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(54) Title: GENETIC TRANSFORMATION OF JATROPHA CURCAS

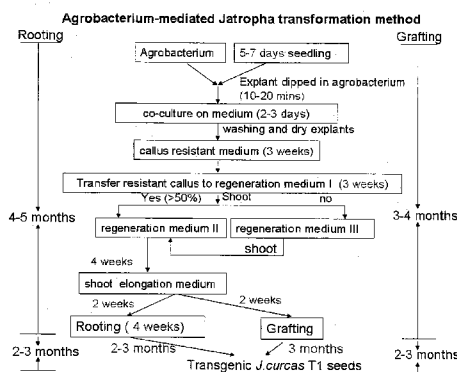


Figure 1

(57) Abstract: The present invention relates to methods for the regeneration and *Agrobacterium*-mediated transformation of plants in the genera of *Jatropha*, more specifically, in *Jatropha curcas*.

GENETIC TRANSFORMATION OF *JATROPHA CURCAS*

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application claims priority to U.S. provisional patent application Serial No. 61/122,454 filed 15 December 2008, which application is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] The present invention relates to the field of plant regeneration and transformation, particularly to methods for the regeneration and transformation of *Jatropha*. More specifically, the present invention relates to a method and media compositions for regeneration and transformation of plants of *Jatropha curcas*.

[0003] The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the Bibliography.

[0004] The world is facing dwindling supply of fossil fuel and worsening Green House Effect. There is an urgent demand to increase production and consumption of renewable energy. Biofuels have been recognized as a national priority for many countries in their search for alternative sources to meet their energy security needs and at the same time help reduce CO₂ emissions that cause the Green House Effect. The demand for biofuel has put increasing pressure on food production. For example, to satisfy the biofuel need for Germany in 2017 as mandated by the German government the entire farm land of this country would have to be used for growing bioenergy crops with no land left for food production. To ease this competition for land and to satisfy our need for renewable fuels, there is a strong need to utilize marginal land for bio-energy production.

[0005] *Jatropha curcas* is a small woody plant belonging to the Euphorbiaceae family. Several unique characters of *Jatropha curcas* make it an ideal plant for biodiesel production. These include its rapid growth, easy propagation, low cost of seeds, high oil content, short gestation period, wide adaptability, drought tolerance and the ability to thrive on degraded soils. Moreover, its plant size renders convenient collection of seed (Jones, 1991; Sujatha et al., 2008).

[0006] However, *Jatropha* suffers from several shortcomings that may limit its wide adoption. The productivity of the plant is constrained by the unfavourable male to female flower ratio and its oil content has not been optimized by breeding. This plant is also sensitive to biotic stresses such as viral (Narayanna et al., 2007), fungal and bacterium pathogens and abiotic stresses, especially cold and drought (<http://www.jatropha.org>). The

presence of several toxic components (e.g. the protein toxin, curcin, and the cancer-causing agent phorbol esters) in seeds and leaves of the plant possess health hazards for farmers and bioprocess workers in the *Jatropha* industry.

[0007] The traditional way to improve on quality traits of plants is by breeding for superior genotypes. However, an assessment of genetic diversity using molecular markers disclosed low inter-accessional variability amongst local *J. curcas* germplasm (Sujatha et al., 2008). Therefore, alternative genetic manipulation tools, such as genetic transformation methods, are urgently required to provide additional strategies for genetic improvement of this crop. *Agrobacterium*-mediated genetic transformation has become the principal choice for generating transgenic plants. However, very few reports have appeared on the use of *Agrobacterium*-mediated transformation of plants belonging to the *Euphorbiaceae* family. The only one reported transformation protocol for *Jatropha* (Li et al., 2008) is not reproducible in our hands.

[0008] Thus, there is a need for methods of transforming *J. curcas* to provide means for genetic improvement in this crop species.

SUMMARY OF THE INVENTION

[0009] The present invention relates to methods for the regeneration and *Agrobacterium*-mediated transformation of plants in the genera of *Jatropha*, more specifically, in *Jatropha curcas*.

[0010] Thus, in one aspect the present invention provides an efficient and reproducible plant regeneration protocol for *J. curcas* by optimizing tissue culture and shoot regeneration conditions. This regeneration protocol has been used in combination with *Agrobacterium*-mediated transformation to produce T₀ transgenic *Jatropha* shoots/plants. The present invention also provides the use of a grafting step using T₀ transgenic shoots as scions and non-transgenic plants as root stocks. This grafting step obviates the need for regenerated plants to produce roots in tissue culture and considerably shortens the time for transgenic shoots to flower and produce T₁ seeds.

[0011] In one embodiment, the present invention provides a method of regenerating *J. curcas* plants. According to this embodiment, explants are obtained from cotyledons from 5-7 day old seedlings. The explants are cultured on callus formation medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose and 6-benzylaminopurine (6-BA) and 1-naphthaleneacetic acid (NAA) as plant hormones. Callus tissue is then transferred to a first shoot regeneration medium which comprises MS mineral salts, B5 vitamins, citric acid,

glutamine, casein hydrolysate, adenine, sucrose and 6-BA and 3-indolebutyric acid (IBA) as plant hormones. Any shoots that regenerated from the callus tissue are transferred to a second shoot regeneration medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose and 6-BA, IBA and gibberellic acid (GA₃) as plant hormones. Callus tissue with no regenerates shoots are transferred to a third shoot regeneration medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose and 6-BA and IBA as plant hormones for further regeneration of shoots. The shoots that have regenerated are transferred to a shoot elongation medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose and 6-BA and GA₃ as plant hormones for elongation and bud multiplication. Elongated shoots are transferred to a rooting medium which comprises MS mineral salts, B5 vitamins, sucrose and IBA. After rooting, the plantlets are transferred to soil. Alternatively, the elongated shoots can be grafted to *J. curcas* root stock.

[0012] In a second embodiment, the present invention provides a method for *Agrobacterium*-mediated transformation of *J. curcas* plants. According to this embodiment, the *Agrobacterium*-mediated transformation of *J. curcas* utilizes the same basic scheme as described above for the regeneration of *J. curcas*. For transformation, the explants are first co-cultured with *Agrobacterium* cells prior to transfer to the callus formation medium with subsequent transfers to the shoot regeneration media, shoot elongation medium and rooting medium as described above. The co-culturing medium comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, glucose, acetosyringone and 6-BA and NAA as plant hormones. The callus formation medium is the same as for regeneration except that it further comprises a selective agent and an *Agrobacterium* eradicator. Similarly, the shoot regeneration media further comprise a selective agent and an *Agrobacterium* eradicator. For transformation, culturing on the callus formation medium is performed in the dark. Conventional selective agents can be used for the *Agrobacterium*-mediated transformation of *J. curcas* plants. Examples of selective agents include, but are not limited to, the herbicide BASTA, hygromycin and the like.

BRIEF DESCRIPTION OF THE FIGURES

[0013] Figure 1 illustrates an *Agrobacterium*-mediated *Jatropha* transformation method in accordance with the present invention. The time scale listed in the left is to use rooting protocol while the left is to use grafting protocol.

[0014] Figure 2 illustrates *Agrobacterium* transformation vectors utilized to demonstrate the transformation method of the present invention.

[0015] Figures 3A-3K show the transformation, regeneration, flowering and seedling of *J. curcas*. Fig. 3A: *J. curcas* MD 5 day's seedling suitable for transformation. Fig. 3B: Callus formation and shoot generation. Left, cotyledons inoculated with *Agrobacterium* without carrying any vector. Right, cotyledons inoculated with *Agrobacterium* carrying a vector with a trait gene. Note shoot regeneration from explants. Fig. 3C: an enlarged view of hygromycin-resistant callus and shoot-like organs on the surface of brownish cotyledons. Fig. 3D: Regeneration of hygromycin-resistant shoots of *J. curcas*. Fig. 3E: Shoot elongation. Fig. 3F: Rooting of transgenic shoots. Fig. 3G: High rooting efficacy for transgenic *J. curcas*. Fig. 3H: Transgenic *J. curcas* grown on soil. Fig. 3I and Fig. 3J: Transgenic *J. curcas* shoots grafted onto non-transgenic rootstock. White arrow indicates the grafting site. Fig. 3K: Transgenic *J. curcas* flowering and seeding. The scale bars indicate 10mm.

[0016] Figure 4 shows PCR analyse of hyg-resistant ubi:GFP *J. curcas* plants. Lane -: wildtype *Jatropha* control; Lane +: plasmid DNA of p1300-GFP; Lanes #1-#10 from hygromycin-resistant *Jatropha* shoot leaves.

[0017] Figures 5A-5P show the expression of GFP in T_0 plant root (Fig. 5B, Fig. 5D), male flower (Fig. 5F, Fig. 5H), and T_1 seeds 3 week after fertilization (Fig. J, Fig. K, Fig. L, Fig. N, Fig. O, Fig. P). Fig. A, Fig. C, Fig. E, Fig. G, Fig. I and Fig. M are wildtype controls for every plant organ. The scale bars indicate 2mm.

[0018] Figure 6 shows PCR analyses of BASTA-resistant 35S:JcWRI1 *J. curcas* plants. Lane M, DNA ladder; Lane #1-#7 from BASTA-resistant *Jatropha* shoot leaves; lane -, wildtype control; lane +, plasmid DNA of pBA002-MYC-JcWRI1.

[0019] Figure 7 shows Western blot analysis of RcFAH12 and JcDGAT1 levels in leaves of transgenic *Jatropha* plants expressing 35S:RcFAH12 and 35S:JcDGAT1 using anti-HA antibody. Bottom panel: Coomassie Bright Blue staining of the RUBL (the large subunit of RUBISCO) which serves as a loading control.

DETAILED DESCRIPTION OF THE INVENTION

[0020] The present invention relates to methods for the regeneration and *Agrobacterium*-mediated transformation of plants in the genera of *Jatropha*, more specifically, in *Jatropha curcas*.

[0021] In one aspect, the present invention provides a method of regenerating *J. curcas* plants. According to this embodiment, explants are obtained from cotyledons from about 5 day to about 12 day old seedlings, preferably about 5-7 day old seedlings. The culturing is performed in the light at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a 16h light ($100\text{ }\mu\text{mol/m}^2\text{S}$)/8h dark cycle. The seedlings are grown in tissue culture. Seed kernels of *J. curcas* are surface sterilized using conventional techniques and immersed in sterile water overnight at 28°C in the dark. The endosperm-free embryos are germinated on hormone free germination medium with the radicals in contact with the medium. The germination medium comprises $\frac{1}{2}$ strength MS mineral salts, B5 vitamins and sucrose. The concentration of sucrose is about 5% (w/v). The germination medium may further comprise a buffer. In one embodiment, the buffer is 2-(4-morpholino)ethanesulfonic acid (MES) at about 0.5 g/L at a pH of about 5.6. The germination medium is solidified with agar or phytogel. The culturing is performed in the light at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in a 16h light ($100\text{ }\mu\text{mol/m}^2\text{S}$)/8h dark cycle.

[0022] The explants are cultured on callus formation medium in the dark for about 2 weeks to about 3 weeks, preferably about three weeks. The callus formation medium comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose and 6-benzylaminopurine (6-BA) and 1-naphthaleneacetic acid (NAA) as plant hormones. The concentration of citric acid is about 10 mg/L to about 30 mg/L, preferably about 10 mg/L. The concentration of glutamine is about 150 mg/L to about 200 mg/L, preferably about 150 mg/L. The concentration of casein hydrolysate is about 100 mg/L. The concentration of sucrose is about 3%. The concentration of 6-BA is about 1.5 mg/L. The concentration of NAA is about 0.05 mg/L. The callus formation medium preferably further comprises MgCl_2 at a concentration of about 0.5 g/L to about 0.95 g/L, preferably 0.5 g/L. The callus formation medium has a pH of about 5.8 to about 6.0. The callus formation medium is solidified with agar or phytogel, preferably phytogel at a concentration of about 2.5 g/L to about 3 g/L, preferably 2.5 g/L.

[0023] Callus tissue is then transferred to a first shoot regeneration medium and cultured in the light for about 2 weeks to about 3 weeks, preferably about three weeks. The first shoot regeneration medium comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, adenine, sucrose and 6-BA and 3-indolebutyric acid (IBA) as plant hormones. The concentrations of the citric acid, glutamine, casein hydrolysate and 6-BA are the same as in the callus formation medium. The concentration of adenine is about 2 mg/L to about 4 mg/L, preferably about 2 mg/L. The concentration of IBA is about 0.05 mg/L. The first shoot regeneration medium preferably further comprises MgCl_2 at a concentration of about 0.5 g/L to

about 0.95 g/L, preferably 0.5 g/L. The first shoot regeneration medium has a pH of about 5.8 to about 6.0. The first shoot regeneration medium is solidified with agar or phytigel, preferably phytigel at a concentration of about 2.5 g/L to about 3.0 g/L, preferably 2.5 g/L.

[0024] Any shoots that regenerated from the callus tissue are transferred to a second shoot regeneration medium and cultured in the light for about 3 weeks to about 4 weeks, preferably about four weeks. The second shoot regeneration medium comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose and 6-BA, IBA and gibberellic acid (GA₃) as plant hormones. The concentrations of the citric acid, glutamine, casein hydrolysate, 6-BA and IBA are the same as in the first shoot regeneration medium. The concentration of GA₃ is about 0.05 mg/L to about 0.5 mg/L, preferably about 0.5 mg/L. The second shoot regeneration medium preferably further comprises MgCl₂ at a concentration of about 0.5 g/L. The second shoot regeneration medium has a pH of about 5.8 to about 6.0. The second shoot regeneration medium is solidified with agar or phytigel, preferably agar at a concentration of about 6.5 g/L to about 7 g/L, preferably 7 g/L.

[0025] Callus tissue with no regenerates shoots are transferred to a third shoot regeneration medium and cultured in the light for about 4 weeks to about 5 weeks, preferably about 4 weeks. The third shoot regeneration medium comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose and 6-BA and IBA as plant hormones for further regeneration of shoots. The concentration of the citric acid, glutamine, casein hydrolysate, 6-BA and IBA are the same as in the first shoot regeneration medium. The third shoot regeneration medium preferably further comprises MgCl₂ at a concentration of about 0.5 g/L to about 0.95 g/L, preferably 0.5 g/L. The third shoot regeneration medium has a pH of about 5.8 to about 6.0. The third shoot regeneration medium is solidified with agar or phytigel, preferably phytigel at a concentration of about 2.5 g/L to about 3 g/L, preferably 2.5 g/L.

[0026] The shoots that have regenerated on the second shoot regeneration medium are transferred to a shoot elongation medium and cultured in the light for about 2 weeks to about 3 weeks, preferably about two weeks. The shoot elongation medium comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose and 6-BA and GA₃ as plant hormones for elongation and bud multiplication. The concentrations of the citric acid, glutamine and casein hydrolysate are the same as in the first shoot regeneration medium. The concentration of 6-BA is about 0.3 mg/L. The concentration of GA₃ is about 0.1 mg/L to about 0.5 mg/L, preferably about 0.1 mg/L. The shoot elongation medium has a pH of about 5.8 to

about 6.0. The shoot elongation medium is solidified with agar or phytigel, preferably agar at a concentration of about 6.5 g/L to about 7 g/L, preferably 7 g/L.

[0027] Elongated shoots are transferred to a rooting medium and cultured in the light for about 3 weeks to about 4 weeks, preferably about four weeks. The rooting medium comprises MS mineral salts, B5 vitamins, sucrose and IBA. The concentration of sucrose is about 3%. The concentration of IBA is about 0.07 g/L. The rooting medium has a pH of about 5.6. The rooting medium is solidified with agar or phytigel, preferably phytigel at a concentration of about 2.2 g/L. After rooting, the plantlets are transferred to soil. Alternatively, the elongated shoots can be grafted to *J. curcas* root stock using conventional techniques instead of being transferred to the rooting medium.

[0028] In a second aspect, the present invention provides a method for *Agrobacterium*-mediated transformation of *J. curcas* plants. According to this embodiment, the *Agrobacterium*-mediated transformation of *J. curcas* utilizes the same basic scheme as described above for the regeneration of *J. curcas*. Vectors containing DNA of interest are introduced into *Agrobacterium* using conventional techniques, such as electroporation. Transformed *Agrobacterium* cells are cultured prior to use using conventional techniques. In accordance with one such technique, *Agrobacterium* cells are inoculated into LB medium supplemented with kanamycin and carbicillin. The concentration of kanamycin is about 25 mg/L to about 100 mg/L; preferably about 50 mg/L. The concentration of carbicillin is about 50 mg/L to about 100 mg/L, preferably about 100 mg/L. The *Agrobacterium* cells are grown overnight at 28° C, 250 rpm. The *Agrobacterium* cells are collected by centrifugation and re-suspended in liquid MS medium supplemented with sucrose, glucose, acetosyringone (AS), 6-BA and NAA. The concentration of sucrose is about 30 g/L. The concentration of glucose is about 10 g/L. The concentration of AS is about 20 mg/L. The concentration of 6-BA is about 1.5 mg/L. The concentration of NAA is about 0.05 mg/L to about 0.1 mg/L, preferably about 0.1 mg/L.

[0029] For transformation, the explants are first co-cultured with *Agrobacterium* cells prior to transfer to the callus formation medium with subsequent transfers to the shoot regeneration media, shoot elongation medium and rooting medium as described above. The co-culturing is performed in the dark for about 2-3 days. The co-culturing medium comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, AS and 6-BA and NAA as plant hormones. The concentrations of citric acid, glutamine, casein hydrolysate and sucrose are the same as in the callus formation medium. The concentration of AS is about 20 mg/L. The concentration of 6-BA is about 1.5 mg/L. The concentration of NAA is about 0.05 mg/L to

about 0.1 mg/L, preferably about 0.05 mg/L. The co-culturing medium may further comprise a suitable buffer. In one embodiment, the buffer is MES. The concentration of MES is about 0.5 g/L at a pH of about 5.0 to about 5.2.

[0030] The callus formation medium used for the *Agrobacterium*-mediated transformation of *J. curcas* is the same as that used for regeneration except that it further comprises a selective agent and an *Agrobacterium* eradicator. The selective agent may be any selective agent for which a marker gene, such as described below, has been included in the transformed *Agrobacterium*. In one embodiment, the selective agent is hygromycin at a concentration of about 3 mg/L to about 5 mg/L, preferably 3.5 mg/L. In another embodiment, the selective agent is glufosinate ammonium at a concentration of about 1 mg/L. The *Agrobacterium* eradicator may be any conventional eradicator, such as cefotaxime, and the like. In one embodiment, the *Agrobacterium* eradicator is cefotaxime at a concentration of about 100 mg/L to about 150 mg/L, preferably 100 mg/L. Culturing on the callus formation medium for *Agrobacterium*-mediated transformation is performed in the dark for about 2 weeks to about 3 weeks, preferably about 3 weeks.

[0031] The callus tissue is then treated as described above with respect to the regeneration of *J. curcas* with transfers and culturing in the light as described above to the first shoot regeneration medium, second shoot regeneration medium, third shoot regeneration medium, shoot elongation medium, rooting medium or grafting. The first shoot regeneration medium used for the *Agrobacterium*-mediated transformation of *J. curcas* is the same as that used for regeneration except that it further comprises a selective agent and an *Agrobacterium* eradicator. The selective agent may be any selective agent for which a marker gene, such as described below, has been included in the transformed *Agrobacterium*. In one embodiment, the selective agent is hygromycin at a concentration of about 3 mg/L to about 5 mg/L, preferably 3.5 mg/L. In another embodiment, the selective agent is glufosinate ammonium at a concentration of about 1 mg/L. The *Agrobacterium* eradicator may be any conventional eradicator, such as cefotaxime. In one embodiment, the *Agrobacterium* eradicator is cefotaxime at a concentration of about 100 mg/L to about 150 mg/L, preferably 100 mg/L.

[0032] The second shoot regeneration medium used for the *Agrobacterium*-mediated transformation of *J. curcas* is the same as that used for regeneration except that it further comprises a selective agent and an *Agrobacterium* eradicator. The selective agent may be any selective agent for which a marker gene, such as described below, has been included in the transformed *Agrobacterium*. In one embodiment, the selective agent is hygromycin at a

concentration of about 4 mg/L to about 5 mg/L, preferably 4 mg/L. In another embodiment, the selective agent is glufosinate ammonium at a concentration of about 1 mg/L. The *Agrobacterium* eradicator may be any conventional eradicator, such as cefotaxime. In one embodiment, the *Agrobacterium* eradicator is cefotaxime at a concentration of about 100 mg/L to about 150 mg/L, preferably 100 mg/L.

[0033] The third shoot regeneration medium used for the *Agrobacterium*-mediated transformation of *J. curcas* is the same as that used for regeneration except that it further comprises a selective agent and an *Agrobacterium* eradicator. The selective agent may be any selective agent for which a marker gene, such as described below, has been included in the transformed *Agrobacterium*. In one embodiment, the selective agent is hygromycin at a concentration of about 3 mg/L to about 5 mg/L, preferably 3.5 mg/L. In another embodiment, the selective agent is glufosinate ammonium at a concentration of about 1 mg/L. The *Agrobacterium* eradicator may be any conventional eradicator, such as cefotaxime. In one embodiment, the *Agrobacterium* eradicator is cefotaxime at a concentration of about 100 mg/L to about 150 mg/L, preferably 100 mg/L.

[0034] The shoot elongation medium and the rooting medium used for the *Agrobacterium*-mediated transformation of *J. curcas* are the same as that used for regeneration.

[0035] The DNA that is inserted (the DNA of interest) into plants of the genera *Jatropha* is not critical to the transformation process. Generally the DNA that is introduced into a plant is part of a construct. The DNA may be a gene of interest, e.g., a coding sequence for a protein, or it may be a sequence that is capable of regulating expression of a gene, such as an antisense sequence, a sense suppression sequence or a miRNA sequence. The construct typically includes regulatory regions operatively linked to the 5' side of the DNA of interest and/or to the 3' side of the DNA of interest. A cassette containing all of these elements is also referred to herein as an expression cassette. The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. The regulatory regions (i.e., promoters, transcriptional regulatory regions, and translational termination regions) and/or the polynucleotide encoding a signal anchor may be native/analogous to the host cell or to each other. Alternatively, the regulatory regions and/or the polynucleotide encoding a signal anchor may be heterologous to the host cell or to each other. See, U.S. Patent No. 7,205,453 and U.S. Patent Application Publication Nos. 2006/0218670 and 2006/0248616. The expression cassette may additionally contain selectable marker genes. See, U.S. Patent No. 7,205,453 and U.S. Patent Application Publication Nos. 2006/0218670 and 2006/0248616.

[0036] Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Usually, the plant selectable marker gene will encode antibiotic resistance, with suitable genes including at least one set of genes coding for resistance to the antibiotic spectinomycin, the streptomycin phosphotransferase (spt) gene coding for streptomycin resistance, the neomycin phosphotransferase (nptII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (hpt or aphiv) gene encoding resistance to hygromycin, acetolactate synthase (als) genes. Alternatively, the plant selectable marker gene will encode herbicide resistance such as resistance to the sulfonylurea-type herbicides, glufosinate, glyphosate, ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D), including genes coding for resistance to herbicides which act to inhibit the action of glutamine synthase such as phosphinothricin or basta (e.g., the bar gene). See generally, WO 02/36782, U.S. Patent No. 7,205,453 and U.S. Patent Application Publication Nos. 2006/0248616 and 2007/0143880, and those references cited therein. This list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used.

[0037] A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. That is, the nucleic acids can be combined with constitutive, tissue-preferred, or other promoters for expression in the host cell of interest. Such constitutive promoters include, for example, the core promoter of the Rsyn7 (WO 99/48338 and U.S. Patent No. 6,072,050); the core CaMV^{35S} promoter (Odell et al., 1985); rice actin (McElroy et al., 1990); ubiquitin (Christensen and Quail, 1989 and Christensen et al., 1992); pEMU (Last et al., 1991); MAS (Velten et al., 1984); ALS promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, those disclosed in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

[0038] Other promoters include inducible promoters, particularly from a pathogen-inducible promoter. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. Other promoters include those that are expressed locally at or near the site of pathogen infection. In further embodiments, the promoter may be a wound-inducible promoter. In other embodiments, chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. The promoter may be a chemical-inducible promoter, where application of the chemical induces

gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. In addition, tissue-preferred promoters can be utilized to target enhanced expression of a polynucleotide of interest within a particular plant tissue. Each of these promoters are described in U.S. Patent Nos. 6,506,962, 6,575,814, 6,972,349 and 7,301,069 and in U.S. Patent Application Publication Nos. 2007/0061917 and 2007/0143880.

[0039] Where appropriate, the DNA of interest may be optimized for increased expression in the transformed plant. That is, the coding sequences can be synthesized using plant-preferred codons for improved expression. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, 5,436,391, and 7,205,453 and U.S. Patent Application Publication Nos. 2006/0218670 and 2006/0248616.

[0040] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992), *Current Protocols in Molecular Biology* (John Wiley & Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Russell, 1984, *Molecular biology of plants: a laboratory course manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu *et al.* eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Fire *et al.*, *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge

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EXAMPLES

[0041] The present invention is described by reference to the following Examples, which is offered by way of illustration and is not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Materials and Methods

[0042] *Plant materials and culture methods: Jatropha curcas* (L.) MD seeds were obtained from Indonesia. After removing the outer seed coat, seeds kernel were surface sterilized for 60 seconds with 75% (v/v) ethanol, following immersed in 10% (v/v) H₂O₂ for 1h, then rinsed with sterile water for two times, finally immersed in sterile water overnight at 28° C in darkness. The endosperm-free embryos were germinated on hormone-free half-strength Murashige and Skoog salt (1/2 MS) medium (Murashige and Skoog, 1962) containing B5 Vitamins (Gamborg et al., 1968), 5 g/L sucrose, 0.5 g/L 2-(4-morpholino)ethanesulfonic acid (MES) and 2.2 g/L phytigel (Sigma), pH 5.6, with the radicals in contact with the medium, and cultured in a tissue culture room, at 25° C $\pm$ 2° C in a 16h light (100 μ mol/m²S)/8h dark cycle.

[0043] *Media:* The media used in the present invention are as follows.

[0044] *Medium I (basal medium):* MS major salts, MS minor salts and B5 vitamins, 10 mg/L citric acid, 150 mg/L glutamine, 100 mg/L casein enzymatic hydrolysate, 3 % (w/v) sucrose, 0.5 g/L MgCl₂ (only used in phytigel-containing medium) in combination with plant growth regulators was used. Medium I was adjusted to pH 5.8-6.0 with 1 N KOH, solidified with 2.5 g/L phytigel and autoclaved at 121° C for 20 min. All plant growth regulators were filter sterilized before being added to autoclaved medium.

[0045] *Co-cultivation medium:* basal medium plus 20 mg/L acetosyringone (AS), 0.5 g/L MES, 1.5 mg/L 6-benzylaminopurine (6-BA) and 0.05 mg/L 1-naphthaleneacetic acid (NAA), pH 5.0-5.2.

[0046] *Callus formation medium*: basal medium plus 1.5 mg/L 6-BA, 0.05 mg/L NAA, 3.5 mg/L hygromycin (hyg, A.G scientific, SanDiego, CA) as selective agent for plant transformation or 1 mg/L glufosinate ammonium (BASTA, Crescent Chemical, NY) and 100 mg/L cefotaxime (Cef) for elimination of *Agrobacteria* cells.

[0047] *Shoot regeneration medium I*: basal medium plus 1.5 mg/L 6-BA, 0.05 mg/L 3-indolebutyric acid (IBA), 2 mg/L adenine (adenine hemisulfate salt, SIGMA), 3.5 mg/L Hyg or 1 mg/L glufosinate ammonium and Cef 100 mg/L.

[0048] *Shoot regeneration medium II*: basal medium plus 1.5 mg/L 6-BA, 0.05 mg/L IBA, 0.5 mg/L gibberellic acid (GA₃), 4 mg/L Hyg or 1 mg/L glufosinate ammonium and 100 mg/L Cef 100, change phytagel to 7 g/L agar.

[0049] *Shoot regeneration medium III*: basal medium plus 1.5 mg/L 6-BA, 0.05 mg/L IBA, 3.5 mg/L Hyg or 1 mg/L glufosinate ammonium and 100 mg/L Cef.

[0050] *Shoot elongation medium*: basal medium plus 0.3 mg/L 6-BA, 0.1 mg/L GA₃, change phytagel to 7 g/L agar.

[0051] *Rooting medium*: MS major salts, MS minor salts and B5 vitamins, 3 % sucrose, 0.5 g/L MES, 0.07 g/L IBA, 2.2 g/L phytagel, pH5.6.

[0052] *Medium II*: liquid MS medium supplemented with 10 g/L glucose, 0.5 g/L MES, 20mg/L AS, 1.5 mg/L 6-BA, 0.1 mg/L NAA, pH 5.0-5.2.

[0053] *RNA extraction and analysis*: Fresh leaf or seed tissue (100 mg) was ground in liquid nitrogen and extracted with plant RNA purification reagent (Invitrogen). RNA concentration was measured by Nanodrop (Thermo, USA). DNase treatment and reverse transcription (RT) reaction were performed as described (Qu et al., 2007).

[0054] *Agrobacterium strain and vectors*: *J.curcas* WRINKLE1 (*JcWRI1*) and DGAT1 sequences were identified by sequencing a *Jatropha* seed cDNA library. The *JcWRI1* full-length cDNA was amplified from *J. curcas* seed first stranded cDNA product with two primers 5'-AATCGGATCCTAATGAAGAGGTCTTCTGCT-3' (SEQ ID NO:1) and 5'-TCATGTTAATT AATCAAACAGAATAGTTACAAGAAA-3' (SEQ ID NO:2) (underlined nts are enzyme recognition sites). The PCR product was further inserted into the pBA002-MYC vector treated with the BamHI and PaeI to form pBA002-MYC-JcWRI1. The JcDGAT1 full-length cDNA was amplified from *J.curcas* seed first stranded cDNA product with two primers 5'-CAATATCTAGACCATGACGATTTTGGAGACCACT-3' (SEQ ID NO:3) and 5'-TATTAGATCTGGTCTTAATTCAGCATTGCC-3' (SEQ ID NO:4) (underlined nts are enzyme recognition sites). The PCR product was further inserted into the pBA002-HA vector treated

with XbaI and BamHI to form pBA002-JcDGAT1-HA. The RcFAH12 full-length cDNA was amplified from castor bean seed first stranded cDNA product with two primers: 5'-CAATATCTAGACCATGGGAGGTGGTGGTC-3' (SEQ ID NO:5) and 5'-TGTAGGATCCGGATACTTGTTCGGTACCAG-3' (SEQ ID NO:6) (underlined nts are enzyme recognition sites). The PCR product was further inserted into the pBA002-HA vector treated with XbaI and BamHI to form pBA002-RcFAH12-HA. Vectors were introduced into *Agrobacterium* strain AGL1 by electroporation (BIO-RAD, CA, USA). Transformed *Agrobacterium* cells were used to inoculate liquid LB medium supplemented with 50 mg/L kanamycin (for pCAMBIA 1300-GFP) or 50 mg/L spectimycin (for pBA002-MYC-WRI1, pBA002-JcDGAT1-HA, pBA002-RcFAH12-HA) and 100 mg/L carbicillin and were grown overnight at 28° C, 250 rpm to a final OD₅₉₅=0.7-1. *Agrobacterium* cells were collected by centrifugation at 4200 rpm for 10 min at 20° C. The cell pellet was re-suspended with Medium II and adjusted to an OD₅₉₅ of 0.25-0.35 (only *Agrobacterium* AGL1) prior to co-cultivation.

[0055] *Isolation of DNA from J. curcas leaves and genotype analysis:* Fifty mg of fresh *J. curcas* leaves were disrupted in liquid nitrogen and incubated at 65° C for one hour after addition of 400 µL CTAB extraction buffer (100 mM Tris, pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2% cetyl trimethylammonium bromide (CTAB)). After two times extraction with chilled-chloroform, DNA was precipitated with isoprepanol and collected by centrifugation. For hygromycin gene genotyping, the primers used were hyg5: 5'-CGATGTAGGAGGGCGTGG-3' (SEQ ID NO:7), hyg3: 5'-ACTTCTACACAGCCATCGGT CC-3' (SEQ ID NO:8). For bar gene genotyping, the primers used were bar5: 5'-GTCTGCAC CATCGTCAACC-3' (SEQ ID NO:9), bar3: 5'-GAAGTCCAGCTGCCAGAAAC-3' (SEQ ID NO:10).

[0056] *Antibodies and protein gel blot analysis:* Curcin protein antibody was prepared by Dr. Yin Zhongcao's lab. Western blot analysis was performed as previously described (Qu et al., 2007). Total plant proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. ECL peroxidase conjugated donkey anti-rabbit immunoglobulin G was used as a secondary antibody. Immunoreactive bands were visualized using ECL Western blotting Detection Reagents (GE healthcare).

EXAMPLE 2

J. curcas Cotyledon Explant Transformation

[0057] Figure 1 illustrates the *Agrobacterium*-mediated *Jatropha* transformation method as set forth in further detail in this Example. Figure 2 illustrates the *Agrobacterium* transformation vectors that were used in this Example.

[0058] *Co-cultivation*: Cotyledons from 5-7 day old seedlings (Example 1; Fig. 3A) were cut into small pieces (5 X 5 mm) and incubated with *Agrobacterium* cells (Example 1) harboring the target expression cassette in 20 ml of medium II for 10-20 min at 25° C. Explants were then transferred to the co-cultivation medium for 2-3 days at 22° C in the dark. Following co-cultivation, explants were rinsed several times with sterile water, following one wash with 300 mg/L cefotaxime. Cotyledon tissues were blotted dry by putting them on a pad of sterilized paper to remove excess surface water.

[0059] *Selection of hygromycin-resistant or glufosinate ammonium-resistant calli*: After co-cultivation, the explants were plated onto the callus formation medium plate and transferred to darkness at 25° C $\pm$ 1° C for three weeks. The nontransformed and transformed explants form callus tissue (Fig. 3B) and some form callus upon culturing (Fig. 3B, right panel; Fig. 3C). Nontransformed explants normally will turn brown when cultured in the dark.

[0060] *Shoot regeneration*: Explants with newly emerged hygromycin-resistant or glufosinate ammonium-resistant callus were transferred onto the shoot regeneration medium I for 3 weeks at 25 °C with 16h light (100 μ mol/m²S¹)/8h dark cycles. The methods described here are based on direct shoot induction from transformed callus by adding adenine. While the term "regeneration" is used here to describe the re-creation of a whole plant from such transformed calli. Although 6-BA (6-benzyladenine) has similar effect on shoot regeneration, it can not be used in this special step during the methods described here. Furthermore, higher or lower concentration, early adding or later adding adenine will make the shoot regeneration more difficult or unnormal shooting. In an alternative embodiment, the method for obtaining shoot regeneration involves adding 2 mg/L plus normal 6-BA or other adenine derivative such as 2-isopentenyl adenine. During this period, any shoots regenerated from calli (about 40-50%) were transferred to the shoot regeneration medium II (Fig. 3D). Calli with no regenerated shoots were transferred to the shoot regeneration medium III for further culturing and regeneration of shoots.

[0061] *Shoot elongation*: After 4 weeks, regenerated shoots were transferred onto shoot elongation medium for elongation and bud multiplication (Fig. 3E).

[0062] *Rooting*: The elongated shoots about 2.5 cm in length were rooted in rooting medium (Fig. 3F). Normally it takes more than one month to get roots such as shown in Fig. 3F. Our rooting protocol can provide high rooting efficacy about 45% (Fig. 3G) and one main root length longer than 10mm can be successfully transferred into soil and get more than 90% live (Fig. 3H).

[0063] *Grafting*: Elongated, transgenic shoots can also be used as scions for grafting onto non-transgenic root stocks. Healthy and vigorously growing *J. curcas* plants were chosen to be rootstocks. Both scions and rootstocks were cut into the cambium region so that phloem tissues from both will connect after joining. The graft joint was wrapped with parafilm and secured by a tape. Grafted *J. curcas* plants were maintained under low light intensity (28 °C with 16h light (50 $\mu\text{mol}/\text{m}^2\text{S}^{-1}$)/8h dark cycles) and 85% humidity for 7 days. Transgenic *J. curcas* shoots grafted onto non-transgenic root stock are shown in Figs. 3I and 3J. Transgenic *J. curcas* plant showed normal flowering and seeding in greenhouse (Fig. 3K).

EXAMPLE 3

Transformation and Analysis of Transgenic *J. curcas*

[0064] Examples of *Jatropha* transformation and regeneration of BASTA or hygromycin plants from the transformed cells using the method of the present invention are detailed below. Briefly, the method requires that a heterologous DNA construct comprising a plant promoter, a DNA sequence encoding a protein that confers a selective advantage, such as BASTA or hygromycin tolerance, and a 3' untranslated transcriptional terminator region be provided. The DNA constructs comprise a plant promoter operably connected to a DNA coding region encoding a protein that confers BASTA or hygromycin tolerance, and a 3' termination signal. Preferably, the DNA construct encodes an additional gene of interest. For example, the DNA construct may include a gene the expression of which results in increased yields or altered fatty acid content in transformed plants.

[0065] In the example below, hygromycin tolerant *Jatropha* plants expressing green fluorescent protein (GFP) were obtained from tissue that was transformed with DNA constructs that included a GFP gene. This GFP gene and other genes such as GUS, *luciferase* gene, which can serve as easily screenable markers, were used in some of the examples described below, simply because their phenotypes can be readily detected in the transformed plants. It is reasonable to expect that by using DNA constructs created by standard molecular biological techniques, the present invention may be employed to obtain a *Jatropha* plants expressing

virtually any other gene. In an alternative embodiment, the method for obtaining transformed *Jatropha* plants involves the cotransformation of two DNA constructs, one of which comprises a selectable marker, such as a BASTA or hygromycin tolerance marker, and the other of which comprises a gene of interest.

[0066] The transformation and shoot regeneration of hygromycin resistant putative GFP transgenic *Jatropha* plants was accomplished according to the method described in Example 2. Genomic DNA of hygromycin resistant shoots was extracted with the method described in Example 1. Genotyping was performed with the hygromycin gene primer pair (SEQ ID NO:7 and SEQ ID NO:8). Nine of 10 events were PCR positive, while the non-transformation control shows no band in the CK lane (Fig.4). GFP expression was fast screened when transgenic *Jatropha* roots were excited with ultraviolet light (Fig 3B). The fluorescence indicated that this new introduced GFP expression cassette was expressed in T₀ *Jatropha* plants. After ubi:GFP transgenic *Jatropha* flowering, we checked the GFP expression in the inflorescence. The male flower, especially the pollen has some weak green fluoresce (Fig. 5H). We also checked GFP expression in the 3 week after fertilization seeds. Strong GFP expression can be seen in the whole transgenic T₁ seed both from outside (Fig. 5N, Fig. 5O) or inside (Fig. 5P). This indicates GFP also express well in the progeny seeds in transgenic *Jatropha*.

[0067] Triacylglycerols (TAG) is the main energy storage form after the plant converts solar energy into chemical energy. But the standard biochemical route for its synthesis was thought to be quite wasteful when plants use a variation of glycolysis as an intermediate. *WRINKLED1* (WRI1), a transcription factor of the AP2/EREB family, has an impact on more specific aspects of the seed storage process especially transcriptional control conversion of sugar variants into TAG, and therefore, shows a very important role in control seed oil content. Expression of the *Arabidopsis* WRI1 cDNA under the control of the cauliflower mosaic virus 35S-promoter led to increased 10–20% seed oil content. Moreover, the ectopic expression of the *WRINKLED1* cDNA caused the accumulation of triacylglycerols in developing seedlings (Cernac and Benning, 2004). We proposed that ectopic expression of *Jatropha* WRI1 gene in *Jatropha* would lead to higher oil content. In addition, the transgenic seedling can develop into embryos or embryo-like organ producing oil when fed with sugars, just like a lipid reactor that can be supplied with sugar-containing liquid substrate for the constitutive CaMV 35S promoter-driven to WRI1 strong expression in vegetative organs.

[0068] We cloned the full-length cDNA of *Jatropha* WRI1 (*JcWRI1*) PCR amplified from *Jatropha* seed RT-PCR products using PCR primers (SEQ ID NO:1 and SEQ ID NO:2) for the

JcWRI1 clone sequence derived from the *Jatropha* seeds cDNA library sequencing. The full length JcWRI1 cDNA sequence is set forth in SEQ ID NO:11. The overexpression vector (pBA002-MYC-JcWRI1) having the JcWRI1 cDNA under the control of CaMV 35S promoter was constructed and transformed into the *Agrobacterium* AGL1 strain. The proposed 6xMYC tag fusion WRI1 could be detected with MYC tag antibody. Transformation and shoot regeneration of BASTA resistant putative JcWRI1 overexpression transgenic *Jatropha* plants was accomplished according to the method described in Example 2. Genomic DNA of hygmycin resistant shoots was extracted with the method described in Example 1. Genotyping was performed with the BASTA gene primer pair (SEQ ID NO:9 and SEQ ID NO:10). All events we tested were PCR positive, while the non-transformation control showed no band in the CK (Fig.6).

[0069] Plant and animal diacylglycerol acyltransferases (DGAT) are responsible for packaging of nascent fatty acids into TAGs, which subsequently accumulate in oil bodies that bud off from the endoplasmic reticulum. Plant type 1 DGAT (DGAT1) genes have been shown to contribute significantly to seed oil content, both by overexpression and through mutational downregulation studies (Zou et al., 1999; Jako et al., 2001). We proposed that ectopic expression of *Jatropha* DGAT1 gene in *Jatropha* would leads to higher level oil content.

[0070] We cloned the full-length *Jatropha* DGAT1 cDNA from *Jatropha* seed RT-PCR products using PCR primers (SEQ ID NO:3 and SEQ ID NO:4) according to the DGAT1 clone sequence. The full length JcDGAT1 cDNA sequence is set forth in SEQ ID NO:13. The overexpression vector (pBA002-JcDGAT1-HA) having the JcDGAT1 cDNA under the control of CaMV 35S promoter was constructed and transformed into the *Agrobacterium* AGL1 strain. The proposed 3xHA tag fusion DGAT1 could be detected with HA tag antibody. Transformation and shoot regeneration of BASTA resistant putative JcDGAT1 transgenic *Jatropha* plants was accomplished according to the method described in Example 2. 35S-JcDGAT1 expression was proved by Western blot based on HA-antibody with the method describing in Example 1 (Fig. 7). HA-specific band can be seen in two lanes out of three transgenic *Jatropha* lines.

[0071] Plant oils (and their derivatives) can be used in numerous cases and applications for plant-derived industrial feedstocks. When compared with non-renewable petroleum, the renewable nature making them especially attractive for many industrial applications for total loss applications where environmental concerns are an issue. Castor (*Ricinus communis*) oil has numerous applications in transportation, cosmetics and pharmaceuticals, and manufacturing

industries. Castor oil contains more than 90% ricinoleic acid, which is a monounsaturated, 18-carbon fatty acid. It is unusual in that it has a hydroxyl functional group on the twelfth carbon. This functional group causes ricinoleic acid (and castor oil) to be unusually polar (http://en.wikipedia.org/wiki/Castor_oil). One specific enzyme: fatty acid hydroxylase 12 (FAH12) is responsible for adding the hydroxyl group instead of normal FAD2 function to introduce unsaturated band on the twelfth carbon (van de Loo et al., 1995). Compared to other seed oils which lack the hydroxyl group, castor oil demands a higher price. Despite a widespread demand for castor oil, however, cultivation of this crop is restricted due to the presence of a toxin (ricin) and allergenic proteins, and thus the cost of castor oil is relatively high. Transgenic exogenous FAH12 can produce hydroxyl-castor oil in *Arabidopsis* seeds (Lu et al., 2006). We proposed that ectopic expression of castor bean FAH12 gene in *Jatropha* would lead to the production of castor oil.

[0072] We cloned the full-length castor bean FAH12 cDNA (*RcFAH12*) from castor bean seed RT-PCR products using PCR primers (SEQ ID NO:5 and SEQ ID NO:6) according to the *FAH12* CDS sequence. The full length *RcFAH12* cDNA sequence is set forth in SEQ ID NO:15. The overexpression vector (pBA002-*RcFAH12*-HA) having the *RcFAH12* cDNA under the control of CaMV 35S promoter was constructed and transformed into the *Agrobacterium* AGL1 strain. The proposed 3xHA tag fusion *RcFAH12* could be detected with HA tag antibody. Transformation and shoot regeneration of BASTA resistant putative *RcFAH12* transgenic *Jatropha* plants was accomplished according to the method described in Example 2. 35S-*RcFAH12* expression was proved by Western blot based on HA-antibody with the method describing in Example 1. HA-specific band can be seen in 5 lanes out of 7 transgenic *Jatropha* lines (Fig. 7). Two lines, #2 and #5, have very high FAH12-HA fusion expression protein level.

[0073] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For example, if the range 10-15 is disclosed, then 11, 12, 13, and 14

are also disclosed. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0074] It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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WHAT IS CLAIMED IS:

1. A method for regenerating plants of *Jatropha curcas* comprising:
 - (a) culturing cotyledon explants on a solid callus formation medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, 6-benzylaminopurine (6-BA) and 1-naphthaleneacetic acid (NAA) for a period of time to produce explants with callus tissue;
 - (b) culturing the callus tissue on a solid first shoot regeneration medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, adenine, 6-BA and 3-indolebutyric acid (IBA) for a period of time to produce callus tissue with shoots and callus tissue;
 - (c) culturing the shoots on a solid second shoot regeneration medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, 6-BA, IBA and gibberellic acid (GA₃) for a period of time to produce shoots;
 - (d) culturing shoots from step (c) on a solid shoot elongation medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, 6-BA and GA₃ for a period of time to produce elongated shoots; and
 - (e) rooting or grafting the elongated shoots, wherein rooting is performed by culturing the elongated shoots on a solid rooting medium which comprises MS mineral salts, B5 vitamins, sucrose and IBA for a period of time to produce plantlets or wherein grafting is performed by grafting the elongated shoots to *J. curcas* rootstock.
2. The method of claim 1, which further comprising culturing the callus tissue from step (b) on a solid third shoot regeneration medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, 6-BA and IBA for a period of time to produce callus tissue with shoots which is then cultured in step (c).
3. The method of claim 1, wherein the concentration of media components are:
 - (a) citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L;
 - glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L;
 - casein hydrolysate: about 100 mg/L;
 - sucrose: about 3%;
 - 6-BA: about 1.5 mg/L; and
 - NAA: is about 0.05 mg/L to about 0.1 mg/L, preferably about 0.05 mg/L in the callus formation medium;

(b) citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein hydrolysate: about 100 mg/L; sucrose: about 3%; adenine: about 2 mg/L to about 4 mg/L, preferably about 2 mg/L; 6-BA: about 1.5 mg/L; and IBA: about 0.05 mg/L in the first shoot regeneration medium;

(c) citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein hydrolysate: about 100 mg/L; sucrose: about 3%; 6-BA: about 1.5 mg/L; IBA: about 0.05 mg/L and GA₃: about 0.05 mg/L to about 0.5 mg/L, preferably about 0.5 mg/L in the second shoot regeneration medium;

(d) citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein hydrolysate: about 100 mg/L; sucrose: about 3%; 6-BA: about 0.3 mg/L; and GA₃: about 0.1 mg/L to about 0.5 mg/L, preferably about 0.1 mg/L in the shoot elongation medium; and

(e) sucrose: about about 3% and IBA: is about 0.07 g/L in the rooting medium.

4. The method of claim 2, wherein the concentration of the media components in the third shoot regeneration medium are citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein hydrolysate: about 100 mg/L; sucrose: about 3%; 6-BA: about 1.5 mg/L; IBA: about 0.05 mg/L.

5. The method of claim 1, wherein periods of time for the culturing are:

(a) about two weeks to about three weeks, preferably about three weeks on the callus formation medium;

(b) about three weeks to about four weeks, preferably about three weeks on the first shoot regeneration medium;

(c) about three weeks to about four weeks, preferably about four weeks on the second shoot regeneration medium;

(d) about two weeks to about three weeks, preferably about two weeks on the shoot elongation medium; and

- (e) about three weeks to about four weeks, preferably about four weeks on the rooting medium.
6. The method of claim 2, wherein the period of time for culturing on the third shoot regeneration medium is about four weeks to about five weeks, preferably about four weeks.
7. The method of claim 3, wherein periods of time for the culturing are:
- (a) about two weeks to about three weeks, preferably about three weeks on the callus formation medium;
 - (b) about three weeks to about four weeks, preferably about three weeks on the first shoot regeneration medium;
 - (c) about three weeks to about four weeks, preferably about four weeks on the second shoot regeneration medium;
 - (d) about two weeks to about three weeks, preferably about two weeks on the shoot elongation medium; and
 - (e) about three weeks to about four weeks, preferably about four weeks on the rooting medium.
8. The method of claim 4, wherein the period of time for culturing on the third shoot regeneration medium is about four weeks to about five weeks, preferably about four weeks.
9. A method for *Agrobacterium*-mediated transformation of plants of *Jatropha curcas* comprising:
- (a) co-culturing cotyledon explants with *Agrobacterium* on a solid medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, acetospringone (AS), 6-benzylaminopurine (6-BA) and 1-naphthaleneacetic acid (NAA) for a period of time to produce transformed cotyledon explants
 - (b) culturing transformed cotyledon explants on a solid callus formation medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, 6-BA, NAA, a selective agent and an *Agrobacterium* eradicator in the dark for a period of time to produce explants with transformed callus tissue;

(c) culturing the transformed callus tissue on a solid first shoot regeneration medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, adenine, 6-BA, 3-indolebutyric acid (IBA), a selective agent and an *Agrobacterium* eradicator for a period of time to produce transformed callus tissue with transformed shoots and transformed callus tissue;

(d) culturing the transformed shoots on a solid second shoot regeneration medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, 6-BA, IBA, gibberellic acid (GA₃), a selective agent and an *Agrobacterium* eradicator for a period of time to produce transformed shoots;

(e) culturing transformed shoots from step (c) on a solid shoot elongation medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, 6-BA and GA₃ for a period of time to produce elongated transformed shoots; and

(f) rooting or grafting the elongated transformed shoots, wherein rooting is performed by culturing the elongated transformed shoots on a solid rooting medium which comprises MS mineral salts, B5 vitamins, sucrose and IBA for a period of time to produce transformed plantlets or wherein grafting is performed by grafting the elongated transformed shoots to *J. curcas* rootstock.

10. The method of claim 9, which further comprising culturing the transformed callus tissue from step (c) on a solid third shoot regeneration medium which comprises MS mineral salts, B5 vitamins, citric acid, glutamine, casein hydrolysate, sucrose, 6-BA, IBA a selective agent and an *Agrobacterium* eradicator for a period of time to produce callus tissue with shoots which is then cultured in step (d).

11. The method of claim 9, wherein the concentration of media components are:

(a) citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein hydrolysate: about 100 mg/L; sucrose: about 3%; AS: about 20 mg/L; 6-BA: 1.5 mg/L; and NAA: is about 0.05 mg/L to about 0.1 mg/L, preferably about 0.05 mg/L in the co-culturing medium;

(b) citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein

hydrolysate: about 100 mg/L; sucrose: about 3%; 6-BA: about 1.5 mg/L; and NAA: is about 0.05 mg/L to about 0.1 mg/L, preferably about 0.05 mg/L in the callus formation medium;

(c) citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein hydrolysate: about 100 mg/L; sucrose: about 3%; adenine: about 2 mg/L to about 4 mg/L, preferably about 2 mg/L; 6-BA: about 1.5 mg/L; and IBA: about 0.05 mg/L in the first shoot regeneration medium;

(d) citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein hydrolysate: about 100 mg/L; sucrose: about 3%; 6-BA: about 1.5 mg/L; IBA: about 0.05 mg/L and GA₃: about 0.05 mg/L to about 0.5 mg/L, preferably about 0.5 mg/L in the second shoot regeneration medium;

(e) citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein hydrolysate: about 100 mg/L; sucrose: about 3%; 6-BA: about 0.3 mg/L; and GA₃: about 0.1 mg/L to about 0.5 mg/L, preferably about 0.1 mg/L in the shoot elongation medium; and

(f) sucrose: about 3% and IBA: is about 0.07 g/L in the rooting medium.

12. The method of claim 10, wherein the concentration of the media components in the third shoot regeneration medium are citric acid: about 10 mg/L to about 30 mg/L, preferably about 10 mg/L; glutamine: about 150 mg/L to about 200 mg/L, preferably about 150 mg/L; casein hydrolysate: about 100 mg/L; sucrose: about 3%; 6-BA: about 1.5 mg/L; IBA: about 0.05 mg/L.
13. The method of claim 9, wherein periods of time for the culturing are:
 - (a) about 2-3 days;
 - (b) about two weeks to about three weeks, preferably about three weeks on the callus formation medium;
 - (c) about three weeks to about four weeks, preferably about three weeks on the first shoot regeneration medium;

- (d) about three weeks to about four weeks, preferably about four weeks on the second shoot regeneration medium;
 - (e) about two weeks to about three weeks, preferably about two weeks on the shoot elongation medium; and
 - (f) about three weeks to about four weeks, preferably about four weeks on the rooting medium.
14. The method of claim 10, wherein the period of time for culturing on the third shoot regeneration medium is about four weeks to about five weeks, preferably about four weeks.
15. The method of claim 11, wherein periods of time for the culturing are:
- (a) about 2-3 days;
 - (b) about two weeks to about three weeks, preferably about three weeks on the callus formation medium;
 - (c) about three weeks to about four weeks, preferably about three weeks on the first shoot regeneration medium;
 - (d) about three weeks to about four weeks, preferably about four weeks on the second shoot regeneration medium;
 - (e) about two weeks to about three weeks, preferably about two weeks on the shoot elongation medium; and
 - (f) about three weeks to about four weeks, preferably about four weeks on the rooting medium.
16. The method of claim 12, wherein the period of time for culturing on the third shoot regeneration medium is about four weeks to about five weeks, preferably about four weeks.

1 / 5

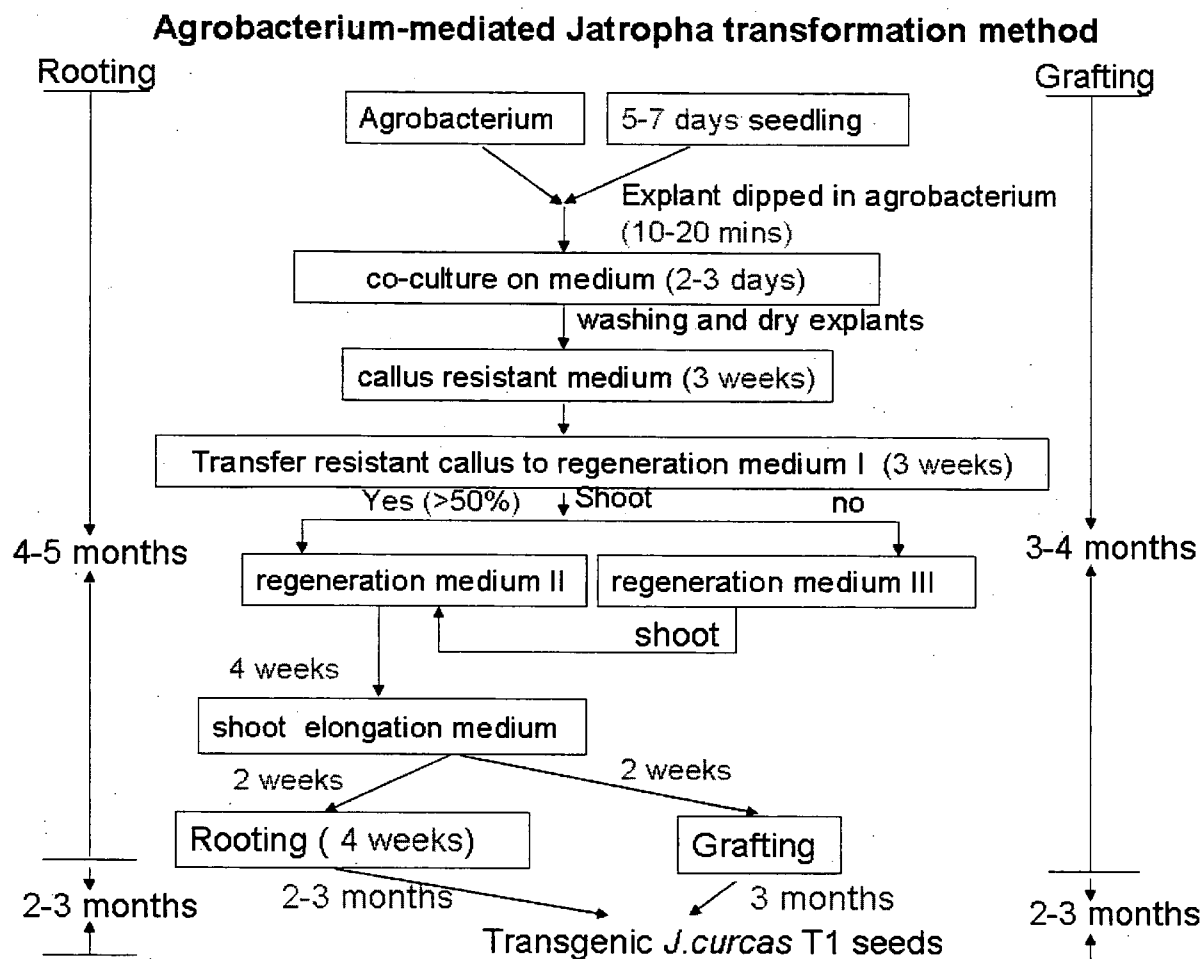


Figure 1

2 / 5

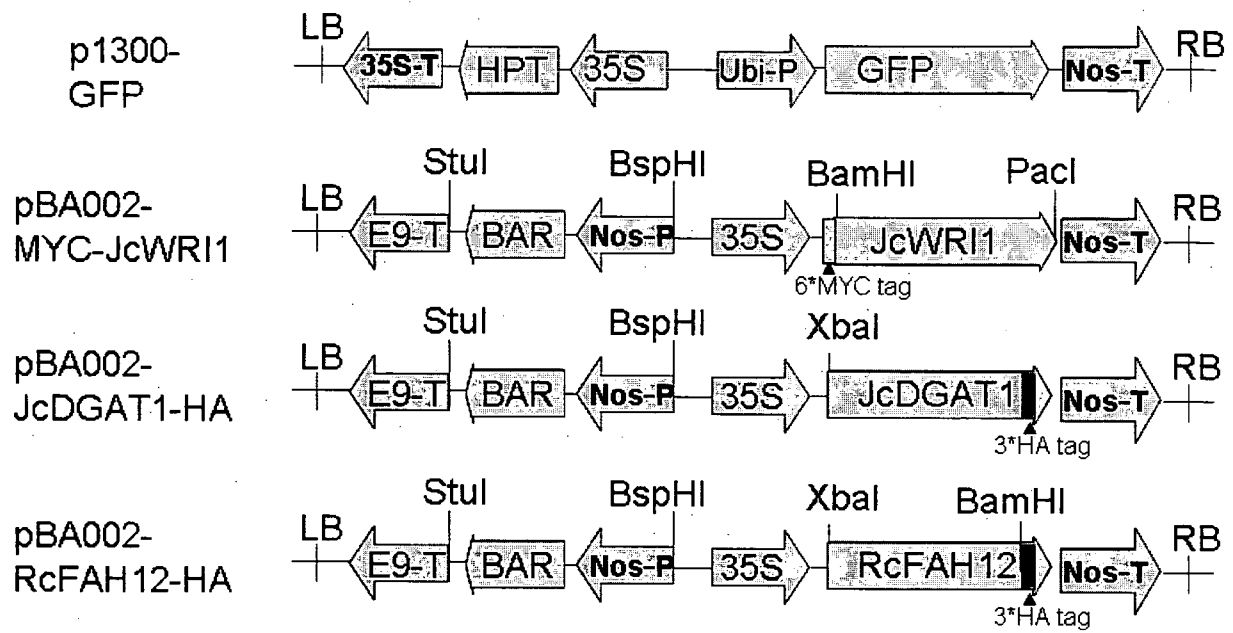


Figure 2

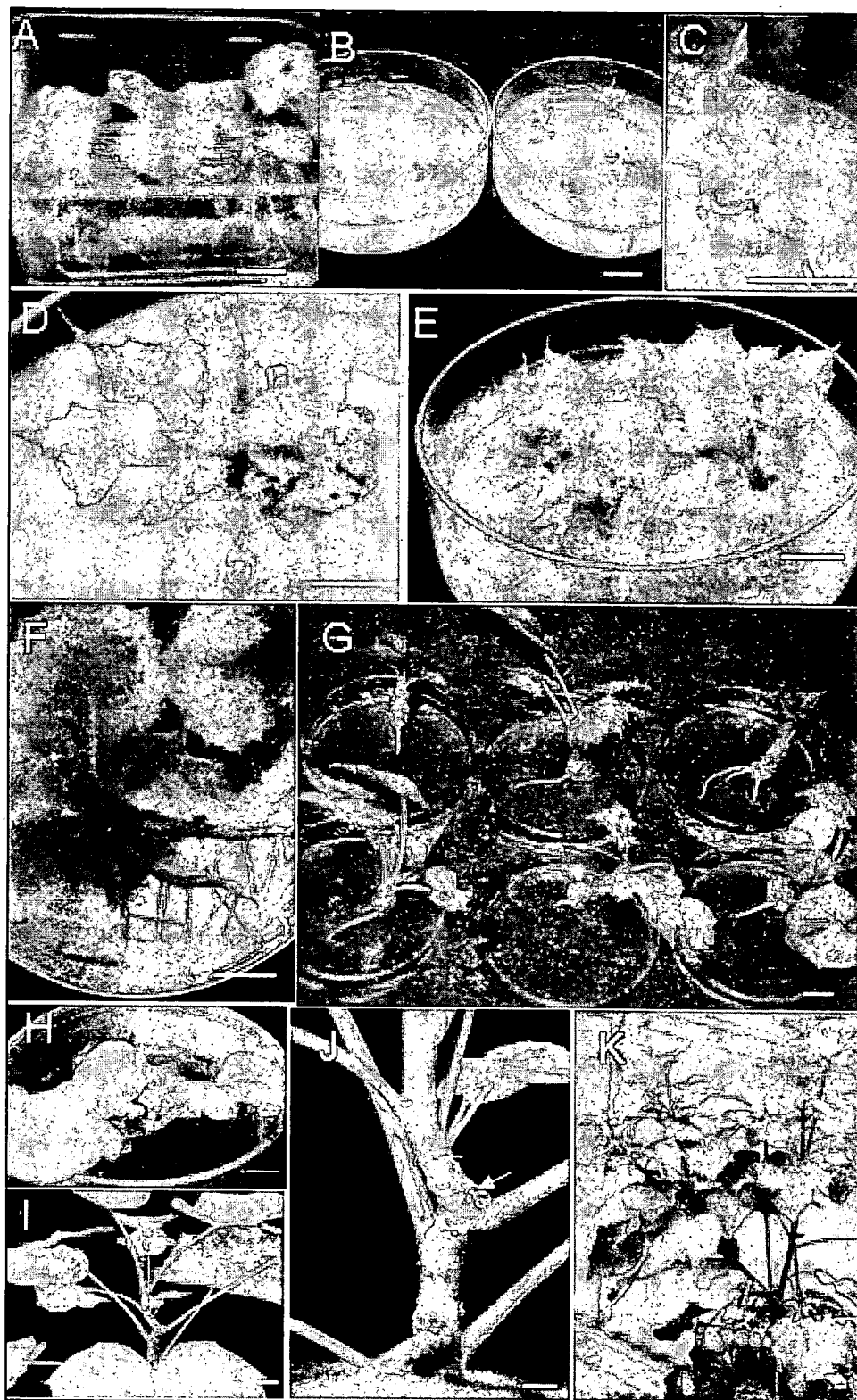


Figure 3



Figure 4

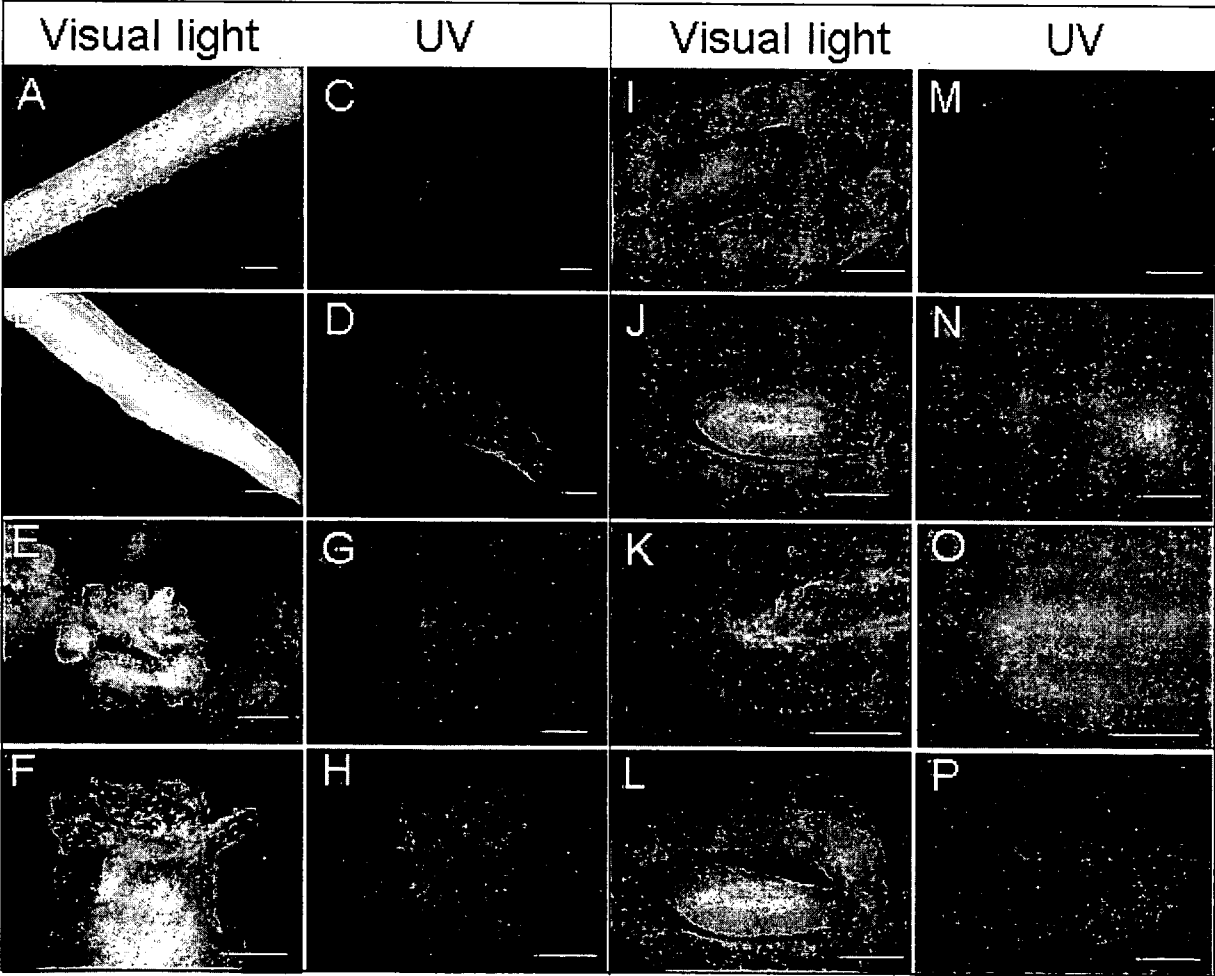


Figure 5

5 / 5

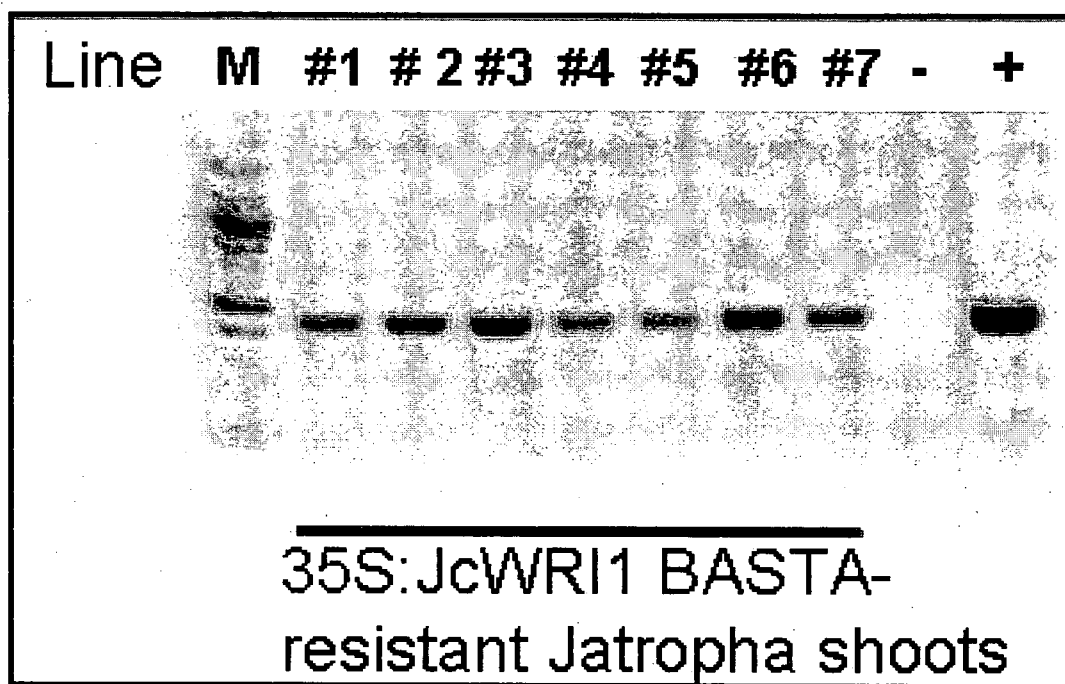


Figure 6

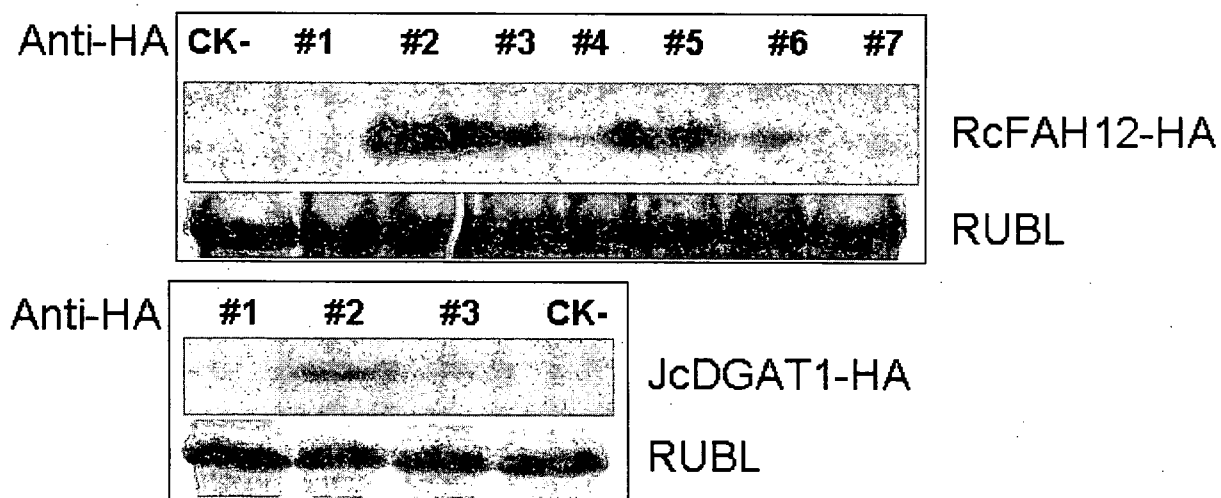


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2009/000479

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A01H 1/00 (2006.01)

C12N 15/05 (2006.01)

C12N 15/84 (2006.01)

A01H 4/00 (2006.01)

C12N 15/82 (2006.01)

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)

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

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

MEDLINE, BIOSIS, HCA, AGRICOLA, EPODOC, WPI: *Jatropha curcas*, regenerate, transformation, engineer, modify, recombinant, transgenic, cotyledon, explant, leaves, shoots, roots, graft, and similar terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SUJATHA, M. <i>et al.</i> 'Role of biotechnological interventions in the improvement of castor (<i>Ricinus communis</i> L.) and <i>Jatropha curcas</i> L.' Biotechnology Advances. October 2008, vol. 26(5), pages 424-435. See whole document. In particular p. 430: para. 5.2, table 2, and p. 432: para. 6.3.	1-16
X	RAJORE, S. & BATRA, A. 'An alternative source for regenerable organogenic callus induction in <i>Jatropha curcas</i> L.' Indian Journal of Biotechnology. October 2007, vol. 6(4), pages 545-548. See whole document. In particular abstract and materials and methods.	1-8
X	US 2008/0196121 A1 (MURALI, K.S. <i>et al.</i>) 14 August 2008. See whole document. In particular para. [0015]-[0023].	1-8

☒ 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

"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

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

"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

"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)

"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

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

"&" document member of the same patent family

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

Date of the actual completion of the international search

12 February 2010

Date of mailing of the international search report

18 FEB 2010

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2009/000479

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
 - a. (means)
☐ on paper
☐ in electronic form
 - b. (time)
☐ in the international application as filed
☐ together with the international application in electronic form
☐ subsequently to this Authority for the purposes of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing 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:
Please note, although the specification contains a sequence listing, it was not used for the purposes of this search.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2009/000479

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LI, M. <i>et al.</i> 'Establishment of an <i>Agrobacterium</i> -mediated cotyledon disc transformation method for <i>Jatropha curcas</i> ' Plant Cell, Tissue and Organ Culture. 2008, vol. 92, pages 173-181. See whole document. In particular Materials and methods	9-16
X	KR 10-0856930 B1 (NEXGEN BIOTECHNOLOGIES, INC.) 11 September 2008. Abstract only.	9-16
P,X	HE, Y. <i>et al.</i> ' <i>Agrobacterium tumefaciens</i> -mediated Transformation of <i>Jatropha curcas</i> : Factors Affecting Transient Transformation Efficiency and Morphology Analysis of Transgenic Calli.' Silvae Genetica. 3 Dec. 2009, vol. 58(3), pages 123-128. See whole document. In particular Materials and methods.	9-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2009/000479

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member	
US	2008/0196121	US	20080194026
KR	10-0856930		NONE
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			
END OF ANNEX			