U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety.

Agrobacterium-mediated transformation is most efficient in dicotyledonous plants and is the preferable method for transformation of dicots, including *Arabidopsis*, tobacco, tomato, alfalfa and potato. Indeed, while *Agrobacterium*-mediated transformation has been routinely used with dicotyledonous plants for a number of years, it has only recently become applicable to monocotyledonous plants. Advances in *Agrobacterium*-mediated transformation techniques have now made the technique applicable to nearly all monocotyledonous plants. For example, *Agrobacterium*-mediated transformation techniques have now been applied to rice (Hiei *et al.*, *Plant Mol. Biol.* 35:205-218, 1997; U.S. Patent No. 5,591,616), wheat (McCormac *et al.*, *Euphytica* 99:17-25, 1998), barley (Tingay *et al.*, *Plant J.* 11:1369-1376, 1997), alfalfa (*e.g.* Thomas *et al.*, *Pl. Sci.* 69:189-198, 1990; McKersie *et al.*, *Pl. Physiol.* 103:1155-1163, 1993; Chabaud *et al.*, *Pl. Cell Rep.* 22:46-51, 1993) and maize (Ishida *et al.*, *Naute Biotechnol.* 14:745-750, 1996).

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, *Bio/Technol.* 3:637-642, 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate the construction of vectors capable of expressing various

polypeptide coding genes. The vectors described (Rogers *et al.*, *ibid.* 1987) have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant strains where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

B. Electroporation

To effect transformation by electroporation, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation of intact cells include maize (U.S. Patent No. 5,384,253; Rhodes *et al.*, *Methods Mol. Biol.* 55:121-131, 1995; D'Halluin *et al.*, *Plant Cell* 4:1495-1505, 1992), wheat (Zhou *et al.*, *Pl. Cell Rep.* 12:612-616, 1993), tomato (Hou and Lin, *Pl. Physiol.* 111:166, 1996), soybean (Christou *et al.*, *PNAS* 84:3962-3966, 1987) and tobacco (Lee *et al.*, *Korean J. Genet.* 11:65-72, 1989).

One also may employ protoplasts for electroporation transformation of plants (Bates, *Mol. Biotechnol.* 2:135-145, 1994; Lazzeri, *Methods Mol. Biol.* 49:95-106, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and Widholm in Intl. Patent Appl. Publ. No. WO 9217598 (specifically incorporated herein by reference). Other examples of species for which protoplast transformation has been described include barley (Lazzeri, *ibid.*, 1995), sorghum (Battraw *et al.*, *TAG* 82:161-168, 1991), maize (Bhattacharjee *et al.*, *J. Plant Biochem. Biotechnol.* 6:69-73, 1997), wheat (He *et al.*, *Pl. Cell Rep.* 14:192-196, 1994) and tomato (Tsukada, *Pl. Cell Physiol.* 30:599-604, 1989).

C. Microprojectile Bombardment

Another method for delivering transforming DNA segments to plant cells in accordance with the invention is microprojectile bombardment (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,880; U.S. Patent No. 5,610,042; and PCT Application WO 94/09699; each of which is

specifically incorporated herein by reference in its entirety). In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold. It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. However, it is contemplated that particles may contain DNA rather than be coated with DNA. Hence, it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate.

An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is the Biolistics® Particle Delivery System (Dupont), which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or nylon screen (*e.g.* NYTEX screen; Sefar America, Depew, NY USA), onto a filter surface covered with plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. Microprojectile bombardment techniques are widely applicable, and may be used to transform virtually any plant species. Examples of species for which have been transformed by microprojectile bombardment include monocot species such as maize (PCT Application WO 95/06128), barley (Ritala *et al.*, *Pl. Mol. Biol.* 24:317-325, 1994), wheat (U.S. Patent No. 5,563,055), and sorghum (Casa *et al.*, *PNAS* 90:11212-11216, 1993); as well as a number of dicots including tobacco (Tomes *et al.*, *Plant Mol. Biol.* 14:261-268, 1990; Buising and Benbow, *Mol. Gen. Genet.* 243:71-81, 1994), soybean (U.S. Patent No. 5,322,783), sunflower (Knittel *et al.*, *Pl. Cell Rep.* 14:81-86, 1994), peanut (Singsit *et al.*, *Transgenic Res.* 6:169-176, 1997), cotton (McCabe and Martinell, *Bio/Technol.* 11:596-598, 1993), tomato (VanEck *et al.*, *Pl. Cell Rep.* 14:299-304, 1995), and legumes in general (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety).

D. Other Transformation Methods

Transformation of protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, e.g., Potrykus *et al.*, 1985, *ibid.*; Lorz *et al.*, *Mol. Gen. Genet.* 199:178-182, 1985; Omirulleh *et al.*, 1993, *ibid.*; Fromm *et al.*, 1986, *ibid.*; Uchimiya *et al.*, *Mol. Gen. Genet.* 204:204, 1986; Callis *et al.*, 1987, *ibid.*; Marcotte *et al.*, *Nature* 335:454, 1988).

Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of plants from protoplasts have been described (Toriyama *et al.*, *TAG* 73:16, 1986; Yamada *et al.*, *Pl. Cell Rep.* 4:85, 1986; Abdullah *et al.*, *Bio/Technol.* 4:1087, 1986; Omirulleh *et al.*, 1993, *ibid.* and U.S. Patent No. 5,508,184). Examples of the use of direct uptake transformation of protoplasts include transformation of rice (Ghosh-Biswas *et al.*, *J. Biotechnol.* 32:1-10, 1994), sorghum (Battraw and Hall, 1991, *ibid.*), barley (Lazerri, 1995, *ibid.*), oat (Zheng and Edwards, *J. Gen. Virol.* 71:1865-1868, 1990) and maize (Omirulleh *et al.*, 1993, *ibid.*).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1989, *ibid.*). Also, silicon carbide fiber-mediated transformation may be used with or without protoplasting (Kaeppler, *Pl. Cell Rep.* 9:415-418, 1990; Kaeppler *et al.*, *TAG* 84:560-566, 1992; U.S. Patent No. 5,563,055). Transformation with this technique is accomplished by agitating silicon carbide fibers together with cells in a DNA solution. DNA passively enters as the cells are punctured. This technique has been used successfully with, for example, the monocot cereals maize (PCT Application WO 95/06128; (Thompson, *NAR* 25:4876-4882, 1995) and rice (Nagatani, *Biotech. Tech.* 11:471-473, 1997).

V. Production and Characterization of Stably Transformed Plants

After effecting delivery of exogenous DNA to recipient cells, the next steps generally concern identifying the transformed cells for further culturing and plant regeneration. In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene with a transformation vector prepared in accordance with the invention. In this case, one would then generally assay the potentially transformed cell population by

exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

A. Selection

It is believed that DNA is introduced into only a small percentage of target cells in any one experiment. In order to provide an efficient system for identification of those cells receiving DNA and integrating it into their genomes one may employ a means for selecting those cells that are stably transformed. One exemplary embodiment of such a method is to introduce into the host cell, a marker gene which confers resistance to some normally inhibitory agent, such as an antibiotic or herbicide. Examples of antibiotics which may be used include the aminoglycoside antibiotics neomycin, kanamycin and paromomycin, or the antibiotic hygromycin. Resistance to the aminoglycoside antibiotics is conferred by aminoglycoside phosphotransferase enzymes such as neomycin phosphotransferase II (NPT II) or NPT I, whereas resistance to hygromycin is conferred by hygromycin phosphotransferase.

Potentially transformed cells then are exposed to the selective agent. In the population of surviving cells will be those cells where, generally, the resistance-conferring gene has been integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA.

One herbicide which constitutes a desirable selection agent is the broad spectrum herbicide bialaphos. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of the L-alanine residues by intracellular peptidases, the PPT is released and is a potent inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (Ogawa *et al.*, *Sc. Rep.* 13:42-48, 1973). Synthetic PPT, the active ingredient in the herbicide Liberty™ also is effective as a selection agent. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the plant cells.

The organism producing bialaphos and other species of the genus *Streptomyces* also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the *bar* gene in *Streptomyces hygroscopicus* and the *pat* gene in *Streptomyces viridochromogenes*. The

use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (PAT) is referred to in DE 3642 829 A, wherein the gene is isolated from *Streptomyces viridochromogenes*. In the bacterial source organism, this enzyme acetylates the free amino group of PPT preventing auto-toxicity (Thompson *et al.*, *EMBO J.* 6:2519-2523, 1987). The *bar* gene has been cloned (Murakami *et al.*, 1986, *ibid.*; Thompson *et al.*, 1987, *ibid*) and expressed in transgenic tobacco, tomato, potato (De Block *et al.*, *EMBO J.* 6:2513-2518, 1987) *Brassica* (De Block *et al.*, *Pl. Physiol.* 91:694-701, 1989) and maize (U.S. Patent No. 5,550,318). In previous reports, some transgenic plants which expressed the resistance gene were completely resistant to commercial formulations of PPT and bialaphos in greenhouses.

Another example of a herbicide which is useful for selection of transformed cell lines in the practice of the invention is the broad spectrum herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS which is active in the aromatic amino acid biosynthetic pathway. Inhibition of this enzyme leads to starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived thereof. U.S. Patent No. 4,535,060 describes the isolation of EPSPS mutations which confer glyphosate resistance on the *Salmonella typhimurium* gene for EPSPS, *aroA*. The EPSPS gene was cloned from *Zea mays* and mutations similar to those found in a glyphosate resistant *aroA* gene were introduced *in vitro*. Mutant genes encoding glyphosate resistant EPSPS enzymes are described in, for example, International Patent WO 97/4103. The best characterized mutant EPSPS gene conferring glyphosate resistance comprises amino acid changes at residues 102 and 106, although it is anticipated that other mutations will also be useful (PCT/WO97/4103).

To use the *bar*-bialaphos or the EPSPS-glyphosate selective system, transformed tissue is cultured for 0 - 28 days on nonselective medium and subsequently transferred to medium containing from 1-3 mg/l bialaphos or 1-3 mM glyphosate as appropriate. While ranges of 1-3 mg/l bialaphos or 1-3 mM glyphosate will typically be preferred, it is proposed that ranges of 0.1-50 mg/l bialaphos or 0.1-50 mM glyphosate will find utility.

It further is contemplated that the herbicide DALAPON, 2,2-dichloropropionic acid, may be useful for identification of transformed cells. The enzyme 2,2-dichloropropionic acid dehalogenase (*deh*) inactivates the herbicidal activity of 2,2-dichloropropionic acid and therefore

confers herbicidal resistance on cells or plants expressing a gene encoding the dehalogenase enzyme (Buchanan-Wollaston *et al.*, *Pl. Cell Rep.* 11:627-631, 1992; U.S. Patent No. 5,508,468).

Alternatively, a gene encoding anthranilate synthase, which confers resistance to certain amino acid analogs, *e.g.*, 5-methyltryptophan or 6-methyl anthranilate, may be useful as a
5 selectable marker gene. The use of an anthranilate synthase gene as a selectable marker was described in U.S. Patent No. 5,508,468.

An example of a screenable marker trait is the enzyme luciferase. In the presence of the substrate luciferin, cells expressing luciferase emit light which can be detected on photographic or x-ray film, in a luminometer (or liquid scintillation counter), by devices that enhance night
10 vision, or by a highly light sensitive video camera, such as a photon counting camera. These assays are nondestructive and transformed cells may be cultured further following identification. The photon counting camera is especially valuable as it allows one to identify specific cells or groups of cells which are expressing luciferase and manipulate those in real time. Another screenable marker which may be used in a similar fashion is the gene coding for green
15 fluorescent protein.

It further is contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cells or tissue types, a selection agent, such as bialaphos or glyphosate, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants
20 and nontransformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as bialaphos or glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as luciferase would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. It is proposed
25 that combinations of selection and screening may enable one to identify transformants in a wider variety of cell and tissue types. This may be efficiently achieved using a gene fusion between a selectable marker gene and a screenable marker gene, for example, between an NPTII gene and a GFP gene.

B. Regeneration and Seed Production

Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. In an exemplary embodiment, MS and N6 media may be modified by including further substances
5 such as growth regulators. One such growth regulator is dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or picloram. Media improvement in these and like ways has been found to facilitate the growth of cells at specific developmental stages. Tissue may be maintained on a basic media with growth regulators until sufficient tissue is available to begin plant regeneration efforts, or following repeated rounds of
10 manual selection, until the morphology of the tissue is suitable for regeneration, at least 2 wk, then transferred to media conducive to maturation of embryoids. Cultures are transferred every 2 wk on this medium. Shoot development will signal the time to transfer to medium lacking growth regulators.

The transformed cells, identified by selection or screening and cultured in an appropriate
15 medium that supports regeneration, will then be allowed to mature into plants. Developing plantlets are transferred to soil-less plant growth mix, and hardened, *e.g.*, in an environmentally controlled chamber, for example, at about 85% relative humidity, 600 ppm CO₂, and 25-250 microeinsteins m⁻² s⁻¹ of light. Plants are preferably matured either in a growth chamber or greenhouse. Plants can be regenerated from about 6 wk to 10 months after a transformant is
20 identified, depending on the initial tissue. During regeneration, cells are grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels are petri dishes and Plantcon™ containers (MP-ICN Biomedicals, Solon, OH, USA). Regenerating plants are preferably grown at about 19 to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

Seeds on transformed plants may occasionally require embryo rescue due to cessation of
25 seed development and premature senescence of plants. To rescue developing embryos, they are excised from surface-disinfected seeds 10-20 days post-pollination and cultured. An embodiment of media used for culture at this stage comprises MS salts, 2% sucrose, and 5.5 g/l agarose. In embryo rescue, large embryos (defined as greater than 3 mm in length) are
30 germinated directly on an appropriate media. Embryos smaller than that may be cultured for 1

wk on media containing the above ingredients along with 10^{-5} M abscisic acid and then transferred to growth regulator-free medium for germination.

C. Characterization

To confirm the presence of the exogenous DNA or “transgene(s)” in the regenerating plants, a variety of assays may be performed. Such assays include, for example, “molecular biological” assays, such as Southern and northern blotting and PCR; “biochemical” assays, such as detecting the presence of a protein product, *e.g.*, by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

D. DNA Integration, RNA Expression and Inheritance

Genomic DNA may be isolated from cell lines or any plant parts to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art. Note that intact sequences will not always be present, presumably due to rearrangement or deletion of sequences in the cell. The presence of DNA elements introduced through the methods of this invention may be determined, for example, by polymerase chain reaction (PCR). Using this technique, discreet fragments of DNA are amplified and detected by gel electrophoresis. This type of analysis permits one to determine whether a gene is present in a stable transformant, but does not prove integration of the introduced gene into the host cell genome. It is typically the case, however, that DNA has been integrated into the genome of all transformants that demonstrate the presence of the gene through PCR analysis. In addition, it is not typically possible using PCRTM techniques to determine whether transformants have exogenous genes introduced into different sites in the genome, *i.e.*, whether transformants are of independent origin. It is contemplated that using PCR techniques it would be possible to clone fragments of the host genomic DNA adjacent to an introduced gene.

Positive proof of DNA integration into the host genome and the independent identities of transformants may be determined using the technique of Southern hybridization. Using this technique specific DNA sequences that were introduced into the host genome and flanking host DNA sequences can be identified. Hence the Southern hybridization pattern of a given transformant serves as an identifying characteristic of that transformant. In addition it is possible through Southern hybridization to demonstrate the presence of introduced genes in high

molecular weight DNA, *i.e.*, confirm that the introduced gene has been integrated into the host cell genome. The technique of Southern hybridization provides information that is obtained using PCR, *e.g.*, the presence of a gene, but also demonstrates integration into the genome and characterizes each individual transformant.

5 Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA will only be expressed in particular cells or tissue types and hence it will be necessary to prepare RNA for analysis from these tissues. PCR techniques also may be used for detection and quantitation of RNA produced from introduced genes. In this application of PCR it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse
10 transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species also can be determined using
15 dot or slot blot northern hybridizations. These techniques are modifications of northern blotting and will only demonstrate the presence or absence of an RNA species.

E. Gene Expression

While Southern blotting and PCR may be used to detect the gene(s) in question, they do not provide information as to whether the corresponding protein is being expressed. Expression
20 may be evaluated by determining expression via transcript-profiling techniques such as by use of a microarray, and by specifically identifying the protein products of the introduced genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-
25 chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of
30 approaches may be employed with even greater specificity such as western blotting in which

antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Assay procedures also may be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed and may include assays for PAT enzymatic activity by following production of radiolabeled acetylated phosphinothricin from phosphinothricin and ^{14}C -acetyl CoA or for anthranilate synthase activity by following loss of fluorescence of anthranilate, to name two.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of genes encoding enzymes or storage proteins which change amino acid composition and may be detected by amino acid analysis, or by enzymes which change starch quantity which may be analyzed by near infrared reflectance spectrometry. Morphological changes may include greater stature or thicker stalks. Most often changes in response of plants or plant parts to imposed treatments are evaluated under carefully controlled conditions termed bioassays.

VI. Breeding Plants of the Invention

In addition to direct transformation of a particular plant genotype with a construct prepared according to the current invention, transgenic plants may be made by crossing a plant having a selected DNA of the invention to a second plant lacking the construct. For example, a selected CT biosynthesis gene can be introduced into a particular plant variety by crossing, without the need for ever directly transforming a plant of that given variety. Therefore, the current invention not only encompasses a plant directly transformed or regenerated from cells which have been transformed in accordance with the current invention, but also the progeny of

such plants. As used herein the term “progeny” denotes the offspring of any generation of a parent plant prepared in accordance with the instant invention, wherein the progeny comprises a selected DNA construct prepared in accordance with the invention. “Crossing” a plant to provide a plant line having one or more added transgenes relative to a starting plant line, as disclosed herein, is defined as the techniques that result in a transgene of the invention being introduced into a plant line by crossing a starting line with a donor plant line that comprises a transgene of the invention. To achieve this one could, for example, perform the following steps:

- (a) plant seeds of the first (starting line) and second (donor plant line that comprises a transgene of the invention) parent plants;
- (b) grow the seeds of the first and second parent plants into plants that bear flowers;
- (c) pollinate a flower from the first parent plant with pollen from the second parent plant; and
- (d) harvest seeds produced on the parent plant bearing the fertilized flower.

Backcrossing is herein defined as the process including the steps of:

- (a) crossing a plant of a first genotype containing a desired gene, DNA sequence or element to a plant of a second genotype lacking the desired gene, DNA sequence or element;
- (b) selecting one or more progeny plant containing the desired gene, DNA sequence or element;
- (c) crossing the progeny plant to a plant of the second genotype; and
- (d) repeating steps (b) and (c) for the purpose of transferring a desired DNA sequence from a plant of a first genotype to a plant of a second genotype.

Introgression of a DNA element into a plant genotype is defined as the result of the process of backcross conversion. A plant genotype into which a DNA sequence has been introgressed may be referred to as a backcross converted genotype, line, inbred, or hybrid. Similarly a plant genotype lacking the desired DNA sequence may be referred to as an unconverted genotype, line, inbred, or hybrid.

VII. Definitions

Expression: The combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule such as a structural gene to produce a polypeptide.

5 **Genetic Transformation:** A process of introducing a DNA sequence or construct (*e.g.*, a vector or expression cassette) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

10 **Heterologous:** A sequence which is not normally present in a given host genome in the genetic context in which the sequence is currently found. In this respect, the sequence may be native to the host genome, but be rearranged with respect to other genetic sequences within the host sequence. For example, a regulatory sequence may be heterologous in that it is linked to a different coding sequence relative to the native regulatory sequence.

15 **Obtaining:** When used in conjunction with a transgenic plant cell or transgenic plant, obtaining means either transforming a non-transgenic plant cell or plant to create the transgenic plant cell or plant, or planting transgenic plant seed to produce the transgenic plant cell or plant. Such a transgenic plant seed may be from an R₀ transgenic plant or may be from a progeny of any generation thereof that inherits a given transgenic sequence from a starting transgenic parent plant.

20 **Over-expression:** As defined herein, "over-expression" is defined as an increased level of mRNA encoding a gene of interest, or an increased level of the translational product thereof within the plant. For instance in a transgenic plant this may be measured relative to an otherwise isogenic plant which does not comprise a transgene encoding the gene of interest.

25 **Promoter:** A recognition site on a DNA sequence or group of DNA sequences that provides an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene. A "constitutive promoter" allows for continual transcription of its associated gene. The term "constitutive" as used herein does not necessarily indicate that a gene is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types, although some variation in abundance is often observed. An "inducible promoter" is a promoter that is capable activating

transcription of one or more DNA sequences or genes in response to an inducer. The inducer can be, for instance, a chemical agent, a physiological stress or condition, or a pathogen.

R₀ transgenic plant: A plant that has been genetically transformed or has been regenerated from a plant cell or cells that have been genetically transformed.

5 **Regeneration:** The process of growing a plant from a plant cell (*e.g.*, plant protoplast, callus or explant).

Selected DNA: A DNA segment which one desires to introduce into a plant genome by genetic transformation.

10 **Transformation construct:** A chimeric DNA molecule which is designed for introduction into a host genome by genetic transformation. Preferred transformation constructs will comprise all of the genetic elements necessary to direct the expression of one or more exogenous genes. In particular embodiments of the instant invention, it may be desirable to introduce a transformation construct into a host cell in the form of an expression cassette.

15 **Transformed cell:** A cell the DNA complement of which has been altered by the introduction of an exogenous DNA molecule into that cell.

20 **Transgene:** A segment of DNA which has been incorporated into a host genome or is capable of autonomous replication in a host cell and is capable of causing the expression of one or more coding sequences. Exemplary transgenes will provide the host cell, or plants regenerated therefrom, with a novel phenotype relative to the corresponding non-transformed cell or plant. Transgenes may be directly introduced into a plant by genetic transformation, or may be inherited from a plant of any previous generation which was transformed with the DNA segment.

25 **Transgenic plant:** A plant or progeny plant of any subsequent generation derived therefrom, wherein the DNA of the plant or progeny thereof contains an introduced exogenous DNA segment not naturally present in a non-transgenic plant of the same strain. The transgenic plant may additionally contain sequences which are native to the plant being transformed, but wherein the “exogenous” gene has been altered in order to alter the level or pattern of expression of the gene, for example, by use of one or more heterologous regulatory or other elements.

Vector: A DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

VIII. Examples

5 The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate
10 that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to
15 those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

EXAMPLE 1

Mutation of *AtELP4* Compromises Basal Resistance to a Bacterial Plant Pathogen

A pathogen growth assay was performed on an *elo1-1/Atelp4* mutant *Arabidopsis* line which contains a mutation (G to A) at the acceptor splice site of the third intron of the *AtELP4/ELO1* gene in Landsberg *erecta* (*Ler*) background (Nelissen *et al.*, *PNAS* 102:7754-7759, 2005). As shown in FIG. 1(a), *elo1-1/Atelp4* mutant plants supported significantly more pathogen growth than wild-type plants 3 days after infection with the bacterial strain *Pseudomonas syringae* pv. *maculicola* (“*Psm*”) ES4326 (Davis *et al.*, *Mol. Plant Microbe Interact* 4:477-488, 1991). To further confirm this result, a loss-of-function T-DNA insertion *Atelp4* mutant in Columbia background (SALK_079193; Alonso *et al.*, *Science* 301:653-657, 2003; *Arabidopsis* Biological Resource Center, Ohio State University, Columbus, OH, USA), which exhibits a similar morphological phenotype as other Elongator mutants (FIG. 1(c)), was also tested for its resistance to *Psm* ES4326 (FIG. 1(b)). This knockout mutant was also more susceptible to *Psm* ES4326 than the wild type, as measured by pathogen multiplication. These results demonstrate that ELP4 is a positive regulator of plant immunity and that the accessory subcomplex of Elongator contains functions in plant defense.

EXAMPLE 2

The *elo1-1* mutation restores SA tolerance to *npr1-L* and suppresses hyperaccumulation of SA in *npr1-L*

To examine the role of ELP4 in plant defense responses, including tolerance of seedlings to SA, an *Arabidopsis* double mutant was created via genetic cross between an *elo1-1/Atelp4* line and *npr1-L*, which is a loss-of-function T-DNA insertion *npr1* mutant in *Ler* background. As shown in FIG. 2(a), *npr1-L* seedling failed to develop beyond the cotyledon stage on media containing high concentrations (0.5 mM) of SA, which is consistent with previous studies (Cao *et al.* *Cell* 88:57-63, 1997; DeFraia *et al.*, *Plant J.* 64:511-523, 2010, DeFraia *et al.*, *BMC Plant Biol.* 13, 102, 2013; Kinkema *et al.*, *Plant Cell* 12:2339-2350, 2000). In contrast, the *elo1-1 npr1-L* double mutant showed partial tolerance to the SA toxicity, suggesting that *AtELP4* functions similarly as *AtELP2* and *AtELP3* in suppressing SA tolerance. SA levels in the *elo1-1 npr1-L* double mutant were measured and it was found that the presence of the *elo1-1* mutation

along with the *npr1-L* mutation reduced SA accumulation in *npr1-L* after *Psm* ES4326 infection, relative to the presence of *npr1-L* alone (FIG. 2(b)). Moreover, simultaneous removal of *AtELP4* and *NPR1* resulted in plants more susceptible to *Psm* ES4326 and also lacking resistance to two different ETI-inducing pathogens, *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000/*avrRpt2* and *Pst* DC3000/*avrRps4*. Thus, *AtELP4* has a role in plant defense phenotypes, demonstrating that the accessory subcomplex (ELP4-ELP6) of Elongator is essential for the function of the complete Elongator complex in plant immunity.

EXAMPLE 3

The *elo1-1* mutation genetically interacts with *eds1-2*

EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) is an essential regulator of basal resistance to (hemi)biotrophic pathogens by controlling defense amplification and the accumulation of the defense signaling molecule SA. Since Elongator also controls defense responses and regulates accumulation of SA, the genetic relationship between *Elongator* and *EDS1* in plant immunity was studied. A double mutant *Arabidopsis* line, *elo1-1 eds1-2*, was created by a genetic cross in *Ler* background and pathogen-induced SA accumulation in *elo1-1/Atelp4*, *eds1-2* and the double mutant *elo1-1 eds1-2* was followed. As shown in FIG. 3(a), (b), the *elo1-1* single mutant exhibited a small decrease in SA levels after pathogen infection. However, *eds1* appears to be epistatic to *npr1* since the *npr1 eds1-2* double mutant did not differ from *eds1-2* in pathogen-induced SA accumulation.

To investigate the relationship between *Elongator* and *EDS1* in disease resistance, pathogen growth in *elo1-1/Atelp4*, *eds1-2* and the double mutant *elo1-1 eds1-2* was also examined. As shown in FIG. 3(c), (d), the *elo1-1* mutation allowed for more pathogen growth in both the wild-type and *eds1-2* backgrounds, indicating that *elo1-1* and *eds1-2* are largely independent and additive in contributing to disease susceptibility.

EXAMPLE 4

Overexpression of *AtELP4* confers disease resistance

Transcriptome (mRNA abundance) analysis of the six ELP subunits based on signal intensity on the Affymetrix GeneChip® *Arabidopsis* ATH1 Genome Array (Affymetrix, Santa

Clara, CA, USA) using GENEVESTIGATOR (Zimmermann et al., *Plant Physiol.* 136:2621-2632, 2004) indicated that *AtELP4* is the lowest expressed gene throughout plant life of the genes encoding the plant Elongator complex (FIG. 4(a)). This suggested that overexpression of ELP4 may result in a detectable phenotype. Therefore the effect of overexpression of *AtELP4* on plant disease resistance was tested. The T-DNA vector pBI1.4T (Mindrinos et al., *Cell* 78:1089-1099) was utilized, which contains the neomycin phosphotransferase II (nptII) gene, conferring resistance to kanamycin for plant selection. The coding region of *AtELP4* (SEQ ID NO:13) was amplified from cDNA using PCR, cloned into pBI1.4T and then transformed into the *elo1-1/Atelp4* mutant plants using the *Agrobacterium* strain GV3101 following the floral-dip method (Clough and Bent, *Plant J.* 16:735-743). An *AtELP4*-GFP fusion gene was also transformed into the *elo1-1/Atelp4* mutant plants. The *AtELP4* coding sequence was cloned into the plasmid pRTL2-mGFP (von Arnim et al., *Gene* 221:35-43, 1998), which contains the cauliflower mosaic virus (CaMV) 35S promoter, a green fluorescent protein (GFP) gene and a CaMV 35S terminator. The 35S promoter-*AtELP4*-GFP-35S terminator cassette was cloned into the T-DNA vector pCB302 (Xiang et al., *Plant Mol. Biol.* 40:711-717, 1999), which confers resistance to Basta for plant selection, and then transformed into the *elo1-1/Atelp4* mutant plants using the *Agrobacterium* strain GV3101 following the floral-dip method (Clough and Bent, *Plant J.* 16:735-743). Both transgenes *AtELP4/ELO1* and *AtELP4/ELO1-GFP* complemented the mutant morphology of the *elo1-1/Atelp4* mutant (FIG. 4(b)). Two independent 35S::*AtELP4* lines with high *AtELP4* expression and one 35S::*AtELP4*-GFP were selected and their homozygous progenies containing a single insert were used for further experiments (FIG. 4). No spontaneous lesion formation was observed in these lines. Resistance of *Arabidopsis* to bacterial pathogen *Psm* ES4326 was tested. Three days after infection, transgenic lines exhibited significantly enhanced resistance to *Psm* ES4326 as compared to wild-type plants (FIG. 4(c), (d)) as determined by multiplication of bacteria in infected leaves.

EXAMPLE 5

Overexpression of *AtELP4* leads to constitutive activation of SA signaling

Elongator associates with elongating RNAP II and positively regulates transcription. In order to understand the mechanism underlying enhanced disease resistance in *AtELP4* overexpressing plants, microarray analysis was performed to investigate global transcriptional

profiling changes in *AtELP4* overexpressing plants. Triplicate experiments were performed independently, and the data were analyzed to identify genes that were differentially expressed between *35S::AtELP4* line #1 and the wild type. *q*-values were used to identify differentially expressed candidate genes. It was found that overexpressing *AtELP4* had a significant impact on the expression levels of genes involved in the SA signaling pathway (Table 1).

Table 1. Defense genes that are differentially expressed between *35S::AtELP4* and wild-type *Ler* plants.

AGI Locus	Gene Name	<i>35S::AtELP4/Ler</i>		AGI Description
		Log ₂ (FC)	<i>q</i> Value	
At3g11220	<i>ELO1</i>	5.54	0	ELONGATA 1/Elongator subunit 4
At1g02450	<i>NIMIN1</i>	1.089	0	NIM-INTERACTING 1
At3g25882	<i>NIMIN2</i>	1.593	0.0001	NIM-INTERACTING 2
At4g39030	<i>EDS5</i>	1.301	0	ENHANCED DISEASE SUSCEPTIBILITY 5
At5g13320	<i>PBS3</i>	2.345	0.0001	AVRPPHB SUSCEPTIBLE 3
At3g52430	<i>PAD4</i>	1.145	0	PHYTOALEXIN DEFICIENT 4
At4g14400	<i>ACD6</i>	1.122	0	ACCELERATED CELL DEATH 6
At2g13810	<i>ALD1</i>	1.265	0.0005	AGD2-LIKE DEFENSE RESPONSE PROTEIN 1
At1g19250	<i>FMO1</i>	4.168	0	FLAVIN_DEPENDENT MOMOOXYGENASE 1
At2g14610	<i>PR1</i>	2.251	0	PATHOGENESIS-RELATED GENE 1
At3g57260	<i>PR2</i>	2.122	0.0007	PATHOGENESIS-RELATED GENE 2
At1g75040	<i>PR5</i>	2.652	0.0010	PATHOGENESIS-RELATED GENE 5
At4g31800	<i>WRKY18</i>	1.252	0	WRKY DNA-binding protein 18
At5g22570	<i>WRKY38</i>	1.374	0.0002	WRKY DNA-binding protein 38
At2g04450	<i>NUDT6</i>	2.348	0	NUDIX HYDROLASE HOMOLOG 6
At4g12720	<i>NUDT7</i>	1.103	0	NUDIX HYDROLASE HOMOLOG 7
At2g19190	<i>FRK1</i>	1.613	0	FLG22-INDUCED RECEPTOR-LIKE KINASE 1
At1g45145	<i>TRX5</i>	1.361	0	THIOREDOXIN H-TYPE 5

To ensure validity of the microarray data, the expression levels of pathogenesis-related genes (*PR1*, *PR2*, and *PR5*) in the *AtELP4* overexpressing plants were investigated further by

RT-qPCR. It was found that *PR1*, *PR2*, and *PR5* mRNAs were constitutively activated in *35S::AtELP4* plants, further confirming the microarray data.

To determine the possible link in SA levels to the activated defense phenotype observed in *35S::AtELP4* plants, we measured the SA levels using HPLC. As shown in FIG. 5, the endogenous SA levels in *35S::AtELP4* plants were elevated in comparison with those in the wild type. Taken together, our observations suggest that overexpressing *AtELP4* contributes to disease resistance by constitutively activating SA-related defense signaling.

EXAMPLE 6

Induction of defense genes after *Psm* ES4326 infection is stronger and faster in *AtELP4*-overexpressing plants

The speed of pathogen-induced transcriptional reprogramming in plants is a determining factor of plant defense. Previous studies showed that disruption of Elongator results in delayed and/or decreased induction of a group of defense genes compared to wild type during pathogen infection, suggesting that the plant Elongator complex may serve as an accelerator in plant immunity (DeFraia *et al.*, *Plant J.* 64:511-523, 2010, DeFraia *et al.*, *BMC Plant Biol.* 13, 102, 2013; Wang *et al.*, *Plant Cell* 25:762-776, 2013). To investigate whether overexpression of the Elongator complex can accelerate the induction of defense genes, a time course experiment was performed to monitor the induction kinetics of *PR* genes during the infection of the virulent pathogen *Psm* ES4326 in *35S::AtELP4* transgenic plants. Similarly to *Atelp2* and *Atelp3*, *elo1/Atelp4* plants exhibited a delayed induction of *PR1*, *PR2*, and *PR5* compared to the wild type during *Psm* ES4326 infection. In contrast, induction of the defense genes *WRKY18*, *WRKY38*, and *PR1* is stronger and faster in *AtELP4* overexpressing plants (FIG. 6). These results show that Elongator affects the speed and amplitude of defense gene induction during *Psm* ES4326 infection, explaining the enhanced defense phenotype in *AtELP4* overexpressing plants.

EXAMPLE 7

Materials and methods for construction and testing of transgenic strawberry plants

A. Plasmid construction and plant transformation

The Gateway® T-DNA vector pK7WG2D.1 (Life Technologies, Grand Island, NY, USA) was utilized, which contains the neomycin phosphotransferase II (nptII) gene, conferring resistance to kanamycin for plant selection, and the enhanced green fluorescent protein (GFP) for visual selection (Karimi *et al. Trends Plant Sci.* 7:193-195, 2002). The coding regions of *AtELP3* and *AtELP4* were amplified from cDNA using PCR, cloned into pK7WG2D.1 and then transformed into the diploid strawberry *Fragaria vesca* L. using the *Agrobacterium* strain GV3101. Leaf explants of *F. vesca* accession ‘Hawaii-4’ were transformed using a regeneration protocol (Oosumi *et al. Planta* 223:1219-1230, 2006) optimized by Silva *et al. Transgenic Res.* 1-12, doi: 10.1007/s11248-015-9869-5, 2015). Transgenic calli and shoots were visually screened for GFP six weeks after co-culture, and well-developed and rooted shoots were transplanted into soil and placed in a growth chamber with a 12 hour photoperiod. The plants were considered to be independent transgenic lines when regenerated from independent calli. ‘Hawaii-4’ seedlings, also derived through leaf regeneration, were used as a control in all experiments of the study. Plants were propagated by runners, and watered and fertilized as needed. Pesticides were applied as necessary to control insects and mites. After two months of growth in soil, well-developed plants were used in the experiments.

B. Salicylic acid measurement

Basal levels of total and free SA were quantified. For free SA accumulation after pathogen challenge, transgenic strawberry lines and the control were inoculated with *C. gloeosporioides* (see below), and leaf samples were collected 24 hours after infection. SA content in strawberry leaf tissues was measured by HPLC as described by Verberne *et al. Phytochemical Anal.*, 13:45-50, 2002).

C. Assessment of disease resistance

To evaluate the effect of *AtELP3* and *AtELP4* on disease resistance, the pathogens *Colletotrichum gloeosporioides*, *Podosphaera aphanis* and *Xanthomonas fragariae*, causal agents of strawberry crown rot, powdery mildew and angular leaf spot, respectively, were utilized.

Three isolates of the hemibiotrophic pathogen *C. gloeosporioides* (CG#13-01, GG#98-285 and CG#97-15A) were obtained from diseased strawberry crowns. Colonies were maintained on Potato-dextrose-agar (PDA) for 6 to 8 d at 24°C. Conidial suspension and plant inoculation was performed according to Silva *et al.*, 2015, *ibid.* Incidence of Anthracnose crown

5 rot was scored as the number of individual plants collapsed at 20 dpi. Disease incidence (DI) was also assessed every 2 d, allowing the calculation of the area under the disease progress curve (AUDPC) (Shaner and Finney, *Phytopathol.* 67:1051-1066, 1977):

$$\text{AUDPC} = \sum_{i=1}^{N_i-1} \frac{(y_i + y_{i+1})}{2} (t_{i+1} - t_i), \text{ where } N_i \text{ is number of assessments; } (y_i + y_{i+1}) \text{ is the sum of}$$

initial and consecutive disease incidence; and $(t_{i+1} - t_i)$ is time interval between two consecutive

10 assessments. The experiment was conducted three times with five plants per strawberry line each time.

Powdery mildew inoculum was originally obtained from strawberry cv. 'Festival' (*Fragaria x ananasa* Duch.) and identified as *Podosphaera aphanis* based on the examination of conidiophores and/or chasmothecia detected on leaves surface. Selected inoculum (PM#15-31)

15 was used to infect non-transformed *F. vesca* accession Hawaii-4. Inoculated plants were kept in growth chamber for 8 days at 80 % RH and 22°C. After incubation, plants were assigned disease reaction (DR) scores according to the method described (Göllner *et al.*, *New Phytol.* 177:725-742, 2008), where a DR score of 2 describes fully susceptible plants, indicating that extensive pathogen growth was observed; a DR score of 0 refers to fully resistant plants, on which no

20 fungal structure and disease symptoms could be observed; and a DR score of 1 denotes plants with intermediate susceptibility, which show fungal structure on less than 10% of the leaf surface. At the end of the experiment, three samples of approximately 500 mg of leaves were harvested per genotype for spore count according to the methodology described by Silva *et al.*, 2015, *ibid.* Spore counts were normalized to the initial sample weight. The experiment was done

25 with five plants per line each time, and repeated three times with similar results.

The bacterial pathogen *X. fragariae* (XF#11-15) was initially isolated from leaves of strawberry 'Festival' and kept on solid sucrose peptone agar (SPA) (5% peptone, 0.5% K₂HPO₄, 0.25% MgSO₄·7H₂O, 10% sucrose and 18% agar) at ± 29°C for 4 d. Suspension preparation and

inoculation were performed according to Silva *et al.*, 2015, *ibid.*, adapted from Maas *et al.*, *HortSci.* 35:4, 2000).

Inoculation sites were evaluated and rated at the end of the fourth week using the following scale: 0 = no reaction, transient water-soaking from inoculation no longer evident; 1 = transient water-soaking evident in the inoculation site; 2 = slight chlorosis or necrosis in the center of the inoculation site; 3 = water-soaking expanding beyond inoculation site and often bacterial exudate evident; 4 = necrosis spreading beyond the inoculation site and/or secondary infections evident; and 5 = total necrosis of the inoculation area and leaflet changing color from chlorosis to reddish-brown. Plants were considered susceptible on a whole-plant basis if bacterial exudate was produced at any inoculation site, if inoculation sites remained translucent, or if secondary infection sites were apparent on inoculated leaves. Plants were considered resistant if inoculation sites lost translucency and/or developed necrotic centers that did not progress beyond inoculation sites. At the end of the test, inoculation sites were cut from the leaves, surface sterilized, ground in 1 mL of sterile water, vortexed, and serially diluted. The dilutions were transferred onto SPA in culture plates. Plates were incubated at room temperature for development of colonies of *X. fragariae* (Roberts *et al.*, *Plant Dis.* 80:1283-1288, 1996). The number of CFU/mL was calculated following the incubation of the plates for 6 d at RT.

All isolates used in these experiments were obtained from plants growing in greenhouses or strawberry fields located in west-central Florida. *X. fragariae* and *C. gloeosporioides* isolates were stored at -80°C in 20% glycerol, and *P. aphanis* accession was stored at the Herbarium and Culture Collection of Gulf Coast Research and Education Center (GCREC) - University of Florida.

D. Gene Expression

Total DNA and RNA were extracted from strawberry leaves (~100 mg) of transgenic lines and the control. In order to verify the presence of the full length of the plasmid, the *AtELP3* and *AtELP4* genes were amplified by PCR (94°C for 5 min, 35 cycles: 94°C for 3min, 65°C for 1 min, 72°C for 2 min, and hold at 72°C for 10 min) using *AtELP3F* and *AtELP3R*, and *AtELP4F* and *AtELP4R* primers. Strawberry genomic DNA was extracted from leaves using a CTAB method optimized for strawberry (Brunings *et al.*, *Euphytica* 173:63-75, 2010; Chang *et al.*,

Plant Mol. Biol. Rep. 11:113-116, 1993). RNA was extracted using an RNeasy[®] Plant Mini-Kit (Qiagen, Valencia, CA, USA) following the manufacture's instruction. Leaves samples were taken at 0, 24, 48 and 72 h after inoculated with *C. gloeosporioides*. One µg RNA was reverse transcribed using the Improm-II Reverse Transcriptase (Promega Inc., Madison, WI). Reverse transcription and real-time quantitative PCR (qPCR) was performed using the StepOne Plus system (Applied Biosystems, USA) based on SYBR Green chemistry. All qPCR primers were designed with the Primer Express 2.0 software (Applied Biosystems, USA). The relative mRNA levels of *AtELP3*, *AtELP4*, *FvPR1*, *FvPR2* and *FvNPR5* were expressed in relation to the strawberry elongation factor-1-alpha (*EF1α*) gene, and calculated using the $2^{-\Delta CT}$ method (Livak and Schmittgen, *Methods* 25:402-408, 2001; *Sehringer et al., Anal. Bioanal. Chem.* 383:768-775, 2005).

E. Statistical analysis.

Statistical analysis was performed using GraphPad Prism[®] software (GraphPad, San Diego, CA, USA) by one-way or two-way analysis of variance (ANOVA), or by Student's t test analysis. All experiments were performed three times with similar results.

EXAMPLE 8

AtELP3 and *AtELP4* expression and plant development

A total of 30 *AtELP3* and 20 *AtELP4* transgenic strawberry plants ("E3" and "E4" plants) were generated and analyzed by PCR. Ten and nine strawberry plants containing the full-length *AtELP3* and *AtELP4* transgene were selected, respectively. Total RNA was extracted from leaves of six-month-old plants and subjected to RT-qPCR analysis to examine the expression of *AtELP3* and *AtELP4*. Results showed that the transgene was expressed at different levels in the transgenic plants (FIG. 7(a), (b)).

In general, E3-plants presented regular canopy, and E4-plants showed elongated and less dense canopy (FIG. 7(d)). Excepting lines E3/69 and E3/76, which did not differ from control, and E3/88, the shortest plant in the set, all transgenic plants were higher (FIG. 7(e)), and most plants produced fruits significantly smaller compared to the control (FIG. 7(f)). Whereas all transgenic lines formed flowers, one E3-plant (line E3/88) and two E4-plants (line E4/01 and

E4/06) had aborted or irregular fruits (FIG. 7(c), (d)). Altered runner development was sometimes observed. Most of E4-plants did not grow runners, except for lines E4/57 and E4/90 that presented low transgene expression (FIG. 7(b)), whereas only two E3-plants did not grow runners (lines E3/65 and E3/88). Although not correlated to transcript levels, the presence of the
5 *AtELP3* and *AtELP4* transgenes may affect growth and development in strawberry plants.

EXAMPLE 9

Disease resistance of transgenic strawberry plants

A. Anthracnose crown rot resistance

Crown rot symptoms, characterized by initial signs of water stress and subsequently
10 collapse, were observed 5 days after infection on control plants. These plants had their tissue aggressively invaded and collapsed before 20 dpi, whereas transgenic plants displayed reduced symptoms (FIG. 8(a)). Disease Incidence (DI) was lower in E3- and E4-transgenic lines presenting higher transgene levels (FIG. 8(b)). E4/06 presented the lowest percentage of collapsed plants (40%) compared to the control. Resistance to *C. gloeosporioides* was also
15 reflected by AUDPC results (FIG. 8(c)). AUDPC of all tested lines were significantly reduced compared to the non-transgenic plants, with E4-lines displaying the best results (lower AUDPC ranging from 75% to 98% compared to the control).

B. Powdery mildew resistance

Eight days following fungal inoculation, leaves from transgenic plants were slightly
20 affected while those from control showed vigorous patches of white powdery fungus, showing a DS score = 2 (FIG. 9(a)). Little mycelium was produced on lines E3/76, E3/65 and E4/06 (Mean DR score = 1; FIG. 9(a), (b)), and no visible symptoms were observed in the transgenic line E4/01 (Mean DR score = 0; FIG 9(a), (b)), demonstrating enhanced resistance to *P. aphanis* in these lines. The number of spores was also drastically reduced on strawberry transgenic lines,
25 being significantly different from the control (FIG. 9(c)). Reductions from 89.9% (E4/85) up to 95.5% (E3/76) were observed and the high expression lines were clearly more resistant. These results suggest that the signs and symptoms, and the susceptibility to the pathogen decrease with

the increase of transgene expression for both studied genes, and E4-lines were more resistant to powdery mildew.

C. Angular leaf spot resistance

AtELP3- and *AtELP4*-expressing lines were tested for their resistance to angular leaf spot. At 7 dpi control plants showed water-soaking lesions still evident (Score 1) that eventually expanded beyond inoculation site and exposed bacterial exudate (Score 3) (FIG. 10(a)). At the end of the experiment, no plants showed symptoms of total necrosis, chlorosis, and/or secondary infections. However, control plants exhibited the highest DR scores, and E3/74 developed slight necrosis in the center of the inoculation site, being also described as susceptible (FIG. 10(a)). E3/65 and E4-lines were considered resistant to *X. fragariae* (FIG. 10(b)) as their inoculation sites lost translucency and did not develop any other symptoms (Score 0). Bacterial populations were also determined by colony counts of each inoculated area. As shown in FIG. 10(c), with exception of E4/85, the bacterial populations were significantly decreased in all tested lines. Highest disease resistance was observed in lines E3/65, E4/06 and E4/01. These results demonstrate that overexpression of *AtELP3* and *AtELP4* in strawberry enhances resistance to the bacterial pathogen *X. fragariae*.

D. Salicylic acid accumulation in transgenic strawberry

The effect of ectopic expression of *AtELP3* and *AtELP4* on SA accumulation in strawberry was also tested. Basal levels of free SA in transgenic plants, and the accumulation of free SA 24 hours after pathogen challenge were determined. Ectopic expression of *AtELP3* and *AtELP4* in strawberry did not change basal levels of free SA (FIG. 10(a)); however, significant accumulation of free SA was observed in transgenic lines E4/06 and E4/01 (FIG. 11(b)).

EXAMPLE 10

Disease resistance of transgenic tomato plants

25 Generation of transgenic tomato plants

The T-DNA plasmid pK7WG2D,1-*AtELP4* was also used for production of transgenic tomato plants. The *AtELP4* gene was transformed into the tomato cultivar 'MoneyMaker' using the *Agrobacterium* strain GV3010. The genetic transformation was conducted by the University

of Nebraska's Plant Transformation Core Research Facility. A total of 25 independent transgenic R1 lines were generated and 10 single T-DNA insertion homozygous lines were identified in the R2 and R3 generations. These homozygous lines were used for the disease resistance test.

5 **Disease resistance test**

One-month-old *AtELP4* transgenic plants and the control 'Moneymaker' plants were spray-inoculated with a suspension of the bacterial pathogen *P. syringae* pv. *tomato* (*Pst*) DC3000 in 10 mM MgCl₂ (OD₆₀₀ = 0.3). *Pst* DC3000 causes tomato bacterial speck disease. After inoculation, the plants were covered with plastic bags for 36 hours. Pathogen growth
10 inside the plants was determined by collecting leaf tissues at different time points (day 0, 3, 6, and 9), grinding the tissue in 10 mM MgCl₂, plating the suspension on growth medium, and counting the colonies. The experiment was repeated four times.

Bacterial Speck resistance

Out of the 10 independent transgenic homozygous lines, three lines (23-1, 37-3, and 61-
15 5) exhibited enhanced resistance to *Pst* DC3000 (FIG. 12(a)). At nine days post inoculation, the bacterial titer in the most resistant *AtELP4* transgenic line 61-5 was 50 fold less than that in the control 'Moneymaker' plants. Consistently, while the control plants were significantly damaged by the bacterial pathogens, as indicated by the burning of leaves on the control plant in FIG. 12(b), plants of the exemplary transgenic line 61-5 stayed mildly affected. The photo in FIG. 12
20 (b) was taken six days post inoculation. These results demonstrate that overexpression of *AtELP4* in tomato increases resistance to the bacterial pathogen *Pst* DC3000.

WHAT IS CLAIMED IS:

- Claim 1. A transgenic plant comprising a heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the transgenic plant, and exhibits disease resistance as a result of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence.
- Claim 2. The plant of claim 1, wherein the nucleic acid sequence encoding ELP3 or ELP4 is operably linked to a heterologous promoter which is a developmentally-regulated, organelle-specific, inducible, tissue-specific, constitutive, cell-specific, or seed specific promoter.
- Claim 3. The plant of claim 2, wherein the promoter is a constitutive or inducible promoter.
- Claim 4. The plant of claim 1, wherein the plant is a member of the Brassicaceae, Cucurbitaceae, Fabaceae, Poaceae, Rosaceae, Rutaceae, Malvaceae, or Solanaceae.
- Claim 5. The plant of claim 4, wherein the plant is a Brassica.
- Claim 6. The plant of claim 4, wherein the plant is a *Fragaria sp.*
- Claim 7. The plant of claim 4, wherein the plant is a tomato plant.
- Claim 8. The plant of claim 4, wherein the plant is a Citrus plant.
- Claim 9. The plant of claim 1, wherein the plant displays an additional altered agronomic property relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence, wherein the altered agronomic property is selected from the group consisting of: enhanced yield; altered leaf area; altered leaf shape, and altered production of runners.
- Claim 10. The plant of claim 1, wherein the plant pathogen is a bacterial plant pathogen or a fungal plant pathogen.
- Claim 11. The plant of claim 1, further defined as an R₀ transgenic plant.

Claim 12. The plant of claim 1, further defined as a progeny plant of any generation of an R₀ transgenic plant, wherein the transgenic plant has inherited the selected DNA from the R₀ transgenic plant.

Claim 13. The plant of claim 1, wherein the heterologous nucleic acid sequence comprises at least 90% sequence identity to a sequence selected from the group consisting of SEQ ID NOs:1-24.

Claim 14. A seed that produces the plant of claim 1.

Claim 15. A plant part of the plant of claim 1.

Claim 16. The plant part of claim 15, further defined a protoplast, cell, meristem, root, leaf, pistil, anther, flower, seed, fruit, embryo, stalk, or petiole.

Claim 17. A recombinant nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

(a) a nucleic acid sequence that hybridizes to a nucleic acid sequence complementary to the sequence of any of SEQ ID NOs: 1-24, under wash conditions of 1X SSC and 65°C;

(b) a nucleic acid comprising the sequence complementary to SEQ ID NOs: 1-24 or a fragment thereof; and

(c) a nucleic acid sequence exhibiting at least 80% sequence identity to any of SEQ ID NOs: 1-24;

wherein the nucleic acid sequence is operably linked to a heterologous promoter sequence and wherein expression of the nucleic acid molecule in a plant cell results in over-expression of ELP3 or ELP4 relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence.

Claim 18. The nucleic acid molecule of claim 17, wherein the DNA molecule comprises a nucleic acid sequence exhibiting at least 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a complement of any of SEQ ID NOs: 1-24.

Claim 19. The nucleic acid molecule of claim 17, wherein the heterologous promoter sequence is a developmentally-regulated, organelle-specific, inducible, tissue-specific, constitutive, cell-specific, or seed specific promoter.

Claim 20. A transgenic plant cell comprising the nucleic acid molecule of claim 17.

Claim 21. A transgenic plant or plant part comprising the nucleic acid molecule of claim 17.

Claim 22. A method of conferring disease resistance to a plant comprising: over-expressing a heterologous nucleic acid sequence that encodes ELP3 or ELP4 in a transgenic plant; wherein the transgenic plant exhibits disease resistance, as a result of the over-expression of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence.

Claim 23. The method of claim 22, wherein the disease is a bacterial plant disease or a fungal plant disease.

Claim 24. The method of claim 22, wherein the disease is caused by a fungus.

Claim 25. The method of claim 24, wherein the disease is Strawberry Anthracnose or Strawberry Powdery Mildew.

Claim 26. The method of claim 23, wherein the disease is caused by a bacterium.

Claim 27. The method of claim 26, wherein the disease is caused by a Pseudomonad, a Xanthomonad, or an *Erwinia sp.*

Claim 28. The method of claim 27, wherein the disease is Strawberry Angular Leaf Spot.

Claim 29. The method of claim 27, wherein the disease is Bacterial Speck of Tomato.

Claim 30. A method of plant breeding comprising:

(a) crossing a first plant, which is over-expressing a heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the first plant, and wherein the first plant exhibits disease resistance as a result of the heterologous nucleic acid

sequence relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence, with a second plant;

(b) obtaining one or more progeny plants, and

(c) selecting a progeny plant which is over-expressing the heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the progeny plant, and wherein the progeny plant exhibits disease resistance as a result of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence.

FIG. 1

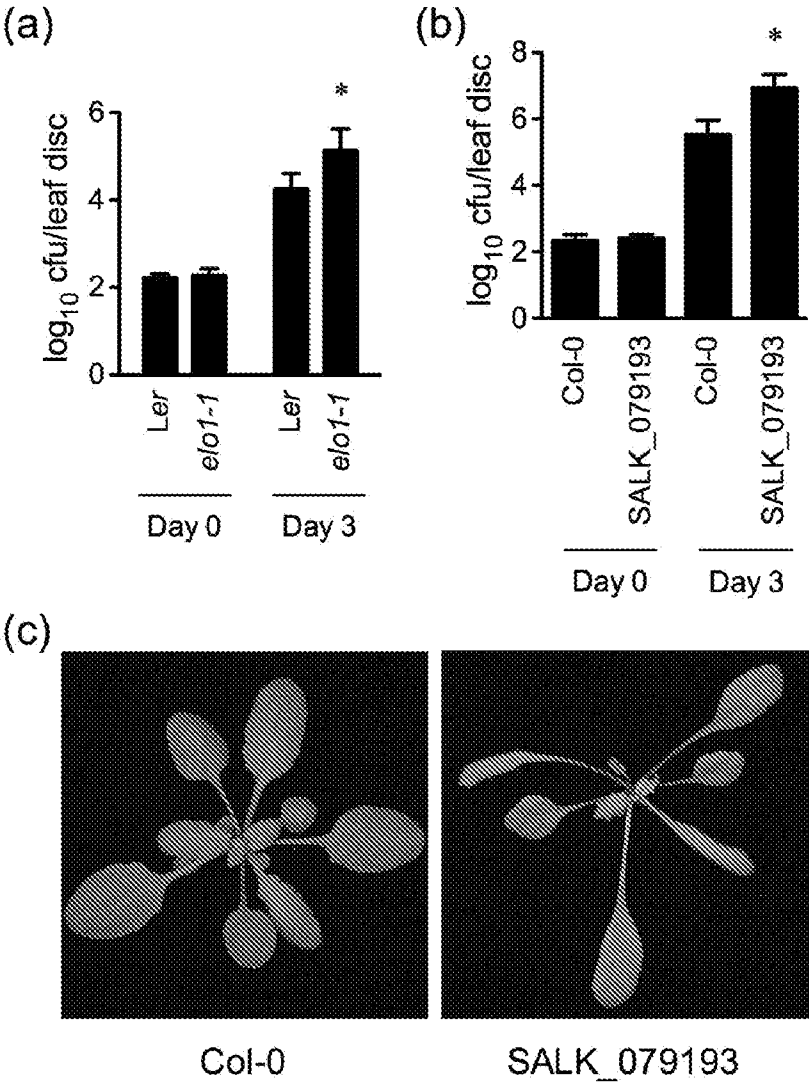


FIG. 2

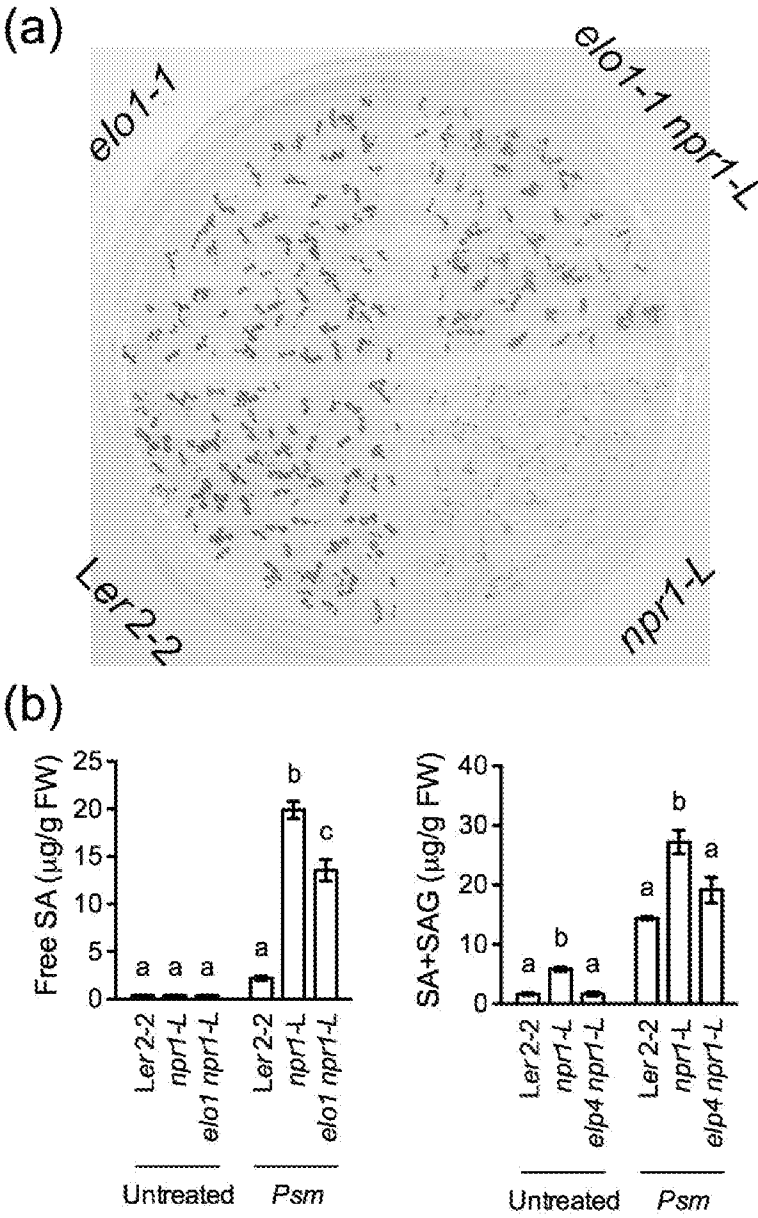


FIG. 3

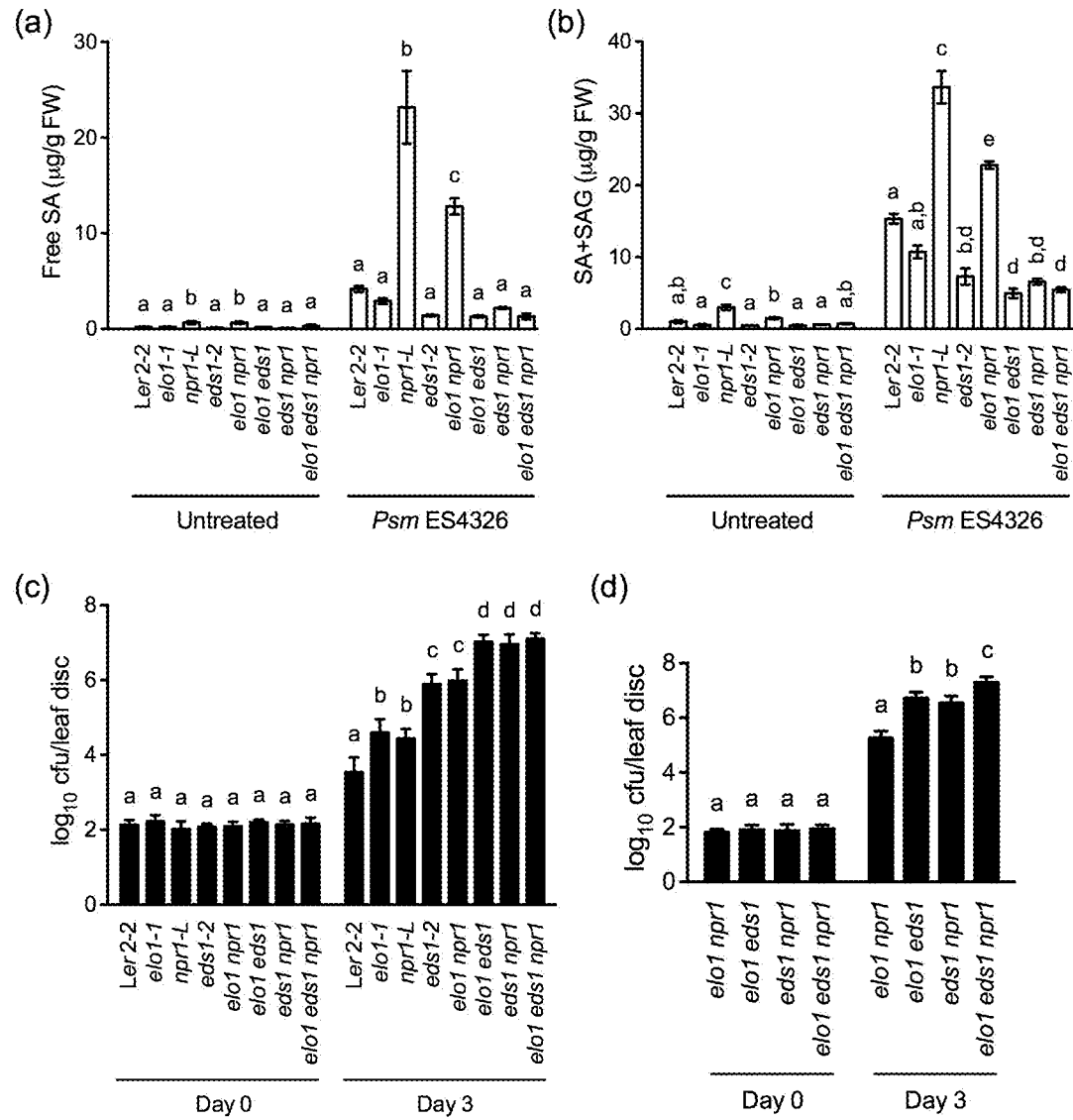


FIG. 4

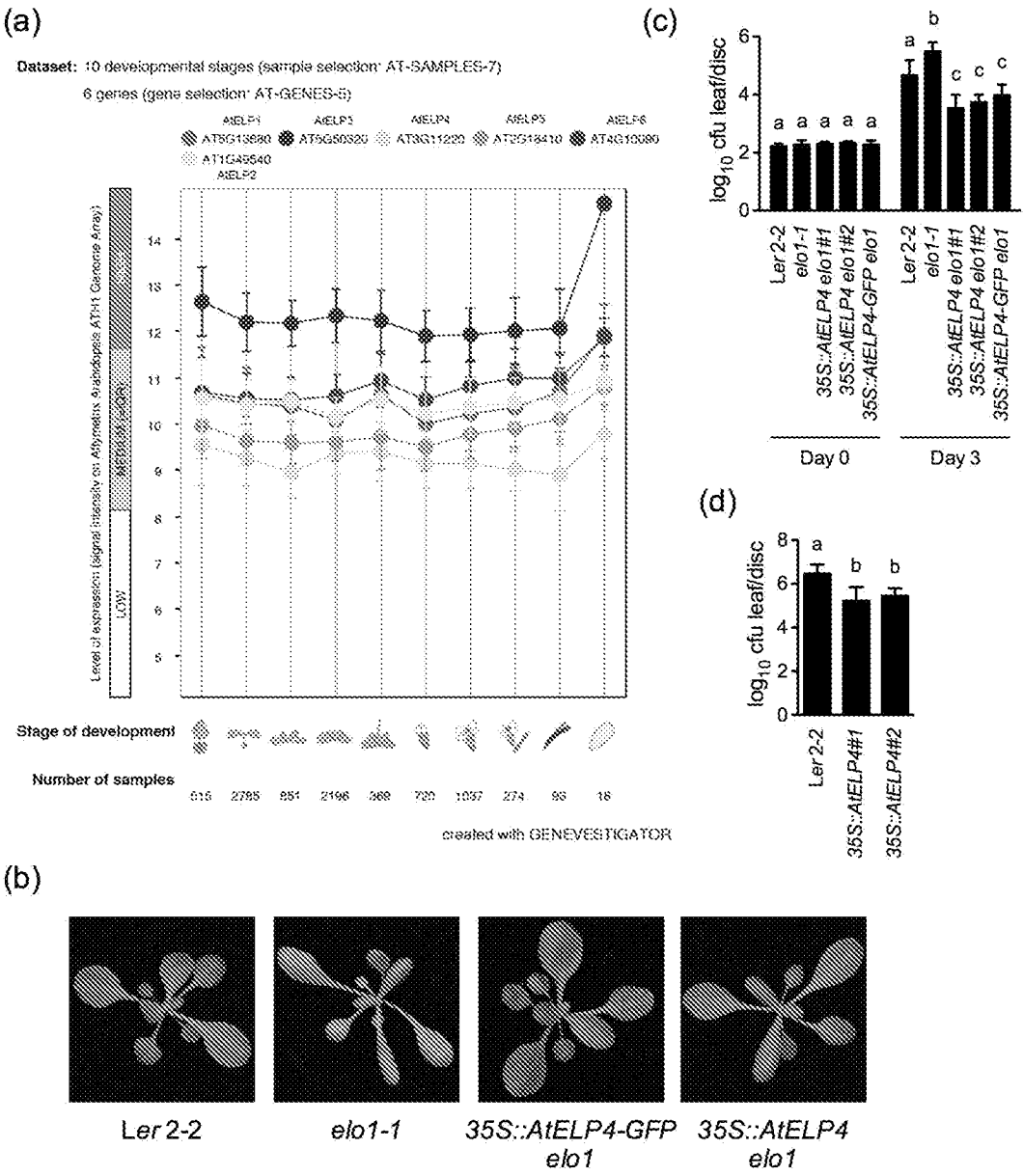


FIG. 5

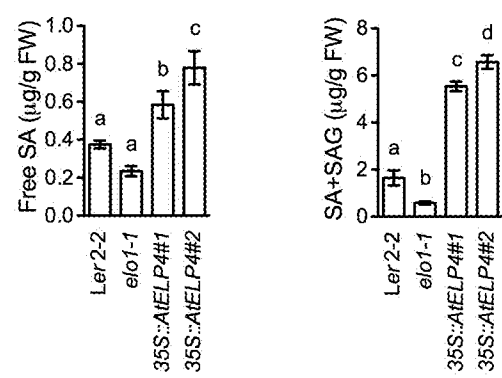
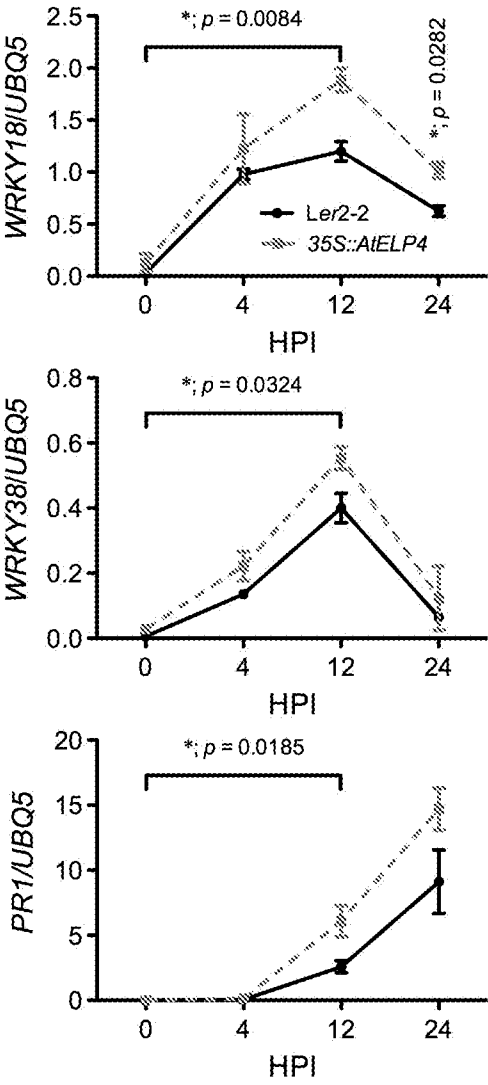


FIG. 6



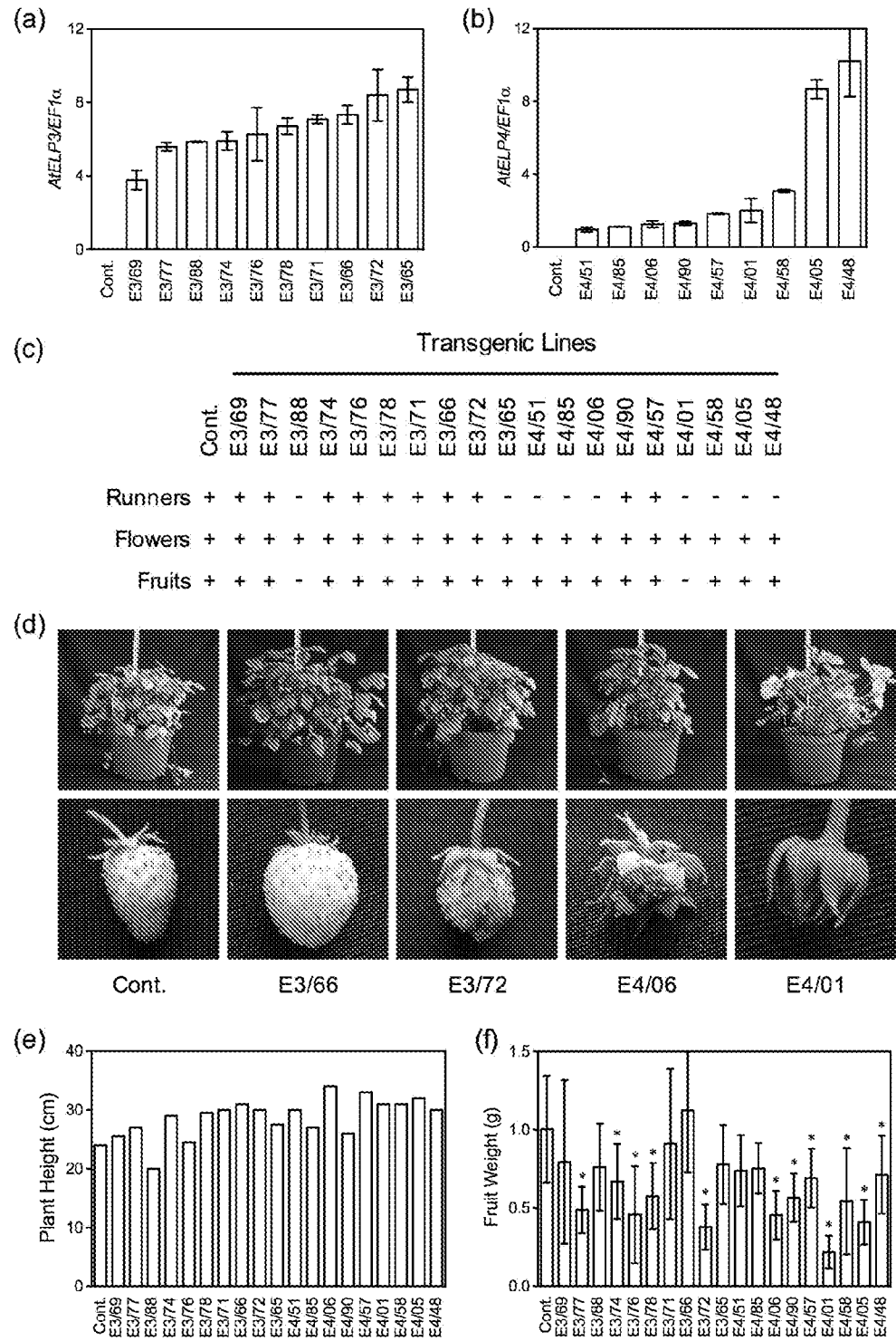


FIG. 7

FIG. 8

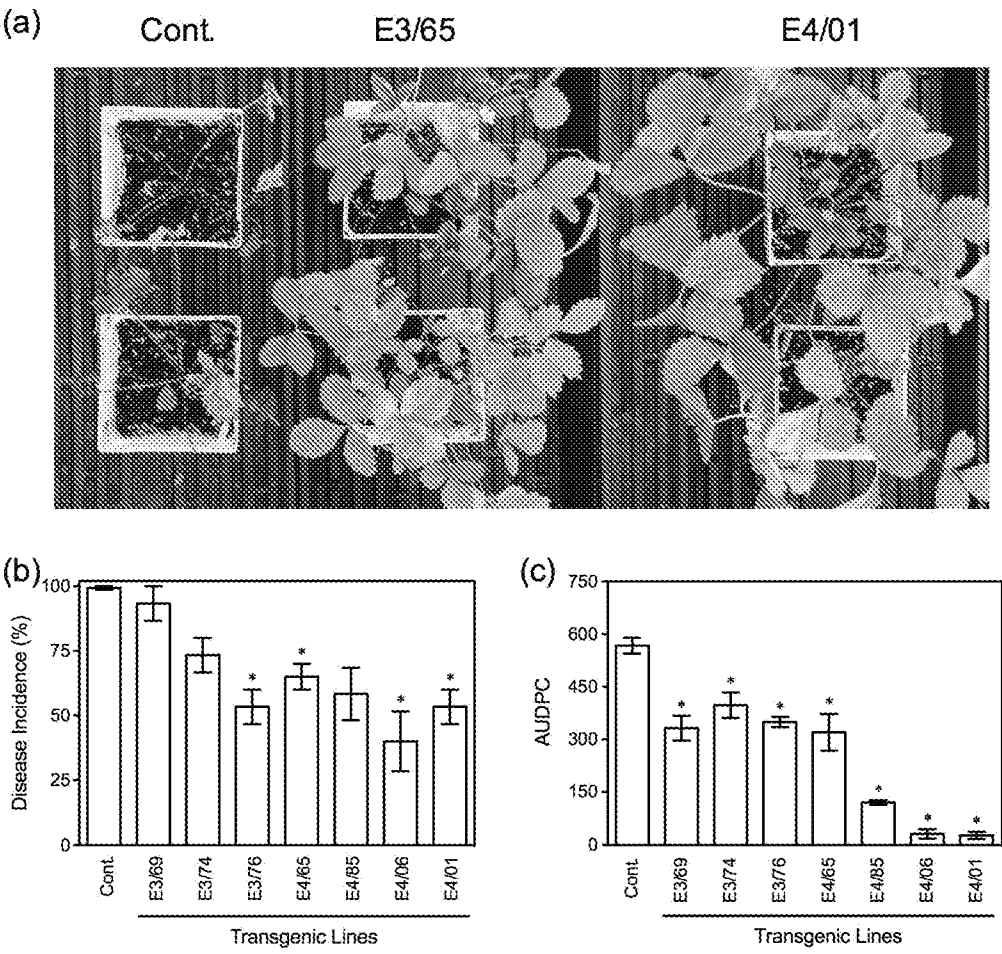


FIG. 9

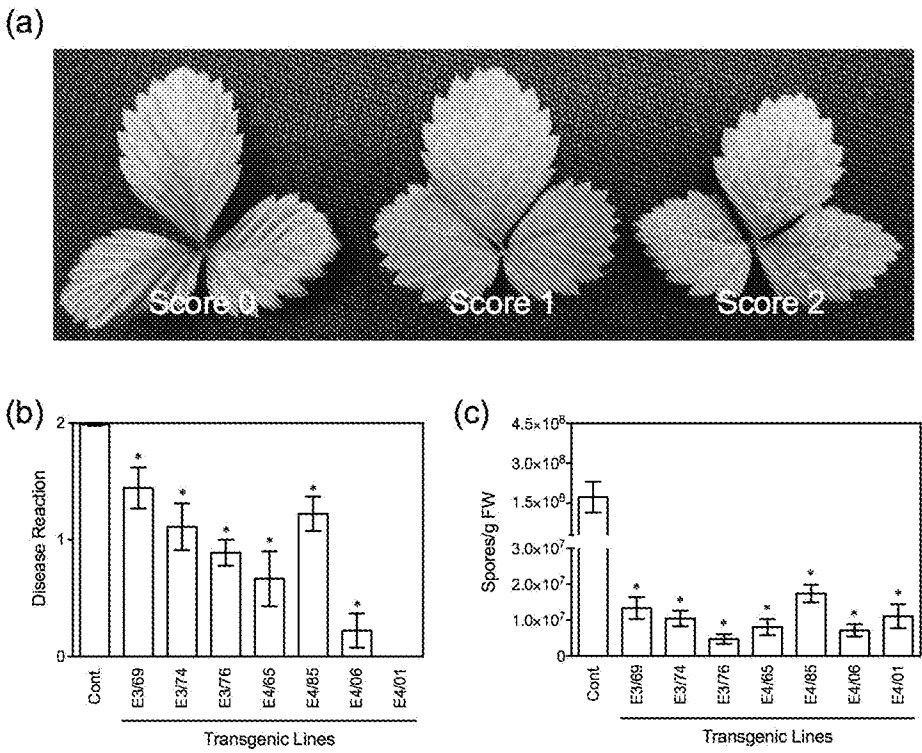


FIG. 10

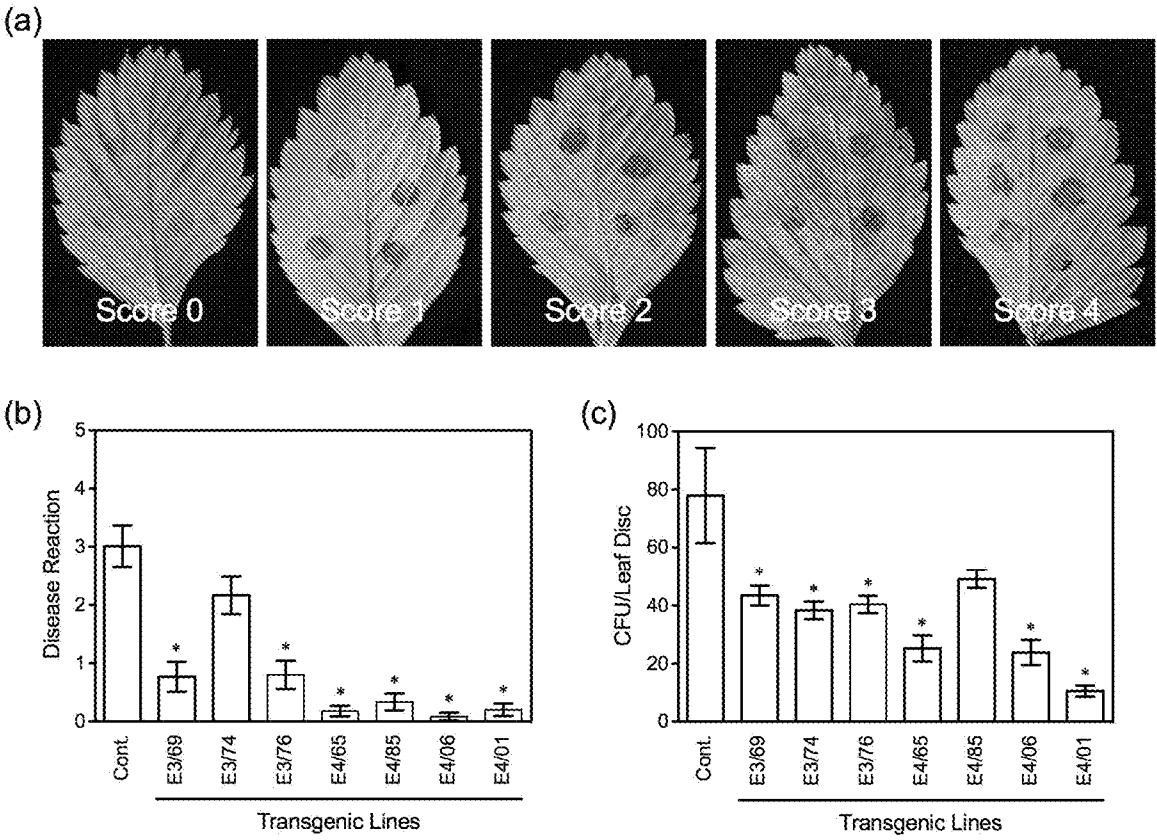


FIG. 11

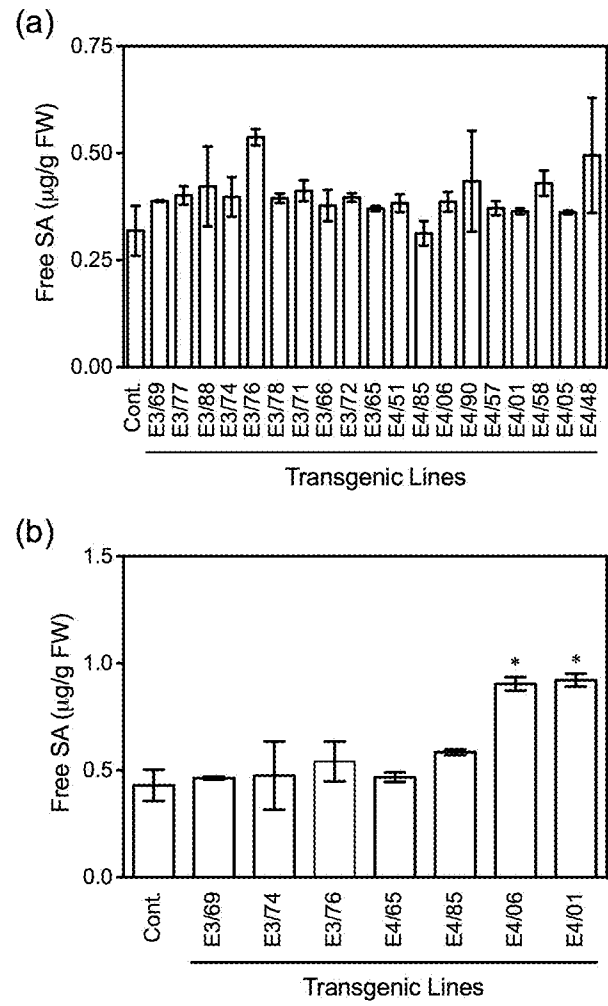
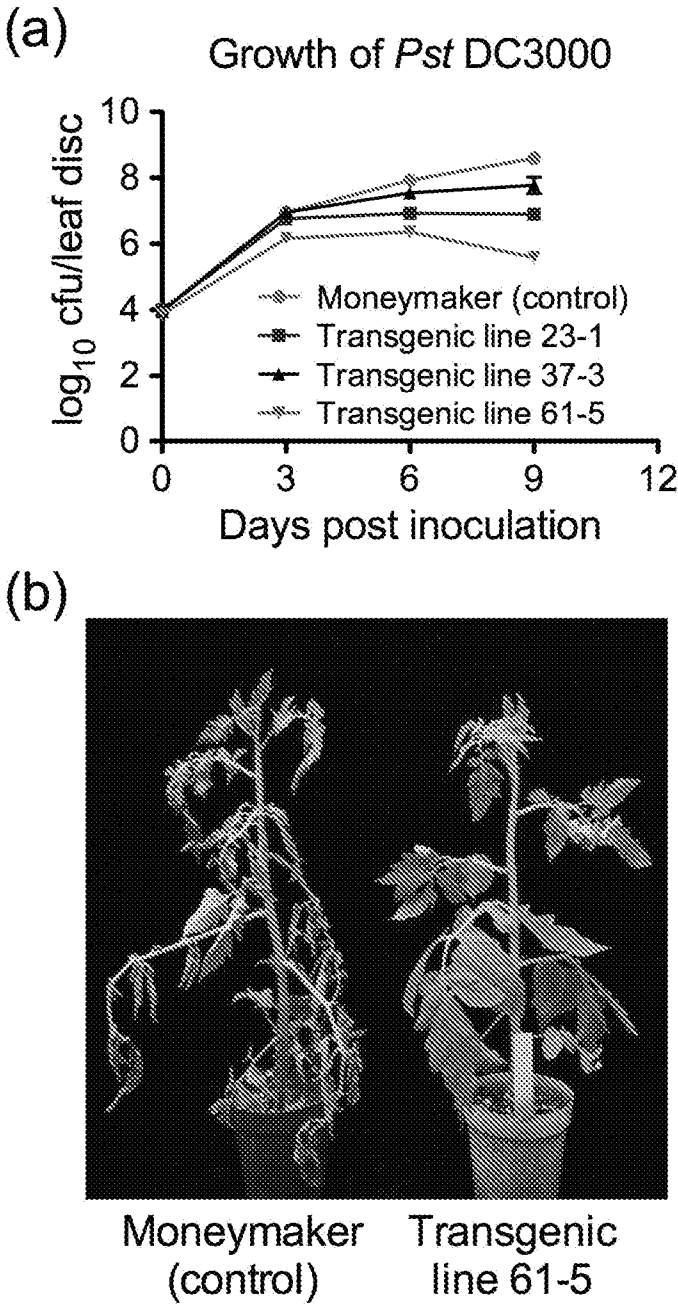


FIG. 12



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/41907

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/82; C07K 14/415; A01H 5/00 (2016.01)

CPC - C12N 15/82, 15/8201; C07K 14/415; A01H 5/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) Classifications: C12N 15/82; C07K 14/415; A01H 5/00, 5/10, 5/12 (2016.01)

CPC Classifications: C12N 15/82, 15/8201, 15/5205; C07K 14/415; A01H 5/00, 5/10, 5/12

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)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google; Google Scholar; PubMed; EBSCO; Transgen*, Plant*, crop*, Hetero*, Nucle*, acid*, DNA, RNA, deoxyribonucle*, ribonucle*, Overexpress*, upregulat*, Diseases*, Virus*, Infect*, Disorder*, condition*, Resist*, immun*, defens*, ELP3, ELP4, ELO1, 'Elongator complex subunit', KAT9

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	WO 2006/114434 A1 (VIB VZW, et al.) 2 November 2006; page 1, lines 2-5; page 2, lines 5-8 and 13; page 3, lines 2-14 and 22-27; page 4, lines 27-31; page 8, lines 17-37; claim 2.	1-4, 9, 11-12, 14-16, 22 --- 5-8, 10, 13, 17-21, 23-24, 26-27, 29-30
Y	MOU, Z. Generation Of Transgenic Citrus Plants For Resistance Test. Citrus Advanced Technology Program Quarterly and Final Reports: Control Of Citrus Greening, Canker and Emerging Diseases of Citrus. 15 April 2012; page 1, first paragraph.	8, 10, 23, 26-27, 29
Y	NELISSEN, et al. The Elongata Mutants Identify A Functional Elongator Complex In Plants With A Role In Cell Proliferation During Organ Growth. PNAS. 24 May 2005, Vol. 102, pages 7754-7759, doi:10.1073/pnas.0502600102; page 7754, data deposition, column 2, second paragraph.	13, 17-21
Y	DEFRAIA, et al. Elongator Subunit 3 Positively Regulates Plant Immunity Through Its Histone Acetyltransferase And Radical S-adenosylmethionine Domains. BMC Plant Biology. 2013, Vol. 13, pages 1-13; page 5, second column, third paragraph.	29
Y	US 2014/0304849 A1 (MONSANTO TECHNOLOGY LLC) 9 October 2014; paragraph [0014].	30
Y	DING, et al. Elongator And Its Epigenetic Role In Plant Development And Responses To Abiotic And Biotic Stresses. Frontiers in Plant Science. 29 April 2015, Vol. 6, pages 1-8, doi:10.3389/fpls.2015.00296; page 4, second column, third paragraph.	7
Y	LEE, et al. ELP3 Is Involved In Sexual And Asexual Development, Virulence, And The Oxidative Stress Response In Fusarium graminearum. Molecular Plant-Microbe Interactions. 2014, Vol. 27, pages 1344-1355, doi.org/10.1094/MPMI-05-14-0145-R; abstract.	24



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

31 October 2016 (31.10.2016)

Date of mailing of the international search report

12 DEC 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/41907

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YUSUF, et al. Overexpression Of γ -tocopherol Methyl Transferase Gene In Transgenic Brassica Juncea Plants Alleviates Abiotic Stress: Physiological And Chlorophyll A Fluorescence Measurements. Biochimica et Biophysica Acta. 6 February 2010, pages 1428-1438, doi:10.1016/j.bbabo.2010.02.002; abstract.	5
Y	HUSAINI, et al. Development Of Transgenic Strawberry (Fragaria x ananassa Dusch.) Plants Tolerant To Salt Stress. Plant Science. 2008 Vol. 174, pages 446-455, doi:10.1016/j.plantsci.2008.01.007; abstract.	6
A	MOU, Z. Application Of A Natural Inducer Of Systematic Acquired Resistance And Engineering Non-host Resistance In Citrus For Controlling Citrus Diseases. Citrus Advanced Technology Program Quarterly and Final Reports: Control Of Citrus Greening, Canker and Emerging Diseases of Citrus. 13 February 2015; page 2, second paragraph.	25, 28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/41907

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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:

- Please See Supplemental Page-

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.:
Groups I+, Claims 1-30; SEQ ID NO: 1 (nucleic acid sequence)

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/US16/41907

****-Continued from Box No. III: Observations Where Unity Of Invention Is Lacking:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-30 and SEQ ID NO: 1 are directed toward a transgenic plant comprising a heterologous nucleic acid sequence that encodes ELP3 or ELP4; a recombinant nucleic acid molecule comprising a sequence that encodes ELP3 or ELP4; and methods associated therewith.

The plant, recombinant nucleic acid and methods will be searched to the extent they encompass SEQ ID NO: 1 (first exemplary nucleic acid sequence). Applicant is invited to elect additional nucleic acid sequence(s), with specified SEQ ID NO: for each, to be searched. Additional nucleic acid sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1-12, 13 (in-part), 14-16, 17 (in-part), 18 (in-part), 19 (in-part), 20 (in-part), 21 (in-part) and 22-30 encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 (nucleic acid sequence). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be a nucleic acid sequence encompassing SEQ ID NO: 2 (first exemplary elected nucleic acid sequence).

No technical features are shared between the nucleic acid sequences of Groups I+ and, accordingly, these groups lack unity a priori.

Groups I+ share the technical features including: a transgenic plant comprising a heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the transgenic plant, and exhibits disease resistance as a result of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence; a seed that produces the plant; a plant part of the plant; a recombinant nucleic acid molecule comprising a nucleic acid sequence; wherein the nucleic acid sequence is operably linked to a heterologous promoter sequence and wherein expression of the nucleic acid molecule in a plant cell results in overexpression of ELP3 or ELP4 relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence; a transgenic plant cell comprising the nucleic acid molecule; a transgenic plant or plant part comprising the nucleic acid molecule; a method of conferring disease resistance to a plant comprising: over-expressing a heterologous nucleic acid sequence that encodes ELP3 or ELP4 in a transgenic plant; wherein the transgenic plant exhibits disease resistance, as a result of the over-expression of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence; and a method of plant breeding comprising: (a) crossing a first plant, which is over-expressing a heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the first plant, and wherein the first plant exhibits disease resistance as a result of the heterologous nucleic acid sequence relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence, with a second plant; (b) obtaining one or more progeny plants, and (c) selecting a progeny plant which is over-expressing the heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the progeny plant, and wherein the progeny plant exhibits disease resistance as a result of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence.

****-Continued Within the Next Supplemental Box-****

***-Continued from Previous Supplemental Page:

However, these shared technical features are previously disclosed by WO 2006/114434 A1 to VIB VZW (hereinafter 'VIB VZW') in view of US 2014/0304849 A1 to Monsanto Technology LLC (hereinafter 'Monsanto').

VIB VZW discloses a transgenic plant (a transgenic plant; page 3, lines 22-27) comprising a heterologous nucleic acid sequence (comprising a heterologous nucleic acid sequence; page 3, lines 2-14 and lines 22-27) that encodes ELP3 or ELP4 (that encodes an elongator complex protein, including ELP3 or ELP4 (that encodes ELP3 or ELP4); page 1, lines 9-11; page 2, lines 7-9), wherein ELP3 or ELP4 is over-expressed in the transgenic plant (wherein ELP3 or ELP4 is over-expressed in the transgenic plant; page 1, lines 9-11; page 2, lines 7-9, page 3, lines 2-14 and lines 22-27), and exhibits disease resistance as a result of the heterologous nucleic acid sequence (and exhibits stress resistance as a result of the heterologous nucleic acid sequence; page 1, lines 3-4; wherein disease resistance, although not specifically disclosed, would have been demonstrable in the transgenic plants, absent evidence to the contrary, based on the overexpression of said genes in said plants), relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence (relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence; page 3, lines 22-27); a seed that produces the plant (T0 seeds from transgenic plants (a seed that produces the plant); page 8, lines 10-23); a plant part of the plant (a vegetative part (plant part) of the plant; page 9, lines 30-34); a recombinant nucleic acid molecule (a recombinant nucleic acid molecule; page 3, lines 8-9); wherein the nucleic acid sequence is operably linked to a heterologous promoter sequence (wherein the nucleic acid sequence is operably linked to a transgenic (heterologous) promoter sequence; page 3, lines 2-14, lines 22-27) and wherein expression of the nucleic acid molecule in a plant cell results in overexpression (wherein expression of the nucleic acid molecule in a plant cell results in overexpression; page 3, lines 2-14) of ELP3 or ELP4 (of ELP3 or ELP4; page 1, lines 9-11; page 2, lines 7-9) relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence; page 3, lines 22-27); a transgenic plant, plant part, and plant cell comprising the nucleic acid molecule (a transgenic plant, plant part, and plant cell comprising the nucleic acid molecule; page 3, lines 22-27; page 9, lines 30-34); a method of conferring disease resistance to a plant (a method of increasing stress tolerance in a plant (a method of conferring disease resistance to a plant); page 1, lines 2-5; wherein disease resistance, although not specifically disclosed, would have been demonstrable in the transgenic plants, absent evidence to the contrary, based on the overexpression of ELP3 or ELP4 genes in said plants) comprising: over-expressing a heterologous nucleic acid sequence (comprising: over-expressing a heterologous nucleic acid sequence; page 3, lines 1-14, lines 22-27) that encodes ELP3 or ELP4 (that encodes an elongator complex protein, including ELP3 or ELP4 (that encodes ELP3 or ELP4); page 1, lines 9-11; page 2, lines 7-9) in a transgenic plant (in a transgenic plant; page 3, lines 22-27); wherein the transgenic plant exhibits disease resistance (wherein the transgenic plant exhibits stress (disease) resistance; page 1, lines 2-5, page 3, lines 22-27; wherein disease resistance, although not specifically disclosed, would have been demonstrable in the transgenic plants, absent evidence to the contrary, based on the overexpression of ELP3 or ELP4 genes in said plants), as a result of the over-expression of the heterologous nucleic acid sequence (as a result of the over-expression of the heterologous nucleic acid sequence; page 1, lines 2-5; page 2, lines 2-14), relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence (relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence; page 3, lines 22-27).

VIB VZW does not disclose a method of plant breeding comprising: (a) crossing a first plant, which is over-expressing a heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the first plant, and wherein the first plant exhibits disease resistance as a result of the heterologous nucleic acid sequence relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence, with a second plant; (b) obtaining one or more progeny plants, and (c) selecting a progeny plant which is over-expressing the heterologous nucleic acid sequence that encodes ELP3 or ELP4, wherein ELP3 or ELP4 is over-expressed in the progeny plant, and wherein the progeny plant exhibits disease resistance as a result of the heterologous nucleic acid sequence, relative to an otherwise isogenic plant which does not comprise the heterologous nucleic acid sequence.

Monsanto discloses a method of plant breeding (a method of producing progeny plants (plant breeding); paragraph [0014]) comprising: (a) crossing a first plant, which is over-expressing a heterologous nucleic acid sequence (a first plant expressing a cassette that confers glyphosate tolerance (crossing a first plant, which is over-expressing a heterologous nucleic acid sequence); paragraph [0014]), and, wherein the first plant exhibits a phenotypic change as a result of the heterologous nucleic acid sequence (wherein the first plant exhibits tolerance to the application of glyphosate (wherein the first plant exhibits a phenotypic change as a result of the heterologous nucleic acid sequence); paragraph [0014]), with a second plant (with a second plant that lacks said tolerance (with a second plant); paragraph [0014]); (b) obtaining one or more progeny plants (producing (obtaining) one or more progeny plants; paragraph [0014]), and (c) selecting a progeny plant which is over-expressing the heterologous nucleic acid sequence, wherein the progeny plant exhibits a phenotypic change as a result of the heterologous nucleic acid sequence (selecting progeny plants that tolerate the application of glyphosate (selecting a progeny plant which is over-expressing the heterologous nucleic acid sequence, wherein the progeny plant exhibits a phenotypic change as a result of the heterologous nucleic acid sequence); paragraph [0014]).

It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of VIB VZW to have included the breeding method of Monsanto in order to enable a person of ordinary skill in the art at the time the invention was made to have performed successful outcrossing with other strains of plants, and selection of plants demonstrating a desirable phenotype, such as disease resistance, based on the disclosure of Monsanto.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the VIB VZW and Monsanto references, unity of invention is lacking.