

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Dirección de Nuevas Creaciones

E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 11-025439- -00009-0000

Fecha: 2013-05-14 15:58:01 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 523 RESPUESTA Folios: 73

Expediente: 11.025.439

Titular: BAYER CROPSCIENCE N.V.

Asunto: CONTESTACIÓN A REQUERIMIENTO.

MAURICIO PATIÑO BONNET, mayor de edad, vecino de esta ciudad, identificado como aparece al pie de mi firma, portador de la tarjeta profesional número 73.583 del Consejo Superior de la Judicatura, en mi calidad de apoderado de **BAYER CROPSCIENCE N.V.**, por medio del presente memorial acudo a la Dirección de Nuevas Creaciones de la Superintendencia de Industria y Comercio con el fin de **DAR RESPUESTA** al Oficio No. **23670**, de acuerdo como sigue:

Desde el principio, respetuosamente quisiera enfatizar que el punto crucial de la invención es el aislamiento por primera vez de macro moléculas biológicas distintas de lignina y polisacáridos tales como celulosa o hemicelulosa en fibras de plantas (algodón) maduras y en fibras de plantas (algodón) procesadas. Las macro moléculas son de manera predominante ADN, pero también incluyen ARN y cadenas dobles de ARN y hay evidencia de aislamiento de material polipéptido (ver página 53, Ejemplo 7).

Como se mencionó en la página 4, líneas 7 a 15, las células de fibra de algodón en desarrollo son células vivas y por consiguiente contienen todas las macromoléculas esperadas en una célula viva incluyendo ADN. Las fibras maduras por el contrario están muertas, las células huecas consisten principalmente en celulosa, y algunos otros polisacáridos y algunas veces lignina. De hecho ellas consisten principalmente de la pared de la célula de la planta (secundaria) sin "protoplastos" restantes para el desarrollo de células de plantas.

Es generalmente aceptado que las células de fibra maduras, incluyendo las fibras de células de algodón no contienen ácidos nucleicos o proteínas que se

puedan extraer y muestras de algodón son usadas para tomar muestras para análisis de ADN en análisis forenses, basados en la inactividad de las fibras (de algodón).

Las células de fibras procesadas como aquellas de tejidos de punto o hiladas hechas de fibras de plantas maduras han sufrido duros tratamientos químicos, mecánicos y de calor, por lo que no se espera encontrar ningún ácido nucleico intacto en dichas células de fibras procesadas.

Por otra parte, la solicitud resalta que el ADN que es aislado es una representación del genoma completo (ver ejemplo 6) incluyendo la secuencia plástida (ver ejemplo 5).

Una vez las macromoléculas, particularmente el ácido nucleico / ADN, es aislado, pueden por supuesto ser procesadas de cualquier manera y usadas de otra manera. Por ejemplo, pueden ser usadas para detectar secuencias específicas de ácido nucleico, ser las secuencias endógenas (ver por ejemplo, Ejemplo 1, página 37, líneas 19 a 25- glucanasas endógenas; Ejemplo 1, página 37, líneas 16 a 24 - expansión de otras genes endógenos; Ejemplo 6, página 51 y 52, líneas 16 a 24, y 1 y 2 - detección de 40 SNPs) o secuencias transgénicas (Ejemplo 3, página 41, líneas 4 a 12 - NodC; Ejemplo 6, página 52, líneas 3 a 13 - evento Mon 531 transgénico, Evento Mon15985 y Evento Roundup ready Flex MON88913).

En consecuencia, la invención no está dirigida hacia un método específico para la detección de secuencias específicas, y de hecho ni siquiera requiere métodos de aislamiento muy elaborados y novedosos (excepto para algunos largos tiempos de incubación de las fibras de las plantas en comparación con la técnica). Por el contrario, es el primer entendimiento de que dichas fibras aún contienen macromoléculas, de manera predominante ADN que permite las diferentes aplicaciones descritas.

CLARIDAD Y USOS

Con el fin de superar las objeciones que por claridad y usos planteó su Despacho, presento nuevo pliego reivindicatorio compuesto por veintiún (21)

reivindicaciones, en el cual se han realizado las siguientes modificaciones. A saber:

1. La reivindicación 7 ha sido objetada por no estar soportada por la descripción. Dicha reivindicación ha sido eliminada.
2. La reivindicación 10 ha sido objetada por estar soportada únicamente en la mención de un gen codificado de N-acetil glucosamina transferasa.

Primero que todo y en vista de las numerosas secuencias de ADN identificadas y ejemplificadas en esta solicitud, no hay razón para sospechar que habría alguna restricción en el tipo de ácido nucleico que puede ser amplificado desde fibras maduras o procesadas.

Segundo, hay secuencias de genes distintos al gen NodC (el cual es conocido del estado de la técnica anterior) el cual ha sido ejemplificado en la solicitud, en extenso: Gen glucanasa endógeno - Ejemplo 1, Gen expansina endógeno - Ejemplo 1, Evento Mon531; Boliguard I es por ejemplo gen proteína cristal; Evento Mon15985; Boliguard II por ejemplo gen proteína cristal BT; Evento MON88913; Flex por ejemplo gen EPSPS, los últimos tres todos han sido descritos en el Ejemplo 6.

La reivindicación 10 ha sido limitada a esos genes únicamente para acelerar el proceso.

3. Finalmente, las anteriores reivindicaciones 1, 2, 14, 19, 21 y 24 han sido objetadas por no ser claras por qué no incluyen una descripción de los reactivos y material necesario para la extracción de macro moléculas, ni hace el recuento de cualquiera de las primeras secuencias usadas para las reacciones de amplificación.

Todas las reivindicaciones independientes han sido modificadas para incluir la etapa de aislamiento/incubación en un regulador de lisis que contiene detergentes y proteasas. Limitar los reactivos a un tipo particular sería restringir innecesariamente la protección a la que tiene derecho el mi mandante.

Más aún, como se mencionó anteriormente, la solicitud revela y ejemplifica numerosos aislamientos y amplificaciones de fragmentos de ADN, y no hay razón para limitar el ensayo de detección a secuencias de ácido nucleico específicos o aun a un tipo específico de ensayo de detección. El solicitante demuestra el uso de ensayos basados en PCR, ensayos basados en la detección de hibridación de ácido nucleico (Taqman®; ver Ejemplo 4, página 32) y toda la amplificación del genoma (ver Ejemplo 6), la inclusión de imprimantes específicos en los métodos reivindicados sería una restricción indebida al alcance de la protección. Esto sería similar a requerir que un método para secuenciar usando, por ejemplo, dideoxireagentes sea limitado a la secuencia particular usada para ejemplificar el método. Los métodos reivindicados reflejan la contribución inventiva de la solicitud, es decir, que los ácidos nucleicos pueden ser aislados de fibras de plantas maduras y procesadas (contrario a la creencia común en este momento). La naturaleza exacta de este ensayo de detección o de los procesos adicionales de ADN aislado es inmaterial para esta invención.

4. La reivindicación 23 ha sido objetada por reivindicar el uso del método. Dicha reivindicación ha sido eliminada.
5. Las reivindicaciones 2 a 6, 8 a 22, 24 y 25 han sido objetadas por pretender proteger un uso distinto al originalmente comprendido el patente inicial (la anterioridad citada **D1**) conforme el artículo 21.

En este sentido, respetuosamente pongo de presente que esta objeción es desafortunada toda vez que la anterioridad citada no revela un método patentado sino el estado de la técnica general y dichas reivindicaciones no pretenden atribuirle un uso distinto.

Adicionalmente, el método de reivindicación difiere del método revelado en **D1** en la opción del material de inicio y en el tiempo de incubación en un regulador de lisis tal y como es introducido en las presentes modificaciones (ver también los argumentos de la sección de novedad).

6. Finalmente, las nuevas reivindicaciones 2, 3 y 10 (antiguas reivindicaciones 3, 4 y 13) son claras toda vez que adicionan una característica que limita el objeto de protección¹.

En este sentido, nótese por demás que las modificaciones realizadas al pliego reivindicatorio no amplían la materia objeto de protección, sino por el contrario aclaran y limitan la misma, permitiendo una interpretación inequívoca del alcance de protección y su comparación con el estado del arte. Así, las mismas se encuentran en concordancia con los lineamientos del artículo 34 de la D.486.

En consecuencia, las modificaciones al nuevo pliego reivindicatorio dan por superadas las objeciones que por claridad y usos planteó su Despacho, toda vez que los compuestos reivindicados se encuentran plenamente sustentados por la descripción de conformidad con el artículo 30 de la D. 486.

Por consiguiente, atentamente solicito al señor Examinador acepte las modificaciones propuestas y proceda con el respectivo estudio de patentabilidad de la solicitud de la referencia.

NOVEDAD

El Despacho considera que **D1** afecta la novedad de la presente solicitud de patente.

Establece el artículo 16:

"Una invención se considerará nueva cuando no está comprendida en el estado de la técnica [...]".

En este sentido, el instructivo para el Examen de Solicitudes de Patentes de Invención y Modelos de Utilidad de la Superintendencia de Industria y Comercio (Instructivo), establece:

¹ Superintendencia de Industria y Comercio **"Examen de Solicitudes de Patentes de Invención y Modelos de Utilidad"**, Bogotá D.C., Febrero 8, 2012. Pág. 37.

"El examinador debe evaluar si la invención es nueva o no, de la siguiente manera:

- Definir cuáles son las características técnicas esenciales de la primera reivindicación independiente*
- Comparar, elemento por elemento, las características técnicas esenciales con las características de la materia divulgada en cada documento del estado del arte*
- Verificar, según la comparación anterior, si la invención reivindicada es idéntica a lo revelado en el estado de la técnica, en cuyo caso se considera que no tiene novedad*
- Examinar de la misma manera las otras reivindicaciones independientes, y*
- Revisar si las reivindicaciones dependientes mencionan elementos nuevos"².*

Por lo tanto, a partir de los lineamientos del artículo 16 y los conceptos expuestos por el Instructivo procedo a demostrar que la invención de mi representada es novedosa a la luz de **D1**. Veamos:

La diferencia principal entre la reivindicación 1 (así como las otras reivindicaciones independientes) y **D1** es el uso de cualquiera de las dos fibras maduras o procesadas (incluyendo telas que comprenden dichas fibras) para aislar ADN.

D1 usa fibras vivas, es decir en desarrollo, del algodón para aislar el ADN. Tal y como indica el examinador, dicha anterioridad en la página 1299 columna de la izquierda, primer párrafo, establece que el total de ARN fue aislado de "fibras de diferentes etapas de desarrollo de plantas maduras". (Nótese que esto no es lo mismo que fibras maduras; las plantas tienen que estar maduras para florecer y formar cuadrados de algodón y cápsulas).

El ARN total de óvulos 4DPA (4 días antes de la floración) con fibras fue usado para aislar el cADN del GhDWF1 (ver página 1299 columna de la izquierda, primer párrafo). **D1**, figura 5, página 1303 menciona las otras etapas de

² Íbidem, Pág. 62.

desarrollo de la fibra (fibras 0DPA; 12 DPA; y 23DPA) desde las cuales el ARN ha sido aislado.

Adjunto al presente escrito una publicación de Chen y Guan (ver Anexo ii) en donde la figura 1 representa muy bien las diferentes fases del desarrollo de las fibras de algodón. Como se puede ver la maduración solo inicia en 50 DPA. La última etapa de desarrollo desde la cual se ha aislado ácido nucleico descrito por **D1** es 23DPA, es decir, elongación tardía, biosíntesis de celulosa temprana y claramente aun una célula viva de fibras de algodón.

Por otra parte, todas las reivindicaciones independientes han sido modificadas para decir que el aislamiento ocurre por medio de la incubación de dichas fibras de planta en un regulador de lisis que contiene detergentes, proteasas y sales por un tiempo prolongado, de manera preferente por un periodo de 4 a 100 horas, el cual además diferencia el procedimiento reivindicado de aquel revelado en **D1**.

En consecuencia, la invención de mi representada goza de novedad a la luz de **D1**, toda vez que realizada la comparación elemento por elemento de las características técnicas esenciales y las modificaciones realizadas en virtud de la presente objeción, se puede concluir que la invención en estudio no es idéntica a lo revelado en el estado de la técnica.

PRUEBAS DE PATENTABILIDAD

En virtud de lo establecido en el artículo 1.2.2.5.2 de la Circular Única, modificado por la Resolución No. **54093** del 18 de septiembre de 2012; y en concordancia con la posición del Consejo de Estado consagrada en la sentencia del 13 de agosto de 2009³, solicito de manera atenta a la Dirección que tenga en cuenta los precedentes jurídicos respecto de la patentabilidad de las reivindicaciones de mí representada.

Teniendo en cuenta lo anterior, presento copia simple de las patentes concedidas EP 2 318 541 B y MX.302009, las cuales reconocen la contribución a la técnica hecha por la invención de mi representada. Nótese que aunque

³ Consejo de Estado, Sala de lo Contencioso Administrativo, Sección Primera. Expediente 2003-00256. Sentencia de 13 de agosto de 2009.

existen diferencias en el pliego reivindicatorio, debido a los requerimientos específicos en cada jurisdicción, todas las reivindicaciones independientes se han mantenido.

Adicional a lo anterior, comedidamente llamo la atención del Despacho a que el documento **D1** fue citado en el Reporte de Búsqueda Internacional (International Search Report – PTC/ISA/210), el cual tiene por objetivo descubrir el estado de la técnica y categorizar los documentos con el fin de determinar si la invención reivindicada es o no es nueva y si implica o no nivel inventivo a la luz de los documentos citados. Un objetivo adicional de la búsqueda internacional es evitar, o al menos minimizar, una búsqueda adicional en la fase nacional⁴.

En dicho Reporte, el documento **D1** fue citado como documento de categoría "A", es decir, documento que define el estado de la técnica general, el cual no es considerado de particular relevancia (ver Anexo v).

La Opinión Escrita expedida por la Autoridad Internacional de Búsqueda (Written Opinion of the International Searching Authority – PCT/ISA/237) reconoce la novedad de la invención de mí representada a la luz de **D1** (correspondiente a D2) y en referencia a dicho documento cita las diferencias existentes entre la invención objeto de protección y el estado de la técnica, conforme los argumentos presentados en el presente escrito (ver anexo vi).

ANEXOS

- i. Nuevo pliego reivindicatorio compuesto por veintiún (21) reivindicaciones.
- ii. Z Jeffrey Chen & Xueying Guan. "**Auxina impulso de algodón**". Nature Biotechnology 29, 407–409 (2011) doi:10.1038/nbt.1858. Published online. 06 May 2011, junto con su respectiva traducción al español.

⁴ World Intellectual Property Organization "**PCT INTERNATIONAL SEARCH AND PRELIMINARY EXAMINATION GUIDELINES**", Ginebra, Octubre 3, 2011.

- iii. Copia simple del texto de patente EP 2 318 541 B en su idioma original.
- iv. Copia simple del texto de patente MX.302009 en su idioma original.
- v. Reporte de Búsqueda Internacional (International Search Report – PTC/ISA/210).
- vi. Opinión Escrita expedida por la Autoridad Internacional de Búsqueda (Written Opinion of the International Searching Authority – PCT/ISA/237).

De acuerdo con todo lo expuesto, solicito en forma muy comedida y respetuosa al Sr. Jefe de la División de Nuevas Creaciones se sirva tener en cuenta las manifestaciones y documentos aportados junto con el presente memorial y, de conformidad con ello, se sirva continuar con el trámite de la solicitud de patente de la referencia.

Del señor Jefe de la Dirección de Nuevas Creaciones,



MAURICIO PATIÑO BONNET

C.C. 11.342.699 de Zipaquirá

T.P. 73583 del C.S. de la J.

Reivindicaciones

1. un método para la identificación de una fibra de una planta madura una fibra de una planta procesada que comprende los pasos de:
 - a. aislar desde dicha fibra de planta una macro molécula biológica que ocurre de manera natural en dicha fibra de planta de aquellos polisacáridos o lignina seleccionados del grupo de ácido nucleico, ADN, ARN, ARN de cadena única y ARN de doble cadena por medio de la incubación de dicha fibra de planta en un regulador de lisis que contiene detergente, proteasas y sales por un tiempo prolongado, preferiblemente un periodo de 4 a 100 horas y el procesamiento de dicho regulador de lisis de acuerdo con los métodos estándares de aislamiento de ácido nucleico y de aislamiento de dicho ADN; y
 - b. someter dicha macro molécula biológica a un ensayo de detección específico para dicha fibra de planta.
2. El método de la reivindicación 1, en donde dicha fibra de planta es una fibra de semilla.
3. El método de la reivindicación 1, en donde dicha fibra de planta es una fibra de algodón.
4. El método de cualquiera de las reivindicaciones 1 a 3, en donde dicho ensayo de detección es un ensayo de detección basado en una reacción en cadena de polimerasa.
5. El método de cualquiera de las reivindicaciones 1 a 3, en donde dicho ensayo de detección es un ensayo de detección basado en la hibridación de un ácido nucleico.
6. El método de cualquiera de las reivindicaciones 1 a 5, en donde dicho ensayo de detección detecta la presencia o ausencia de genes quiméricos presentes en el genoma de la planta que produce las fibras.
7. El método de la reivindicación 6, en donde dichos genes quiméricos comprenden una región codificada o parte de una porción en contrasentido de la misma seleccionada de N- acetilglucosamina transferasa, EPSPS, una porción de insecticida de proteína *Bacillus thuringiensis* cristal, glucanasa, o expansina.

8. El método de la reivindicación 6, en donde dicho ensayo de detección es un ensayo de detección de evento específico.
9. El método de cualquiera de las reivindicaciones 1 a 6, en donde dicho ensayo de detección detecta la presencia o ausencia de alelos específicos presentes en el genoma de la planta que produce las fibras.
10. El método de cualquiera de las reivindicaciones 1 a 9, en donde dicha fibra está presente en hilados, telas, tejidos o prendas de vestir terminadas.
11. Un método para analizar el genoma de una planta que produce la fibra que comprende los pasos de
 - a. aislar los ácidos nucleicos de fibras maduras o procesadas de dicha planta, siendo dichos ácidos nucleicos de origen natural en dicha fibra de planta por medio de la incubación de dichas fibras de planta en un regulador de lisis que contiene detergentes, proteasas y sales un tiempo prolongado, preferiblemente por un período de 4 a 100 horas y el procesamiento de dicho regulador de lisis de acuerdo con los métodos estándares de aislamiento de ácido nucleico y de aislamiento de dicho ácido nucleico; y
 - b. someter dicho ácido nucleico a un protocolo de análisis de genoma.
12. Un método de acuerdo con la reivindicación 11, en donde dicho ácido nucleico es seleccionado del grupo de ADN, ARN, ARN de cadena única y ARN de doble cadena.
13. El método de acuerdo con la reivindicación 11, en donde dicho ácido nucleico es ADN.
14. El método de acuerdo con la reivindicación 13, en donde dicho ADN aislado está sujeto a todo el paso de amplificación del genoma antes de someter dicho ácido nucleico al protocolo de análisis del genoma.
15. El método de acuerdo con cualquiera de las reivindicaciones 11 a 14, en donde dicho método es aplicado a fibras maduras viejas y, hilados, telas, tejidos o prendas de vestir terminadas viejos.

16. Un método para aislar ácido nucleico de fibras de plantas maduras o fibras de plantas procesadas que comprende los pasos de
 - a. remover hojas y material de basura del tallo de las fibras de plantas maduras cosechadas; y
 - b. incubar dichas fibras de plantas en un regulador de lisis que contiene detergentes, proteasas y sales un tiempo prolongado, preferiblemente por un período de 4 a 100 horas; y
 - c. procesar dicho regulador de lisis de acuerdo con los métodos de aislamiento estándares de ácido nucleico y aislar dicho ácido nucleico.
17. Un método de acuerdo a la reivindicación 16, en donde dicho ácido nucleico es ADN.
18. Un método para aislar un ácido nucleico de tela tejida o tela que comprende fibras de planta, dicho método comprende los pasos de
 - a. destejer los hilos de dicha tela;
 - b. incubar dichos hilos en un regulador de lisis que contiene detergentes, proteasas y sales un tiempo prolongado, preferiblemente por un período de 4 a 100 horas; y
 - c. procesar dicho regulador de lisis de acuerdo con los métodos de aislamiento estándares de ácido nucleico y aislar dicho ácido nucleico.
19. Un método de acuerdo a la reivindicación 18, en donde dicho ácido nucleico es ADN.
20. Un método para determinar las cantidades relativas de diferentes fibras de plantas en una mezcla de fibras de plantas maduras o procesadas que comprende los pasos de
 - a. aislar de dicha mezcla de fibras de plantas una macro molécula biológica que ocurre de manera natural en dichas fibras de planta distintas de aquellos polisacáridos o lignina seleccionados del grupo de ácido nucleico, ADN, ARN, ARN de cadena única y ARN de doble cadena por medio de la incubación de dicha fibra de planta en un regulador de lisis que contiene detergente, proteasas y sales por un tiempo prolongado, preferiblemente un periodo de 4 a 10 horas y el procesamiento de dicho regulador de lisis de acuerdo con los métodos estándares de aislamiento de ácido nucleico y de aislamiento de dicho ADN

- b. someter dicha macro molécula biológica a ensayos de detección específicos para cada una de dichas fibras de planta; y
- c. determinar la cantidad relativa de cada una de las fibras

21. Un método para certificar la identidad de las fibras de algodón comercializadas, dicho método comprende:

- a. registrar las compras de semilla de algodón certificada por medio del registro de los productores, dichas semillas de algodón comprenden una composición específica de genoma y la producción de una marca particular de fibras de algodón;
- b. registrar pacas de materia prima de fibras de algodón producidas por dichos productores registrados a partir de dicha semilla de algodón certificada como aquella marca particular de fibras de algodón;
- c. autenticar dichas pacas por medio de un chequeo cruzado dichos registros de compra de semillas;
- d. proporcionar dichas pacas registradas a las fábricas para producir hilados, telas y prendas de vestir terminadas desde dicha marca de fibras de algodón, preferiblemente de una manera predominante, particularmente de una manera exclusiva desde dicha marca de fibras de algodón;
- e. auditar la identidad de dicha marca de fibras de algodón en uno o más pasos usando un método de acuerdo con cualquiera de las reivindicaciones 1 a 15.

suppresses DAMP-mediated signaling from TLRs remains uncertain.

The importance of this inhibitory circuit in intestinal sepsis suggested the possibility that bacterial sialidases may exacerbate inflammation in wild-type mice by cleaving sialic acids on CD24 that are required for recognition by SiglecG/10 (Fig. 1). Indeed, bacterial sialidases in the blood of mice after intestinal puncture reduced sialic acid levels on the surfaces of dendritic cells. *In vitro*, sialidase treatment of wild-type dendritic cells amplified cytokine production in response to HMGB1 but had no effect on the elevated levels of cytokines produced by dendritic cells from CD24 or SiglecG null mice. Moreover, sepsis induced by administration of the sialidase-producing *Streptococcus pneumoniae* caused a more profound pathogenicity than a mutant lacking its two sialidase genes.

These findings motivated Chen *et al.*² to assess the potential of sialidase inhibitors as a way to preserve the CD24/SiglecG/10 inhibitory circuit and moderate the effects of sialidase. Two sialidase inhibitors, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid and 2,3-dehydro-2-deoxy-*N*-glycolylneuraminic acid, protected wild-type mice, but not *Cd24*^{-/-} or *siglecg*^{-/-} mice, from experimental sepsis by cecal ligation and puncture. These results suggest that sialidase inhibitors might reduce the virulence and morbidity of sepsis involving bacterial pathogens that produce sialidases, such as *Streptococcus pneumoniae*, or polybacterial infections such as those produced from punctured intestine.

Siglec is not the first glycan receptor linked to sepsis caused by *S. pneumoniae*. Platelets are especially susceptible to loss of sialic acid by sialidase, resulting in clearance of the desialylated platelets by the asialo-glycoprotein receptor (ASGR) of liver hepatocytes¹⁰. ASGR-null mice exhibit more profound mortality as a result of increased levels of desialylated platelets and increased coagulopathy. In contrast to the results of Chen *et al.*², Grewal *et al.*¹⁰ found that a sialidase-deficient strain of *S. pneumoniae* produced increased mortality in wild-type mice. The apparently contradictory results may reflect the different levels of sialidase that affect the activities of two different receptor systems—one that clears desialylated platelets and reduces coagulation, and the other that is required for dampening the inflammatory response but is destroyed by sialidase.

Sialidase inhibitors are not new to the pharmaceutical industry. The influenza virus medicines Tamiflu and Relenza inhibit the influenza virus sialidase, which is required for spreading of the virus from infected cells. The results of

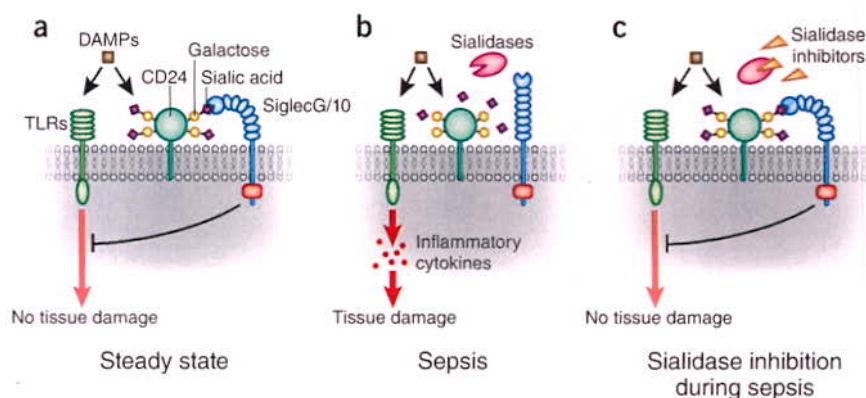


Figure 1 Sialidase (neuraminidase) from bacterial pathogens disrupts the Siglec circuit that suppresses TLR signaling by DAMPs. (a) Normally, DAMPs attenuate pro-inflammatory TLR signaling by forming a ternary complex with sialylated CD24 and SiglecG/10. (b) During sepsis, bacterial sialidases cleave sialic acids on CD24, disrupting the CD24/SiglecG/10 inhibitory circuit and leading to enhanced cytokine production. (c) Sialidase inhibitors block the desialylation of CD24 that occurs during sepsis, preserving the Siglec inhibitory circuit and suppressing the inflammatory response.

Chen *et al.*² may stimulate further investigations to broaden the utility of sialidase inhibitors to the treatment of polybacterial sepsis.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Auxin boost for cotton

Z Jeffrey Chen & Xueying Guan

Increasing auxin levels at the right time and place during ovule and fiber development improves the yield and quality of cotton fibers.

Cotton fiber is the largest renewable source of textile materials, with a global annual consumption of ~27 million metric tons worth ~\$500 billion. Biotech has succeeded in raising cotton yields through the introduction of herbicide and insecticide transgenes, but improving the quality of cotton itself has not been possible without a penalty in fiber yield or seed size or number. In this issue, Zhang *et al.*¹ simultaneously improve the yield and quality of cotton fiber. The key to their success

in achieving this elusive goal lies in regulated overexpression of a gene responsible for the synthesis of the auxin indole-3-acetic acid (IAA). The study, validated by field trials spanning four years, underscores the importance of precise temporal and spatial regulation in harnessing plant growth regulators for crop improvement.

The development of cotton fibers involves four partially overlapping stages^{2–4} (Fig. 1). Whereas lint fibers are long and can be spun into yarn, shorter fuzz fibers have no value for the textile industry. For upland cotton (*Gossypium hirsutum* L.), the epidermal cells that give rise to lint fibers appear before or on the day of anthesis (0 days post anthesis, DPA), and cells that initiate after approximately +4 or +5 DPA become fuzz fibers. Each cotton fiber is a single-celled seed trichome or hair,

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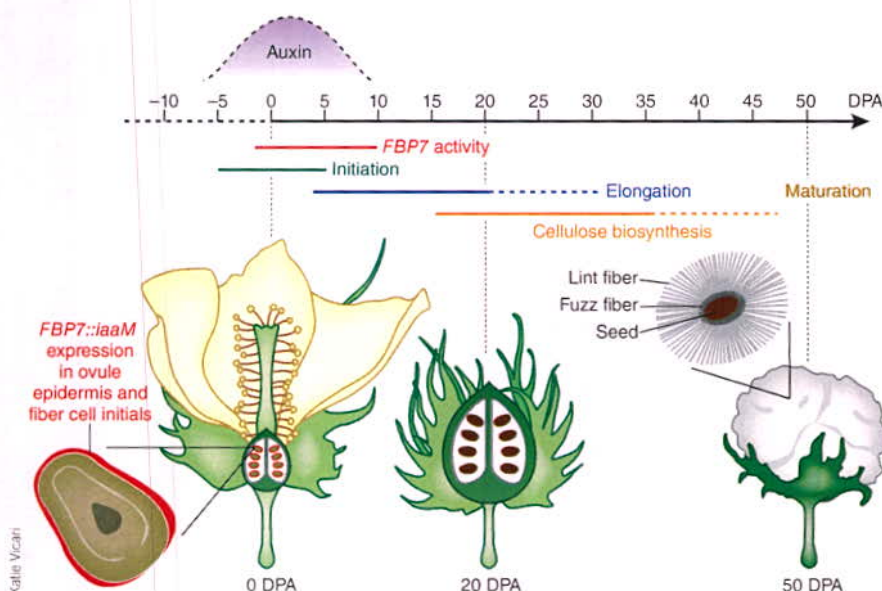


Figure 1 The role of auxin in cotton fiber development. Auxin accumulates in the ovule epidermis and fiber cells from -5 to +10 days post-anthesis (DPA) with a peak at +2 DPA. The effects of other plant growth regulators (e.g., brassinosteroids, cytokinins, ethylene and gibberellins) and transcription factors (e.g., members of the MYB family) on fiber cell development *in planta* are largely unclear. The time scale (black) indicates the duration of fiber cell initiation (green), elongation (blue), cellulose biosynthesis (orange) and maturation. Broken lines indicate overlapping stages or the time before 0 DPA. Zhang *et al.*¹ show that restricted expression of an auxin biosynthetic gene in the epidermis and fiber cell initials of cotton ovules between -2 and +10 DPA (red) increases the number and length of useful lint fibers at the expense of the number of fuzz fibers, which cannot be used in textile manufacture. The increase in lint percentage does not compromise the fiber quality.

and >20,000 fibers may develop semisynchronously on each seed. This tight spatiotemporal regulation is mediated by phytohormones. Fertilized ovules produce fibers *in vitro* but require both auxin and gibberellin for maximal fiber growth. In contrast, unfertilized ovules cannot produce fibers unless auxin and gibberellin are present in culture media⁵. Pre-anthesis treatment with IAA alone, or post-anthesis treatment with gibberellin alone can lead to efficient *in vitro* fiber production. Thus, the timing and duration of hormone supply are critical for fiber development.

In developing cotton ovules, auxin accumulates before anthesis, peaks at +2 DPA and gradually decreases to basal levels by +10 DPA¹ (Fig. 1). The increase appears to result at least in part from transport of endogenous auxin, as the IAA transport inhibitor 1-naphthylphthalamic acid dramatically reduces IAA levels in the cultured ovules¹. To test whether accumulation of auxin within the days immediately before and after anthesis might be causally linked to lint-fiber development, Zhang *et al.*¹ placed the *iaaM* gene—encoding an enzyme that converts tryptophan to indole-3-acetamide, which is then hydrolyzed to IAA—under the control of each of 16 promoter fragments reported to drive expression in ovules and/or fibers¹. Of the promoters tested, *FBP7*, an epidermal-specific promoter derived from petunia and active in

cotton ovule epidermis from -2 to +10 DPA¹, gave the most promising effects.

A steady supply of IAA within this time window both increased the number of fiber cell initials (14–19%) and reduced the number of fuzz fibers. As a result, the transgenic plants produced 20–40% more lint fibers per seed. Micronaire indices, a measure of fiber coarseness, were 6.1–6.3 in controls and 4.3–4.8 in transgenic plants, indicating that the lint fibers were thinner and thus of higher quality in the latter. Use of the *FBP7* promoter to drive expression of *iaaL*, which encodes an enzyme that converts IAA to a biologically inactive auxin, significantly reduced the number of fiber initials in 0 DPA ovules. This verifies the role of IAA in fiber cell initiation.

Data from field trials spanning four growth seasons revealed increases of at least 19% and 14% in lint percentage and lint yield, and a 13–15% decrease in micronaire values. It is, however, worth noting that the transgenic cotton used in these trials was not in the same genetic background as the best commercial varieties, and that the increased fiber yield and fineness came at the cost of smaller seed size, which is probably a result of the redistribution of carbohydrate reserves from the seed to the fibers.

Placing the *iaaM* gene under the control of most of the other promoters tested caused

growth abnormalities¹. The exceptions were *E6*, a promoter that is expressed relatively late in fibers (from +5 to +24 DPA^{1,6}), and the *BAN* promoter from *Arabidopsis thaliana*, which is expressed between -1 and +10 DPA in cotton¹. Although no abnormalities were found in the *E6::iaaM* transgenic plants, there was no effect on IAA accumulation in fiber cell initials or fiber yield, consistent with the previously published data⁶. Preliminary analysis of *BAN::iaaM* plants revealed similar improvements in the lint percentage and micronaire scores as those seen in *FBP7::iaaM* plants¹.

This is not the first demonstration of the agricultural potential of regulated auxin action. For example, elevated *iaaM* expression and auxin content, driven by the promoter of *DefH9*, an *Antirrhinum majus* MADS box-containing gene expressed specifically in ovules, promote parthenocarpic fruit development in eggplant⁷ and increase fruit yield ~180% in cultivated strawberry and ~100% in raspberry⁸. However, as in the study by Zhang *et al.*¹, the effect of auxin was not straightforward in these studies because phytohormones normally interact with one another to regulate plant growth and development^{4,9}.

Much remains to be learned about how auxin, brassinosteroids, ethylene and gibberellins affect the developmental transitions from protodermal cells to fiber cells and from short-fuzz to long-lint fiber cells. Because cotton fibers are seed trichomes, and seed hair development shares many similarities with leaf trichome development in *A. thaliana*^{3,4}, a better understanding of trichome development in this tractable model system may be instructive. Overexpression of cotton genes encoding transcription factors such as MYB2 and MYB25, which are homologs of the genes regulating leaf trichome development, promotes production of occasional seed hairs in *A. thaliana*¹⁰ and trichome development in cotton. However, this has little or no effect on cotton fiber yield¹¹. Many such experiments, usually involving the constitutive cauliflower mosaic virus 35S promoter, lead to negative effects on plant growth and fiber yield. The findings of Zhang *et al.*¹ suggest that it may be necessary to regulate the expression of these genes more tightly if specific agronomic traits, such as fiber yield and quality are to be improved. Fiberless and lintless cotton mutants^{1,12}, such as Xuzhou 142 (*fl*) and the naked seed mutant (*NIN1*), which display delayed fiber cell initiation, may also be helpful in this regard. It remains to be determined whether defects of fiber cell initiation in these mutants are controlled by phytohormones or other factors.

Many have advocated that agricultural biotech graduate from the first generation of transgenic crops, exemplified by the expression of heterologous genes conferring pesticide and herbicide resistance, to a class of crops engineered to meet the growing demand for food, feed, fiber, fuels and biomaterials. The study by Zhang *et al.*¹ illustrates the promise of the next generation of transgenic crops.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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A self-assembling retina

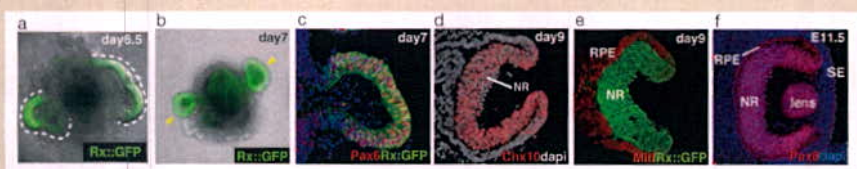


Figure 1 Stages in the generation of a mouse retina *in vitro*. NR, neural retina; SE, surface ectoderm.

Unlike the eye of mollusks, the vertebrate eye is oriented upside down¹. Light-sensitive cells—the rods and cones—are located in the outermost layer of the neurosensory retina, furthest from the eye chamber and the incoming light. The inner retinal layers, closest to the light, contain the interneurons and ganglia that transmit optical signals to the brain, on the far side of the photoreceptor layer. In a new study² that marks an exciting advance in the use of embryonic stem cells (ESCs) to mimic development, this distinctive retinal structure has now been shown to emerge spontaneously *in vitro* starting with little more than mouse ESCs, the growth factor activin/nodal and a bit of extracellular matrix.

ESCs have already been differentiated into cells that resemble many of the cell types in the retina, including photoreceptors, neural cells and retinal pigment epithelium (RPE). But how can cells of different types be assembled *in vitro* into a complex

organ such as the eye? For the self-assembling retina, all that was needed were slight modifications of the differentiation conditions. The critical new component was Matrigel or purified extracellular matrix proteins, which were added because they are known to promote the growth of epithelial structure.

As Eiraku *et al.*² show in time-lapse images taken over 9 days of culture, an *in vitro*-generated retina begins as a floating aggregate of ESCs. Soon, in the presence of extracellular matrix, the cells begin to express Rx, a marker of the retinal anlage. By day 6, the aggregate becomes a hollow sphere of polarized epithelial cells, and the Rx⁺ cells self-associate into distinct Rx⁺ islands (Fig. 1a). By day 7, the Rx⁺ islands form vesicles that project out from the hollow sphere (Fig. 1b) and that express the retinal marker Pax6 (Fig. 1c).

Over the next three days, the vesicle invaginates, creating the double-walled C shape of the developing retina (Fig. 1d). The outer wall expresses markers of RPE (Fig. 1e)

and becomes pigmented, similar to the embryonic outer retina, and the inner wall expresses additional markers of the inner, neurosensory retina (Fig. 1d). Overall, the structure bears a striking resemblance to the mouse embryonic optic cup (Fig. 1f).

In the last stage, over days 20–24, the inner wall stratifies, revealing the characteristic upside-down architecture of the neurosensory retina. Markers of many of the expected cell types appear in their appropriate anatomical locations—photoreceptors (largely rods) on the outside, adjacent to the RPE, and ganglia, bipolar cells, horizontal cells, amacrine cells and Muller glia on the inside.

The generation of self-assembling retinas raises intriguing questions for future research. Are the retinas functional? What drives the self-assembly process at the molecular level? Can the method be adapted to human cells or to other organs? The work is also likely to have therapeutic implications if *in vitro*-generated retinas can be used for disease modeling and drug screening or as a source of cells for transplantation.

Kathy Aschheim

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Auxina impulso de algodón

Z Jeffrey Chen & Xueying Guan

El incremento de los niveles de Auxina en el momento y lugar correctos durante la ovulación y el desarrollo de las fibras mejora el rendimiento y calidad de las fibras de algodón.

La fibra de algodón es la más importante fuente renovable de textiles materiales, un consumo anual global de 27 millones de toneladas métricas con valor de \$500 mil millones. Biotech ha sido exitoso en aumentar el rendimiento del algodón a través de la introducción de herbicidas e insecticidas transgénicos, pero la mejora de la calidad del algodón en sí misma no ha sido posible sin una penalidad en el rendimiento de las fibras con el tamaño o número de las semillas. En esta edición, Zhang y otros¹ de manera simultánea mejoraron el rendimiento y la calidad de las fibras de algodón. La clave para su éxito en alcanzar esta meta difícil de alcanzar radica en la sobre expresión regulada de un gen responsable de la síntesis del ácido auxina indol-3 acético (1AA). El estudio, validado por ensayos de campo que abarca cuatro años, subraya la importancia de la precisa regulación temporal y espacial del aprovechamiento de los reguladores de crecimiento de la planta para el mejoramiento de los cultivos.

El desarrollo de fibras de algodón incluye cuatro etapas²⁻⁴ que se sobreponen (Fig 1). En donde fibras sin pelusa son largas pueden girar en el hilado, fibras con pelusa más cortas no tienen valor para la industria textil. Para el algodón americano (*Gossypium hirsutum* L.), las células de la epidermis que dan lugar a fibras con pelusa es aparecen antes o en el día de la floración (0 días después de la floración, DPA) y las células que inician después de aproximadamente +4 o +5 DPA vienen a ser fibras con pelusa. Cada fibra de algodón es una semilla unicelular tricoma o cabello, y >20.000 fibras se pueden desarrollar de manera casi sincrónica en cada semilla. Esta apretada regulación espacio temporal es mediada por fito hormonas. Los óvulos fertilizados producen fibras indito pero requieren tanto auxina como gibberelina para el máximo crecimiento de las fibras. En contraste, óvulos no fertilizados no pueden producir tiras a menos que auxina y gibberelina estén presentes en el medio de cultivo². El tratamiento pre antesis únicamente con 1AA, o el tratamiento post antesis únicamente con gibberelina pueden llevar a una producción indito eficiente. Por lo tanto tiempo y la duración de la administración de la hormona son críticos para el desarrollo de la fibra.

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En el desarrollo de óvulos de algodón, auxina acumula antes de la antesis, picos en el momento +2 DPA y gradualmente disminuye a niveles basales para el momento +10 DPA¹ (Fig 1). El incremento aparece para resultar al menos en parte desde el transporte de auxina endógena, como el transporte 1AA inhibidor ácido 1-naftilftalámico reduce de manera dramática los niveles de 1AA en los óvulos cultivados¹. Las pruebas en donde la acumulación de auxina dentro de los días inmediatamente anteriores y después de la antesis pueden estar vinculados de manera casual al desarrollo de fibras con pelusa, Zhang y otros¹ situaron el gen 1AAM - codificando una enzima que convierte triptofan en indol-3.acetamida, la cual es entonces hibridizada en 1AA - bajo el control de cada uno de los 16 fragmentos impulsados reportados para llevar la expresión en óvulos y/o fibras¹. De los promotores probados, FBP7, un promotor específico epidérmico derivado de la petunia y activo en la epidermis de los óvulos de algodón desde el momento -2 hasta el momento +10 DPA¹, dieron los efectos más prometedores.

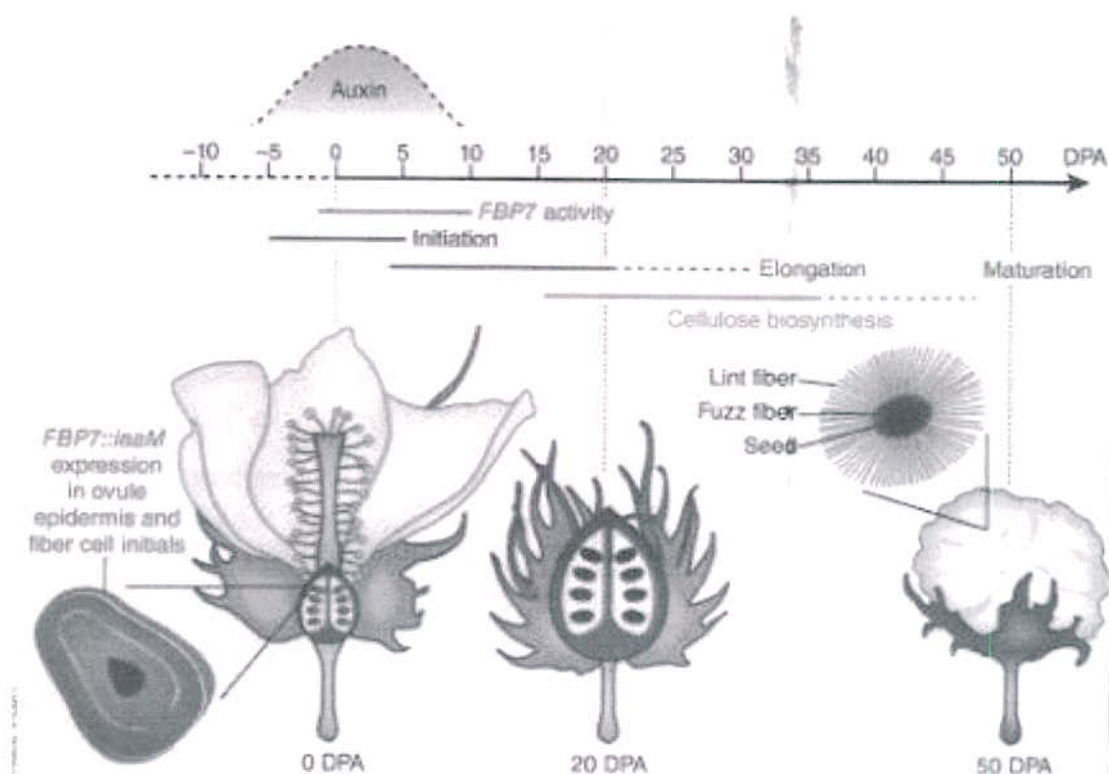


Figura 1 el rol de la auxina en el desarrollo de las fibras de algodón. La auxina acumula en la epidermis del óvulo y en las células de fibra desde los días -5 hasta +10 post antesis (DPA) con un pico en +2 DPA. Los efectos de otros reguladores de crecimiento de las plantas (por ejemplo brasinoesteriodes, citoquininas, etileno y giberelinas) y factores de transcripción (por ejemplo miembros de la familia MYB) en el desarrollo de las células de fibra en la planta son en gran medida poco claras. La escala de tiempo (negro) indica la duración de la iniciación de las células de fibra (verde), elongación (azul), biosíntesis de la celulosa (naranjas) y

maduración. Las líneas punteadas indican las etapas que se sobreponen o el tiempo antes de 0 DPA. Zhang y otros¹, muestran que la expresión restringida de un gen auxina yo sintético en la epidermis de las células de fibra iniciales de óvulos de algodón entre -2 y +10 DPA (rojo) incrementa el número y la longitud de fibras sin pelusa útiles a costa del número de fibras con pelusa, las cuales no pueden ser usados en la manufactura de textiles. El incremento del porcentaje de fibras sin pelusa no compromete la calidad de la fibra.

Un suministro constante de 1AA dentro de esta ventana de tiempo incrementa el número de células de fibra inicial (14-19%) a la vez que reduce el número de fibras con pelusa. Como resultado, las plantas transgénicos producen 20-40% más fibras con pelusa por semilla. El índice Micronaire, una medida para la aspereza de las fibras, fue 6.1 - 6.3 en los controles y 4.3 - 4.8 en las plantas transgénicas, indicando que las fibras sin pelusa fueron más delicadas por lo tanto más alta calidad en este último. El uso del promotor FBP7 para conducir la expresión de *iaaL*, la cual codifica una enzima que convierte 1AA en una auxina biológicamente inactiva, reduce de manera significativa el número de fibras iniciales en los óvulos 0 DPA. Esto modifica el rol de 1AA en la iniciación de las células de fibras.

Los datos de los ensayos de campo llevados a cabo a través del cuatro temporadas de crecimiento revelaron el incremento de al -1 19% del 14% en el porcentaje de la fibra y de rendimiento de la fibra, y una disminución de 13 – 15% en los valores de Micronaire. Esto es, sin embargo, sin ningún un valor que el algodón transgénico usado ensayos no tuvo los mismos antecedentes genéticos como las mejores variedades comerciales, y el incremento del rendimiento de la fibra y la fineza vienen de un costo de un menor tamaño de las semillas, lo cual probablemente es un resultado de la distribución de las reservas del carbohidrato desde la semilla a las fibras.

Localizando el gen *iaaM* bajo el control de la mayoría de los otros promotores probados se causan anomalías de crecimiento¹. Las excepciones fueron E6, promotor que es expresado de manera relativamente tardía en las fibras (desde +5 hasta +24 DPA^{1, 6}), y el promotor BAN desde *Arabidopsis thaliana*, el cual es expresado entre -1 y +10 DPA el algodón¹. Si bien no fueron encontradas anomalías en las plantas transgénicas E6::*iaaM*, no hubo efecto en la acumulación de 1AA en las células iniciales de fibra o en el rendimiento de la fibra, consistentes con los datos publicados con anterioridad⁶. Los análisis preliminares de plantas BAN::*iaaM* revelaron mejoras similares en el porcentaje de luces y las puntuaciones Micronaire que aquellas vistas en las plantas FBP7::*iaaM*¹.

Esta no es la primera demostración del potencial agrícola de una acción de auxina regulada. Por ejemplo, la expresión elevada de *iaaM* y de contenido de auxina, conducida por el promotor *DefH9*, un gen *Antirrhinum majus* *MADS* contenido en una caja expresada de manera significativa los óvulos, promueve el desarrollo de frutos partenocárpicos en la berenjena⁷ e incrementa el rendimiento de los frutos ~180% en fresas cultivadas y ~100% en frambuesas⁸. Sin embargo, como el estudio de Zhang y otros¹, el efecto de la auxina no fue directo en esos estudios porque las fito hormonas normalmente interactúan una con la otra para regular el crecimiento de las plantas y el desarrollo^{4,9}.

Aún queda mucho por aprender acerca de cómo la auxina, los brasinoesteroides, el etileno y las gibberelinas afectan la transición del desarrollo desde las células proto dérmicas hasta las células de fibra y desde las células de fibra de pelusa corta hasta las células de fibra de pelusa larga. Porque las fibras de algodón son semillas tricomas, y semillas de cabello que desarrollan formas con muchas similitudes con el desarrollo de hojas tricomas en *A. taliana*^{3, 4}, un mejor entendimiento del desarrollo de tricomas en este sistema de modelo manejable puede ser instructivo. La sobre expresión de los factores de transcripción codificados de los genes de algodón tal como *MYB2* y *MYB25*, los cuales son homólogos de los genes que regulan el desarrollo de hojas tricomas, promueve la producción de cabellos de semilla ocasionales en *A. taliana*¹⁰ y el desarrollo de tricomas en el algodón. Sin embargo, esto tiene un efecto muy pequeño o ninguno en el rendimiento de las fibras de algodón¹¹. Muchos de estos experimentos, usualmente incluyen el promotor del virus 35S constitutivo del mosaico de la coliflor, produciendo efectos negativos en el crecimiento de las plantas y en el rendimiento de las fibras. Los hallazgos de Zhang y otros¹, sugieren que esto puede ser necesario para regular la expresión de estos genes más estrechamente si rasgos agronómicos específicos, tales como el rendimiento de la fibra y la calidad van a ser mejorados. Algodones mutantes libres de fibra y libres de pelusa^{1, 12}, tal como el Xuzhou 142 (fl) y la semilla desnuda mutante (N1N1), la cual muestra una emisión demorada de las células de fibra, también pueden ser útiles en este sentido. Aún queda por ser determinado si los defectos de la iniciación de las células de fibra en estas mutantes están controlados por las fito hormonas u otros factores.

Muchos han defendido que la agricultura biotecnológica graduada desde la primera generación de cultivos transgénicos, ejemplificada por la expresión de genes heterólogos confiere resistencia a la pesticida y a los herbicidas, a una clase de cultivos diseñados para alcanzar las demandas de crecimiento para

comida, alimentos, fibras, combustibles y bio materiales. Los estudios de Zhang y otros¹, ilustran las promesas de esta nueva generación de cultivos transgénicos.

Intereses financieros correspondientes.

Los autores declaran no tener intereses financieros correspondientes.

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12. Lee, J.J y otros Planta 223, 418 – 432 (2006)



(11) **EP 2 318 541 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

- | | |
|--|--|
| (45) Date of publication and mention of the grant of the patent:
19.09.2012 Bulletin 2012/38 | (51) Int Cl.:
C12Q 1/68 (2006.01) |
| (21) Application number: 09777873.2 | (86) International application number:
PCT/EP2009/005891 |
| (22) Date of filing: 06.08.2009 | (87) International publication number:
WO 2010/015423 (11.02.2010 Gazette 2010/06) |

(54) **METHODS FOR PLANT FIBER CHARACTERIZATION AND IDENTIFICATION**
VERFAHREN ZUR CHARAKTERISIERUNG UND IDENTIFIZIERUNG VON PFLANZENFASERN
PROCÉDÉS DE CARACTÉRISATION ET IDENTIFICATION DE FIBRES VÉGÉTALES

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| <p>(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK SM TR
Designated Extension States:
AL BA RS</p> <p>(30) Priority: 08.08.2008 EP 08014211
11.08.2008 US 188613 P</p> <p>(43) Date of publication of application:
11.05.2011 Bulletin 2011/19</p> <p>(73) Proprietor: Bayer CropScience NV
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• ENGELN, Steven
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Description

Field of the invention

5 [0001] The current invention relates to methods for isolating biological macromolecules including peptides, proteins, and nucleic acids from mature plant fibers such as cotton fibers. The plant fibers may have been processed into yarns, which in turn may be woven or knitted to fabric or finished apparel, prior to the isolation of the biological macromolecules. Since the isolated macromolecules contain information encoded in the sequence of their building blocks, these macromolecules isolated from plant fibers according to the currently described methods may be conveniently used to characterize the plant fiber or identify the origin or source of the plant fiber. The resulting information may be used e.g. in identity preservation programs for special plant fibers, certification of origin of plant fibers or even in reconstruction of breeding pedigrees using historical sources of plant fibers.

Background of the invention

15 [0002] Plant fibers form a source for economically important products such as paper, cordage (cords and ropes) and textiles. A fiber is botanically defined as a long narrow tapering cell, dead and hollow at maturity with a rigid thick cell wall composed mostly of cellulose and usually lignin. Soft or bast fibers are found in the phloem (inner bark) of dicotyledonous stems (flax, jute, hemp, ramie). Hard or leaf fibers are found in monocot leaf vascular bundles (sisal, manilla hemp, pineapple). Surface fibers grown from the surface of seeds (cotton), leaves or fruits (coconut coir).

20 [0003] Cotton provides much of the high quality fiber for the textile industry and major efforts have been invested in obtaining cotton fibers with characteristics which suit the requirements of the industry through breeding by either classical methods or by genetically altering the genome of cotton plants.

[0004] Cotton fiber originates as a seed trichome, more specifically a single cell that initiates from the epidermis of the outer integument of the ovules, at or just prior to anthesis. The morphological development of cotton fibers has been well documented (Basra and Malik, 1984, Int Rev of Cytology 89: 65-113; Graves and Stewart, 1988, *supra*; Ramsey and Berlin, 1976, American Journal of Botany 63 (6): 868-876; Ruan and Chourey, 1998, Plant Physiology 118: 399-406; Ruan et al. 2000, Aust. J. Plant Physiol. 27:795-800; Stewart, 1975, Am. J. Bot. 62, 723-730). Cotton fibers, in particular from *Gossypium hirsutum*, undergo four overlapping developmental stages: fiber cell initiation, elongation, secondary cell wall biosynthesis, and maturation. Fiber cell initiation is a rapid process. White fuzzy fibers begin to develop immediately after anthesis and continue up to about 3 days post-anthesis (DPA), which is followed by fiber cell elongation (until about 10 to about 17 DPA). Depending upon growth conditions, secondary cell wall biosynthesis initiates and continues to about 25 to about 40 DPA, followed by a maturation process until about 45 to about 60 DPA. The secondary cell wall synthesis and maturation phase are commonly considered the "fiber strength building phase". Only about 25 to 30% of the epidermal cells differentiate into the commercially important lint fibers (Kim and Triplett, 2001). The majority of cells does not differentiate into fibers or develop into short fibers or fuzz. During fiber elongation and secondary wall metabolism, the fiber cells elongate rapidly, synthesize secondary wall components, and show dramatic cellular, molecular and physiological changes. Fiber elongation is coupled with rapid cell growth and expansion (Seagull, 1991. In Biosynthesis and biodegradation of cellulose (Haigler, C. H. & Weimer, P. J., eds) pp. 143-163, MarcelDekker, New York) and constant synthesis of a large amount of cell metabolites and cell wall components such as cellulose. About 95% of the dry-weight in mature cotton fibers is cellulose (Pfluger and Zambryski, 2001, Curr Biol 11: R436-R439; Ruan et al., 2001, Plant Cell 13: 47-63). Non-celluloid components are also important to fiber cell development (Hayashi and Delmer, 1988, Carbohydr. Res. 181: 273-277; Huwylar et al., 1979, Planta 146: 635-642; Meinert and Delmer, 1977, Plant Physiol 59: 1088-1097; Peng et al., 2002, Science 295: 147-150). Compared to other plant cells, cotton fibers do not contain lignin in secondary walls but have large vacuoles that are presumably related to rapid cell growth and expansion (Basra and Malik, 1984, *supra*; Kim and Triplett, 2001, Plant Physiology 127: 1361-1366; Mauney, 1984, *supra*; Ruan and Chourey, 1998, *supra*; Ruan et al., 2000, *supra*; Van 't Hof, 1999, American Journal of Botany 86: 776-779).

50 [0005] Developing cotton fiber cells are living cells and accordingly contain biological macromolecules such as peptides, proteins, and nucleic acids such as DNA and RNA. In contrast, mature fibers are dead, hollow cells, consisting mainly of cellulose. It is generally accepted that fiber cells, particularly cotton fiber cells, no longer contain extractable nucleic acids or proteins. Based on this inertness, cotton swabs are used as an applicator for medical substances as well as to take DNA samples from, most commonly, the inner cheek in forensic investigations.

55 [0006] In addition, plant fibers are subject to extensive processing prior to their use in industry, particularly prior to their use in the textile industry. In the case of cotton, processing from raw fiber in the field to finished apparel for sale involves numerous steps that vary greatly depending on the end product desired. The various possible steps in these processes can be divided generally into mechanical and chemical steps.

[0007] The process of harvesting and ginning cotton can subject the fiber to thermal and mechanical damage. No

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chemicals are applied during the ginning process. Heat is often utilized to dry cotton during the ginning process if it contains more than 8% moisture. Heat is usually closely regulated to avoid fiber damage. Mechanical damage can occur during the picking and ginning operations. This mechanical damage should affect a very low % of the total fibers.

[0008] During spinning, the process steps are primarily mechanical. Short fibers (immature and/or mechanically damaged fibers and foreign material are removed in the spinning process). The first chemical treatments in processing are made toward the end of the spinning process when yarn for knitting is treated with a lubricant and about half the yarn for weaving is treated with sizing material. Cotton yarn for knitting is most often treated with wax as a lubricant to facilitate movement of the yarn through the knitting equipment. Synthetic lubricants are also available but it is believed wax is still the most common treatment. Sizing, also called slashing, is applied to the yarn for weaving that will be used as the lengthwise strands in the finished fabric (the warp). The lateral (fill) yarn is not treated with sizing. The common material used for sizing is starch. Polyvinyl alcohol is also used for sizing and blends of starch and polyvinyl alcohol are common. Additives such as binders, humectants, softeners, wetting agents, defoamers and other adjuvants may be added to the sizing treatment.

[0009] Knitting and weaving are both mechanical processes involving no chemical or heat applications. At the end of the knitting and weaving processes, steps to prepare the resulting fabric for finishing involve the application of chemicals and in some cases, high temperatures. The preparation process objective of the finishing step is to remove impurities that will interfere with processing through dyeing, printing or other finishing steps. Primarily, the wax must be removed from knitted fabric (scouring) and the sizing must be removed from woven fabric (desizing). Other impurities that may be removed at this step include, seed husks, pectins and other chemicals. Chemicals used for desizing include enzymes to remove starch and soda, ash or detergents to remove polyvinyl alcohol. Scouring is done with organic solvents that will dissolve the wax or other lubricants used. After scouring or desizing, fabric is usually bleached using chlorine or peroxide bleach. Goals of bleaching are to remove any remaining non-fibrous material, hydrolysis, oxidation and removal of residual sizing and improve absorbancy of dye. Bleaching with peroxide may involve steam or water bath at near boiling water temperatures and the bleach bath may contain additives such as stabilizers and sequestering agents. Bleaching with chlorine is done at lower temperatures (40 to 50 degrees Centegrade) and cellulose degradation is less than with peroxide. If chlorine is used for bleaching, an antichlor treatment is also required. Singeing with a controlled flame is carried out on some fabrics to clean the fabric surface and remove or reduce pilling. Optical brighteners may be added to fabrics or garments to be sold as white undyed products. Anionic organic optical brighteners are typically used on cotton. Several compounds are available and some can be added during bleaching.

[0010] Mercerization is a process that can be applied to cotton yarn or fabric to improve absorption of or reaction to dye and other chemical finishing treatments, improve breaking strength, improve dimensional stability, improve fabric smoothness and luster and cover immature cotton fibers. It is a process that involves treatment with NaOH, washing, acid scouring, rinsing and drying.

[0011] The most common finishing treatment is dyeing. It can be done at the yarn, fabric or garment stage. Dyes used for cotton are numerous and of many different types. The most common dye product for cotton is indigo but it represents only 4% of dyed fabric. Dyeing can be done as a continuous process or batch process. While the chemicals used for dyeing are numerous, the process customarily involves the use of high temperature, near boiling, in the dye vat to which the fabric, yarn or garments may be submersed for extended time. Printing is the other major process for adding color or design to textiles. Printing involves mostly a mechanical process for applying coloring inks to the surface of fabric or garments. Both dyeing and printing may involve the application of chemical fixing agents at the end of the process.

[0012] Another common finishing treatment, especially after dyeing is aggressive laundering. This would include use of detergents and other cleaning agents to remove residual finishing materials from the finished fabric or garment.

[0013] Many other mechanical and chemical finishing steps may be applied to fabric, yarn or garments involving numerous options of chemicals and treatment processes. Softeners alone may be of three different types and each type may involve several different chemicals.

[0014] Although the above overview of converting raw cotton fiber into end use textile products is highly generalized, it will be clear that a person skilled in the art would not expect to be able to extract peptides, proteins or nucleic acids from yarns, textiles or finished apparel products after the numerous chemical and thermal treatment steps customarily involved.

[0015] There is a need in the industry to be able to track the source and/or origin of plant fibers, particularly of mature and/or processed plant fibers. Such source and/or origin identification of plant fibers may be important for certification processes; guaranteeing production and processing of fibers of specified quality produced from specific germplasm, such as the FiberMax® Certification Program (www.certifiedfibermax.com). The capacity to track source and/or origin in mature and/or processed fibers also allows identification of plant fibers which have special characteristics, such as the fibers with improved chemical reactivity or dyeability as described in W02006/136351.

[0016] Existing certification programs are based on recordation by growers or seed retailers of purchase of seeds of specific germplasm of a fiber crop, such as cotton, and identification of the produced fibers of the registered growers (in the case of cotton by permanent USDA bale identification numbers).

[0017] The ability to track source and/or origin of plant fibers, particularly of mature and/or processed fibers would be greatly improved if it would be possible to extract biological macromolecules from mature and or processed plant fibers which contain biological information related to the source plant of the fibers, such as peptides, proteins, nucleic acids, DNA or RNA. In this way, auditing of existing certification programs would be made possible. Additionally it would become possible to identity fibers at a later stage in the supply chain, beyond the growers or suppliers of fibers, such as e.g. at spinner, weaver or retail customer level.

[0018] Agricultural Research Service, USDA and Applied DNA Sciences worked together to develop a tagging system based on DNA embedded technologies to trace US-sourced cotton and textile components.
(<http://seedquest.com/News/releases/2004/February/7829.htm>).

[0019] It would thus be advantageous to be able to extract naturally occurring biological macromolecules from plant fibers, such as cotton fibers, particularly from mature and/or processed fibers, textiles, yarns or apparel, and further to be able to derive plant source related information from such plant fibers.

[0020] The methods described hereinafter in the different embodiments, examples, figures and claims provide a solution to the above mentioned problem, by allowing to extract natural biological macromolecules from plant fibers, such as cotton fibers, particularly from mature and/or processed fibers, textiles, yarns or apparel, and further to be able to derive plant source related information from such plant fibers.

Summary of the invention

[0021] In one embodiment, the invention provides a method for characterizing a mature plant fiber or a processed plant fiber, such as a seed fiber or cotton fiber comprising the steps of isolating from said plant fiber a biological macromolecule naturally occurring in said plant fiber other than polysaccharides or lignin; and characterizing the information encoded in the sequence of the monomers of said biological macromolecule.

[0022] In another embodiment of the invention, the invention provides a method for identifying a mature plant fiber or a processed plant fiber, such as a seed fiber or cotton fiber comprising the steps of isolating from said plant fiber a biological macromolecule naturally occurring in said plant fiber other than polysaccharides or lignin; and subjecting said biological macromolecule to a detection assay specific for said plant fiber.

[0023] In one embodiment the biological macromolecule may be a polypeptide, protein, nucleic acid, DNA, RNA, single stranded RNA or double-stranded RNA.

[0024] In another embodiment the detection assay may be a polymerase chain reaction based detection assay, an antibody based detection assay, or a nucleic acid hybridization based detection assay.

[0025] In yet another embodiment, the detection assay may detect the presence or absence of chimeric genes present in the genome of the plant producing the fibers. The chimeric gene may comprise a coding region or part or an antisense portion thereof selected from N-acetylglucosamine transferase, phosphotransacetyltransferase, EPSPS, hydroxyphenylpyruvatedioxygenase, an insecticidal portion of Bacillus thuringiensis crystal protein, poly(ADP-ribose) polymerase, poly(ADP-ribose) glucohydrolase, sucrose synthase, sucrose phosphate synthase, glucanase, cellulose synthase, chitinase, expansin, callose synthase, kinase, nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenylyl transferase, nicotinamide adenine dinucleotide synthetase or nicotine amide phosphorybosyltransferase. The detection assay may also be an event-specific detection assay.

[0026] In still another embodiment of the invention, the detection assay may detect the presence or absence of specific alleles present in the genome of the plant producing the fibers.

[0027] In yet another embodiment of the invention, the fiber from which the biological macromolecule is isolated is present in yarns, fabric, tissue or finished apparel.

[0028] The invention further provides a method for analyzing the genome of a fiber producing plant comprising the steps of isolating nucleic acids from mature or processed fibers of said plant, said nucleic acids being naturally occurring in said plant fiber; and subjecting said nucleic acid to a genome analysis protocol. The method may be applied to old mature fibers, or old yarns, fabrics or finished apparel.

[0029] Yet another embodiment of the invention is a method for isolating nucleic acid such as DNA from mature plant fibers or processed plant fibers comprising the steps of incubating said plant fibers in a buffer containing detergents, proteases and salts for a prolonged time, preferably for a period of 4 to 100 hours; and processing said lysis buffer according to standard nucleic acid isolation methods and isolating said DNA.

[0030] In another embodiment of the invention, a method is provided for isolating nucleic acid, such as DNA, from woven or knitted fabric comprising the steps of unweaving the threads of said fabric; incubating said threads in a buffer containing detergents, proteases and salts for a prolonged time, preferably for a period of 4 to 100 hours; and processing said lysis buffer according to standard nucleic acid isolation methods and isolating said nucleic acid.

[0031] In yet another embodiment of the invention, a method is provided for isolating nucleic acid, such as DNA, from harvested mature plant fibers comprising the steps of removing leaf and stem trash material from the harvested mature plant fibers; incubating said plant fibers in a buffer containing detergents, proteases and salts for a prolonged time,

preferably for a period of 4 to 100 hours; and processing said lysis buffer according to standard nucleic acid isolation methods and isolating said nucleic acid.

[0032] The invention also provides for the use of a method according to the invention in a process to certify the identity of traded plant fibers.

[0033] The invention further provides a method to determine the relative amounts of different plant fibers in a mixture of mature or processed plant fibers comprising the steps of isolating from said mixture of plant fibers a biological macromolecule naturally occurring in said plant fibers other than polysaccharides or lignin; subjecting said biological macromolecule to detection assays specific for each of said plant fibers; and determining the relative amount of each of the fibers.

[0034] In another embodiment of the invention, a method is provided to certify the identity of traded cotton fibers, said method comprising recording purchase of certified cotton seeds by registered growers, said cotton seed comprising a specific genome composition and producing a particular brand of cotton fibers; registering bales of raw cotton fibers produced by said registered growers from said certified cotton seed as said particular brand of cotton fibers; authenticating said bales by cross checking with said seed purchase records; providing said registered bales to mills to produce yarns, fabrics or apparel from said brand of cotton fibers, preferably predominantly, particularly exclusively from said brand of cotton fibers; and auditing the identity of said brand of cotton fibers at one or more steps using a protocol as herein described.

Brief description of the drawings

[0035]

Figure 1: PCR amplification of GhGluc 1 on nucleic acids templates isolated from mature cotton fiber material (*Gossypium hirsutum* FM966). Lane 1: molecular weight marker; lane 2: template DNA from purified fibers (1 µl); lane 3: template DNA from unpurified fibers containing leaf trash (1 µl); lane 4: template DNA from leaf trash only (1 µl); lane 5: template DNA from purified fibers (5 µl); lane 6: template DNA from unpurified fibers containing leaf trash (5 µl); lane 7: template DNA from leaf trash only (1 µl); lane 8: no template control; lane 9: positive control - genomic DNA isolated from FiberMax966 (FM966) plant material.

Figure 2: PCR amplification of GhGluc1 on nucleic acids templates isolated from mature *Gossypium hirsutum* FM966 cotton fiber material and from 4 year old *Gossypium barbadense* Pima Y5 cotton fiber material. Lane 1: molecular weight marker; lanes 2-3: template DNA from FM966 purified fibers containing no leaf trash (5 µl); lanes 4-5: template DNA from FM966 unpurified fibers containing leaf trash (5 µl); lane 6: template DNA from FM966 leaf trash containing no fiber material (5 µl); lanes 7-8: template DNA from Pima Y5 purified fibers containing no leaf trash (5 µl); lane 9: no template control; lane 10: positive control - genomic DNA isolated from FiberMax966 plant material.

Figure 3: PCR amplification of GhGluc1 on nucleic acids templates isolated through various protocols. Lane 1: molecular weight marker; lane 2: template DNA isolated by DNeasy® Plant Mini Kit (5 µl); lane 3: template DNA isolated by DNeasy® Plant Mini Kit (1 µl); lane 4: template DNA isolated by DNeasy® Plant Mini Kit (1 µl); lane 5: template DNA isolated by DNeasy® Plant Mini Kit (5 µl); lane 6: template DNA isolated by Wizard® Genomic DNA Purification Kit (1 µl); lane 7: template DNA isolated by Wizard® Genomic DNA Purification Kit (5 µl); lane 8: template DNA isolated by Wizard® Genomic DNA Purification Kit (1 µl); lane 9: template DNA isolated by Wizard® Genomic DNA Purification Kit (5 µl); lane 10: template DNA isolated by CTAB-procedure (1 µl); lane 11: template DNA isolated by CTAB procedure (5 µl); lane 12: no template control; lane 13: positive control - genomic DNA isolated from FiberMax966 plant material.

Figure 4: PCR amplification of GhGluc1 on nucleic acids templates isolated cotton cloth. Lanes 1 and 8: molecular weight marker; lane 2: template DNA from cotton cloth (ON incubation) (1 µl); lane 3: template DNA from cotton cloth (30' incubation) (1 µl); lane 4: template DNA from cotton cloth (ON incubation) (5 µl); lane 5: template DNA from cotton cloth (30' incubation) (5 µl); lane 6: no template control; lane 7: positive control - genomic material isolated from FiberMax966 plant material.

Figure 5: PCR amplification of GhGluc1 on nucleic acids templates isolated from cotton shirts with different weaves (knitted or woven). Lane 1: molecular weight marker; lane 2: template DNA from woven cotton shirt, ON incubation, (1 µl); lane 3: template DNA from woven cotton shirt, ON incubation, (5 µl); lane 4: template DNA from woven cotton shirt, ON incubation, (1 µl); lane 5: template DNA from woven cotton shirt, ON incubation, (5 µl); lane 6: template DNA from knitted cotton shirt, ON incubation, (1 µl); lane 7: template DNA from knitted cotton shirt, ON incubation,

(5µl); lane 8: template DNA from knitted cotton shirt, ON incubation, (1µl); lane 9: template DNA from knitted cotton shirt, ON incubation, (5µl); lane 10: template DNA from woven cotton shirt, 6 days incubation, (1µl); lane 11: template DNA from woven cotton shirt, 6days incubation, (5µl); lane 12: template DNA from woven cotton shirt, 6 days incubation, (1µl); lane 13: template DNA from woven cotton shirt, 6days incubation, (5µl); lane 14: template DNA from knitted cotton shirt, 6 days incubation, (1µl); lane 15: template DNA from knitted cotton shirt, 6days incubation, (5µl); lane 16: template DNA from knitted cotton shirt, 6days incubation, (1µl); lane 17: template DNA from knitted cotton shirt, 6 days incubation, (5µl); lane 18: no template control; lane 19: positive control - genomic DNA isolated from FiberMax966 plant material.

Figure 6: PCR amplification of NodC on nucleic acids templates isolated from mature transgenic cotton fiber material containing a chimeric N-acetylglucosamine transferase encoding gene. Lane 1: template DNA from transgenic fiber material, duplicate 1 (1µl); Lane 2: template DNA from transgenic fiber material, duplicate 1 (5 µl); Lane 3: template DNA from transgenic fiber material, duplicate 2 (1 µl); Lane 4: template DNA from transgenic fiber material, duplicate 2 (5µl); lane 5: no template control; lane 6: positive control - genomic DNA isolated from transgenic cotton plant material; lane 7: molecular weight marker.

Figure 7: Graphic representation of the endpoint Taqman assay discriminating between the GhGlucI A subgenome allele from *Gossypium hirsutum* and from *Gossypium barbadense*. Genomic DNA samples prepared from Pima (*G. barbadense*) or FM966 (*G. hirsutum*) plant (leaf) material, either in pure form, or in mixes of various ratio's (75/25, 50/50 and 25/75), subjected to a TaqMan detection assay. The X axis indicates the presence of the *hirsutum* allele of Gluc, while the Y-axis indicates the presence of the *barbadense* allele of Gluc1. In the mixed DNA samples , the Taqman analysis allows the detection of the various ratio's of both alleles.

Figure 8: Graphic representation of the endpoint Taqman assay discriminating between the GhGlucI A subgenome allele from *Gossypium hirsutum* and from *Gossypium barbadense* on fiber material and polo shirt textile. Genomic DNA samples prepared from Pima (*G. barbadense*) or FM966 (*G. hirsutum*) plant (leaf) material, either in pure form, or in mix of 50/50 as control samples. Nucleic acids prepared from fiber material (FM966 Bale) or from textile material (polo) was subjected to a TaqMan detection assay. The X axis indicates the presence of the *hirsutum* allele of Gluc1, while the Y-axis indicates the presence of the *barbadense* allele of Gluc1. In the analysis only the *hirsutum* allele was detected, indicating that all fiber material and all textile material contains only *hirsutum* type fibers.

Detailed description of embodiments of the invention

[0036] The currently described methods are based on the unexpected finding that it is possible to extract biological macromolecules (other than polysaccharides) such as peptides, proteins, nucleic acids, DNA or RNA from mature and/or processed plant fibers, particularly from seed fibers such as cotton fibers. Moreover, the isolated DNA proved to be of sufficient quality to be usable for further processing allowing characterization of the fibers e.g. using detection assays, such as polymerase chain reaction based amplifications.

[0037] Thus, in a first embodiment, the invention provides a method for a mature plant fiber or a processed plant fiber comprising the steps of isolating from said plant fiber a biological macromolecule naturally occurring in said plant fiber other than polysaccharides or lignin; and characterizing the information encoded in the sequence of the monomers of said biological macromolecule.

[0038] As used herein, a "mature plant fiber" is a fiber which has completed its developmental cycle and is considered to be the remnant of a dead cell. In particular, a mature plant fiber is a plant fiber after it has been harvested from the fiber crop producing the plant fiber. For cotton e.g., a mature fiber would be considered the fibers as they are present in the cotton balls picked at harvest. It will be clear that a mature plant fibers comprises all plant fibers processed by the fiber industry.

[0039] A "processed plant fiber" as used herein, is a mature plant fiber as harvested from the plant crop, which has undergone additional mechanical, heat and/or chemical treatments including waxing, dying, carding, spinning, weaving, sizing, mercerization etc. (see background section).

[0040] "Plant fibers", as used herein, are long narrow tapering cells of plant origin, dead and hollow at maturity with a rigid thick cell wall composed mostly of cellulose and lignin.

[0041] "Seed fibers" as used herein are fibers grown from the surface of seeds such as cotton. "Cotton" as used herein includes *Gossypium hirsutum* or *Gossypium barbadense* including "Cotton progenitor plants" such as *Gossypium arboreum*, *Gossypium herbaceum* and *Gossypium raimondii* and *Gossypium longicalyx*.

[0042] A "biological macromolecule naturally occurring in a plant fiber cell" is a biological macromolecule which has not been added exogenously to said plant fiber, e.g. through incubation or injection with a molecule such as a foreign DNA molecule, peptide or protein to tag the plant fibers, and which includes all macromolecules, i.e. molecules of

polymeric nature which have an informational content. The informational content will usually be encoded in the sequence of the building blocks or monomers. Examples of biological macromolecules are polypeptides, proteins, nucleic acids such as DNA or RNA, be they single-stranded or double stranded.

5 [0043] "Characterizing a biological macromolecule" as used herein means to decode the informational content of the biological macromolecule. This can be conveniently done by subjecting the biological macromolecule to a series of analyses, including determining the sequence of monomers of which the macromolecule is composed, but also subjecting the biological macromolecule to one or more specific detection assays.

10 [0044] A "detection assay" as used herein is a method or protocol targeted at finding a specific sequence of monomers in a biological macromolecule. This may include detection assays based on polymerase chain reaction based amplification, antibody detection or nucleic acid hybridization.

[0045] It has been demonstrated that the particular extraction methods, while having an influence on the quality of the extracted biological macromolecule are not critical to the ability of being able to extract such biological macromolecules from plant fibers.

15 [0046] Nevertheless, it has been unexpectedly found that the quality and/or the quantity of the biological macromolecules, particularly the nucleic acid fraction thereof, isolated from mature and/or processed plant fibers can be greatly by several measures.

[0047] It may e.g. be advantageous to leave the plant fibers to be analyzed for a prolonged time in a lysis buffer containing detergents and possibly other constituents. In particular it has been found that incubating mature and/or processed plant fibers for a period of at least 30 minutes, but more preferably for a period of at least 4 hrs up to 120 hrs in a lysis buffer, preferably at room temperature will improve the yield of recovered biological macromolecules, particularly the nucleic acid fraction thereof.

20 [0048] It has also be determined that carefully removing impurities, including leaf remnants, resins and other trashes from the plant fibers, such as harvested and/or ginned cotton fibers, prior to isolation of the biological macromolecules, greatly improves the quality of the isolated nucleic acids, particularly with regard to further analysis and processability of the isolated nucleic acids

25 [0049] The methods of the current invention can be advantageously used to identify cotton fibers derived from plants containing transformation events, or combination of transformation events, that may be the subject of petitions for non-regulated status, in the United States of America, to the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) whether such petitions are granted or are still pending, including the following events:

30

Transformation event	Petition	Transgenic phenotype
COT67B	07-108-01p	lepidopteran resistant
GHB614	06-332-01p	glyphosate tolerant
MON88913	04-086-01p	glyphosate tolerant
COT102	03-155-01p	lepidopteran resistant
281-24-236	03-036-01p	lepidopteran resistant
3006-210-23	03-036-02p	lepidopteran resistant
LLCotton25	02-042-01p	phosphinotricin tolerant
MON15985	00-342-01p	lepidopteran resistant
31807&31808	97-013-01p	bromoxynil tolerant & lepidopteran resistant
19-51a	95-256-01p	Sufonylurea tolerant
MON 1445, 1698	95-045-01p	glyphosate tolerant
MON 531,757,1076	94-308-01p	lepidopteran resistant
BXN	93-196-01p	bromoxynil tolerant

55 [0050] To this end the biological macromolecules, particularly the nucleic acid such as DNA may be subjected to a detection assay specifically designed to detect the transformation events. Such detection assays include the assays published on crl.jrc.it/statusofdoss.htm at least for events MON1445, MON531, MON15985, LLCotton25, 3006-210-23 and 281-24-236. Additional information such as nucleotide sequences of the plant flanking sequence adjacent to the inserted DNA in these transformation events (upon which information specific DNA detection assays can be designed)

can be found in the following patent applications:

Event name	Phenotype	Patent application
CE43-67B	Insect resistance (CryIAb)	WO2006/128573
CE46-02A	Insect resistance (CryIAb)	WO2006/128572
CE44-69D	Insect resistance (CryIAb)	WO2006/128571
1143-14A	Insect resistance (CryIAb)	WO2006/128569
T342-142	Insect resistance (CryIAb)	WO2006/128568
event3006-210-23	Insect resistance (CryIAc)	WO2005103266
PV-GHGT07 (1445)	Glyphosate tolerance	US2004-148666
MON88913	Glyphosate tolerance	WO2004/072235
EE-GH3	Glyphosate tolerance	WO2007/017186
T303-40	Insect-resistance	PCT/EP2008/002667
GHB 119	Insect-resistance	PCT/EP2008/004652
Cot202	Insect resistance (VIP3)	US2007-067868
LLcotton25	Glufosinate resistance	WO2007/017186
event 281-24-236	Insect resistance (Cry 1F)	WO2005103266
Cot102	Insect resistance (Vip3A)	US2006-130175
MON 15985	Insect resistance	US2004-250317

[0051] The methods of the current invention can be advantageously used to identify cotton fibers derived from cotton plants containing genetic material which imparts particularly advantageous, useful traits to these plants. Examples of such traits are better plant growth, increased tolerance to abiotic stresses such as high or low temperatures, drought, extreme water or salt or mineral content in the soil, increased flowering performance, easier harvesting, accelerated maturation, higher harvest yields of commercially relevant plant parts (e.g. seeds), higher quality and/or a higher nutritional value of the harvested products, better storage stability and/or processability of the harvested products. Further and particularly emphasized examples of such traits are resistance against biotic stresses i.e. a better defense of the plants against animal and microbial pests, such as against insects, mites, phytopathogenic fungi, bacteria and/or viruses, and also increased tolerance of the plants to certain herbicidally active compounds.

[0052] The methods of the current invention can be advantageously used to identify cotton fibers derived from cotton plants containing genetic material which imparts herbicide-resistance for example glyphosate-tolerant plants, i.e. plants made tolerant to the herbicide glyphosate or salts thereof. Plants can be made tolerant to glyphosate through different means. For example, glyphosate-tolerant plants can be obtained by transforming the plant with a gene encoding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Examples of such EPSPS genes are the AroA gene (mutant CT7) of the bacterium Salmonella typhimurium (Comai et al., 1983, Science 221, 370-371), the CP4 gene of the bacterium Agrobacterium sp. (Barry et al., 1992, Curr. Topics Plant Physiol. 7, 139-145), the genes encoding a Petunia EPSPS (Shah et al., 1986, Science 233, 478-481), a Tomato EPSPS (Gasser et al., 1988, J. Biol. Chem. 263, 4280-4289), or an Eleusine EPSPS (WO 01/66704). It can also be a mutated EPSPS as described in for example EP 0837944, WO 00/66746, WO 00/66747 or WO02/26995. Glyphosate-tolerant plants can also be obtained by expressing a gene that encodes a glyphosate oxido-reductase enzyme as described in U.S. Patent Nos. 5,776,760 and 5,463,175. Glyphosate-tolerant plants can also be obtained by expressing a gene that encodes a glyphosate acetyl transferase enzyme as described in for example WO 02/36782, Wo 03/092360, WO 05/012515 and WO 07/024782. Glyphosate-tolerant plants can also be obtained by selecting plants containing naturally-occurring mutations of the above-mentioned genes, as described in for example WO 01/024615 or WO 03/013226. Other herbicide resistant plants are for example plants that are made tolerant to herbicides inhibiting the enzyme glutamine synthase, such as bialaphos, phosphinothricin or glufosinate. Such plants can be obtained by expressing an enzyme detoxifying the herbicide or a mutant glutamine synthase enzyme that is resistant to inhibition. One such efficient detoxifying enzyme is an enzyme encoding a phosphinothricin acetyltransferase (such as the bar or pat protein from Streptomyces species). Plants expressing an exogenous phosphinothricin acetyltransferase are for example described in U.S. Patent Nos. 5,561,236; 5,648,477; 5,646,024; 5,273,894; 5,637,489; 5,276,268; 5,739,082; 5,908,810 and 7,112,665. Further herbicide-tolerant plants are also plants

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that are made tolerant to the herbicides inhibiting the enzyme hydroxyphenylpyruvatedioxygenase (HPPD). Hydroxyphenylpyruvatedioxygenases are enzymes that catalyze the reaction in which para-hydroxyphenylpyruvate (HPP) is transformed into homogentisate. Plants tolerant to HPPD-inhibitors can be transformed with a gene encoding a naturally-occurring resistant HPPD enzyme, or a gene encoding a mutated HPPD enzyme as described in WO 96/38567, WO 99/24585 and WO 99/24586. Tolerance to HPPD-inhibitors can also be obtained by transforming plants with genes encoding certain enzymes enabling the formation of homogentisate despite the inhibition of the native HPPD enzyme by the HPPD-inhibitor. Such plants and genes are described in WO 99/34008 and WO 02/36787. Tolerance of plants to HPPD inhibitors can also be improved by transforming plants with a gene encoding an enzyme prephenate deshydrogenase in addition to a gene encoding an HPPD-tolerant enzyme, as described in WO 2004/024928. Still further herbicide resistant plants are plants that are made tolerant to acetolactate synthase (ALS) inhibitors. Known ALS-inhibitors include, for example, sulfonylurea, imidazolinone, triazopyrimidines, pyrimidinyoxy(thio)benzoates, and/or sulfonylaminocarbonyl triazolinone herbicides. Different mutations in the ALS enzyme (also known as acetohydroxyacid synthase, AHAS) are known to confer tolerance to different herbicides and groups of herbicides, as described for example in Tranel and Wright (2002, Weed Science 50:700-712), but also, in U.S. Patent No. 5,605,011, 5,378,824, 5,141,870, and 5,013,659. The production of sulfonylurea-tolerant plants and imidazolinone-tolerant plants is described in U.S. Patent Nos. 5,605,011; 5,013,659; 5,141,870; 5,767,361; 5,731,180; 5,304,732; 4,761,373; 5,331,107; 5,928,937; and 5,378,824; and international publication WO 96/33270. Other imidazolinone-tolerant plants are also described in for example WO 2004/040012, WO 2004/106529, WO 2005/020673, WO 2005/093093, WO 2006/007373, WO 2006/015376, WO 2006/024351, and WO 2006/060634. Further sulfonylurea- and imidazolinone-tolerant plants are also described in for example WO 07/024782.

[0053] The methods of the current invention can be advantageously used to identify cotton fibers derived from cotton plants containing genetic material which imparts resistance to attack by certain target insects. Such plants can be obtained by genetic transformation, or by selection of plants containing a mutation imparting such insect resistance and may contain at least one transgene comprising a coding sequence encoding:

- 1) an insecticidal crystal protein from *Bacillus thuringiensis* or an insecticidal portion thereof, such as the insecticidal crystal proteins listed by Crickmore et al. (1998, Microbiology and Molecular Biology Reviews, 62: 807-813), updated by Crickmore et al. (2005) at the *Bacillus thuringiensis* toxin nomenclature, online at: http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/, or insecticidal portions thereof, e.g., proteins of the Cry protein classes CryIAb, CryIAc, CryIF, Cry2Ab, Cry3Aa, or Cry3Bb or insecticidal portions thereof; or
- 2) a crystal protein from *Bacillus thuringiensis* or a portion thereof which is insecticidal in the presence of a second other crystal protein from *Bacillus thuringiensis* or a portion thereof, such as the binary toxin made up of the Cry34 and Cry35 crystal proteins (Moellenbeck et al. 2001, Nat. Biotechnol. 19: 668-72; Schnepf et al. 2006, Applied Environm. Microbiol. 71, 1765-1774); or
- 3) a hybrid insecticidal protein comprising parts of different insecticidal crystal proteins from *Bacillus thuringiensis*, such as a hybrid of the proteins of 1) above or a hybrid of the proteins of 2) above, e.g., the Cry1A.105 protein produced by corn event MON98034 (WO 2007/027777); or
- 4) a protein of any one of A1) to A3) above wherein some, particularly 1 to 10, amino acids have been replaced by another amino acid to obtain a higher insecticidal activity to a target insect species, and/or to expand the range of target insect species affected, and/or because of changes introduced into the encoding DNA during cloning or transformation, such as the Cry3Bb1 protein in corn events MON863 or MON88017, or the Cry3A protein in corn event MIR604;
- 5) an insecticidal secreted protein from *Bacillus thuringiensis* or *Bacillus cereus*, or an insecticidal portion thereof, such as the vegetative insecticidal (VIP) proteins listed at: http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html, e.g., proteins from the VIP3Aa protein class; or
- 6) a secreted protein from *Bacillus thuringiensis* or *Bacillus cereus* which is insecticidal in the presence of a second secreted protein from *Bacillus thuringiensis* or *B. cereus*, such as the binary toxin made up of the VIP1A and VIP2A proteins (WO 94/21795); or
- 7) a hybrid insecticidal protein comprising parts from different secreted proteins from *Bacillus thuringiensis* or *Bacillus cereus*, such as a hybrid of the proteins in 1) above or a hybrid of the proteins in 2) above; or
- 8) a protein of any one of 1) to 3) above wherein some, particularly 1 to 10, amino acids have been replaced by another amino acid to obtain a higher insecticidal activity to a target insect species, and/or to expand the range of target insect species affected, and/or because of changes introduced into the encoding DNA during cloning or transformation (while still encoding an insecticidal protein), such as the VIP3Aa protein in cotton event COT102.

[0054] The methods of the current invention can be advantageously used to identify cotton fibers derived from cotton plants containing genetic material which imparts tolerance to abiotic stresses. Such plants can be obtained by genetic transformation and may contain one or more of the following transgenes

- a. a transgene capable of reducing the expression and/or the activity of poly(ADP-ribose)polymerase (PARP) gene in the plant cells or plants as described in WO 00/04173 or EP 04077984.5 or EP 06009836.5.
- b. a stress tolerance enhancing transgene capable of reducing the expression and/or the activity of the PARG encoding genes of the plants or plants cells, as described e.g. in WO 2004/090140.
- c. a stress tolerance enhancing transgene coding for a plant-functional enzyme of the nicotineamide adenine dinucleotide salvage synthesis pathway including nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase, nicotinamide adenine dinucleotide synthetase or nicotine amide phosphorybo-syltransferase as described e.g. in EP 04077624.7 or WO 2006/133827 or PCT/EP07/002433.

[0055] The methods of the current invention can be advantageously used to identify cotton fibers derived from cotton plants containing genetic material which can modify the characteristics of the fiber such as strength, length, micronaire etc. including the chimeric genes described in WO05/017157 (glucanase silencing genes) WO04/0655571 (chitinase encoding genes), WO02/45485 and WO08/012058 (sucrose synthase genes), WO01/17333 (sucrose phosphate syn-thetase genes) WO98/00549 (cellulose synthase genes) WO06/133827 (N-acetylglucosamine transferase genes such a chitin synthase or NODC encoding genes).

[0056] A further application for the methods of the current invention is to identify plant fibers, such as cotton fibers from plants which comprise specific allelic variations of particular genes. The methods for identifying such allelic variations are well known in the art. In particular, such allelic variations may be used to differentiate between fibers from particular plant species such as e.g. differentiating between fibers from *G. barbadense* and *G. hirsutum*. As an example, the polymorphism between glucanase1 A subgenome allele from *Gossypium barbadense* and from *Gossypium hirsutum* (by the presence of a stop codon in the coding region of Gluc1 A subgenome allele in *G. barbadense*.) may be used to differentiate fibers originating from *Gossypium barbadense* or *Gossypium hirsutum*.

[0057] The methods herein described may also be used to isolate nucleic acid templates which can be used for any genome characterization protocols, including AFLP, READS and the like. The nucleic acid templates isolated from the mature or processed fibers, may be amplified using methods known in the art such as whole genome amplification, prior to application of a genome characterization protocol.

[0058] The methods described herein may also be used to assist in certification programs, guaranteeing the supply of commercially traded plant fibers, such as cotton fibers, having particularly specified characteristics. Usually, such certification programs include recordation of the purchase of certified seeds by registered growers, whereby the cotton seed comprise a specific genome composition and produce a particular brand of plant fibers. Balese of the harvested plant fibers produced by the registered growers from the said certified seeds are registered and authenticated by cross checking with said seed purchase records and are provided to mills to produce preferably predominantly, particularly exclusively yarns, fabrics or apparel from said brand of fibers. The methods describe herein allow to perform audits at different points in the supply chain.

[0059] The methods described herein can also be applied to isolate biological macromolecules from hair-like (trichome-like) structures on plant seeds, such as the hairs on tomato seeds or the bear hairs on the basis of wheat seeds.

[0060] The methods described herein can also be applied to characterize the nucleic acid material of old fibers and old textile material (e.g. fibers and textiles which are over 100 years old). Without limiting the invention to a specific mechanism or theory it is believed that one possibility that biological material is preserved in old fibers is because we have observed that mature fibers are twisted such that biological material is 'trapped' (and thereby 'preserved') in mature fibers.

[0061] The methods described herein can also be applied in forensic applications because fiber or textile material identified at a crime scene can be characterized by for example preceding a whole genome amplification step as shown in example 6 in this application.

[0062] As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region, which is functionally or structurally defined, may comprise additional DNA regions etc.

[0063] The following non-limiting Examples describe the methods for isolating biological macromolecules from mature plant fibers and from processed fibers and their use for characterizing or identifying plant fibers. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by

R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.
[0064] Throughout the description and Examples, reference is made to the following sequences represented in the sequence listing:

- SEQ ID No 1: oligonucleotide 1 for PCR amplification of Ghgluc1
- SEQ ID No 2: oligonucleotide 2 for PCR amplification of Ghgluc1
- SEQ ID No 3: oligonucleotide 1 for PCR amplification of NodC
- SEQ ID No 4: oligonucleotide 2 for PCR amplification of NodC
- SEQ ID No 5: oligonucleotide 1 for PCR amplification of expansin
- SEQ ID No 6: oligonucleotide 2 for PCR amplification of expansin
- SEQ ID No 7: oligonucleotide 1 for Taqman detection assay of GhGluc 1 (forward primer)
- SEQ ID No 8: oligonucleotide 2 for Taqman detection assay of GhGluc1 (reverse primer)
- SEQ ID No 9: oligonucleotide 3 for Taqman detection assay of subgenomic A GhGluc 1 allele (VIC marked oligonucleotide detecting the G. hirsutum allele of Gluc1)
- SEQ ID No 10: oligonucleotide 4 for Taqman detection assay of subgenomic A GhGluc1 allele (FAM marked oligonucleotide detecting the G. barbadense allele of Gluc1)
- SEQ ID No 11: oligonucleotide 1 for PCR amplification of rp116
- SEQ ID No 12: oligonucleotide 2 for PCR amplification of rp116
- SEQ ID No 13: oligonucleotide 1 for PCR amplification of matK
- SEQ ID No 14: oligonucleotide 2 for PCR amplification of matK
- SEQ ID No 15: oligonucleotide 1 for PCR amplification of trnT-trnL
- SEQ ID No 16: oligonucleotide 2 for PCR amplification of trnT-trnL
- SEQ ID No 17: oligonucleotide 1 for PCR amplification of ndhF
- SEQ ID No 18: oligonucleotide 2 for PCR amplification of ndhF

Examples

Example 1: Isolation of nucleic acids from raw cotton fiber material.

A. Isolation of nucleic acids from *Gossypium hirsutum* FM966 fiber material.

[0065] Harvested cotton fibers from FM966, which had been ginned and baled, were subjected to DNA extraction using the conventional CTAB (Hexadecyl trimethylammonium bromide) procedure (Doyle and Doyle, 1987, Phytochem. Bull, 19, 11) for isolation of genomic DNA from plant material. Although a pellet of nucleic acids was obtained, this material could not be used for any further application including PCR amplification, or restriction enzyme digestion.

[0066] The harvested baled raw cotton fibers still contain some impurities such as leaf remnants and other trashes. These contaminant materials were carefully removed by manual cleaning to obtain a purified fiber fraction (and a trash fraction). The raw fiber material, the purified fiber material and the trash fraction were subjected to DNA extraction using the DNeasy®Plant Mini Kit commercialized by QIAGEN according to the manufacturer's recommendations (see DNeasy® Plant Handbook available at www.qiagen.com/HB/DNeasyPlantMiniMaxi) and the concentrations of the isolated nucleic acids was determined using UV spectrophotometry (Table 1)

Table 1.

Sample	ng/ul	A260	A280	260/280	260/230
Pure fiber	3.49	0.07	0.05	1.41	0.52
Pure fiber	4.19	0.084	0.065	1.29	0.51
Raw fiber	14.91	0.298	0.194	1.54	0.43
Raw fiber	17.4	0.348	0.239	1.46	0.43
Trash only	76.55	1.531	1.09	1.4	0.45

[0067] The isolated nucleic acids were used as templates in a standard PCR reaction using the following primers to detect an endogenous cotton glucanase gene (GhGluc1; see EP 08075514.3 herein incorporated by reference for nucleotide sequences of different alleles of GhGluc1):

SE002: ggccgaagccgatcttatctagg (SEQ ID No.: 1)
SE003: cggcaacaatcttccatctccag (SEQ ID No.: 2)

[0068] Both 1 µl and 5 µl of template nucleic acids were used. The results of the PCR amplification are visualized in Figure 1. A 656 bp amplicon (as expected) is clearly detected using pure fiber (5µl template) (lane 4) whereas a faint signal can be observed using small amount (1µl) of nucleic acids isolated from both pure and raw fiber material. No amplification signal can be detected in the nucleic acids isolated from the trash material only nor from the nucleic acids obtained from unpurified material when used in higher concentrations indicating that the extract obtained from contaminant materials appears to inhibit the further processing of nucleic acids obtained from raw fiber material.

[0069] Interestingly, this experiment demonstrates that nucleic acids, which can be further analyzed, can be extracted from mature cotton fiber material. The experiment also indicates that the quality of the isolated nucleic acids - DNA can be further improved by removing the leaf trash and other impurities from the raw harvested and ginned cotton fibers.

B. Isolation of nucleic acids from *Gossypium barbadense* Pima-Y5 fiber material.

[0070] 4 year old raw cotton fiber material from *Gossypium barbadense* Pima5Y was treated along with *Gossypium hirsutum* FM966 cotton fiber material, as described above in 1.A. The obtained nucleic acids were used as templates in PCR reactions as described in 1.A. The results are visualized in Figure 2.

[0071] Although *Gossypium barbadense* fibers are stronger and have a different architecture from *Gossypium hirsutum* fibers, the results demonstrate that nucleic acids including processable DNA can also be isolated from these fibers. Interestingly, these fibers had also been stored for several years under non-optimal conditions indicating that processable DNA or other nucleic acids can be obtained from old fiber material.

C. Amplification of other cotton endogenous genes.

[0072] The isolated nucleic acids were also used as templates in standard PCR reactions using primers specific for other endogenous genes such as the following primers which amplify a 299 bp fragment from the expansin encoding gene:

K151: gggagctgtgttatggaaacc (SEQ ID No.: 5)
K152: cagggacgatcccagctcgatattc (SEQ ID No.: 6)

The expected amplicons could also be detected using these other primers for other endogenous genes.

D. Isolation of nucleic acids from cotton fiber material using other lysis buffers and protocols.

[0073] Raw cotton fiber material was purified as described in section 1.A. by removing the contaminating leaf trash material and other impurities. Nucleic acids were extracted from the purified fiber material using either the DNeasy® Plant Mini Kit commercialized by QIAGEN or the Wizard® Genomic DNA Purification Kit commercialized by PROMEGA CORPORATION or the conventional CTAB procedure (Doyle and Doyle, 1987, Phytochem. Bull. 19, 11). The concentration of the obtained nucleic acids was determined using U.V. spectrophotometry (Table 2).

Table 2.

Sample	ng/ul	A260	A280	260/280	260/230
Qiagen 1	7.66	0.153	0.069	2.21	1.4
Qiagen 2	19.97	0.399	0.241	1.66	0.76
Promega 1	21.34	0.427	0.208	2.05	1.42
Promega 2	38.14	0.763	0.389	1.96	1.06
CTAB*	225.22	4.504	2.235	2.02	1.42
* Note: High concentration readings probably due to absence of RNase treatment.					

[0074] The isolated nucleic acids were used as templates in a standard PCR reaction using the following primers to detect an endogenous cotton glucanase gene (GhGluc1):

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SE002: ggccgaagccgatcttatctagg (SEQ ID No.: 1)

SE003: cggaacaatcttccatctccag (SEQ ID No.: 2)

[0075] Both 1 µl and 5 µl of template nucleic acids were used. The results of the PCR amplification are visualized in Figure 3. The expected DNA amplicon was observed using template nucleic acids isolated by any method, although apparently higher concentrations of the nucleic acids (5 µl reaction) isolated by Wizard® and CTAB procedures had a negative effect on the PCR reaction.

[0076] Albeit with a lower efficiency, we observed that nucleic acids could also be extracted from the cleaned fiber material by incubating this fiber material with water (H₂O) for a certain amount of time (e.g. at least 4 hours incubation of the cleaned fiber material in the presence of a volume of water).

Example 2: Isolation of nucleic acids from textiles.

A. Isolation of nucleic acids from cotton cloth.

[0077] The successful isolation of nucleic acids, such as DNA which can be further processed, from mature cotton fibers, spurred an attempt to isolate biological macromolecules such as nucleic acids, particularly processable DNA from fibers after spinning and weaving. To this end, yarns were isolated by unweaving a piece of cotton cloth, cut into smaller pieces and incubated in 5 ml of lysis buffer (DNeasy® AP 1 buffer) at 65°C for at least 30 minutes or overnight at room temperature. Buffer was squeezed out of the material and 500 µl lysate was transferred to an eppendorf tube and further treated according to the manufacturer's recommendations i.e.:

- add 130 µl AP2 buffer, mix and incubate on ice for 5 minutes
- centrifuge for 5 minutes at 13000 rpm
- transfer the liquid phase to the lilac QIAshredder mini spin column
- centrifuge for 2 minutes at 13000 rpm
- transfer the flow through to a new eppendorf tube without disturbing the pellet;
- add 800 µl AP3/E buffer and mix
- transfer 650 µl to the DNeasy® mini spin column and centrifuge for 1 minute at 8000 rpm, discarding the flow-through
- repeat this step with the remaining sample; discard the collection tube and place the column in a new 2 ml collection tube
- wash the column with 500 µl AW buffer and centrifuge for 1 min at 8000 rpm, discard the flow-through
- wash the column with 500 µl AW buffer and centrifuge for 2 min at 13000 rpm, discard the flow-through
- place the column in a 1.5 ml eppendorf without ethanol carryover.
- Add 50 µl water to the column and incubate at least 5 minutes at room temperature
- Centrifuge for 1 minute at 8000 rpm. Store the DNA at 4 °C.

[0078] 1 and 5 µl samples were used as template DNA in a PCR reaction using the primers of example 1.A. The results are visualized in Figure 4. When nucleic acid samples after overnight incubation were used, the expected amplicon of 669 bp could be detected, indicating that the protocol can be used to isolate nucleic acids from cotton fibers after spinning and weaving. The significantly stronger PCR signal after overnight incubation versus the 30 minutes incubation, indicates the former protocol results in higher concentration of processable DNA.

B. Isolation of nucleic acids from textiles with different weaves.

[0079] The nucleic acid isolation protocol was also applied to finished apparel with different weaves (i.e. which has undergone different processing). To this end, pieces were cut from a cotton long-sleeve shirt (woven) and a cotton polo (knitted) and treated as in Example 2A, with the exception that incubation in lysis buffer was performed either overnight, or even prolonged to 6 days of incubation. A PCR reaction as described in Example 2.A was performed on the isolated nucleic acids and the results are visualized in Figure 5.

[0080] The expected amplicon was detected after PCR amplification using nucleic acid samples isolated from both kinds of fabrics. Again, the signal was stronger in samples in which the incubation in lysis buffer had been prolonged.

Example 3: Isolation of nucleic acids from specialty fibers.

[0081] Mature fibers were isolated and purified from transgenic cotton plants comprising an N-acetylglucosamine transferase chimeric gene ("NodC gene") as described in WO2006/136351 Example 1. Nucleic acids were isolated from the purified fibers extraction using the DNeasy®Plant Mini Kit commercialized by QIAGEN according to the manufacturer's

recommendations (see DNeasy® Plant Handbook available at www.qiagen.com/HB/DNeasyPlantMiniMaxi) and the concentrations of the isolated nucleic acids was determined using UV spectrophotometry (Table 3)

Table 3.

Sample	ng/ul	A260	A280	260/280	260/230
NodC1	7.95	0.159	0.105	1.51	0.56
NodC2	7.62	0.152	0.101	1.51	0.56

[0082] The isolated nucleic acids were also used as templates in standard PCR reactions using primers specific for other endogenous genes such as the following primers which amplify a 1224 bp fragment from the NodC encoding gene:

ldb093: cgttttcactcatcgctgtttcaagtgctgtagatgtgatcggttgcttgcg (SEQ ID No.: 3)
1db126: ggcgcgcttaggaactctcgctgatagccac (SEQ ID No.: 4)

[0083] Both 1 µl and 5 µl of template nucleic acids were used. The results of the PCR amplification are visualized in Figure 6. The expected DNA amplicon indicative of the presence of the transgene could be observed in each sample.

Example 4: Automation and differentiation between different fiber types.

[0084] The glucanase 1 A subgenome allele from *Gossypium barbadense* differs from the glucanase 1 subgenome allele from *Gossypium hirsutum* by the presence of a stop codon in the coding region of Gluc1 A subgenome allele in the former. This polymorphism could be used to differentiate between cotton fibers from plants having the *Gossypium barbadense* Gluc 1 allele or the *Gossypium hirsutum* Gluc 1 allele. To this end, a Taqman® assay was designed, whereby the oligonucleotide primers to amplify a DNA fragment carrying the polymorphism used were:

Forward primer: GCTTTTGAAGCGATATAACATCGA (SEQ ID No.: 7)
Reverse primer: GGCATAGGCAAATAAGGGTACACA (SEQ ID No.: 8)

[0085] The polymorphism was detected by the monitoring of the degradation of the following fluorescence marked primers detecting the polymorphism:

VIC- AATCCTGTGCAACCAG (hirsutum allele) (SEQ ID No.: 9)
FAM- ATCCTGTCAAACCAG (barbadense allele) (SEQ ID No.: 10)

[0086] These primers were used in a qPCR setup, increasing the numbers of cycles up to 60 and determining fluorescence at the endpoint only. As nucleic acid templates, mixes of DNA isolated from either *G. barbadense* fibers (PIMA) or *G. hirsutum* fibers (FM966) were used in the following ratios: 100% PIMA, 75% PIMA/25%FM966, 50% PIMA/50%FM966, 25% PIMA/75%FM966, 100%FM966. The results are schematically represented in Figure 7.

[0087] The X axis indicates the presence of the *G. hirsutum* allele of Gluc1, while the Y-axis indicates the presence of the *G. barbadense* allele of Gluc 1. In DNA isolated from PIMA fibers, only the *G. barbadense* allele of Gluc 1 was detected. In DNA isolated from PIMA fibers, only the *G. hirsutum* allele of Gluc1 was detected. In the mixed DNA samples, the Taqman analysis allows the detection of the various ratio's of both alleles.

[0088] In a further step, the same RT-PCR type analysis was used to analyze nucleic acid samples from cotton textile and fiber material from a FM966 bale. The results are represented in Figure 8. In nucleic acids extracted from the FM966 bale and from the polo shirts, only the *G. hirsutum* allele of Gluc1 was detected, indicating that the cotton material used exclusively originated from *G. hirsutum* type material.

[0089] Although the assays may need further optimization, such optimization is clearly within the realm of the skilled person.

Example 5: Characterization of the plastid genome isolated from cotton fibers.

[0090] Because of the unexpected finding that genomic DNA can be isolated and characterized from mature cotton fibers we investigated the possibility of identifying plastid DNA in the pool of DNA extracted from these fibers.

[0091] Although, leucoplast plastids have been shown to be present in developing cotton fibers (Ryser U. (1985) European journal of cell biology 39, pp. 236-256) they are not expected to be present in mature cotton fibers. Leucoplasts are similar to chloroplasts by having the same genome - because they differentiated from the same progenitor cell type

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- but leucoplasts are specialized with high accumulation of starch content.

[0092] Cronn et al (2002) American Journal of Botany 89(4): 707-725 published primer combinations to amplify 4 genes of the plastid genome (rp116, matK, trnT-trnL and ndhF). In a first step we optimized the published primer combinations for amplification of these four genes by carrying our PCR analysis on DNA isolated from cotton leaves. In a next step, the optimized primer combination per gene was used for amplification on a fiber DNA sample obtained from a cotton mini bale (Certified FiberMax cotton). We surprisingly showed amplification of 4 PCR fragments from this fiber DNA sample. Subcloning in TOPO-blunt vectors followed by sequence analysis of these fragments confirmed that these four fragments originated from the plastid genome. For rp116, matK and trnT-trnL the sequencing resulted in a unique consensus sequence, for ndhF several consensus sequences were obtained.

[0093] Since the complete chloroplast sequences of *Gossypium hirsutum* and *Gossypium barbadense* are available in the EMBL database (entry DQ345959 is the chloroplast DNA sequence of coker 310 FR (*G. hirsutum*) and entry AP009123 is the chloroplast DNA sequence of *G. barbadense*) it was possible to align the obtained sequences with the published sequences. It was found that the obtained sequences were 100% identical to the published *G. hirsutum* sequences (except for the degenerate primer sites). This confirms that the fiber in the cotton bale was derived from *G. hirsutum*.

The successful molecular characterization of these specific plastid sequences now provides novel opportunities for genotyping of fiber DNA. Also older fiber samples can be probed for plastid sequences and their relationship can be interpreted during evolution and breeding.

[0094] PCR conditions:

2µl template
1.5µl forward primer (10µM)
1.5µl reverse primer (10µM)
21µl MQ
1µl dNTP
10 µl 10X HF buffer
0.5µl Phusion
12.5µl MQ
PCR program
30s @ 98°C
10s@98°C
30s @ 60°C
60s @ 72°C
35 cycli
10min @ 72°C
Hold @ 4°C

[0095] Primer combinations :

rp116	F71: gctatgcttagtgtgtgactcgtt (SEQ ID No 11) R1516: cccttcattctcctctatgttg (SEQ ID No 12)
matK	matKF3: ctaatggatcaacagaawcgttg (SEQ ID No 13) trnKR: aactagtcggatggagtag (SEQ ID No 14)
trnT-trnL	trnL2: aatattactgactccmttttkatttckag (SEQ ID No 15) trnB: tctaccgatttcgcatatc (SEQ ID No 16)
ndhF	5'Fnew: gaatatgcatggatcatacc (SEQ ID No 17) 1318R: cgaaacatataaaatgcrgttaatcc (SEQ ID No 18)

Example 6: characterization of the genome of nucleic acids isolated from cotton fibers.

[0096] The previous examples have convincingly shown that DNA can be isolated and characterized from mature cotton fibers and from downstream products thereof. In a next step we questioned if the isolated fiber DNA is a true representation of the whole cotton genome or whether it represents only a fraction of the cotton genome. A partial genome equivalent would not be useful for trait or genotype testing as it would generate unreliable and incomplete assay results. The first challenge was to overcome the limited amount of DNA obtained from cotton fibers as starting material for assay testing. The method of Whole Genome Amplification (WGA) was chosen to overcome this limitation. WGA

has not been used before to amplify a polyploid genome such as cotton, for genotyping and trait testing. A DNA amplification step is important to enhance the amount of starting fiber DNA material but it is critical not to introduce a genome bias which is sometimes inherent to a WGA step as reported by Giardina *et al* (2009) *BMC Genomics* Apr 14, 10:159. We addressed the reliability of a WGA step on isolated fiber DNA in a functional Taqman assay. Thereto the quality of WGA-amplified fiber DNA was compared with WGA-amplified cotyledon DNA of the same commercial cotton fiber source in substantially the same Taqman assay.

[0097] Approximately 500 grams of FM9063B2F fiber (500 grams samples each in 3 small brown bags; 3 replicates were taken from different bales for the sample variety, Bale no 721134350138, 721134350154, 721134350156) was provided by K. Merritt from a CropMark Direct farm. The fiber was from cotton grown in the 2008-9 cotton season and listed for the FiberMax Certification Program.

[0098] Since we had determined that contaminants in fiber can have a significant impact upon the use of fiber DNA for downstream testing we manually cleaned the fibers. This is done by stretching a small amount of fibers (e.g. 1.5 grams) whereby most of the debris is removed with tweezers until no debris can be seen with the naked eye. DNA extraction was carried out with the Qiagen DNeasy Plant Mini kit with the following steps:

- Take 1 gram of cleaned (no leaf trash) mature cotton fiber and put it in a 50ml Falcon tube. Cut the fibers a few times with clean scissors.
- Add 5ml AP1 lysis buffer (Qiagen cat. 1014630) and 100µl RNaseA (10mg/ml). Use a pestle to homogenize the contact between sample and buffer.
- Incubate at 65°C for 20min, use a 15ml tube to mix the material 2 to 3 times during the incubation.
- Use a 15ml tube to squeeze the material in order to transfer 500µl lysate into a 2ml eppendorf tube. 3 aliquots are taken and used as duplicates.
- Add 130µl AP2 buffer, mix and incubate 5min on ice to each aliquot.
- Centrifuge for 5min at 13000rpm.
- Transfer the liquid phase to the lilac QIAshredder mini spin column.
- Centrifuge for 2min at 13000rpm.
- Transfer the flow-through to a new eppendorf tube without disturbing the pellet.
- Add 800µl AP3/E buffer and mix.
- Transfer 650µl to the DNeasy mini spin column and centrifuge for 1 min at 8000rpm, discard the flow-through.
- Repeat this step with the remaining sample. Discard the collection tube and place the column in a new 2ml collection tube.
- Wash the column with 500µl AW buffer and centrifuge for 1 min at 8000 rpm, discard the flow-through.
- Wash the column with 500µl AW buffer and centrifuge for 2 min at 13000 rpm, discard the flow-through and collection tube.
- Place the column in a 1.5ml eppendorf tube without ethanol carryover.
- Add 50µl MQ water to the column and incubate at least 5min at room temperature.
- Centrifuge for 1min at 8000rpm. Store the DNA at 4°C (short term) or -20°C (long term).

[0099] Cotyledon DNA extraction of FM9063 B2F was carried out as follows. 50 seeds of FM9063 B2F were germinated with germination paper and water in a 28°C germinator. Cotyledons were collected and dried with silicon. Cotyledon DNA was extracted. Extracted cotyledon DNA was combined into five 1.52ml tube.

[0100] Whole Genome Amplification was carried out with the Sigma's GenomePlex® Complete Whole Genome Amplification (WGA) Kit (WGA2) with the following steps:

- Add 1µl of 10x Fragmentation Buffer to 10µl of extracted DNA in a PCR tube.
- Incubate in a thermal cycler at 95°C for 2 minutes and cool on ice.
- Add 2µl of 1x Library Preparation buffer.
- Add 1µl of Library Stabilization Solution and mix.
- Incubate in a thermal cycler at 95°C for 2 minutes and cool on ice.
- Add 1 µl of Library Preparation Enzyme and mix.
- Incubate in thermal cycler using program WGA1
 - 16°C for 20 minutes
 - 24°C for 20 minutes
 - 37°C for 20 minutes
 - 75°C for 5 minutes
 - 4°C forever

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- Add the following master mix:
 - 7.5µl 10x Amplification Master Mix
 - 47.5µl nuclease-free water
 - 5µl WGA polymerase
- Incubate in thermal cycler using program WGA2
 - 95°C for 3 minutes
 - 14 cycles of: 94°C for 15 seconds
 - 65°C for 5 minutes
 - 4°C forever
- Put 5µl on a 1% agarose gel to check the quality of the WGA DNA. A smear between 100 and 1000bp should be present.

[0101] All the WGA reactions (fiber and cotyledon) proved to be successful.

[0102] Optionally the WGA-amplified DNA of cotyledon and fiber of FM9063 B2F was further purified in the following way. Combine WGA DNA from 5 samples into 2ml tube (about 240µl DNA), add 24µl 3M sodium acetate pH5.2 and mix, add 800µl 100% ice cold ethanol and mix thoroughly and overnight at -20°C, centrifuge at -20°C for 10min at 12000rpm, carefully remove supernatant, wash DNA pellet with 800ul 70% ice cold ethanol, centrifuge at -20C for 2min at 12000rpm, carefully remove supernatant, put tube in 65°C incubator for 15 min to dry, resuspend DNA in 65ul 1x TE buffer.

[0103] Two different Taqman assays were carried out on the amplified DNA samples. One Taqman assay aimed at identifying 40 different SNPs and another Taqman assay aimed at identifying 3 different traits: Bollgard II (Mon531), Bollgard (MON15985) and Flex (MON 88913). Each of these Taqman assays were carried out on 4 different samples of DNA: 1) WGA-amplified DNA extracted from FM9063 B2F fiber, 2) WGA-amplified DNA extracted from cotyledon FM9063 B2F, 3) purified WGA-amplified DNA of FM9063 B2F fiber and 4) purified WGA-amplified DNA of FM9063 B2F cotyledon. Because it is known that the cotton A-subgenome (At) is 50% larger than the cotton D-subgenome (Dt) in physical size, 23 SNPs were selected from the A-genome and 17 were selected from the D-genome.

[0104] It was observed that all 40 SNPs could be successfully detected in the Taqman assay in purified FM9063 B2F WGA-amplified fiber DNA. In addition, an identical result for the detection of the same 40 SNPs was obtained with the purified FM9063 B2F cotyledon WGA DNA and with raw (i.e. unpurified) FM9063 B2F cotyledon DNA. The Taqman assay for the detection of Bollgard II (Mon531), Bollgard (MON15985), and Flex (MON 88913) also proved to give identical results between the WGA-amplified DNA samples of fiber and cotyledon. Mon531 was assayed according to Yang et al (2005) J. Agric. Food Chem. 53, 6222-6229. Mon15985 and Mon88913 were assayed according to Lee et al (2007) J. Agric. Food Chem. 55, 3351-3357.

[0105] Thus, WGA-amplified cotton fiber DNA can be used as a reliable source for successful molecular marker determination since it provided identical results as a Taqman assay carried out on WGA-amplified leaf DNA. In addition, WGA-amplified cotton fiber DNA can be used as a reliable source for successful trait determination since it provided the same results as a Taqman assay carried out on WGA-amplified leaf DNA. Although usually a higher number of SNPs (about 300) should be used for the characterization and identification of a specific cotton variety, the present example shows that this approach is feasible by using WGA-amplified cotton fiber DNA.

Example 7: Other biological macromolecules isolated from cotton fiber material.

[0106] UV spectrophotometry of the extracts using the different protocols for the isolation indicate that these extracts do not consist purely of DNA and may contain other biological macromolecules. Using a Bradford analysis, a low concentration of proteins (about 10 ng/µl) was found. In addition, the fiber extracts contained various concentrations of small RNAs, resistant to RNase I, which we identified as micro RNAs.

SEQUENCE LISTING

- [0107]
- <110> Bayer Bioscience N.V. Arioli, Tony Engelen, Steven
- <120> Methods for plant fiber characterization and identification

<130> BCS 08-2011

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Claims

1. A method for characterizing a mature plant fiber or a processed plant fiber comprising the steps of
- a. isolating from said plant fiber a biological macromolecule naturally occurring in said plant fiber other than polysaccharides or lignin; and
- b. characterizing the information encoded in the sequence of the monomers of said biological macromolecule.
2. A method for identifying a mature plant fiber or a processed plant fiber comprising the steps of
- a. isolating from said plant fiber a biological macromolecule naturally occurring in said plant fiber other than polysaccharides or lignin; and
- b. subjecting said biological macromolecule to a detection assay specific for said plant fiber.
3. The method of claim 2, wherein said plant fiber is a seed fiber, preferably a cotton fiber.
4. The method of claim 2 or 3, wherein said biological macromolecule is selected from the group of polypeptides, proteins, nucleic acids, DNA, RNA, single stranded RNA and double-stranded RNA.
5. The method of any one of claims 2 to 4, wherein said detection assay is
- a. a polymerase chain reaction based detection assay;
- b. an antibody based detection assay; or
- c. a nucleic acid hybridization based detection assay.

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6. The method of any one of claims 2 to 5, wherein said detection assay detects
 - a. the presence or absence of chimeric genes present in the genome of the plant producing the fibers; or
 - b. the presence or absence of specific alleles present in the genome of the plant producing the fibers.
7. The method of claim 6, wherein said chimeric gene comprises a coding region or part or an antisense portion thereof selected from N-acetylglucosamine transferase, phosphotricinacetyltransferase, EPSPS, hydroxyphenylpyruvate dioxygenase, an insecticidal portion of *Bacillus thuringiensis* crystal protein, poly(ADP-ribose) polymerase, poly(ADP-ribose) glucosyltransferase, sucrose synthase, sucrose phosphate synthase, glucanase, cellulose synthase, chitinase, expansin, callose synthase, kinase, nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenylyl transferase, nicotinamide adenine dinucleotide synthetase or nicotine amide phosphorybosyltransferase.
8. The method of claim 6, wherein said detection assay is an event-specific detection assay.
9. The method of any one of claims 2 to 8, wherein said fiber is present in yarns, fabric, tissue or finished apparel.
10. A method for analyzing the genome of a fiber producing plant comprising the steps of
 - a. isolating nucleic acids from mature or processed fibers of said plant, said nucleic acids being naturally occurring in said plant fiber; and
 - b. subjecting said nucleic acid to a genome analysis protocol.
11. A method for isolating nucleic acid from mature plant fibers or processed plant fibers comprising the steps of
 - a. removing leaf and stem trash material from the harvested mature plant fibers; and
 - b. incubating said plant fibers in a lysis buffer containing detergents, proteases and salts for a prolonged time, preferably for a period of 4 to 100 hours; and
 - c. processing said lysis buffer according to standard nucleic acid isolation methods and isolating said DNA.
12. A method for isolating a nucleic acid from woven or knitted fabric comprising the steps of
 - a. unweaving the threads of said fabric;
 - b. incubating said threads in a lysis buffer containing detergents, proteases and salts for a prolonged time, preferably for a period of 4 to 100 hours; and
 - c. processing said lysis buffer according to standard nucleic acid isolation methods and isolating said nucleic acid.
13. Use of a method according to any one of claims 1 to 10 in a process to certify the identity of traded plant fibers.
14. A method to determine the relative amounts of different plant fibers in a mixture of mature or processed plant fibers comprising the steps of
 - a. isolating from said mixture of plant fibers a biological macromolecule naturally occurring in said plant fibers other than polysaccharides or lignin;
 - b. subjecting said biological macromolecule to detection assays specific for each of said plant fibers; and
 - c. determining the relative amount of each of the fibers.
15. A method to certify the identity of traded cotton fibers, said method comprising:
 - a. recording purchase of certified cotton seeds by registered growers, said cotton seed comprising a specific genome composition and producing a particular brand of cotton fibers;
 - b. registering bales of raw cotton fibers produced by said registered growers from said certified cotton seed as said particular brand of cotton fibers;
 - c. authenticating said bales by cross checking with said seed purchase records;
 - d. providing said registered bales to mills to produce yarns, fabrics or apparel from said brand of cotton fibers, preferably predominantly, particularly exclusively from said brand of cotton fibers;
 - e. auditing the identity of said brand of cotton fibers at one or more steps using a method according to any one of claims 1 to 10.

Patentansprüche

1. Verfahren zur Charakterisierung einer reifen Pflanzenfaser oder einer verarbeiteten Pflanzenfaser, umfassend die folgenden Schritte:
 - a. Isolieren eines biologischen Makromoleküls, das auf natürliche Weise in der Pflanzenfaser vorkommt, bei dem es sich jedoch nicht um Polysaccharide oder Lignin handelt, aus der Pflanzenfaser; und
 - b. Charakterisieren der in der Monomersequenz des biologischen Makromoleküls codierten Information.
2. Verfahren zur Charakterisierung einer reifen Pflanzenfaser oder einer verarbeiteten Pflanzenfaser, umfassend die folgenden Schritte:
 - a. Isolieren eines biologischen Makromoleküls, das auf natürliche Weise in der Pflanzenfaser vorkommt, bei dem es sich jedoch nicht um Polysaccharide oder Lignin handelt, aus der Pflanzenfaser; und
 - b. Durchführen eines für die Pflanzenfaser spezifischen Nachweis-Assays mit dem biologischen Makromolekül.
3. Verfahren nach Anspruch 2, wobei es sich bei der Pflanzenfaser um eine Samenfaser, vorzugsweise eine Baumwollfaser, handelt.
4. Verfahren nach Anspruch 2 oder 3, wobei das biologische Makromolekül aus der Gruppe Polypeptide, Proteine, Nukleinsäuren, DNA, RNA, einzelsträngige RNA und doppelsträngige RNA ausgewählt ist.
5. Verfahren nach einem der Ansprüche 2 bis 4, wobei es sich bei dem Nachweis-Assay um
 - a. einen Nachweis-Assay auf Polymerasekettenreaktionbasis;
 - b. einen Nachweis-Assay auf Antikörperbasis; oder
 - c. einen Nachweis-Assay auf Nukleinsäurehybridisierungsbasis
 handelt.
6. Verfahren nach einem der Ansprüche 2 bis 5, wobei man in dem Nachweis-Assay
 - a. das Vorliegen oder Fehlen von chimären Genen, die im Genom der Pflanze, die die Fasern produziert, vorliegen; oder
 - b. das Vorliegen oder Fehlen von spezifischen Allelen, die in dem Genom der Pflanze, die die Fasern produziert, vorliegen,
 nachweist.
7. Verfahren nach Anspruch 6, wobei das chimäre Gen eine Codierregion oder einen Teil oder einen antisense-Abschnitt davon aus der folgenden Gruppe umfasst: N-Acetylglucosamintransferase, Phosphotricinacetyltransferase, EPSPS, Hydroxyphenylpyruvatdioxygenase, insektizider Abschnitt des Kristallproteins von *Bacillus thuringiensis*, Poly(ADP-ribose)-Polymerase, Poly(ADP-ribose)-Glucohydrolase, Saccharosesynthase, Saccharosephosphatsynthase, Glucanase, Cellulosesynthase, Chitinase, Expansin, Callosesynthase, Kinase, Nicotinamidase, Nicotinatphosphoribosyltransferase, Nicotinsäuremononukleotidadenyltransferase, Nicotinamidadenindinukleotidsynthase oder Nicotinamidphosphoribosyltransferase.
8. Verfahren nach Anspruch 6, wobei es sich bei dem Nachweis-Assay um einen eventspezifischen Nachweis-Assay handelt.
9. Verfahren nach einem der Ansprüche 2 bis 8, wobei die Faser in Garnen, Stoff, Gewebe oder fertiger Kleidung vorliegt.
10. Verfahren zum Analysieren des Genoms einer faserproduzierenden Pflanze, umfassend die folgenden Schritte:
 - a. Isolieren von Nukleinsäuren aus reifen oder verarbeiteten Fasern der Pflanze, wobei die Nukleinsäuren in der Pflanzenfaser auf natürliche Weise vorkommen; und
 - b. Durchführen eines Genomanalyseprotokolls mit der Nukleinsäure.

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11. Verfahren zum Isolieren von Nukleinsäure aus reifen Pflanzenfasern oder verarbeiteten Pflanzenfasern, umfassend die folgenden Schritte:
- Entfernen von Blatt- und Stengelabfallmaterial von den geernteten reifen Pflanzenfasern; und
 - Inkubieren der Pflanzenfasern in einem Lysepuffer, der Detergentien, Proteasen und Salze enthält, über einen längeren Zeitraum, vorzugsweise über einen Zeitraum von 4 bis 100 Stunden; und
 - Verarbeiten des Lysepuffers nach standardmäßigen Nukleinsäureisolationsverfahren sowie Isolieren der DNA.
12. Verfahren zum Isolieren einer Nukleinsäure aus Gewebe oder Gestrick, umfassend die folgenden Schritte:
- Auftrennen der Fäden des Gewebes/Gestricks;
 - Inkubieren der Fäden in einem Lysepuffer, der Detergentien, Proteasen und Salze enthält, über einen längeren Zeitraum, vorzugsweise über einen Zeitraum von 4 bis 100 Stunden; und
 - Verarbeiten des Lysepuffers nach standardmäßigen Nukleinsäureisolationsverfahren sowie Isolieren der Nukleinsäure.
13. Verwendung eines Verfahrens nach einem der Ansprüche 1 bis 10 in einem Verfahren zum Zertifizieren der Identität von gehandelten Pflanzenfasern.
14. Verfahren zum Bestimmen der relativen Mengen von unterschiedlichen Pflanzenfasern in einer Mischung von reifen oder verarbeiteten Pflanzenfasern, umfassend die folgenden Schritte:
- Isolieren eines biologischen Makromoleküls, das in diesen Pflanzenfasern auf natürliche Weise vorkommt und bei dem es sich nicht um Polysaccharide oder Lignin handelt, aus der Pflanzenfasermischung;
 - Durchführen von Nachweis-Assays, die für jede der Pflanzenfasern spezifisch sind, mit dem biologischen Makromolekül; und
 - Bestimmen der relativen Mengen von jeder der Fasern.
15. Verfahren zum Zertifizieren der Identität von gehandelten Baumwollfasern, wobei das Verfahren Folgendes umfasst:
- Aufzeichnen des Ankaufs von zertifiziertem Baumwollsaatgut durch registrierte Landwirte, wobei das Baumwollsaatgut eine spezifische Genomzusammensetzung umfasst und bestimmte Markenbaumwollfasern produziert;
 - Registrieren von Ballen von Rohbaumwollfasern, die von den registrierten Landwirten aus dem zertifizierten Baumwollsaatgut als bestimmte Markenbaumwollfasern produziert wurden;
 - Authentifizieren der Ballen durch vergleichendes Prüfen mit den Aufzeichnungen des Saatgutankaufs;
 - Liefern der registrierten Ballen an Fabriken zur Produktion von Garnen, Stoffen oder Kleidung aus den Markenbaumwollfasern, vorzugsweise in erster Linie, insbesondere ausschließlich, aus den Markenbaumwollfasern;
 - Prüfen der Identität der Markenbaumwollfasern in einem oder mehreren Schritten unter Verwendung eines Verfahrens nach einem der Ansprüche 1 bis 10.
- Revendications**
1. Procédé de caractérisation d'une fibre végétale mature ou d'une fibre végétale traitée, comprenant les étapes consistant à :
- isoler, à partir de ladite fibre végétale, une macromolécule biologique existant naturellement dans ladite fibre végétale autre que des polysaccharides ou de la lignine ; et
 - caractériser les informations codées dans la séquence des monomères de ladite macromolécule biologique.
2. Procédé d'identification d'une fibre végétale mature ou d'une fibre végétale traitée, comprenant les étapes consistant à :
- isoler, à partir de ladite fibre végétale, une macromolécule biologique existant naturellement dans ladite fibre végétale autre que des polysaccharides ou de la lignine ; et

- b. soumettre ladite macromolécule biologique à un dosage par détection spécifique de ladite fibre végétale.
3. Procédé selon la revendication 2, dans lequel ladite fibre végétale est une fibre de graine, de préférence une fibre de coton.
4. Procédé selon la revendication 2 ou la 3, dans lequel ladite macromolécule biologique est choisie dans le groupe des polypeptides, protéines, acides nucléiques, ADN, ARN, ARN en simple brin et ARN en double brin.
5. Procédé selon l'une quelconque des revendications 2 à 4, dans lequel le dosage par détection est :
 - a. un dosage par détection basé sur une réaction en chaîne par polymérase ;
 - b. un dosage par détection basé sur un anticorps ; ou
 - c. un dosage par détection basé sur une hybridation d'acide nucléique.
6. Procédé selon l'une quelconque des revendications 2 à 5, dans lequel le dosage par détection détecte :
 - a. la présence ou l'absence de gènes chimères présents dans le génome de la plante qui produit les fibres ; ou
 - b. la présence ou l'absence d'allèles spécifiques présents dans le génome de la plante qui produit les fibres.
7. Procédé selon la revendication 6, dans lequel ledit gène chimère comprend une région codante ou bien une partie ou une partie antisens de celle-ci choisie parmi la N-acétylglucosamine transférase, la phosphotricinacétyltransférase, l'EPSPS, l'hydroxyphénylpyruvatedioxygénase, une partie insecticide de la protéine cristal de *Bacillus thuringiensis*, la poly(ADP-ribose) polymérase, la poly(ADP-ribose) glucohydrolase, la saccharose synthase, la saccharose-phosphate synthase, une glucanase, une cellulose synthase, une chitinase, une expansine, une callose synthase, une kinase, une nicotinamidase, une nicotinate phosphoribosyltransférase, une acide nicotinique mononucléotide adényl transférase, une nicotinamide adénine dinucléotide synthétase ou une nicotine amide phosphoribosyltransférase.
8. Procédé selon la revendication 6, dans lequel ledit dosage par détection est un dosage par détection spécifique d'un événement.
9. Procédé selon l'une quelconque des revendications 2 à 8, dans lequel ladite fibre est présente dans des fils, une étoffe, un tissu ou un textile fini.
10. Procédé destiné à analyser le génome d'une plante productrice de fibres, comprenant les étapes consistant à :
 - a. isoler des acides nucléiques à partir de fibres matures ou traitées de ladite plante, lesdits acides nucléiques existant naturellement dans ladite fibre végétale ; et
 - b. soumettre ledit acide nucléique à un protocole d'analyse du génome.
11. Procédé d'isolement d'un acide nucléique à partir de fibres végétales matures ou de fibres végétales traitées, comprenant les étapes consistant à
 - a. retirer les matières résiduelles des feuilles et tige à partir des fibres végétales matures récoltées ;
 - b. incuber lesdites fibres végétales dans un tampon pour lyse contenant des détergents, protéases et sels pendant une durée prolongée, de préférence pendant une période de 4 à 100 heures ; et
 - c. traiter ledit tampon pour lyse selon des procédés standards d'isolement d'acides nucléiques et isoler ledit ADN.
12. Procédé d'isolement d'un acide nucléique à partir de d'un tissu tissé ou à mailles, comprenant les étapes consistant à :
 - a. défaire les fils dudit tissu ;
 - b. incuber lesdits fils dans du tampon pour lyse contenant des détergents, protéases et sels pendant une durée prolongée, de préférence pendant une période de 4 à 100 heures ; et
 - c. traiter ledit tampon pour lyse selon des procédés standards d'isolement d'acides nucléiques et isoler ledit acide nucléique.
13. Utilisation d'un procédé, selon l'une quelconque des revendications 1 à 10, dans un processus de certification de l'identité de fibres végétales commercialisées.

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14. Procédé destiné à déterminer les quantités relatives de différentes fibres végétales dans un mélange de fibres végétales matures ou traitées, comprenant les étapes consistant à :
- a. isoler, à partir dudit mélange de fibres végétales, une macromolécule biologique existant naturellement dans lesdites fibres végétales autre que des polysaccharides ou de la lignine ;
 - b. soumettre ladite macromolécule biologique à des dosages par détection spécifiques de chacune desdites fibres végétales ; et
 - c. déterminer la quantité relative de chacune des fibres.
15. Procédé destiné à certifier l'identité de fibres de coton commercialisées, ledit procédé comprenant les étapes consistant à :
- a. enregistrer l'achat de graines de coton certifiées par des producteurs homologués, ladite graine de coton comprenant une composition spécifique du génome et produisant une marque particulière de fibres de coton ;
 - b. enregistrer les balles de fibres de coton brut produites par lesdits producteurs homologués à partir desdites graines de coton certifiées comme étant ladite marque particulière de fibres de coton ;
 - c. authentifier lesdites balles en les recoupant avec lesdits registres d'achats de graines ;
 - d. fournir lesdites balles enregistrées à des usines pour produire des fils, tissus ou textiles à partir de ladite marque de fibres de coton, de préférence de manière prédominante, en particulier exclusivement à partir de ladite marque de fibres de coton ;
 - e. contrôler l'identité de ladite marque de fibres de coton au niveau d'une ou plusieurs étape(s) en utilisant un procédé selon l'une quelconque des revendications 1 à 10.

1 2 3 4 5 6 7 8 9



EP 2 318 541 B1

Figure 1

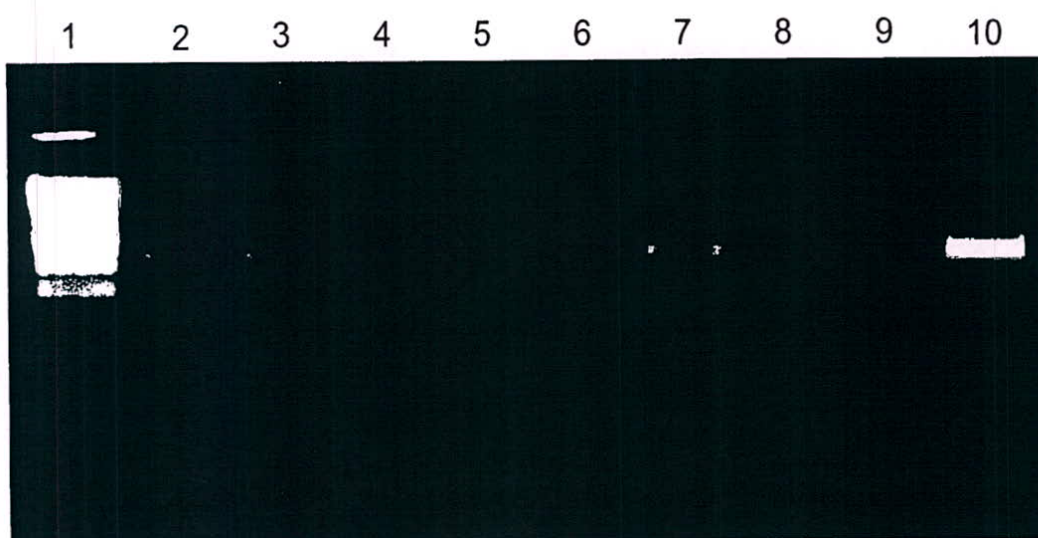
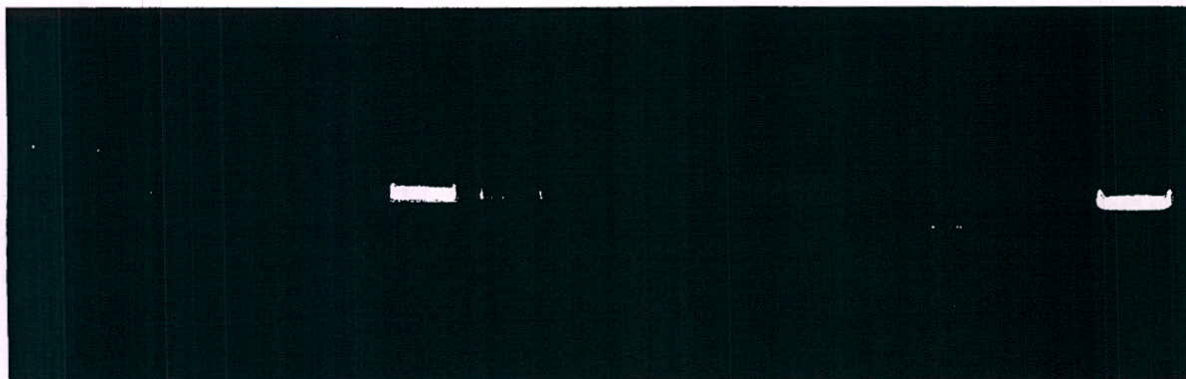


Figure 2

1 2 3 4 5 6 7 8 9 10 11 12 13



EP 2 318 541 B1

Figure 3

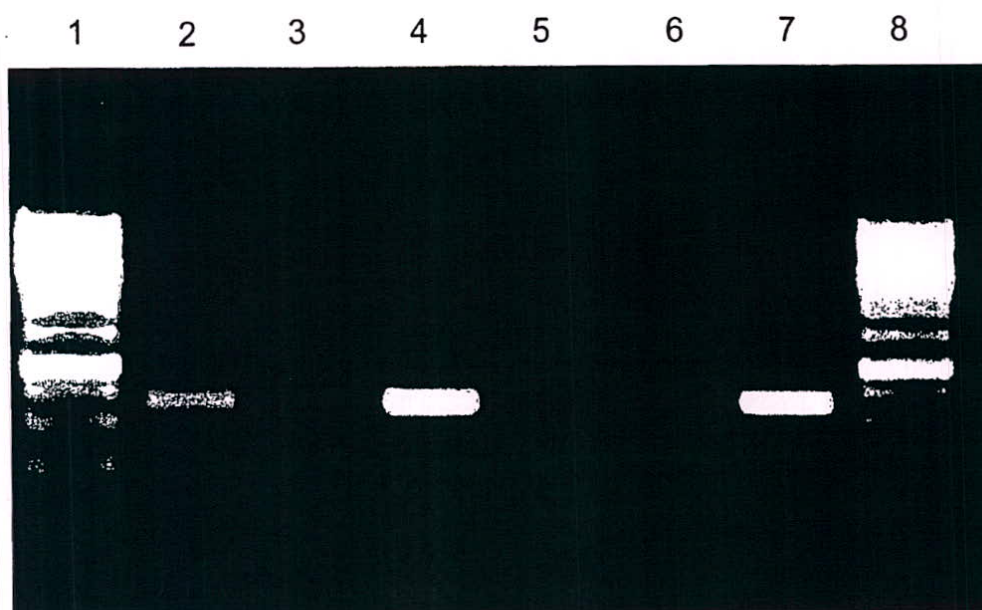
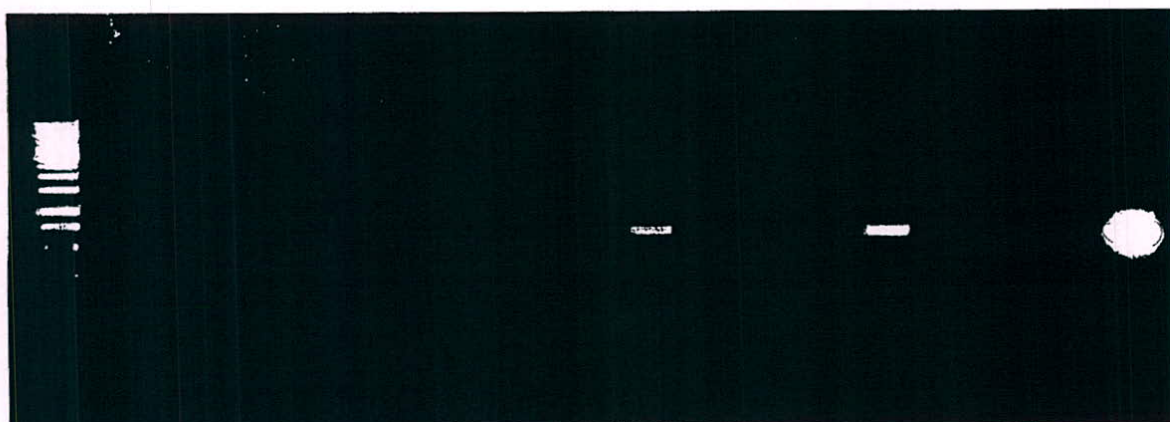


Figure 4

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



EP 2 318 541 B1

Figure 5

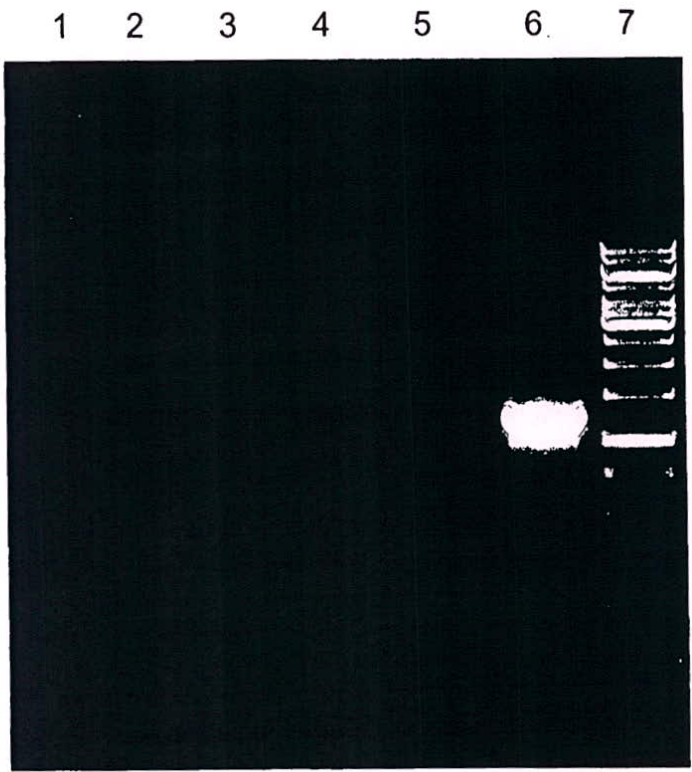
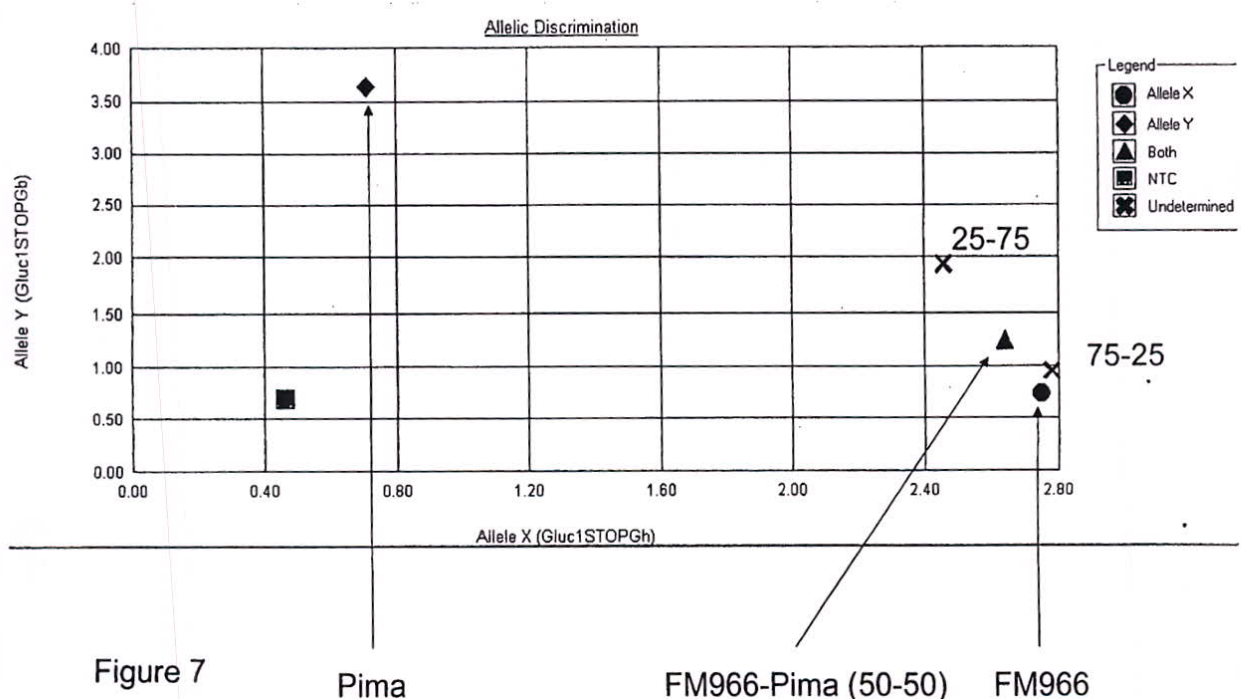


Figure 6



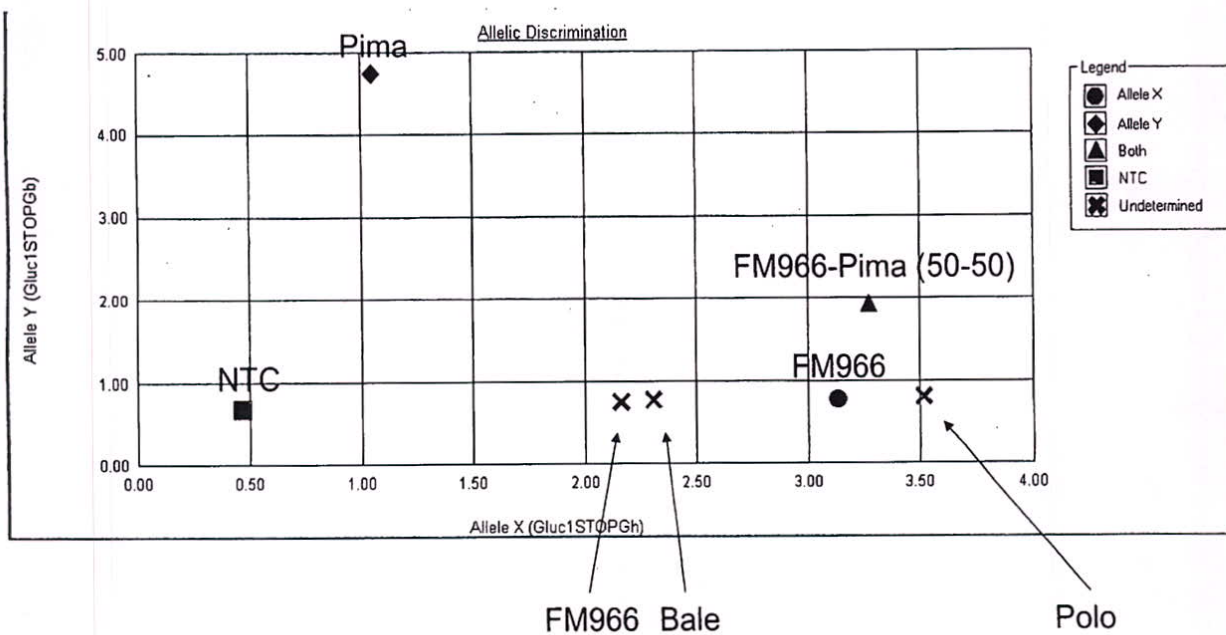


Figure 8

REFERENCES CITED IN THE DESCRIPTION

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TÍTULO DE PATENTE NO. 302009 P11/56 F

Titular(es): BAYER CROPSOURCE NV
Domicilio(s): J.E. Mommaertsiaan 14, 1831 Diegem, BELGICA
Denominación: METODOS PARA LA CARACTERIZACIÓN E IDENTIFICACIÓN DE FIBRAS VEGETALES.
Clasificación: Int.Cl.8: C12Q1/68
Inventor(es): ANTONIO ARIOLI; STEVEN ENGELIN



Número:

MX/a/2011/001427

País:

EP
US

SOLICITUD

Fecha de presentación internacional:

06 de Agosto de 2009

PRIORIDAD

Fecha:

8 de agosto de 2008
14 de agosto de 2008

Número:

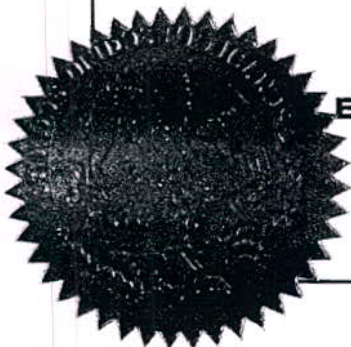
08014211.0
61/188,613

Vigencia: Veinte años

Fecha de Vencimiento: 6 de agosto de 2029

LA VIGENCIA DE ESTA PATENTE ES IMPRORRÓGABLE Y ESTÁ SUJETA AL PAGO DE LA TARIFA PARA MANTENER VIGENTES LOS DERECHOS.

Fecha de expedición: 3 de agosto de 2012



EL DIRECTOR DIVISIONAL DE PATENTES

QUÍM. FABIÁN R. SALAZAR GARCÍA

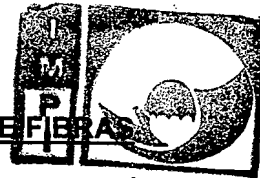


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2011/1427

MÉTODOS PARA LA CARACTERIZACIÓN E IDENTIFICACIÓN DE FIBRASInstituto
Mexicano
de la Propiedad
IndustrialVEGETALESCAMPO DE LA INVENCIÓN

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La presente invención se refiere a métodos para aislar macromoléculas biológicas que incluyen péptidos, proteínas y ácidos nucleicos, de fibras vegetales maduras tales como fibras de algodón. Las fibras vegetales pueden haber sido procesadas en hilos, los cuales a su vez pueden ser tejidos o tricotados hasta tela o indumentaria acabada, antes del aislamiento de las macromoléculas biológicas. Puesto que las macromoléculas aisladas contienen información codificada en la secuencia de sus bloques estructurales, estas macromoléculas aisladas de fibras vegetales de conformidad con los métodos actualmente descritos, pueden usarse convenientemente para caracterizar la fibra vegetal o identificar el origen o la fuente de la fibra vegetal. La información resultante puede usarse, por ejemplo, en programas de preservación de identidad para fibras vegetales especiales, certificación del origen de las fibras vegetales, o incluso en la reconstrucción del mejoramiento genético de pedigrís usando fuentes

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históricas de las fibras vegetales.



NOVEDAD DE LA INVENCION

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REIVINDICACIONES

5 1.- Un método para caracterizar una fibra vegetal madura o una
fibra vegetal procesada, que comprende los pasos de: a. aislar de dicha fibra
vegetal una macromolécula biológica de ocurrencia natural en dicha fibra
vegetal diferente de polisacáridos o lignina; y b. caracterizar la información
codificada en la secuencia de los monómeros de dicha macromolécula
10 biológica.

 2.- Un método para identificar una fibra vegetal madura o una
fibra vegetal procesada, que comprende los pasos de: a. aislar de dicha fibra
vegetal una macromolécula biológica de ocurrencia natural en dicha fibra
vegetal diferente de polisacáridos o lignina; y b. someter dicha macromolécula
15 biológica a una prueba de detección específica para dicha fibra vegetal.

 3.- El método de conformidad con la reivindicación 2,
caracterizado además porque dicha fibra vegetal es una fibra de semilla.

 4.- El método de conformidad con la reivindicación 2,
caracterizado además porque dicha fibra vegetal es una fibra de algodón.

20 5.- El método de conformidad con cualquiera de las
reivindicaciones 2 a 4, caracterizado además porque dicha macromolécula
biológica se selecciona del grupo de polipéptidos, proteínas, ácidos nucleicos,
ADN, ARN, ARN de cadena sencilla y ARN de doble cadena.



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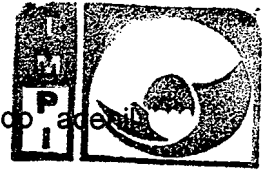
6.- El método de conformidad con cualquiera de las reivindicaciones 2 a 5, caracterizado además porque dicha prueba de detección es una prueba de detección basada en reacción en cadena de polimerasa.

5 7.- El método de conformidad con cualquiera de las reivindicaciones 2 a 5, caracterizado además porque dicha prueba de detección es una prueba de detección basada en anticuerpos.

8.- El método de conformidad con cualquiera de las reivindicaciones 2 a 5, caracterizado además porque dicha prueba de detección es una prueba de detección basada en hibridación de ácidos nucleicos.

9.- El método de conformidad con cualquiera de las reivindicaciones 2 a 8, caracterizado además porque dicha prueba de detección detecta la presencia o ausencia de genes quiméricos presentes en el genoma de la planta que produce las fibras.

10.- El método de conformidad con la reivindicación 9, caracterizado además porque dicho gen quimérico comprende una región codificante o parte de una porción antisentido de la misma seleccionada de N-acetilglucosamina transferasa, fosfinotricinacetiltransferasa, EPSPS, hidroxifenilpiruvatodioxigenasa, una porción insecticida de proteína cristalina de *Bacillus thuringiensis*, poli(ADP-ribosa) polimerasa, poli(ADP-ribosa) glucohidrolasa, sacarosa sintasa, sacarosa fosfato sintasa, glucanasa, celulosa sintasa, quitinasa, expansina, calosa sintasa, cinasa, nicotinamidasas,



nicotinato fosforribosiltransferasa, ácido nicotínico mononucleótido adenín transferasa, nicotinamida adenín dinucleótido sintetasa o nicotinamida fosforribosiltransferasa.

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11.- El método de conformidad con la reivindicación 9,
5 caracterizado además porque dicha prueba de detección es una prueba de detección específica de evento.

12.- El método de conformidad con cualquiera de las
reivindicaciones 2 a 8, caracterizado además porque dicha prueba de
detección detecta la presencia o ausencia de alelos específicos presentes en
10 el genoma de la planta que produce las fibras.

13.- El método de conformidad con cualquiera de las
reivindicaciones 2 a 12, caracterizado además porque dicha fibra está
presente en hilos, tela, tela con hilos metálicos o indumentaria acabada.

14.- Un método para analizar el genoma de una planta que
15 produce fibras, que comprende los pasos de: a. aislar ácidos nucleicos de las fibras maduras o procesadas de dicha planta, dichos ácidos nucleicos siendo de ocurrencia natural en dicha fibra vegetal; y b. someter dicho ácido nucleico a un protocolo de análisis del genoma.

15.- El método de conformidad con la reivindicación 14,
20 caracterizado además porque dicho ácido nucleico se selecciona del grupo de ADN, ARN, ARN de cadena sencilla y ARN de doble cadena.

16.- El método de conformidad con la reivindicación 14,
caracterizado además porque dicho ácido nucleico es ADN.





17.- El método de conformidad con la reivindicación 16, caracterizado además porque dicho ADN aislado es sometido a un paso de amplificación del genoma entero antes de que se someta dicho ácido nucleico a un protocolo de análisis del genoma.

5 18.- El método de conformidad con cualquiera de las reivindicaciones 14 a 17, caracterizado además porque dicho método se aplica a fibras maduras añejas, o hilos añejos, telas o indumentaria acabada.

19.- Un método para aislar un ácido nucleico de fibras vegetales maduras o fibras vegetales procesadas, que comprende los pasos de: a.
10 remover el material de desperdicio del tallo y de la hoja de las fibras vegetales maduras cosechadas; y b. incubar dichas fibras vegetales en un regulador de pH de lisis que contiene detergentes, proteasas y sales por un tiempo prolongado, de preferencia por un período de 4 a 100 horas; y c. procesar dicho regulador de pH de lisis de acuerdo con métodos estándar de
15 aislamiento de ácido nucleicos, y aislar dicho ADN.

20.- El método de conformidad con la reivindicación 19, caracterizado además porque dicho ácido nucleico es ADN.

21.- Un método para aislar un ácido nucleico de tela tejida o tricotada, que comprende los pasos de: a. destejer los hilos de dicha tela; b.
20 incubar dichos hilos en un regulador de pH de lisis que contiene detergentes, proteasas y sales por un tiempo prolongado, de preferencia por un período de 4 a 100 horas; y c. procesar dicho regulador de pH de lisis de acuerdo con





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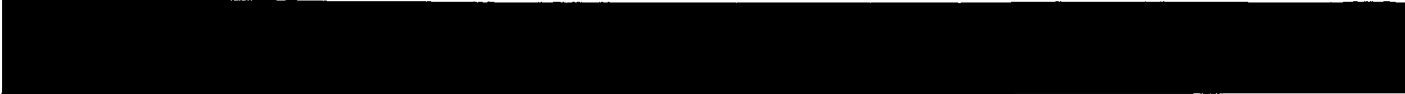
métodos estándar de aislamiento de ácido nucleicos, y aislar dicho ácido nucleico.

22.- El método de conformidad con la reivindicación 1, caracterizado además porque dicho ácido nucleico es ADN.

5 23.- El uso de un método de cualquiera de las reivindicaciones 1 a 18, en un procedimiento para certificar la identidad de fibras vegetales comercializadas.

 24.- Un método para determinar las cantidades relativas de diferentes fibras vegetales en una mezcla de fibras vegetales maduras o
10 procesadas, que comprende los pasos de: a. aislar de dicha mezcla de fibras vegetales una macromolécula biológica de ocurrencia natural en dichas fibras vegetales diferentes de polisacáridos o lignina; b. someter dicha macromolécula biológica a pruebas de detección específicas para cada una de dichas fibras vegetales; y c. determinar la cantidad relativa de cada una de
15 las fibras.

 25.- Un método para certificar la identidad de fibras de algodón comercializadas, dicho método comprendiendo: a. registrar la compra de semillas de algodón certificadas por agricultores registrados, dicha semilla de algodón comprendiendo una composición específica del genoma y
20 produciendo una marca particular de fibras de algodón; b. registrar balas de fibras de algodón en bruto producidas por dichos agricultores registrados de dichas semillas de algodón certificadas como dicha marca particular de fibras de algodón; c. autenticar dichas balas por verificación cruzada con dichos





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- registros de compra de la semilla; d. proveer dichas balas registradas a molinos para producir hilos, telas o indumentaria de dicha marca de fibras de algodón, de preferencia predominantemente, en particular exclusivamente de dicha marca de fibras de algodón; y e. auditar la identidad de dicha marca de
- 5 fibras de algodón en uno o más pasos usando un método de cualquiera de las reivindicaciones 1 a 18.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference BCS08-2011-W	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/EP2009/005891	International filing date (day/month/year) 06/08/2009	(Earliest) Priority Date (day/month/year) 08/08/2008
Applicant BAYER BIOSCIENCE N.V.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed
☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (See Box No. II)

3. ☐ **Unity of invention is lacking** (see Box No. III)

4. With regard to the **title**,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings**,

- a. the figure of the **drawings** to be published with the abstract is Figure No. _____
☐ as suggested by the applicant
☐ as selected by this Authority, because the applicant failed to suggest a figure
☐ as selected by this Authority, because this figure better characterizes the invention
b. ☒ none of the figures is to be published with the abstract

85

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2009/005891

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
- a. type of material
- ☒ a sequence listing
- ☐ table(s) related to the sequence listing
- b. format of material
- ☒ on paper
- ☒ in electronic form
- c. time of filing/furnishing
- ☒ contained in the international application as filed
- ☒ filed together with the international application in electronic form
- ☐ furnished subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/005891

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

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)

C12Q

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

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

EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HSU C Y ET AL: "Transcriptional regulation of the lipid transfer protein gene LTP3 in cotton fibers by a novel MYB protein" PLANT SCIENCE, ELSEVIER IRELAND LTD, IE, vol. 168, no. 1, 1 January 2005 (2005-01-01), pages 167-181, XP025296010 ISSN: 0168-9452 [retrieved on 2005-01-01] figures 4-5 abstract the whole document ----- -/--	1-25



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

9 February 2010

Date of mailing of the international search report

22/02/2010

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Helliot, Bertrand

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/005891

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LUO M ET AL: "Cloning and Expression Analysis of a Brassinosteroid Biosynthetic Enzyme Gene, GhDWF1, from Cotton (Gossypium hirsutum L.)" AGRICULTURAL SCIENCES IN CHINA,, vol. 6, no. 11, 1 November 2007 (2007-11-01), pages 1297-1305, XP022855619 ISSN: 1671-2927 [retrieved on 2007-11-01] figure 5 abstract the whole document	1-25
A	IQBAL SAIMA ET AL: "Identification of differentially expressed genes in developing cotton fibers (Gossypium hirsutum L.) through differential display" ELECTRONIC JOURNAL OF BIOTECHNOLOGY, vol. 11, no. 1, January 2008 (2008-01), page Article No.: 11, XP002567634 ISSN: 0717-3458 figure 1 abstract the whole document	1-25

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43bis.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.
PCT/EP2009/005891

International filing date (day/month/year)
06.08.2009

Priority date (day/month/year)
08.08.2008

International Patent Classification (IPC) or both national classification and IPC
INV. C12Q1/68

Applicant
BAYER BIOSCIENCE N.V.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:



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Date of completion of
this opinion

see form
PCT/ISA/210

Authorized Officer

Helliot, Bertrand

Telephone No. +49 89 2399-7793



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/EP2009/005891

Box No. I	Basis of the opinion
1.	With regard to the language, this opinion has been established on the basis of: ☒ the international application in the language in which it was filed ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).
2.	☐ This opinion has been established taking into account the rectification of an obvious mistake authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))
3.	With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of: a. type of material: ☒ a sequence listing ☐ table(s) related to the sequence listing b. format of material: ☒ on paper ☒ in electronic form c. time of filing/furnishing: ☒ contained in the international application as filed. ☒ filed together with the international application in electronic form. ☐ furnished subsequently to this Authority for the purposes of search.
4.	☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5.	Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/EP2009/005891

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	<u>1-25</u>
	No: Claims	
Inventive step (IS)	Yes: Claims	<u>1-25</u>
	No: Claims	
Industrial applicability (IA)	Yes: Claims	<u>1-25</u>
	No: Claims	

2. Citations and explanations

see separate sheet

ITEM V:

1 INTRODUCTION

1.1 The following documents (D1-D3) are referred to in this communication; the numbering will be adhered to in the rest of the procedure:

- D1 HSU C Y ET AL: "Transcriptional regulation of the lipid transfer protein gene LTP3 in cotton fibers by a novel MYB protein" PLANT SCIENCE, ELSEVIER IRELAND LTD, IE, vol. 168, no. 1, 1 January 2005 (2005-01-01) , pages 167-181, XP025296010 ISSN: 0168-9452 [retrieved on 2005-01-01]
- D2 LUO M ET AL: "Cloning and Expression Analysis of a Brassinosteroid Biosynthetic Enzyme Gene, GhDWF1, from Cotton (Gossypium hirsutum L.)" AGRICULTURAL SCIENCES IN CHINA,, vol. 6, no. 11, 1 November 2007 (2007-11-01) , pages 1297-1305, XP022855619 ISSN: 1671-2927 [retrieved on 2007-11-01]
- D3 IQBAL SAIMA ET AL: "Identification of differentially expressed genes in developing cotton fibers (Gossypium hirsutum L.) through differential display" ELECTRONIC JOURNAL OF BIOTECHNOLOGY, vol. 11, no. 1, January 2008 (2008-01) , page Article No.: 11, XP002567634 ISSN: 0717-3458

1.2 The present application relates to methods for the isolation of biological macromolecules including peptides, proteins, and nucleic acids from mature plant fibers such as cotton fibers and from textiles. The biological macromolecules can be used to characterize the plant fiber (source, origin, genomic composition of producing plant etc.).

2 NOVELTY (Art. 33 (1) and (2) PCT)

No cited prior-art document discloses methods for extracting natural biological macromolecules from mature or processed plant fibers, such as cotton fibers, particularly from textiles, yarns or apparel, and further to be able to derive plant source related information from such plant fibers.

Therefore, the method for characterizing mature and/or processed plant fibers (**claim 1**), the method for identifying a mature and/or processed plant fiber (**claims 2-13**), the method for analysing the genome of a fiber producing plant (**claims 14-18**), the use of said methods (**claim 23**), the method of isolating nucleic acid from mature and or processed plant fibers (**claim 19**) or from woven or knitted fabric (**claims 21-22**), the method to certify the identity of traded cotton fibers (**claim 25**) are considered novel in the sense of Art. 33(1) and (2) PCT.

3 INVENTIVENESS (Art. 33(2) and (3) PCT)

3.1 Moreover, the subject-matter of **claim 1** could involve an inventive step over the prior art (Art. 33 (1) and (3) PCT).

D1 reports the isolation and characterization of two fiber Myb cDNAs (GhMyb 7 and 9) and their corresponding genes from cotton (*G. hirsutum* L. cv. DES119). Northern blot analysis was used to examine the expression pattern of GhMyb 7/9 gene in fibers and other cotton tissues. Total RNA (10 mug) isolated from leaves, flowers, roots, and fibers at 5 DPA, 10 DPA, 15 DPA, and 20 DPA (days after pollination) were electrophoresed on an agarose gel and hybridized with the TRR region of GhMyb 7 (see Fig. 5 and the whole document).

D2 investigates the growth and development mechanism of cotton fibers. The expression level of the GhDWFI gene in various tissues of cotton plants and at different developmental stages of fibers (0, 4, 12 and 23 DPA fibers) is analysed in Fig. 5 (see also the whole document).

D3 performs non-radioactive differential display reverse transcriptase PCR (DDRT-PCR) on purified mRNAs in order to identify genes involved in cotton fiber development. This technique was tested on mRNA isolated from five different developmental stages of cotton fiber including 0, 5, 10, 15 and 20 DPA (see Fig. 1).

The subject-matter of **claim 1** differs from that of D1-D3 in that nucleic acids from mature or processed plant fibers are extracted and analysed.

The morphological development of cotton fibers has been well documented. Thus, cotton fibers, in particular from *Gossypium hirsutum*, undergo four overlapping developmental stages: fiber cell initiation, elongation, secondary cell wall biosynthesis, and maturation. **Fiber cell initiation** is a rapid process. White fuzzy fibers begin to develop immediately after anthesis and continue up to about 3 days post-anthesis (DPA), which is followed by **fiber cell elongation** (until about 10 to about 17 DPA). Depending upon growth

conditions, **secondary cell wall biosynthesis** initiates and continues to about 25 to about 40 DPA, followed by a **maturation** process until about 45 to about 60 DPA (see application, [0004]).

The technical problem to be solved by the subject-matter of said **claim 1** may be regarded as applying a well known characterization method to a cotton fiber at an alternative developmental stage.

Examples 1 and 2 demonstrates that nucleic acids can be extracted from mature cotton fiber material and further analysed (see Fig. 1). When nucleic acid obtained after overnight incubation of textile were used, the expected amplicon of 669 bp could be detected, indicating that the protocol can be used to isolate nucleic acids from cotton fibers after spinning and weaving (see Fig. 4).

The extraction protocol according to the present application appears to solve the problem.

In view of that and since there is no suggestion or hint which could be find in D1-D3 to amend or adapt the method disclosed in the aforecited documents, nor is any incentive to combine the teaching of D1-D4 with any other document of the prior art, an inventive step can be acknowledged for the method of **claim 1** and the requirements of Art. 33 (1) and (3) PCT could be fulfilled.

- 3.2 For the same reasons as set out under item 2-a herein above, an inventive merit can be acknowledged for the method for identifying a mature and/or processed plant fiber (**claims 2-13**), the method for analysing the genome of a fiber producing plant (**claims 14-18**), the use of said methods (**claim 23**), the method of isolating nucleic acid from mature and or processed plant fibers (**claim 19**) or from woven or knitted fabric (**claims 21-22**), the method to certify the identity of traded cotton fibers (**claim 25**). The requirements of Art. 33 (1) PCT could be fulfilled.

4 CLARITY (Art. 6 PCT)

The subject-matter of **claim 19** lacks clarity (Art. 6 PCT) because the term "prolonged time" is vague and leaves the skilled person in doubt as to the scope of said feature (see also PCT International Search and Preliminary Examination Guidelines Chap. 5, Item 5.34).

Moreover, the Applicant is kindly informed that the term "preferably" used in **claim 19** has no limiting effect on the scope of said claims. The features following this expression are entirely optional (see also PCT International Search and Preliminary Examination Guidelines Chap. 5, Item 5.38).