

ANEXO 1.

DECLARACIÓN

Yo, Walter Messier, declaro por la presente lo siguiente:

1. Soy el inventor de la solicitud de patente japonesa n.º 2021-575024 (“la solicitud ‘024”). Mi formación académica y experiencia se exponen a continuación.
2. Tengo un doctorado en biología molecular de la Universidad de Albany, Universidad Estatal de Nueva York, en Albany, Nueva York, Estados Unidos.
3. He sido biólogo molecular durante 26 años. Se adjunta una copia de mi currículum vitae.
4. Soy fundador y actualmente me desempeño como director científico de la empresa a la que se le asignaron los derechos de esta solicitud de patente y la invención divulgada en ella.
5. Estoy familiarizado con la Acción Oficial con fecha del 17 de mayo de 2024.
6. Entiendo que el examinador ha proporcionado la razón 2 (requisito de soporte) y la razón 3 (requisito de habilitación) contra las reivindicaciones 1 a 19 de la solicitud ‘024.
7. Respetuosamente presento los siguientes datos y descripciones que proporcionan ejemplos prácticos del constructo de ácido nucleico de la reivindicación 1 de la solicitud ‘024 y la expresión de resistencia a *Fusarium oxysporum* raza 4 en plantas de banano (*Musa*) utilizando SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11 y SEQ ID NO: 18 con varios promotores y terminadores como se muestra en la Tabla 2 a continuación.
8. El soporte para el uso de las secuencias SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11 y SEQ ID NO: 18 se puede encontrar en la Tabla 1 en el

párrafo [0230] y la Figura 1 de la solicitud '024 y en el Ejemplo 5 a partir del párrafo [0437] de la solicitud '024.

9. Los promotores proporcionados en la Tabla 2 de esta declaración y tal como se utilizan en los ejemplos de trabajo se eligieron de la lista de promotores en la solicitud '024 (véase, por ejemplo, el párrafo [00289] - [00293]) o se ajustan a una de las categorías de promotores enumerados en la solicitud '024 (por ejemplo, promotor constitutivo, promotor específico de tejido, etc.), y si corresponde, el soporte para el uso de un marcador seleccionable se encuentra en el párrafo [00294] de la solicitud '024.

10. El método de transformación que utilizamos para la integración del constructo de la reivindicación 1 en plantas de banano fue el bombardeo con agrobacterium/microproyectiles que se apoya en el párrafo [00296] de la solicitud '024 y en el Ejemplo 5 de la solicitud '024.

11. Las transformaciones de material de planta de banano se llevaron a cabo en Middleton, Wisconsin, Estados Unidos y se realizaron de acuerdo con los procedimientos establecidos en la solicitud '024.

12. Se realizó una evaluación de bananos resistentes a la enfermedad de Panamá, *Fusarium oxysporum* f.sp.cubense II-5 TR4 en Madison, Wisconsin, Estados Unidos, de acuerdo con los procedimientos establecidos en la solicitud '024.

13. El protocolo de detección descrito a continuación sigue una metodología bien conocida por los expertos en la materia, tal como se establece en García-Bastidas, F. A., Van der Veen, A. J. T., Nakasato-Tagami, G., Meijer, H. J. G., Arango-Isaza, R. E. y Kema, G. H. J. (2019). Un protocolo de fenotipado mejorado para la enfermedad de Panamá en el banano. *Frontiers in plant science*, 10, 1006. doi:

10.3389/fpls.2019.01006 (“García-Bastidas et al.”). Esta referencia se publicó el 5 de agosto de 2019, que fue anterior a la fecha de presentación del 7 de octubre de 2019 de la solicitud de patente provisional de EE. UU. n.º 62/912,010, de la que la solicitud '024 reclama prioridad.

14. El material vegetal (normalmente 1,5-2 meses después de que el cultivo de tejidos se traslada al suelo) se transfirió a macetas de 1 litro (L) que contenían medio de cultivo (PRO-MIX HP MYCORRHIZAE, Premier Tech Horticulture, EE. UU.) y se mantuvo en bandejas. Las plantas se cultivaron durante 2 a 3 semanas en un invernadero con ambiente controlado (28 ± 3 °C, 16 h de luz, ~60 % de humedad) antes de la inoculación. Este período de crecimiento tiene como objetivo que las plantas alcancen una edad adecuada (2 a 3 meses) para realizar el ensayo de la enfermedad y aclimatarlas al nuevo entorno. Las plantas se riegan a diario y se fertilizan semanalmente durante los ensayos de la enfermedad.

15. *F. oxysporum* f.sp. *cubense* II-5 Tropical Race 4 (TR4) se obtuvo de la Colección de Cultivos del Servicio de Investigación Agrícola del USDA (consulte la Tabla 1 a continuación). Una vez recibido, utilizando el protocolo proporcionado por García-Bastidas et al citado anteriormente en el párrafo 6, el cultivo fúngico liofilizado se rehidrató y se suspendió en agua estéril. Luego, la suspensión se dejó reposar durante 30 minutos y se inoculó en el papel de filtro humedecido estéril en la superficie del medio de agar dextrosa de papa (PDA) (Neogen Culture Media Co., Michigan, EE. UU.). Después de recuperar el crecimiento a 25 °C durante aproximadamente dos semanas, el papel de filtro completamente cubierto con micelio fúngico se secó durante la noche en una campana laminar. Al día siguiente, el papel de filtro con micelio se cortó en trozos pequeños con tijeras estériles y se almacenó en un

recipiente estéril a -20 °C. Para preparar el inóculo de esporas de conidios, el papel de filtro con micelio fúngico se inoculó en PDA y se mantuvo a 25 °C. Siete días después de la inoculación, se tomaron tapones de hifas de 7 milímetros del borde de la colonia fúngica en crecimiento activo para inocular el medio de esporulación (SM) para producir esporas de conidios.

16. Tabla 1. Origen y características de la cepa de *Fusarium* spp. utilizada en este estudio.

<i>Fusarium</i> spp.	Código	Hospedador	Origen	Proveedor
<i>F. oxysporum</i> f.sp. <i>cubense</i> II-5	TR4	Banana	USDA-ARS-CDL, St. Paul, MN	Jacolyn Morrison

17. Se generó una suspensión de conidios de *F. oxysporum* f.sp. *cubense* II-5 TR4 utilizando un SM modificado que contenía frijol mungo, que ha demostrado inducir una producción rápida y robusta de conidios. Véase el protocolo de García-Bastidas et al. proporcionado en el párrafo 6. Los frijoles mungo frescos se remojaron en agua estéril durante la noche antes de la preparación del medio. Se agregaron tres gramos de frijoles mungo previamente remojados a matraces Erlenmeyer de 1 litro que contenían 500 mililitros (ml) de agua y luego se esterilizaron en un autoclave a 120 °C durante 20 minutos. Después de enfriar a temperatura ambiente durante la noche, los medios SM se inocularon con 5 tapones de hifas de *F. oxysporum* f.sp. *cubense* II-5 en crecimiento activo. Los matraces Erlenmeyer inoculados se incubaron en un agitador a 105 rpm, 25 °C durante 6-8 días. La suspensión de esporas se recolectó en un matraz Erlenmeyer estéril mediante filtración con cuatro capas de estopilla para eliminar las hifas y el contenido del medio. Las concentraciones de las suspensiones de esporas se cuantificaron con un hemocitómetro

bajo microscopio y se normalizaron a una concentración de 10⁷ esporas/ml. Se aplicaron diez microlitros de suspensión de esporas a PDA y se cultivaron a 25 °C durante 7 días para determinar la viabilidad del inóculo.

18. La suspensión de esporas de conidios en el matraz Erlenmeyer se transportó desde el laboratorio hasta el invernadero en una hielera aislada para minimizar el impacto de los factores ambientales adversos. Véase el protocolo de García-Bastidas et al. proporcionado en el párrafo 6. Para cada maceta de planta a inocular, se diluyeron 20 ml de suspensión de esporas a 10⁷ esporas/ml a 200 ml a 10⁶ esporas/ml utilizando agua del invernadero y luego se vertieron uniformemente en la maceta. Las plantas se regaron llenando la bandeja desde el fondo para evitar lavar las esporas del suelo. Cuando la temperatura y la humedad de los invernaderos disminuyen durante la transición de estación (es decir: de verano/otoño a invierno/primavera), las plantas recibieron una segunda inoculación una semana después de la primera inoculación utilizando el mismo protocolo.

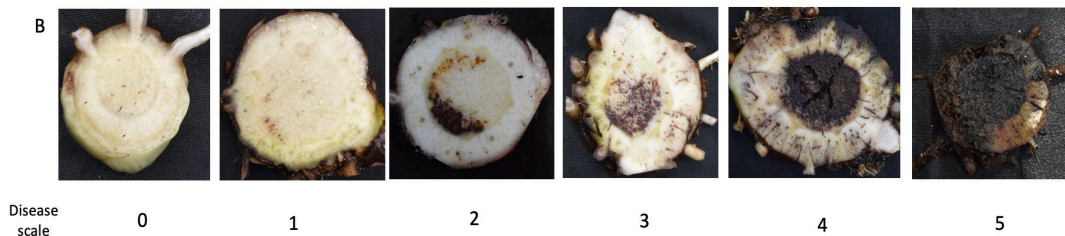
19. Los síntomas de la enfermedad en el material vegetal ensayado se evaluaron tanto externa como internamente siete semanas después de la inoculación utilizando un sistema de puntuación de 5 puntos, similar al sistema de puntuación de 4 puntos descrito en García-Bastidas et al (Figura 4) y en la Solicitud '024 tal como se presentó en [00108] - [00109]. Los síntomas externos de toda la planta se registraron y evaluaron a través de la evaluación categórica del marchitamiento/amarillamiento de las hojas en una escala de 1 a 4 que representa cada uno de los cuartiles en una escala de 0 a 100% de marchitamiento, donde 0 = sin marchitamiento/amarillamiento, 1 = <25% de hojas muestran marchitamiento/amarillamiento, 2 = 25-50%, 3 = 50-75%, 4 = 75-100% y 5 = 100% (ver Figura A a continuación). Después de la evaluación externa, las plantas se sacaron de las macetas y se cortaron horizontalmente a lo largo del rizoma (cormo) de cada planta. La

evaluación interna se cuantificó por el área de decoloración del cormo utilizando una escala de 1 a 4, donde 0 = sin decoloración, 1 = <25 % de cormo con decoloración, 2 = 25-50 %, 3 = 50-75 %, 4 = 75-100 % y 5 = 100 % (consulte la Figura B a continuación).

Figura A



Figura B



20. La Figura A (externa) y la Figura B (interna) muestran los síntomas de las plantas de banano registrados siete semanas después de la inoculación con *Fusarium oxysporum* f.sp. *cubense* II-5, raza tropical 4.

21. La Tabla 2 a continuación ilustra los resultados de la transformación de cultivares de banano con las secuencias de ácido nucleico reclamadas y la resistencia mejorada resultante. Como se describió anteriormente, la evaluación de la enfermedad se realizó tanto internamente (mediante la evaluación del cormo) como externamente (mediante la decoloración/marchitamiento de las plantas). Como se describió anteriormente, la cuantificación de la decoloración del cormo se realizó utilizando una escala de 0 a 5 donde 0 = sin decoloración; 1 = <25 % de cormo que muestra decoloración; 2 = 25-50 % de cormo que muestra decoloración; 3 = 50-75 % de cormo que muestra decoloración; 4 = 75-100 % de cormo que muestra decoloración y 5 = 100 % de cormo que muestra decoloración.

22. Como se muestra a continuación, las puntuaciones de la escala de decoloración del cormo para las plantas modificadas por secuencias de ácido nucleico que codifican la

resistencia a la raza 4 de *Fusarium oxysporum* (en adelante, Puntuación del cormo del evento) fueron entre un 100 % y un 400 % más bajas que las plantas de control (en adelante, Puntuación del cormo del control) no modificadas para la resistencia a la raza 4 de *Fusarium oxysporum*. Se utilizaron plátanos Cavendish para el estudio de selección proporcionado en la Tabla 2 a continuación, tanto para las plantas transformadas como para las plantas de control. Los cultivares de plátano Cavendish se mencionan 47 veces en la Solicitud '024, incluso como un enfoque particular para mejorar la resistencia a las enfermedades. Véase, por ejemplo, [00204] - [00206], [00216], [00273] y en el Ejemplo 3.

TABLA 2

Constructo	Número de plantas probadas	SEQ ID NO	Promotor	Terminador	Puntuación Evento Corm	Puntuación Control Corm
1511	3	9	Constitutivo (banana ACTIN1)	Banana endógeno ACTIN1	1.3	3.6
1511	2	9	Constitutivo (banana ACTIN1)	Banana endógeno ACTIN1	1.5	3.4
1511	2	9	Constitutivo (banana ACTIN1)	Banana endógeno ACTIN1	1.5	4.1
1564	2	9	Tejido endógeno específico del banano (punta de la raíz) TIP2-3_A	Tejido endógeno específico del banano (punta de la raíz) TIP2-3_A	2	3.5
1565	1	9	Tejido específico del maíz (punta de la raíz)	Tejido específico del maíz (punta de la raíz)	1	4.2
1566	1	9	Tejido endógeno específico del banano (punta de la raíz) TIP2-3_B	Tejido endógeno específico del banano (punta de la raíz) TIP2-3_B	2	4.2
1566	1	9	Tejido endógeno específico del banano (punta de la raíz) TIP2-3_B	Tejido endógeno específico del banano (punta de la raíz) TIP2-3_B	1	2.5
1567	3	2	Banana endógeno FusR1	Banana endógeno FusR1	1	2.7
1568	3	5	Banana endógeno FusR1	Banana endógeno FusR1	2.3	3.6
1569	3	18	Banana endógeno FusR1	Banana endógeno FusR1	0.7	2.1
1590	2	9	Banana endo endógeno genous FusR1	Banana endógeno FusR1	2	3.4
1628	2	9	Constitutivo (banana endogenous UBQ1)	Banana endogenous ACTIN1	1	1.8
1629	3	9	Agrobacterium NOS	Banana endógeno ACTIN1	1	2.9
1629	3	9	Agrobacterium NOS	Banana endógeno ACTIN1	1.3	2.4

Constructo	Número de plantas probadas	SEQ ID NO	Promotor	Terminador	Puntuación Evento Corm	Puntuación Control Corm
1630	1	11	Tejido endógeno específico del banano (punta de la raíz) (root-tip) TIP2-3 A	Tejido endógeno específico del banano (punta de la raíz) TIP2-3 A	1	4
1630	1	11	Tejido endógeno específico del banano (punta de la raíz) TIP2-3 A	Tejido endógeno específico del banano (punta de la raíz) TIP2-3 A	1	4
1631	1	2	Constitutivo (banana UBQ1)	Banana endógeno FusR1	0	4
1631	1	2	Constitutivo (banana UBQ1)	Banana endógeno FusR1	1	4
1631	2	2	Constitutivo (banana UBQ1)	Banana endógeno FusR1	1	1.9
1632	1	2	Agrobacterium NOS	Banana endógeno FusR1	3	4
1633	1	2	Tejido específico del maíz (punta de la raíz) TIP2-3 A	Banana endógeno FusR1	1	2.5
1633	1	2	Tejido específico del maíz (punta de la raíz) TIP2-3 A	Banana endógeno FusR1	0	2.5
1633	1	2	Tejido específico del maíz (punta de la raíz) TIP2-3 A	Banana endógeno FusR1	1	2.5
1634	1	18	Constitutivo (banana endógeno UBQ1)	Banana endógeno FusR1	0	1.3
1635	1	18	Tejido específico del maíz (punta de la raíz) TIP2-3 A	Banana endógeno FusR1	1	4
1635	1	18	Tejido específico del maíz (punta de la raíz) TIP2-3 A	Banana endógeno FusR1	0	4

CONCLUSIÓN

1. Respectuosamente, presento que la solicitud '024 respalda la invención reivindicada actualmente y que las reivindicaciones 1 a 19 están habilitadas por la especificación basada en las pruebas y ejemplos reales como se discutió anteriormente.
2. Las plantas de banano se transformaron con las secuencias de ácido nucleico reivindicadas que codifican la expresión de resistencia a *Fusarium oxysporum* TR 4.
3. Como se muestra en los datos proporcionados en la Tabla 2 anterior, las plantas transformadas, utilizando las secuencias y las técnicas proporcionadas en la solicitud '024 mostraron una resistencia significativamente mayor a *Fusarium oxysporum* TR 4 en comparación con las plantas de banano de control que no tenían una construcción de ácido nucleico que codificara la resistencia a *Fusarium oxysporum* TR 4.
4. Declaro que mis declaraciones se hacen con el conocimiento de que las declaraciones falsas intencionales y similares realizadas de esa manera son punibles con multa o prisión, o ambas, según la Sección 1001 del Título 18 del Código de los Estados Unidos, y que dichas declaraciones falsas intencionales pueden poner en peligro la validez de la solicitud o cualquier patente emitida a partir de ella.

Respectfully submitted,

Date: _____

Walter Messier

Anexo A

WALTER MESSIER

Curriculum vitae

Evolutionary Genomics, Inc
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EDUCATION:

- The University at Albany, State University of New York, Albany, NY Ph.D. (Molecular Biology), May 1998
- The College at New Paltz, State University of New York, New Paltz, NY Masters in Biology, May 1992
- The College at New Paltz, State University of New York, New Paltz, NY Bachelor of Science,
May 1990, *Summa cum laude*
- Yale University, New Haven, CT 1973-1975

PRESENT POSITION:

Chief Science Officer, Evolutionary Genomics, Inc.

EMPLOYMENT HISTORY:

- Chief Science Officer, Evolutionary Genomics, August 2003-Present
- Director of Sequencing/Evolutionary Analysis, Evolutionary Genomics, L.L.C., September 2000-August 2003
- Program Director, GenoPlex, Inc. July 2000-September 2000
- Scientist/Project Leader, for Evolutionary Genomics, GenoPlex, Inc., May 2000-July 2000
- Scientist, GenoPlex, Inc. February 1998-May 2000
- Research Associate/Ph.D. Candidate 1992-1998
- Licensed Ophthalmic Dispenser (Optician-Manager) 1973-1992 Sterling Optical

PUBLICATIONS:

- Ferguson, M., Koga, T.M., Johnson, D.A., Koga, K.A., Hirsch, G.N., Becerra Lopez-Lavalle, L.A., and Messier, W. (2015) Identification of genes that have undergone adaptive evolution in cassava (*Manihot esculenta*) and that may confer resistance to cassava brown streak disease. *African Journal of Biotechnology* **14**(2): 96-107 doi: 10.5897/AJB2014.14113.
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- Messier, W., Li, S.-H., and Stewart, C.-B. (1996) The birth of microsatellites. *Nature* **381**:483.
- Turcich, M.P., Bokhari-Riza, A., Hamilton, D.A., He, C., Messier, W., Stewart, C.-B., and Mascarenhas, J.P. (1996) PREM-2, a copia-type retroelement in maize is expressed preferentially in early microspores. *Sexual Plant Reproduction* **9**: 65-74.
- Messier, W., and Stewart, C.-B. (1994) Dissolving the barriers. *Current Biology* **4**: 911-913.

ISSUED U.S. PATENTS (additional issued patents in the European Union, Australia, etc.):

- "EG1117 polynucleotides and uses thereof." Messier, W. Patent No. 7,439,018, Issued 10/21/2008.
- "EG307 polynucleotides and uses thereof." Messier, W. Patent No. 7,252,966, Issued 8/7/2007.
- "Methods to identify polynucleotide and polypeptide sequences which may be associated with physiological and medical conditions." Messier, W. Patent No. 7,247,425, Issued 7/24/2007.

- “Methods to identify polynucleotide and polypeptide sequences which may be associated with physiological and medical conditions.” Messier, W and Sikela, J.M. Patent No. 6,866,996, Issued 3/15/2005.
- “Methods for producing transgenic plants containing evolutionarily significant polynucleotides.” Messier, W and Sikela, J.M. Patent No. 6,743,580, Issued 6/1/2004.
- "Methods to identify polynucleotide and polypeptide sequences which may be associated with physiological and medical conditions." Messier, W and Sikela, J.M. Patent No. 6,280,953, Issued 8/28/2001.
- "Methods to identify evolutionarily significant changes in polynucleotide and polypeptide sequences in domesticated plants and animals." Walter Messier and James Sikela Patent No. 6,274,319, Issued 8/14/2001.
- "Methods to identify polynucleotide and polypeptide sequences which may be associated with physiological and medical conditions." Messier, W and Sikela, J.M. Patent No. 6,228,586, Issued 5/8/2001.

GRANTS AWARDED:

- NSF SBIR Phase I: "Bioinformatic Data Mining for AIDS Resistance Genes." \$99,960 awarded 5/28/03.
- NSF SBIR Phase II: " Bioinformatic Data Mining for AIDS Resistance Genes." \$499,961 awarded 7/8/05.
- NSF SBIR Phase I: “Bioenergy from Genomics: Genes that Increase Corn Kernel Starch and Oil Content.” \$99,129 awarded 4/30/08.
- Bill & Melinda Gates Foundation Grand Challenges Explorations Grant: “Evolutionary-based Host Target Therapeutic Approach Sidesteps HIV/AIDS Drug Resistance.” \$100,000 awarded 9/17/08.
- USDA SBIR Phase I: “Bioenergy from Genomics: Genes that Impact Sorghum Grain, Sugar, and Biomass Yield.” \$79,998 awarded 3/5/09.
- NSF SBIR Phase I: “Increasing Productivity of Microalgae for Biodiesel.” \$99,628 awarded 7/1/09.
- NSF SBIR Phase I: “Identification of salt and drought tolerance genes for crop plant improvement.” \$148,177 awarded 7/1/10.
- NSF SBIR Phase IB: “Identification of salt and drought tolerance genes for crop plant improvement.” \$29,182 awarded 11/30/10.
- Bill & Melinda Gates Foundation Grant: “Discovering cassava genes underlying resistance to Cassava Brown Streak Disease (CBSD) and to whitefly pests.” \$285,000 awarded 10/31/11.
- Bill & Melinda Gates Foundation Grand Challenges Explorations Grant: “Defeating Ug99 wheat stem rust.” \$100,000 awarded 3/29/11.

- United Soybean Board (USB): “Validation in transgenic soybeans of candidate genes for mitigating losses to Soybean Cyst Nematodes (SCN).” \$262,476 awarded 3/1/12.
- Colorado Advanced Industry Accelerator Grant: “Full-plant validation in transgenic soybeans of candidate genes for mitigating losses to Soybean Cyst Nematodes (SCN).” \$250,000 awarded 5/14/15.
- Colorado Advanced Industry Accelerator Grant: “Identification of Resistance Genes in Banana for *Fusarium* TR4 (Panama Disease).” \$250,000 awarded 5/17/18.

REVIEWER FOR:

Nature

Science

Proceedings of the National Academy of Sciences USA

Frontiers in Plant Genetics and Genomics (member of Review Editorial Board)

Oncogene

REFERENCES:

Available upon request.

Atty Docket No. EGEN.09JPU1

Japanese Patent Application No. 2021-575024

Patent Examiner: Harue TANAKA

Office Action Date: 2024-05-17

DECLARATION

I, Walter Messier, hereby declare as follows:

1. I am the inventor for Japanese Patent Application No. 2021-575024 (“the ‘024 Application”). My educational background and experience are set forth below.
2. I have a PhD degree in molecular biology from the University at Albany, State University of New York, in Albany, New York, United States.
3. I have been a molecular biologist for 26 years. A copy of my *curriculum vitae* is attached.
4. I am a Founder and currently serve as the Chief Science Officer of the company assigned the rights to this patent application and the invention disclosed therein.
5. I am familiar with the Office Action dated May 17, 2024.
6. I understand that the Examiner has provided Reason 2 (Support Requirement) and Reason 3 (Enablement Requirement) against Claims 1-19 of the ‘024 Application.
7. I respectfully submit the following data and description which provides working examples of the nucleic acid construct of claim 1 of the ‘024 application and the expression of resistance to *Fusarium oxysporum* race 4 in banana (*Musa*) plants using SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11, and SEQ ID NO: 18 with various promoters and terminators as shown herein in Table 2 below.

8. Support for use of sequences SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11, and SEQ ID NO: 18 can be found in Table 1 in paragraph [0230] and Figure 1 of the '024 application and in Example 5 starting on paragraph [0437] of the '024 application.
9. Promoters provided in Table 2 of this declaration and as used in the working examples were chosen from the list of promoters in the '024 application (see, e.g., paragraph [00289] - [00293]) or fitting one of the categories of promoters listed in the '024 application (e.g., constitutive promoter, tissue-specific promoter, etc.), and if applicable, support for the use of a selectable marker is found in paragraph [00294] of the '024 application.
10. The transformation method we used for the integration of the construct of claim 1 into banana plants was agrobacterium/microprojectile bombardment which is supported in paragraph [00296] of the '024 application and in Example 5 of the '024 application.
11. Transformations of banana plant material were conducted in Middleton, Wisconsin, United States and were performed according to the procedures set forth in the '024 application.
12. Screening of Panama Disease, *Fusarium oxysporum f.sp.cubense* II-5 TR4-resistant bananas was conducted in Madison, Wisconsin, United States according to the procedures set forth in the '024 application.
13. The protocol for screening as described below follows methodology that is well known to those skilled in the art as set forth in García-Bastidas, F. A., Van der Veen, A. J. T., Nakasato-Tagami, G., Meijer, H. J. G., Arango-Isaza, R. E., and Kema, G. H. J. (2019). An Improved Phenotyping Protocol for Panama Disease in Banana. *Frontiers in plant science*, 10, 1006. doi: 10.3389/fpls.2019.01006 ("Garcia-Bastidas et al."). This reference published on August 5, 2019, which was before the October 7, 2019, filing date of U.S. Provisional Patent Application No. 62/912,010, from which the '024 Application claims priority.

14. Plant material (typically 1.5-2 months after tissue culture is moved to soil) were transferred to 1-liter (L) pots containing soil media (PRO-MIX HP MYCORRHIZAE, Premier Tech Horticulture, USA) and kept in trays. The plants were grown for 2 to 3 weeks in an environmentally controlled greenhouse (28 ± 3 °C, 16h light, ~60% humidity) prior to inoculation. This growth period is intended to bring plants to an appropriate age (2 to 3 months) for conducting the disease assay and to acclimate them to the new environment. Plants are watered daily and fertilized weekly throughout the disease assays.
15. *F. oxysporum f.sp. cubense* II-5 Tropical Race 4 (TR4) was obtained from the USDA Agricultural Research Service Culture Collection (see Table 1 below). Upon receipt, using the protocol provided in García-Bastidas *et al* as cited above in paragraph 6, the lyophilized fungal culture is rehydrated and suspended in sterile water. The suspension was then left to rest for 30 mins and inoculated onto the sterile dampened filter paper on the surface of potato dextrose agar (PDA) (Neogen Culture Media Co., Michigan, USA) medium. After recovering growth at 25°C for approximately two weeks, the filter paper fully covered with fungal mycelium was dried overnight in a laminar hood. Next day, filter paper with mycelium was cut into small pieces using sterile scissors and stored in a sterile container at -20 °C. To prepare conidial spore inoculum, filter paper with fungal mycelium was inoculated onto PDA and kept at 25°C. Seven days after inoculation, 7-milimeter hyphal plugs from the edge of the actively growing fungal colony was taken to inoculate sporulation media (SM) to produce conidial spores.

16. **Table 1.** Origin and characteristic of the *Fusarium* spp. strain used in this study.

<i>Fusarium</i> spp.	Code	Host	Origin	Provider
<i>F. oxysporum f.sp. cubense</i> II-5	TR4	Banana	USDA-ARS-CDL, St. Paul, MN	Jacolyn Morrison

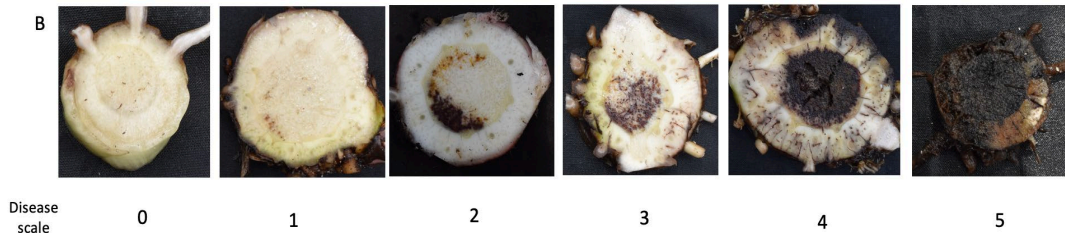
17. A conidial suspension of *F. oxysporum f.sp. cubense* II-5 TR4 was generated using a modified SM containing mung bean, which has been shown to induce rapid and robust production of conidia. *See* Garcia-Bastidas *et al* protocol provided in paragraph 6. Fresh mung beans were soaked in sterile water overnight prior to medium preparation. Three grams of pre-soaked mung beans were added to 1-liter Erlenmeyer flasks containing 500 milliliters (ml) water and then sterilized in an autoclave at 120 °C for 20 mins. After cooling down at room temperature overnight, SM media were inoculated with 5 hyphal plugs of actively growing *F. oxysporum f.sp. cubense* II-5. The inoculated Erlenmeyer flasks were incubated on a shaker at 105 rpm, 25°C for 6-8 days. Spore suspension was collected in sterile Erlenmeyer flask by filtration with four layers of cheesecloth to remove hyphae and media content. The concentrations of spore suspensions are quantified with a hemocytometer under microscope and normalized to a concentration of 10^7 spores/ml. Ten microliters of spore suspension was applied to PDA and grown at 25°C for 7 days to determine the viability of the inoculum.
18. The conidial spore suspension in Erlenmeyer flask was transported from the lab to the greenhouse in an insulated cooler to minimize the impact of adverse environmental factors, *See* Garcia-Bastidas *et al* protocol provided in paragraph 6. For each pot of plant to be inoculated, 20 ml of spore suspension at 10^7 spores/ml was diluted to 200 ml at 10^6 spores/ml using water from the greenhouse and then evenly poured into the pot. Plants were watered by filling the tray from the bottom to avoid washing spores out of soil. When the temperature and humidity of greenhouses decrease during season transition (i.e.: from summer/fall to winter/spring), the plants received a second inoculation one week after the first inoculation using the same protocol.

19. Disease symptoms on assayed plant material were evaluated both externally and internally seven weeks post-inoculation using a scoring 5-point scoring system, similar to the 4-point scoring system described in Garcia-Bastidas *et al* (Figure 4) and in the as-filed '024 Application at [00108] – [00109]. The External symptoms of the whole plant were recorded and evaluated through the categorical assessment of leaf wilting/yellowing on a 1-4 scale representing each of the quartiles on a scale of 0-100% wilting, where 0 = no wilting/yellowing, 1 = <25% leaves show wilting/yellowing, 2 = 25-50%, 3 = 50-75%, 4 = 75-100%, and 5 = 100% (see Figure A below). After external evaluation, plants were removed from pots and cut horizontally along the rhizome (corm) of each plant. Internal evaluation was quantified by the area of discoloration of the corm using 1-4 scale representing where 0 = no discoloration, 1 = <25% corm showing discoloration, 2 = 25-50%, 3 = 50-75%, 4 = 75-100%, and 5 = 100% (see Figure B below).

Figure A



Figure B



20. Figure A (external) and Figure B (internal) show symptoms of banana plants recorded at seven weeks after inoculation with *Fusarium oxysporum f.sp. cubense* II-5 Tropical Race 4.

21. The Table 2 below illustrates the results of transforming banana cultivars with the claimed nucleic acid sequences and the resulting improved resistance. As described above, disease assessment was performed both internally (by corm evaluation) and externally (by discoloring/wilt of the plants). As described above, quantification of discoloration of the corm using a scale of 0-5 where 0 = no discoloration; 1 = <25% corm showing discoloration; 2 = 25-50% corm showing discoloration; 3 = 50-75% corm showing discoloration; 4 = 75-100% corm showing discoloration, and 5 = 100% corm showing discoloration.
22. As shown below, the discoloration scale corm scores for the plants modified by nucleic acid sequences coding for resistance to *Fusarium oxysporum* race 4 (herein Event Corm Score) were over 100% to as much as 400% lower than control plants (herein Control Corm Score) not modified for *Fusarium oxysporum* race 4 resistance. Cavendish bananas were used for the screening study provided in Table 2 below for both the transformed plants and the control plants. Cavendish banana cultivars are mentioned 47 times in the '024 Application, including as a particular focus for improving disease resistance. See, e.g., [00204] - [00206], [00216], [00273], and in Example 3.

TABLE 2

Construct	Number of Plants Tested	SEQ ID NO	Promoter	Terminator	Event Corm Score	Control Corm Score
1511	3	9	Constitutive (banana ACTIN1)	Banana endogenous ACTIN1	1.3	3.6
1511	2	9	Constitutive (banana ACTIN1)	Banana endogenous ACTIN1	1.5	3.4
1511	2	9	Constitutive (banana ACTIN1)	Banana endogenous ACTIN1	1.5	4.1
1564	2	9	Banana endogenous tissue specific (root-tip) TIP2-3_A	Banana endogenous tissue specific (root-tip) TIP2-3_A	2	3.5
1565	1	9	Maize tissue specific (root-tip)	Maize tissue specific (root-tip)	1	4.2
1566	1	9	Banana endogenous tissue specific (root-tip) TIP2-3_B	Banana endogenous tissue specific (root-tip) TIP2-3_B	2	4.2

Construct	Number of Plants Tested	SEQ ID NO	Promoter	Terminator	Event Corm Score	Control Corm Score
1566	1	9	Banana endogenous tissue specific (root-tip) TIP2-3_B	Banana endogenous tissue specific (root-tip) TIP2-3_B	1	2.5
1567	3	2	Banana endogenous FusR1	Banana endogenous FusR1	1	2.7
1568	3	5	Banana endogenous FusR1	Banana endogenous FusR1	2.3	3.6
1569	3	18	Banana endogenous FusR1	Banana endogenous FusR1	0.7	2.1
1590	2	9	Banana endogenous FusR1	Banana endogenous FusR1	2	3.4
1628	2	9	Constitutive (banana endogenous UBQ1)	Banana endogenous ACTIN1	1	1.8
1629	3	9	Agrobacterium NOS	Banana endogenous ACTIN1	1	2.9
1629	3	9	Agrobacterium NOS	Banana endogenous ACTIN1	1.3	2.4
1630	1	11	Banana endogenous tissue specific (root-tip) TIP2-3_A	Banana endogenous tissue specific (root-tip) TIP2-3_A	1	4
1630	1	11	Banana endogenous tissue specific (root-tip) TIP2-3_A	Banana endogenous tissue specific (root-tip) TIP2-3_A	1	4
1631	1	2	Constitutive (banana UBQ1)	Banana endogenous FusR1	0	4
1631	1	2	Constitutive (banana UBQ1)	Banana endogenous FusR1	1	4
1631	2	2	Constitutive (banana UBQ1)	Banana endogenous FusR1	1	1.9
1632	1	2	Agrobacterium NOS	Banana endogenous FusR1	3	4
1633	1	2	Maize tissue specific (root-tip) TIP2-3_A	Banana endogenous FusR1	1	2.5
1633	1	2	Maize tissue specific (root-tip) TIP2-3_A	Banana endogenous FusR1	0	2.5
1633	1	2	Maize tissue specific (root-tip) TIP2-3_A	Banana endogenous FusR1	1	2.5
1634	1	18	Constitutive (banana endogenous UBQ1)	Banana endogenous FusR1	0	1.3
1635	1	18	Maize tissue specific (root-tip) TIP2-3_A	Banana endogenous FusR1	1	4
1635	1	18	Maize tissue specific (root-tip) TIP2-3_A	Banana endogenous FusR1	0	4

CONCLUSION

23. I respectfully submit that the '024 Application provides support for the presently claimed invention and that Claim 1 to 19 are enabled by the specification based upon the actual testing and examples as discussed above.
24. Banana plants were transformed with the claimed nucleic acid sequences which code for the expression of *Fusarium oxysporum* TR 4 resistance.
25. As shown in the data provided in Table 2 above, transformed plants, using the sequences and the techniques as provided in the '024 Application showed significant increased resistance to *Fusarium oxysporum* TR 4 when compared to control banana plants that did not have a nucleic acid construct coding for *Fusarium oxysporum* TR 4 resistance.
26. I declare that my statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Respectfully submitted,

Date: _____

Walter Messier

Appendix A

WALTER MESSIER

Curriculum vitae

Evolutionary Genomics, Inc
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EDUCATION:

- The University at Albany, State University of New York, Albany, NY Ph.D. (Molecular Biology), May 1998
- The College at New Paltz, State University of New York, New Paltz, NY Masters in Biology, May 1992
- The College at New Paltz, State University of New York, New Paltz, NY Bachelor of Science,
May 1990, *Summa cum laude*
- Yale University, New Haven, CT 1973-1975

PRESENT POSITION:

Chief Science Officer, Evolutionary Genomics, Inc.

EMPLOYMENT HISTORY:

- Chief Science Officer, Evolutionary Genomics, August 2003-Present
- Director of Sequencing/Evolutionary Analysis, Evolutionary Genomics, L.L.C., September 2000-August 2003
- Program Director, GenoPlex, Inc. July 2000-September 2000
- Scientist/Project Leader, for Evolutionary Genomics, GenoPlex, Inc., May 2000-July 2000
- Scientist, GenoPlex, Inc. February 1998-May 2000
- Research Associate/Ph.D. Candidate 1992-1998
- Licensed Ophthalmic Dispenser (Optician-Manager) 1973-1992 Sterling Optical

PUBLICATIONS:

- Ferguson, M., Koga, T.M., Johnson, D.A., Koga, K.A., Hirsch, G.N., Becerra Lopez-Lavalle, L.A., and Messier, W. (2015) Identification of genes that have undergone adaptive evolution in cassava (*Manihot esculenta*) and that may confer resistance to cassava brown streak disease. *African Journal of Biotechnology* **14**(2): 96-107 doi: 10.5897/AJB2014.14113.
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- Messier, W., and Stewart, C.-B. (1994) Dissolving the barriers. *Current Biology* **4**: 911-913.

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- "EG1117 polynucleotides and uses thereof." Messier, W. Patent No. 7,439,018, Issued 10/21/2008.
- "EG307 polynucleotides and uses thereof." Messier, W. Patent No. 7,252,966, Issued 8/7/2007.
- "Methods to identify polynucleotide and polypeptide sequences which may be associated with physiological and medical conditions." Messier, W. Patent No. 7,247,425, Issued 7/24/2007.

- “Methods to identify polynucleotide and polypeptide sequences which may be associated with physiological and medical conditions.” Messier, W and Sikela, J.M. Patent No. 6,866,996, Issued 3/15/2005.
- “Methods for producing transgenic plants containing evolutionarily significant polynucleotides.” Messier, W and Sikela, J.M. Patent No. 6,743,580, Issued 6/1/2004.
- "Methods to identify polynucleotide and polypeptide sequences which may be associated with physiological and medical conditions." Messier, W and Sikela, J.M. Patent No. 6,280,953, Issued 8/28/2001.
- "Methods to identify evolutionarily significant changes in polynucleotide and polypeptide sequences in domesticated plants and animals." Walter Messier and James Sikela Patent No. 6,274,319, Issued 8/14/2001.
- "Methods to identify polynucleotide and polypeptide sequences which may be associated with physiological and medical conditions." Messier, W and Sikela, J.M. Patent No. 6,228,586, Issued 5/8/2001.

GRANTS AWARDED:

- NSF SBIR Phase I: "Bioinformatic Data Mining for AIDS Resistance Genes." \$99,960 awarded 5/28/03.
- NSF SBIR Phase II: " Bioinformatic Data Mining for AIDS Resistance Genes." \$499,961 awarded 7/8/05.
- NSF SBIR Phase I: “Bioenergy from Genomics: Genes that Increase Corn Kernel Starch and Oil Content.” \$99,129 awarded 4/30/08.
- Bill & Melinda Gates Foundation Grand Challenges Explorations Grant: “Evolutionary-based Host Target Therapeutic Approach Sidesteps HIV/AIDS Drug Resistance.” \$100,000 awarded 9/17/08.
- USDA SBIR Phase I: “Bioenergy from Genomics: Genes that Impact Sorghum Grain, Sugar, and Biomass Yield.” \$79,998 awarded 3/5/09.
- NSF SBIR Phase I: “Increasing Productivity of Microalgae for Biodiesel.” \$99,628 awarded 7/1/09.
- NSF SBIR Phase I: “Identification of salt and drought tolerance genes for crop plant improvement.” \$148,177 awarded 7/1/10.
- NSF SBIR Phase IB: “Identification of salt and drought tolerance genes for crop plant improvement.” \$29,182 awarded 11/30/10.
- Bill & Melinda Gates Foundation Grant: “Discovering cassava genes underlying resistance to Cassava Brown Streak Disease (CBSD) and to whitefly pests.” \$285,000 awarded 10/31/11.
- Bill & Melinda Gates Foundation Grand Challenges Explorations Grant: “Defeating Ug99 wheat stem rust.” \$100,000 awarded 3/29/11.

- United Soybean Board (USB): “Validation in transgenic soybeans of candidate genes for mitigating losses to Soybean Cyst Nematodes (SCN).” \$262,476 awarded 3/1/12.
- Colorado Advanced Industry Accelerator Grant: “Full-plant validation in transgenic soybeans of candidate genes for mitigating losses to Soybean Cyst Nematodes (SCN).” \$250,000 awarded 5/14/15.
- Colorado Advanced Industry Accelerator Grant: “Identification of Resistance Genes in Banana for *Fusarium* TR4 (Panama Disease).” \$250,000 awarded 5/17/18.

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Available upon request.