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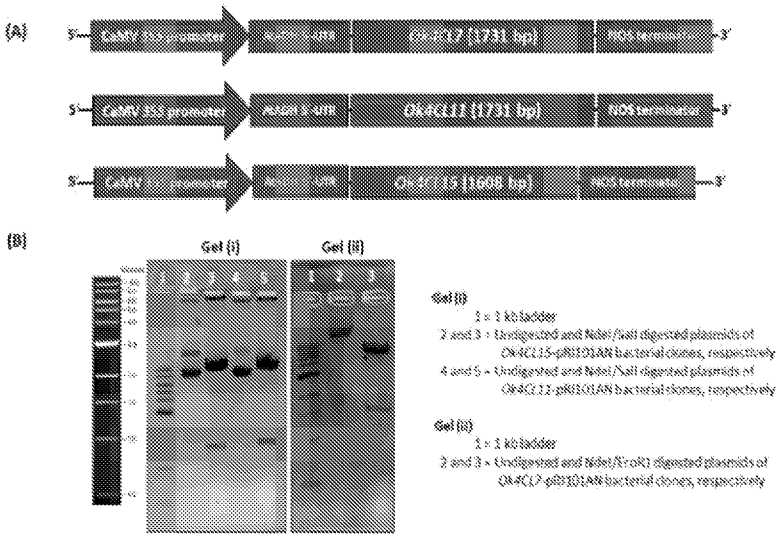


Figure 1

(57) Abstract: The present disclosure relates to a recombinant vector. Particularly, the present disclosure relates to a recombinant vector to overexpress 4-coumarate Co-enzyme A ligase 11 gene (4CL11) in plant cells. Further, the present disclosure relates to a binary expression vector comprising a pRI101AN vector with the 4CL11 ORF sequence from Ocimum kilimandscharicum and regulatory elements for overexpressing host 4CL11 gene in plant cells, which results in the accumulation of flavonoids (kaempferol and quercetin as well as their glycoside derivatives), altered lignin content, increased natural product biosynthesis that eventually leads to reduced auxin levels and decreased expression of auxin transport and signaling genes, causing reduced root growth.

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A RECOMBINANT VECTOR COMPRISING *4CL11* ORF FROM *OCIMUM KILIMANDSCHARICUM*, AND IMPLEMENTATIONS THEREOF

TECHNICAL FIELD OF THE INVENTION

The present disclosure relates to a recombinant vector. Particularly, the present disclosure relates to a recombinant vector to overexpress 4-coumarate Co-enzyme A ligase 11 gene (*4CL11*) in plant cells. Further, the present disclosure relates to a binary expression vector comprising a pRI101AN vector with the *4CL11* ORF sequence from *Ocimum kilimandscharicum* and regulatory elements for overexpressing host *4CL11* gene in plant cells, which results in reduced root growth, altered lignin content, and increased natural product biosynthesis.

BACKGROUND AND PRIOR ART OF THE INVENTION

Secondary metabolites are extensively distributed in the plant kingdom. They play an essential role in the growth, development, and reproduction of plants. Moreover, they are known to impart protection to plants against their competitors *via* allelopathy.

Phenylpropanoids are a large class of plant secondary metabolites derived from aromatic amino acid phenylalanine in most plants or tyrosine in partial monocots, which are produced in response to various biotic or abiotic stress conditions. These stress conditions are comprised of pathogen infections, wounding, UV irradiation, exposure to ozone/pollutants, and other hostile environmental conditions. Several genes and their gene products are enzymes involved in the phenylpropanoid biosynthesis pathway and they show differential molecular responses against numerous stress conditions. Many of them are also known to have or are being explored for medicinal properties.

4-coumarate CoA ligase (EC 6.2.1.12) (*4CL*) is a crucial enzyme involved in the phenylpropanoid pathway as well as lignin biosynthesis. It serves as the main branch point in the pathway. They are encoded by a multigene family of adenylate-forming enzymes. *4CL*s convert hydroxy or methoxy cinnamic acid derivatives to the corresponding activated thioesters. Products of *4CL* are utilized by various oxygenase, reductases, and transferases for the biosynthesis of lignin, flavonoids, anthocyanins, tannins, aurones, stilbenes, coumarins, suberin, cutin, sporopollenin, etc. It has been reported that *4CL*s might play a crucial role in lignin biosynthesis. Lignin is the polymer of H-lignin, G-lignin, and S-lignin monomers. Also, several nonstructural phenylpropanoids synthesized via *4CL*s exhibit diverse functions in plant physiology. *4CL* enzymes contain two conserved peptide motifs: box I (SSGTTGLPKGV) and box II (GEICIRG). Members of the adenylate-forming enzyme family have a conserved adenosine monophosphate binding domain (box I). Crystal structure analysis suggested that

during the catalysis process, enzymes undergo two conformations: adenylate forming and thioester forming. *4CL* isoforms with differential substrate affinities can be used to manipulate metabolite flux. The expression of *4CL* isoforms can be modulated in response to a specific trigger.

Biocatalytic properties of 4-coumarate-CoA ligase (*4CL*s) have been explored in the past for commercial applications in fuel, flavor, and natural product synthesis.

One of the reports is PCT Publication No WO1999024561, which describes a method to genetically alter plants through downregulation of 4-coumarate Coenzyme A ligase (*4CL*).

Another report Yohei Katsuyama et al. in a research study published in Journal of general microbiology, 2008 titled "Production of curcuminoids by *Escherichia coli* carrying an artificial biosynthesis pathway" disclosed that the curcuminoids biosynthesis in *Escherichia coli* can be achieved by expressing *4CL* from *Lithospermum erythrorhizon* and curcuminoid synthase from rice.

Another report, US Patent No. 9187757 covers a method for reducing lignin biosynthesis in a plant by suppressing the expression of 4-coumarate-CoA ligase (*4CL*) genes or *4CL* enzymes.

Another report by Y. Wang et al. in a research study published in Journal of the American Chemical Society, 2011 titled "Structural and Kinetic Analysis of the Unnatural Fusion Protein 4-Coumaroyl-CoA Ligase: Stilbene Synthase" discloses that the resveratrol biosynthesis can be improved in yeast by fusion of At*4CL*1 from *A. thaliana* and stilbene synthase from *Vitis vinifera* (VvSTS).

Another report by J.H. Jung et al. in a research study published in Plant Molecular Biology, 2016 titled "Precision breeding for RNAi suppression of a major 4-coumarate: coenzyme A ligase gene improves cell wall saccharification from field-grown sugarcane" discloses that the silencing of lignin biosynthesis-specific *4CL* isoform can cause a reduction in lignin content.

Based on the literature pertaining to β-glucosidase, it appears there is a need in the art to find an alternative process to overexpress the 4-coumarate-CoA ligase for commercial applications.

OBJECTS OF THE INVENTION

Accordingly, the main objective of the present disclosure is to provide a recombinant vector for overexpressing 4-coumarate Co-enzyme A ligase gene (*4CL*) in host cells.

An objective of the present disclosure is to provide a recombinant vector comprising *4CL11* ORF sequence from *Ocimum kilimandscharicum* for overexpressing the host *4CL11* gene in plant cells.

Another objective of the present disclosure is to provide a recombinant binary expression vector comprising a pRI101AN vector comprising *Ok4CL11* ORF sequence and regulatory elements,

which on integration with the plant cell genome is responsible for overexpressing the host *4CL11* gene in plant cells.

Yet another objective of the present disclosure is to provide a method for transforming the pRI101AN vector comprising *Ok4CL11* ORF sequence and regulatory elements into plant cells.

Yet another objective of the present disclosure is to provide a method for overexpressing host *4CL11* gene in plant cells by transforming the pRI101AN vector comprising *Ok4CL11* ORF sequence and regulatory elements into plant cells.

SUMMARY OF THE INVENTION

Accordingly, aspects of the present disclosure relate to a recombinant vector. Particularly, the present disclosure relates to a recombinant vector to overexpress 4-coumarate Co-enzyme A ligase 11 gene (*4CL11*) in plant cells. Further, the present disclosure relates to a binary expression vector comprising a pRI101AN vector with the *4CL11* ORF sequence from *Ocimum kilimandscharicum* and regulatory elements for overexpressing host *4CL11* gene in plant cells results in reduced root growth, altered lignin content and structure, and increased natural product biosynthesis.

In an aspect of the present disclosure, the host *4CL11* in plant cells is overexpressed by transforming a binary expression vector comprising an open reading frame (1731 bp) of 4-coumarate- Co-enzyme A Ligase 11 gene isolated from *Ocimum kilimandscharicum* (*Ok4CL11*) and one or more transcriptional regulatory sequence into the plant cell, and allowing the vector to integrate into the genome by non-homologous recombination, thus allowing overexpression of the host *4CL11*.

In another aspect of the present disclosure, the host *4CL11* gene is overexpressed by transforming a binary expression vector pRI101AN comprising *Ok4CL11* ORF and one or more transcriptional regulatory sequence into the plant cell, and allowing the vector to integrate into the genome by non-homologous recombination, thus allowing overexpression of the host *4CL11*.

In another aspect of the present disclosure, the *Ok4CL11* ORF sequence is cloned into the pRI101AN vector using the restriction enzymes NdeI and EcoRI to form the pRI101AN-*Ok4CL11*-OE construct.

In yet another aspect of the present disclosure, the pRI101AN-*Ok4CL11*-OE construct is flanked by a 35S CaMV promoter for driving the expression of *Ok4CL11* ORF sequence, followed by the expression of host *4CL11* gene, 5′ untranslated region (UTR) of alcohol dehydrogenase gene from *Arabidopsis thaliana,* and Nopaline synthase terminator.

In still another aspect of the present disclosure, the overexpression of host *4CL11* further helps to increase the level of flavonoids (kaempferol and quercetin-derivatives), lignin, and natural product biosynthesis, which may further lead to a reduced root growth phenotype.

In yet another aspect of the present disclosure, the pRI101AN-*Ok4CL11*-OE construct effects overexpression of host *4CL11* in plant cells selected from but not limited to *Nicotiana benthamiana, Arabidopsis thaliana* or potato plants resulting in rootless or reduced root growth phenotype of said plants that present disclosure. Plants can be multiplied at a large scale in plant tissue culture using stem nodal cuttings and used in hydroponic, aeroponic, and vertical farming for commercial applications.

DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1: depict (A) schematics of *Ok4CL7*, -11 and -15 overexpression constructs in pRI101AN vector under 35S promoter; (B) Gel electrophoresis showing the confirmation of *Ok4CL7*, -11 and -15 genes cloning in pRI101AN vector by EcoRI and NdeI restriction enzymes digestion.

Figure 2: depict the procedure for the generation of transgenic *N. benthamiana* plants using leaf as explant for *Agrobacterium*-mediated transformation protocol.

Figure 3: depict the screening of putative transgenic lines using RT-PCR: (A) *Ok4CL11* and (B) *Ok4CL7* and *Ok4CL15*. Gel A (Well No. 1-9: *Ok4CL11-OE-Nb* (cDNA as template); 10: WT-Nb (cDNA as template); 11: positive control for *Ok4CL11* PCR (Plasmid); 12: negative control for *Ok4CL11* PCR; 13: 100 bp ladder); Gel B (Well No. 1 and 8: 100 bp DNA ladder; 2-4: *Ok4CL11-Nb* (cDNA as template); 5 and 15: (cDNA as template); 6: positive control for *Ok4CL7* PCR (Plasmid); 7: negative control for *Ok4CL7* PCR; 9-14: *Ok4CL15-Nb* plants; 16: positive control for *Ok4CL15* PCR (Plasmid); 17: negative control for *Ok4CL15* PCR)

Figure 4: depict the gene expression of *Ok4CLs* in the individual overexpression lines of *N. benthamiana* using semi-quantitative PCR: (A) *Ok4CL7*, (B) *Ok4CL11*, and (C) *Ok4CL15*. *N. benthamiana L23P* was used as a reference gene. The PCR cycle number used for gel imaging is 25. Complementary DNA (cDNA) prepared from wild-type (WT) *N. benthamiana* plant was used as negative control for PCR.

Figure 5: depict (A) analysis of root phenotypes of *N. benthamiana* plants overexpressing *Ok4CL7*, -11 or -15 gene compared to wild-type plants (WT); (B) the number of primary roots per plant; (C) the lateral roots on the primary root; (D) the primary root length (cm). *Ok4CL11*-OE1 and -2 are the two independent overexpression lines of *Ok4CL11*, whereas wild-type (WT) is a non-transformed plant of *N. benthamiana*. *Ok4CL7*-OE1 and *Ok4CL15*-OE1 are the individual overexpression lines of *Ok4CL7* and -15 constructs, respectively.

Figure 6: depict (A) Relative abundance of auxins (indole-3-acetic acid and indole-3-propionic acid), flavonoids and their glycosides (kaempferol, kaempferol-3-rhamnoside [K3R], kaempferol-3,7-O-bis-alpha-L-rhamnoside [K3,7R], quercetin 3-[2-caffeoylglucuronide]) in *Ok4CL11*-OE lines (OE1 and OE2) and wild-type *N. benthamiana* plants; (B-C) Lignin accumulation in *Ok4CL7*, *-11* and *-15* OE lines and wild-type *N. benthamiana* plants. (D) Expression analysis of phenylpropanoid pathway genes and flavonoid-specific glycosyltransferases. (E) Expression analysis of auxin transport and signaling genes.

Figure 7 (A-E): Depicts the overexpression line confirmation of Ok*4CL*-7 and -11 in potato (*Solanum tuberosum* cv. Désirée), and the resultant reduced root growth phenotypes by *Ok4CL11* overexpression in the potato plant. No such phenotype was observed for *Ok4CL7*-OE lines. Figure 7B Well Nos: 1-4 *Ok4CL7*-OE line confirmation; 8-13: *Ok4CL11*-OE line confirmation; 5: wild type potato plant; 6: Negative control for PCR; 7: positive control *Ok4CL7* (166 bp); 14: positive control *Ok4CL11* (136 bp).

Figure 8: Depicts the schematic diagram of the method of producing rootless phenotype in *Nicontiana benthamiana* plant.

Figure 9 (A-F): Depicts the supplementation of flavonoids (kaempferol and quercetin) and their glycosides (R, K3,7R and Q3G) delays AR emergence in wild-type *Nicotiana benthamiana.*

Figure 10 (A-C): Depicts the reduced root growth phenotype in *Ok4CL11*-OE lines of *Arabidopsis thaliana*, ecotype Col-0.

SOURCE OF BIOLOGICAL MATERIAL:

Nicotiana benthamiana was taken from CSIR-NCL, Pune, Maharashtra, India. *Ocimum kilimandscharicum used* to isolate the 4-coumarate Co-enzyme A ligase 11 (*Ok4CL11*) polynucleotide was obtained from CSIR-NCL, Pune, Maharashtra, India. Binary expression vector pRI101AN was obtained from DSS Takara Bio India Pvt. Ltd., New Delhi, India. Potato (*Solanum tuberosum* cv. Désirée) were obtained from the Indian Institute of Science Education and Research (IISER) Pune, and grown in plant tissue culture facility at the CSIR-NCL, Pune (Maharashtra, India). *Arabidopsis thaliana*, ecotype Col-0 seeds were germinated *in vitro* on MS medium and plantlets obtained were grown in the plant growth room at the CSIR-NCL, Pune (Maharashtra, India).

DETAILED DESCRIPTION OF THE INVENTION:

As used herein, the terms "Ok*4CL*s", when used in the context of the present disclosure, refer to enzyme 4-coumarate-Co-enzyme A ligases (*4CL*s) from *Ocimum kilimandscharicum.*

Accordingly, embodiments of the present disclosure relates to a recombinant vector. Particularly, the present disclosure relates to a recombinant vector to overexpress 4-coumarate Co-enzyme A ligase 11 gene (*4CL11*) in host cells. Further, the present disclosure relates to a binary expression vector comprising a pRI101AN vector with the *4CL11* ORF sequence from *Ocimum kilimandscharicum* and regulatory elements for host *4CL11* overexpression in plant cells resulting in a reduction in root growth, altered lignin content and structure, and increase in natural product biosynthesis.

Accordingly, the present disclosure provides a binary vector to upregulate or overexpress the host *4CL11* to achieve a reduction in root growth, altered lignin content, and increase in natural product biosynthesis.

In an embodiment of the present disclosure, the host *4CL11* overexpression is effected by introducing a binary expression vector comprising an open reading frame (1731bp) of 4-coumarate- Co-enzyme A Ligase 11 gene isolated from *Ocimum kilimandscharicum* (*Ok4CL11*) and one or more transcriptional regulatory sequence into the plant cell, and allowing the vector to integrate into the genome by non-homologous recombination, thus allowing overexpression of the host *4CL11*.

In still another embodiment of the present disclosure, the open reading frame of *Ok4CL11* is represented by SEQ ID NO: 1.

In yet another embodiment of the present disclosure, the *Ok4CL11* coding SEQ ID NO: 1 is

>*Ok4CL11* (1731 bp)

ATGGCTGCATTAATAAAAGCCCACGTCGAGTTGCAGACTGTGAGAGAAGAAAGTG
GAGGGATTGAAGAAATGTCGAAAACTAGAAAGTTGACATGCTCAAACCCTTCTTG
GTATTGCCCTGAAACAGGGATATACTCAAGCAAATACCCTTCCATTAGCCTCCCTT
CTGACCCTTTTCTTGATGTTGTTTCGTTCATTTTCTCACATGAACATGATGGGTTAC
ATGCACTCGTTGATTCAGCATCTGGGCTTTCAGTTCCCTACTCCAAGCTGTTACCTT
TGGTGAAATCCATGGCGGCTGGTCTTCATCATTTGGGTGTGAAGCAAGGTGATGTG
ATCTTGATTCTGCTGCCCAATTCTGTGTGTTTCCCTTTGATTCTTTTAGGTGCTTTGA
GTGTTGGTGCTGTTGTCACCACCCTGAATCCTTTGAGTAGTTTGTTAGAGATAAGA
AAACAGGTTCTTGATAGTAATGCAAGTCTTGTATTCTCTGGTATTGGTAGGGTTGA
TGAATTAGGTAATGCTTTGGAGGGTTGTCTTGTTGTAGGGGTGCCTGAAGTTTTGG
ATTTGAGCTGTGAGAATGTTAATAGTTCTGTTTTTTGTAAGCTCATTTCTAGTGATC
CTGAAATGGCACCTAGGCCTAGAATTAAGCAACAGGACACCGCCGCGATTTTGTA
CTCGTCTGGCACTACTGGGAAGTCTAAAGGAGTCATGTTGACTCATGGGAACTTCA
TAGCCATGATTGAGCTCTTTGTGAGGTTTGAAGCTTCTCTGTATGATTACCCTAGTA

CAGCTAATGTCTACTTGGCTGTTGTGCCTATGTTCCATGTTTACGGGCTGTCTCTTT
TCGTGTTGGGATTGTTGTCGTTGGGGAGTACTGTTGTCACCATGAGAAAGTTTGAT
GGTGATGAAATGGTGAGAGCTATTGATAGATACGGCGTGACTCACCTCCACGCTG
TCCCTCCTATACTGGTTGCGTTGACGAAGAGAGCCAAGAACGGTGATGGTAGTGG
TTTTCGGAGCTTGAGGCAGGTTTCGTGTGGGGCTGCTCCGTTGAGTGAGAAAAGCA
TTGTTGAATTGGTTGAGGCACTCCCCCATGTTGATTTCATTCAGGGTTATGGCATG
ACAGAGTCGACCGCGTTGGCAACTCGCGGCTATAATAATGGTGGAGTCAAGAAAT
ATTCGTCTGTGGGACTTCTTTCTCCGAATATTGAAGCTAAAGTGGTGGACTGGGTT
ACAGGAGCTCTCGTTCCTCCAGGTTCGATCGGTGAGCTTTGGTTGCGCACGCCGGG
GAATATGAAAGGGTACTTGAATAATACTGAAGCAACTGTGGGTGCACTTGACAAA
GAAGGTTGGCTACACACCGGTGACATAGTTTATTTCGACCAAGAAGGATACTTGT
ATGTAATCGATCGCCTGAAAGAAGTAGTCAAGTACAAAGGATTCCAGATTGCTCC
AGCGGATTTGGAGGCGGTATTGATGTCACATCCAGAAGTAGCTGACGCGGCTGTT
ACAGGTGTGAGAGACGAAGAAGCCGGAGAGATCCCTGTAGCATTCGTCGTTCCAA
AAGAAGGTACATCACTCTCCGCGGCCACCCTCATGGACCACGTCGCGAAGCAGGT
TGCACCTTATAAGAAGGTGAGAAAGGTGTATTTTCGGTCGTCGATACCGAGATCG
CCGGCCGGAAAAATACTTCGCAGGGAGCTCAAGAACTTGTTCGTTTCAAGGCTTTA
G

In still another embodiment of the present disclosure, the *Ok4CL11* ORF encodes a polypeptide having the amino acid sequence represented by SEQ ID NO: 2, or a fragment or variant thereof. In a preferred embodiment of the present disclosure, the *Ok4CL11* ORF protein SEQ ID NO: 2 is

>*Ok4CL11* (576 aa)

MAALIKAHVELQTVREESGGIEEMSKTRKLTCSNPSWYCPETGIYSSKYPSISLPSDPFL
DVVSFIFSHEHDGLHALVDSASGLSVPYSKLLPLVKSMAAGLHHLGVKQGDVILILLP
NSVCFPLILLGALSVGAVVTTLNPLSSLLEIRKQVLDSNASLVFSGIGRVDELGNALEG
CLVVGVPEVLDLSCENVNSSVFCKLISSDPEMAPRPRIKQQDTAAILYSSGTTGKSKGV
MLTHGNFIAMIELFVRFEASLYDYPSTANVYLAVVPMFHVYGLSLFVLGLLSLGSTVV
TMRKFDGDEMVRAIDRYGVTHLHAVPPILVALTKRAKNGDGSGFRSLRQVSCGAAPL
SEKSIVELVEALPHVDFIQGYGMTESTALATRGYNNGGVKKYSSVGLLSPNIEAKVVD
WVTGALVPPGSIGELWLRTPGNMKGYLNNTEATVGALDKEGWLHTGDIVYFDQEGY
LYVIDRLKEVVKYKGFQIAPADLEAVLMSHPEVADAAVTGVRDEEAGEIPVAFVVPK
EGTSLSAATLMDHVAKQVAPYKKVRKVYFRSSIPRSPAGKILRRELKNLFVSRL

In still another embodiment of the present disclosure, the *Ok4CL7* gene encodes a polypeptide having the amino acid sequence represented by SEQ ID NO: 3, or a fragment or variant thereof. In yet another embodiment of the present disclosure, the *Ok4CL7* coding SEQ ID NO: 3 is

>*Ok4CL7* (1731 bp)

ATGGCTGCATTAATAAAAGCCCACGTCGAATTGCAGACTGTGAAAGAAGACCGTC
GTGTGATGGAAGAAATGTCGAAAACTAGAAAGTTGACACACTCCAATCCTTCTTG
GTATTGCCCTGAAACAGGAATATACTCCAGCAAATACCCTTCCATCAACCTCCCTT
CTGACCCTTTTCTTGATGTTGTTTCGTTCATTTTCTCACATAAACATGATGGGACAC
ATGCACTCGTTGATTCAGCATCAGGGCTATCACTTCTCTACTCCAAGCTCTTACCTT
TGGTGAAATCCATGGCTGCTGGTCTTCACCATTTGGGTGTCAAGCAAGGTGATGTC
ATCTTGATTCTGCTGCCCAATTCTGTGTGTTTCCCTTTAATTCTCTTGGGTGCTTTGA
GCGTTGGTGCTGTTGTCACCACCCTGAATCCTTTGAGTAGTTTGTTAGAGATCAAA
AAACAGGTTCGTGATAGTAATGCAACTCTAGCATTTTCTGCTATTGGTAGGGTTGA
TGAATTAGGTAATGCATTGGAGGGTTGCCTTATAGTAGGTGTGCCTGAAGTTTTAG
ATTTGAGCTCTCAGCCTATTAGTGGTTCTGTTTTTCATAAGCTCATTTCTAGTGATC
CTGAAATGGCCCCTAGGCCTAGAATTAAGCAGCAGGACACCGCCGCGATTTTATA
CTCATCGGGCACTACTGGTAAGTCTAAAGGAGTCATGTTGACTCATGGGAACTTCA
TAGCCATGATTGAGCTCTTTGTGAGGTTTGAAGCTTCTCTGTATGATTATCCTAGTA
CAGCCAATGTCTACTTGGCTGTTGTGCCTATGTTCCATGTGTATGGGCTGTCTCTTT
TCGTTTTGGGATTGTTGTCGTTGGGGAGTACTGTAGTCACCATGAGAAAGTTTGAT
GGTGATGAAATGGTGAGAGCTATTGATAGATATGGTATAACTCACCTTCATGCTGT
CCCGCCTATACTGATTGCGTTGACGAAGAGAGCCAAGAACGCTGATGGTAATGGT
TTTCAGAGCTTGAAACAGGTTTCGTGTGGGGCTGCTCCGTTGAGTGAGAAAAGCAT
AGTTGAATTGATTGAGGCACTCCCCCATGTTGACTTCATTCAGGGTTATGGCATGA
CAGAATCGACCGCATTGGCAACTCGCGGCTACAATAATGGTGGAGTCAAGAAATA
TTCCTCTGTGGGACTTCTTTCTCCAAATATTGAAGCCAAAGTGGTGGACTGGGTTA
CAGGAGCTCTCGTTCCTCCAGGATCGATCGGTGAGCTTTGGTTGCGCACACCAGGA
AACATGAAAGGGTACTTGAATAATACTGAAGCAACTGTGGGTGCACTTGACAAAG
AAGGCTGGCTACACACTGGTGACATAGTTTATTTTGACCAAGAAGGATACTTGTAT
GTAATCGATCGCCTGAAAGAAGTCATCAAGTACAAAGGATTCCAGATTGCTCCAG
CGGATTTGGAGGCAGTATTGATGTCACATCCAGAAGTAGCTGATGCAGCTGTTAC
AGGTGTGAGGGACGAAGAAGCCGGAGAGATCCCTGTGGCATTCGTCGTTCCAAAA
GAAGGTACATCACTCTCCGCGGCCACCCTCATGGACCACGTAGCGAAGCAGGTTG

CACCTTATAAGAAGGTGAGAAAGGTGTATTTTCGGACATCAATACCGAGATCGCC
TGCCGGAAAAATACTTCGCAGGGAGCTCAAGAACTTGTTTGTTTCCAGGCTTTAG

In a preferred embodiment of the present disclosure, the *Ok4CL7* protein SEQ ID NO: 4 is

>*Ok4CL7* (576 aa)

MAALIKAHVELQTVKEDRRVMEEMSKTRKLTHSNPSWYCPETGIYSSKYPSINLPSDP
FLDVVSFIFSHKHDGTHALVDSASGLSLLYSKLLPLVKSMAAGLHHLGVKQGDVILIL
LPNSVCFPLILLGALSVGAVVTTLNPLSSLLEIKKQVRDSNATLAFSAIGRVDELGNALE
GCLIVGVPEVLDLSSQPISGSVFHKLISSDPEMAPRPRIKQQDTAAILYSSGTTGKSKGV
MLTHGNFIAMIELFVRFEASLYDYPSTANVYLAVVPMFHVYGLSLFVLGLLSLGSTVV
TMRKFDGDEMVRAIDRYGITHLHAVPPILIALTKRAKNADGNGFQSLKQVSCGAAPLS
EKSIVELIEALPHVDFIQGYGMTESTALATRGYNNGGVKKYSSVGLLSPNIEAKVVDW
VTGALVPPGSIGELWLRTPGNMKGYLNNTEATVGALDKEGWLHTGDIVYFDQEGYL
YVIDRLKEVIKYKGFQIAPADLEAVLMSHPEVADAAVTGVRDEEAGEIPVAFVVPKEG
TSLSAATLMDHVAKQVAPYKKVRKVYFRTSIPRSPAGKILRRELKNLFVSRL

In still another embodiment of the present disclosure, the open reading frame of *Ok4CL15* is represented by SEQ ID NO: 5.

>*Ok4CL15* (1608 bp)

ATGGAGCTGAAAGAAGAGAAGGAATACATTTTCCGATCGAAGCTTCCAGATATCT
ACATTCCCCTCCATCTCCCTCTCCACACATACTGCTTCGAAAACCTCTCACTCCACC
GCGCTCGGCCGTACTTAATCAACGCCGTCACCGGCGAAACCTTCACCCATGCCGA
ATTCGAGCTCACCGCGCGCAGAGTCGGCGCCGGTCTCCACAACCTCGGAATCCGC
AAATCCGACGTCGTCATGCTCCTCCTCCACAACTCGCCTGAATTCGCGTTCGCTTTC
CTAGGAGCCTCCTTCGTCGGCGCGGTCGTCACCACCGCGAATCCTCTCTACACCGC
CTCGGAAATCGCGCTGCAGGTTGAGATCTCGAGGCCGAGGCTTATCATCACACAC
GCCTGTCACGTGGAGAAGGTGAGGCAGCACGCCTCCGCGGATGGCGCCAAAATCG
TGACGATCGACGCTCCGCCGTCGCCGGAGATCGTTCATTTCTCGGATTTGACGAGA
TCTGACGAGAATCGACTTCCTGCAGTCGAAATCCGAGGAGAGGACACGGCGGCGC
TGCCGTTCTCCTCCGGAACCACCGGTCTCCCCAAAGGAGTAATGCTGAGTCACAGA
AACATAATCACGTGCATCTCGCAGCAGGTGGACGGTGAAAATCCGGCGAATCACA
TCGACTGCGAGGATCGGCTTCTCTGCGTGTTGCCGCTGTTTCACGTTTACTCTATGG
TGTCGGTGATGCTCTGCAGTCTCCGCGCCGGAGCGGCGATTGTGATTATGCCGAGA
TTTGAACTGAATGAGTTGATGGAGGTGATACAGAAATATAAGGTGACGATCGCGC
CGTTTGTGCCGCCGATATTGTTAGGCATCGCGAAGAGCCAGACGGCGGCGAAGTT
CGATCTGTCGTCGGTGAGGAGAGTCGTCTGCGGCGCGGCGCCGATGGATCGGAAA

CTTGAGCTGTCACTCAAATCAAAACTTCCGAATGCTATTATTGGGCAGGGTTATGG
TATGACAGAAGCCTTAGTCCTATCAATGTGTTTAGGTTTCGCCAAGTTTCCGATGA
AATTCAAGGCTGGCTCGTGTGGAAATGTGATTAAAAACGCCCGTATGAAGATCAT
CGACCCGGCCACCGGTGCCTCCCTCGACCGGAATCAGAGGGGGGAGATTTGCCTC
AAAGGAAGCTCCGTGATGAAAGGTTATTATAAGGATCCGGAGGCGACGAAGAGG
ACTATCGACGAGGAGGGGTGGCTGCACACCGGCGATATAGGGTACATTGACGATG
ACGGTGAAGTGTTCATTGTGGACAGGTTGAAGGAATTGATCAAATACAAAGGATT
TCATGTTGCTCCTGCTGAACTTGAAGCTCTTCTCATAGCTCATCCCTCCATATCTGA
TGCTGCTGTTGTGCCTATGGCCGATGAGGCTGCTGGAGAAGTTCCTGTTGCATTTG
TAGTCAGGGAAAATGGTTCGAAGATTACGGAACTAGAAATCAAGAAATACATCGC
GAGCCAGGTGGTGTCGTACAAGCGTATCAAACATGTATTCTTCATTGACAATATTC
CAAAAGCCCCCTCTGGTAAAATTCTGAGGAAGAATCTGAGAGCTAGAATGTAA

In a preferred embodiment of the present disclosure, the *Ok4CL15* protein SEQ ID NO: 6 is

>*Ok4CL15* (535 aa)

MELKEEKEYIFRSKLPDIYIPLHLPLHTYCFENLSLHRARPYLINAVTGETFTHAEFELT
ARRVGAGLHNLGIRKSDVVMLLLHNSPEFAFAFLGASFVGAVVTTANPLYTASEIALQ
VEISRPRLIITHACHVEKVRQHASADGAKIVTIDAPPSPEIVHFSDLTRSDENRLPAVEIR
GEDTAALPFSSGTTGLPKGVMLSHRNIITCISQQVDGENPANHIDCEDRLLCVLPLFHV
YSMVSVMLCSLRAGAAIVIMPRFELNELMEVIQKYKVTIAPFVPPILLGIAKSQTAAKF
DLSSVRRVVCGAAPMDRKLELSLKSKLPNAIIGQGYGMTEALVLSMCLGFAKFPMKF
KAGSCGNVIKNARMKIIDPATGASLDRNQRGEICLKGSSVMKGYYKDPEATKRTIDEE
GWLHTGDIGYIDDDGEVFIVDRLKELIKYKGFHVAPAELEALLIAHPSISDAAVVPMA
DEAAGEVPVAFVVRENGSKITELEIKKYIASQVVSYKRIKHVFFIDNIPKAPSGKILRKN
LRARM

In an embodiment, the present disclosure provides a recombinant binary expression vector for overexpressing 4-coumarate Co-enzyme A ligase 11 gene (*4CL11*) in host plant cells, the recombinant binary expression vector comprising:

a pRI101AN vector comprising an open reading frame (ORF) of a 4-coumarate- Co-enzyme A ligase gene isolated from *Ocimum kilimandscharicum* (pRI101AN-*Ok4CL11*-overexpression construct); and

at least one transcriptional regulatory element,

wherein the pRI101AN-*Ok4CL11*-overexpression construct comprises the ORF of the *Ok4CL11* gene ligated into a multiple cloning site (MCS) of the pRI101AN vector after

restriction digestion of the pRI101AN vector with NdeI and EcoRI restriction enzyme sites.

In an embodiment of the present disclosure provides, wherein the ORF of the *Ok4CL11* gene comprises a nucleotide sequence with at least 70% similarity to SEQ ID NO: 1.

In an embodiment of the present disclosure provides, wherein the ORF of the *Ok4CL11* gene encodes a polypeptide having an amino acid sequence with at least 70% similarity to SEQ ID NO: 2.

In a preferred embodiment, the present disclosure provides an *Ok4CL11*-ORF nucleotide sequence comprising at least 70% nucleotide sequence identity with the native sequence. For example, at least 70% identity, 75% identity, 80% identity, 85% identity, 90% identity, 91% identity, 92% identity, 93% identity, 94% identity, 95% identity, 96% identity, 97% identity, 98% identity, 99% identity, or 100% identity.

In a preferred embodiment, the present disclosure provides an *Ok4CL11*-ORF polypeptide sequence comprising at least 70% polypeptide sequence identity with the native sequence. For example, at least 70% identity, 75% identity, 80% identity, 85% identity, 90% identity, 91% identity, 92% identity, 93% identity, 94% identity, 95% identity, 96% identity, 97% identity, 98% identity, 99% identity, or 100% identity.

In another preferred embodiment, the present disclosure provides a binary expression vector comprising nucleotide sequences encoding regulatory elements operably linked to a nucleotide sequence encoding the *Ok4CL11*-ORF.

In another embodiment of the present disclosure, the host *4CL11* gene is overexpressed by transforming a binary expression vector pRI101AN comprising *Ok4CL11* ORF and one or more transcriptional regulatory sequence into the plant cell, and allowing the vector to integrate into the genome by non-homologous recombination, thus allowing overexpression of the host *4CL11*.

In still another embodiment of the present disclosure, the binary expression vector pRI101AN may additionally comprises one or more transcriptional regulatory elements selected from but not limited to a promoter, a transcriptional enhancer, a reporter, and a terminator.

In still another exemplary embodiment of the present disclosure, the binary expression vector pRI101AN additionally comprises one or more transcriptional regulatory elements selected from but not limited to 35S CaMV promoter, 5′ untranslated region (UTR) of alcohol dehydrogenase gene from *Arabidopsis thaliana,* and Nopaline synthase terminator.

In an embodiment of the present disclosure, the binary expression vector pRI101AN encoding the *Ok4CL11*-OE operably linked to 35S CaMV promoter, 5′ untranslated region (UTR) of alcohol dehydrogenase gene from *Arabidopsis thaliana,* and Nopaline synthase terminator.

In an embodiment of the present disclosure, the nucleotide construct of at least 70% sequence identity (e.g., 75% identity, 80% identity, 85% identity, 90% identity, 95% identity, 98% identity, 99% identity, or 100% identity) with the nucleotide construct of this invention.

In another embodiment of the present disclosure, the *Ok4CL11* ORF sequence is cloned into the pRI101AN vector using the restriction enzymes NdeI and EcoRI to form the pRI101AN-*Ok4CL11*-OE construct.

In an embodiment, the present disclosure provides a method for expressing host *4CL11* in a suitable plant cell is effected by stably transforming the suitable plant cell with said pRI101AN-*Ok4CL11*-OE construct. In another embodiment, the transforming is effected by *Agrobacterium*-mediated transformation. It is to be noted that the transformation can also be effected by the methods known in the prior art. Any person having ordinary skill in the art would be able to perform the same with the other known transformation methods.

In an embodiment, the present disclosure provides a method for expressing host *4CL11* in a plant cell, said method comprising the steps of

a. providing the pRI101AN-*Ok4CL11*-OE construct as disclosed herein;
b. providing a plant cell;
c. stably transforming the plant cell with said pRI101AN-*Ok4CL11*-OE construct to obtain transformed plant cell;
d. isolating and identifying the transformed plant cells overexpressing *4CL11* gene;
e. regenerating the transformed plant cell overexpressing *4CL11* gene into a plant using plant growth media.

In an embodiment, the present disclosure provides a method for expressing a host *4CL11* gene in a plant, the method comprising:

a. providing the pRI101AN-*Ok4CL11*-overexpression construct as claimed in claim 1;
b. providing a plant cell, a calli tissue, or a leaf explant;
c. stably transforming the plant cell, the calli tissue, or the leaf explant with the pRI101AN-*Ok4CL11*-overexpression construct to obtain a transformed plant cell, a transformed calli tissue, or a transformed leaf explant;
d. isolating and identifying the transformed plant cell, the transformed calli tissue, or the transformed leaf explant overexpressing the host *4CL11* gene; and

e. regenerating the transformed plant cell, the transformed calli tissue, or the transformed leaf explant overexpressing the host *4CL11* gene into the plant using a plant growth media.

In an embodiment of the present disclosure, wherein the plant growth media is defined in Figure *8.*

In an embodiment of the present disclosure, wherein the plant cell derived from a leaf or stem tissue obtained from *Nicotiana benthamiana, Arabidopsis thaliana,* or *Solanum tuberosum* were used as explants for *Agrobacterium*-mediated stable transformation.

In an embodiment of the present disclosure, wherein the stable transformation is effected by an *Agrobacterium*-mediated leaf transformation method.

In an embodiment of the present disclosure, wherein the transformation is effected by an *Agrobacterium tumefaciens* strain (GV3101 strain) using a freeze and thaw transformation method.

In an embodiment of the present disclosure, wherein the pRI101AN-*Ok4CL11*-overexpression construct integrates into the genome of cells of *Nicotiana benthamiana, Arabidopsis thaliana*, or *Solanum tuberosum* by non-homologous recombination.

In an embodiment, the present disclosure provides a transgenic plant is produced by the method as claimed in claim 7, wherein the transgenic plant overexpresses a host *4CL11* gene.

In still another embodiment of the present disclosure, the *4CL11* overexpression lines of *N. benthamiana, Arabidopsis thaliana*, and potato are generated through the *Agrobacterium*-mediated leaf transformation method as disclosed in Horsch et al. (1985), Clough and Bent (1998) and Banerjee et al. (2006), respectively.

In still another embodiment of the present disclosure, five transgenic lines of *Ok4CL11* overexpression in *N. benthamiana* (referred as *Ok4CL11*-OE Nb lines) and 4 lines of potato are developed.

In yet another embodiment of the present disclosure, the overexpression lines of *4CL11* in plants or plant cells help to increase the level of flavonoids (such as kaempferol- and quercetin-derivatives) in the transgenic cells or plant lines of *N. benthamiana* as compared to its wild-type. The increased accumulation of flavonoids and their derivatives) and the reduced auxin content, decreased expression of auxin transport and signaling genes further leads to a reduced root growth phenotype.

In still another embodiment of the present disclosure, the *4CL11* overexpression in *N. benthamiana* plants leads to a reduction in root growth, altered lignin content, and increase in

natural product biosynthesis. Reduced root growth is also observed in case of *Ok4CL11* overexpression in potato or *Arabidopsis thaliana* plants.

In a preferred embodiment of the present disclosure, the *4CL11* overexpressed *N. benthamiana* plants can be further used as feedstock for biofuel production.

In an embodiment of the present disclosure, a pRI101AN vector with the *Ok4CL11* ORF sequence used for the preparation of rootless plants including but not limited to *N. benthamiana* and potato is also disclosed. This is the unique function exhibited by *Ok4CL11*, and not by other two *4CL* isoforms like *Ok4CL7* and -15.

In a preferred embodiment of the present disclosure, the composition can also include variations of *Ok4CL7*, -11 and -15 genes in the pRI101AN vector. The variants are generated by methods selected from but not limited to site-directed mutagenesis, intron removal and the like, wherein the variants of said genes comprise at least 70% sequence identity with the native sequence. For example, at least 70% identity, 75% identity, 80% identity, 85% identity, 90% identity, 91% identity, 92% identity, 93% identity, 94% identity, 95% identity, 96% identity, 97% identity, 98% identity, 99% identity, or 100% identity.

EXAMPLES

Example 1:- Isolation and cloning of *Ok4CL7*, *Ok4CL*11 and *Ok4CL* 15 genes:

cDNA encoding 4-coumarate Co-enzyme A ligase 11 (*Ok4CL11*) were isolated from *Ocimum kilimandscharicum*. The cDNA sequence of *Ok4CL11* has an open reading frame of 1731 bp. A binary expression vector, pRI101AN, was taken as a basic backbone vector to develop the transgenic plants. This vector consists of a 35S CaMV promoter, 5' untranslated region (UTR) of the alcohol dehydrogenase gene from *Arabidopsis thaliana*, and the nopaline synthase terminator (depicted in Figures 1 A and B). The purified *Ok4CL11* was ligated into the multiple cloning site (MCS) of pRI101AN after restriction digestion of the vector with NdeI and EcoRI restriction enzyme sites in forward and reverse primers, respectively. Cloning of *Ok4CL11* gene in pRI101AN vector was confirmed using restriction enzyme digestion (depicted in Figure 1 C), followed by sequencing. Similarly, *Ok4CL7* and *Ok4CL*15 are also cloned individually in pRI101AN vector to generate their respective overexpression constructs.

Example 2:- Generation of gene constructs

For the generation of *Ok4CL7, Ok4CL11* and *Ok4CL15* overexpression gene constructs, the longest open reading frame (ORF) of *Ok4CL7* (1731 bp), *Ok4CL11* (1731 bp) and *Ok4CL15* (1530 bp) were amplified by Polymerase Chain Reaction (PCR) with high-fidelity Phusion DNA Polymerase (Thermo Fisher Scientific, Waltham, United States). Complementary DNA (cDNA)

synthesized from leaf of *O. kilimandscharicum* was used as a template for PCR. Amplified sequences were mobilized individually in a binary vector pRI101AN between the Cauliflower Mosaic Virus (CaMV) 35S promoter and the nopaline synthase terminator (NOST). For cloning purposes, *NdeI* restriction enzyme site was added in the forward primer, whereas *EcoRI or SacI* was added in the reverse primer. Finally, the individual overexpression constructs of these *Ok4CLs* (*35S::Ok4CL7*, *35S::Ok4CL11*, and *35S::Ok4CL15*) were prepared. Similarly, *35S::GUS* construct was used to generate a vector control (VC) line. To develop the RNA*i* construct of *Ok4CL11* (*35S::Ok4CL11-RNAi*), 350 bp unique sequence of sense and antisense strands was cloned on either side of 500 bp intron in the pRI101 binary vector.

Example 3:- Expression of *Ok4CL7*, *Ok4CL11*, and *Ok4CL15* genes:

Transformation of cells:

pRI101AN-*Ok4CL7*, *Ok4CL11*, and *Ok4CL15* constructs were mobilized individually into *Agrobacterium tumefaciens* (GV3101 strain) using the freeze and thaw transformation method. The resulting binary vector containing the *Agrobacterium* strain was used to develop the transgenic lines of *N. benthamiana.* Overexpression lines of three Ok*4CL* isoforms (*Ok4CL7*, *Ok4CL11* and *Ok4CL15*) in *N. benthamiana* were generated through the *Agrobacterium*-mediated leaf transformation method as described by Horsch et al. (1985) (depicted in Figure 2). A total of five independent lines of *Ok4CL11* overexpression (referred to as *Ok4CL11*-OE1 to *Ok4CL11*-OE5) were developed using this method (Fig. 3). Similarly, overexpression lines of *Ok4CL7* and *Ok4CL15* in *N. benthamiana* are generated (Fig. 3).

Transformation of Explants:

In another experiment (Figure 9 A-E), three-week old wild-type *N. benthamiana* seedlings were used to prepare explants (shoot-apex with 2-nodes) for supplementation, whereas in the experiment shown in Figure 9 (F), a single-node explants from the middle region of stem (3-5th nodes from the shoot-apex) from two-month old wild-type *N. benthamiana* were used as an explant.

Example 4:- Screening of transgenic lines:

Respective transgenic lines were confirmed by reverse transcriptase-PCR (RT-PCR) amplification of target gene from cDNA (synthesized from mRNA) and through PCR from genomic DNA isolated from leaves of putative transformants of *N. benthamiana* and wild-type plants (as negative control).

Results:

Transformation of cells:

Out of 9 putative transformants, 5 lines were found to be true transformants (depicted in Figure 3). For *Ok4CL7* overexpression construct, 1 line out of 3 putative transformants is positive, whereas for *Ok4CL15* overexpression construct, 2 lines out of 7 putative transformants are confirmed (depicted in Figure 3). The gene expression of *Ok4CL7*, *Ok4CL11* and *Ok4CL15* in the individual overexpression lines of *N. benthamiana*; wherein two lines of *Ok4CL7*, five lines of *Ok4CL11* and two lines of *Ok4CL15* are shown (Figure 4). Root growth was significantly affected in all transgenic *N. benthamiana* plants compared to wild-type plant (depicted in Figure 5A). Further, transgenic lines, such as *Ok4CL11*-OE1 and *Ok4CL11*-OE2 showed rootless phenotype. The number of primary and lateral roots was significantly less in *Ok4CL11*-OE lines compared to wild-type plant (Figures 5B-C). The length of the primary root was also significantly affected in *Ok4CL11*-OE lines (OE1 and OE2) compared to wild-type *N. benthamiana* plants (depicted in Figure 5D). Root growth parameters, such as root number, lateral root length and the main root length remained similar to wild-type in *Ok4CL15*-OE1 line, whereas in *Ok4CL15*-OE1 line, only root number was increased with the main root length and lateral root numbers being comparable to wild-type *N. benthamiana* plants (Figures 5B-D).

Transformation of Explants:

- The adventitious root (AR) emergence was initiated on day 3 for control plants, whereas the individual flavonoids (K and Q) or their glycosides (R, K3R, K3,7R, Q3G) showed delayed AR initiation (2-3 days). Moreover, the mixture of these aglycon flavonoids and their glycosides (K + Q + R + K3R + K3,7R + Q3G) was the most effective to delay the AR emergence by 4 days Figure 9(A).
- On day 9, the average number of ARs per plant was significantly higher ($p>0.000078$) for control (2.35) compared to 1.36 ARs in the mix treatment. Same for day 12, $p>0.000001$ for control (3.1) compared to 1.84 ARs in the mix treatment. For day 15, $p>0.0001$ for control (3.38) compared to 2.24 ARs in the mix treatment.
- The mixture of the aglycon flavonoids and their glycosides (K + Q + R + K3R + K3,7R + Q3G) appears to be the most effective to affect root number, length, AR biomass as well as shoot biomass Figure 9(B-E). These effects on the shoot biomass are similar to TIBA and NPA treatment (our previous experiment); however, TIBA and NPA results in complete inhibition of root emergence, leading to rootless explants.
- Figure 9(F) shows images of the single-node stem explants of wild-type *N. benthamiana* grown on flavonoids and their glycosides to evaluate their effect on root growth inhibition. All the flavonoids and their glycosides are inhibiting the root growth.

Abbreviations used for flavonoids and their glycosides: Kaempferol (K), Quercetin (Q), Rutin (R), Kaempferol 3-rhamnoside (K3R), Kaempferol-3,7-O-bis-alpha-L-rhamnoside (K3,7R), Quercetin 3-O-β-D-glucuronide (Q3G), Mix (K+Q+R+K3R+K3,7R+Q3G).

Example 5:- *Ok4CL11* overexpression study:

The effect of *Ok4CL11* overexpression on the metabolite contents was studied with the leaf extracts (in 80% methanol) of both transgenic and wild type *N. benthamiana* using an untargeted LC-MS assay.

Results:

LC-MS assay shows that the level of flavonoids (kaempferol- and quercetin-derivatives) increased in *Ok4CL11*-OE lines of *N. benthamiana* compared to wild-type plants (depicted in Figure 6A). The preliminary analysis further shows that the *Ok4CL11* overexpression in *N. benthamiana* alters the lignin content compared to wild-type plants (depicted in Figures 6B-C), further adding its potential application in biofuel production.

Example 6:- *Ok4CL11* overexpression in potato:

Agrobacterium-mediated potato leaf transformation is performed and the overexpression line of potato for *Ok4CL11* gene is generated. The overexpression line is confirmed by reverse transcriptase PCR and root growth phenotype is recorded.

Results:

Of the two potato lines that are tested for *Ok4CL11* overexpression, one line is confirmed (Figure 7). This line showed a reduced root growth phenotype similar to *N. benthamiana* overexpression plants.

ADVANTAGES OF THE INVENTION

- The present technique can be used in hydroponic, aeroponic, and vertical agriculture in order to reduce root growth.
- The present technique can be used in biofuel industries to alter the lignin content by overexpressing the *4CL11* isoform in targeted crop plants.
- The present technique can be applied further in natural product biosynthesis.
- Provides overexpression of a specific Ocimum *4CL* isoform – *Ok4CL11* in *N. benthamiana* leads to a rootless or reduced root growth phenotype, owing to accumulation of specific flavonoids (kaempferol and quercetin) and their glycosides. Further, this phenotype was also shown in potato and *Arabidopsis thaliana* plants.

- Specifically, the invention provides stable overexpression of *Ok4CL11* in *N. benthamiana* causes accumulation of specific flavonoids (kaempferol and quercetin) and their glycosides, leading to the rootless or reduced root growth phenotype.
- More specifically, the invention provides generation of overexpression gene construct of the specific *4CL* isoform from Ocimum - *Ok4CL11* in a binary vector pRI101 and its effect on plant root growth.
- Also, it provides overexpression of *Ok4CL11* in *N. benthamiana* causes accumulation of specific flavonoids (kaempferol and quercetin) and their glycosides that could inhibit auxin transport, leading to the rootless phenotype.
- The effect of overexpressing *Ok4CL11* on root growth was also found in potato and *Arabidopsis thaliana* plants.

We Claim:

1. A recombinant binary expression vector for overexpressing 4-coumarate Co-enzyme A ligase 11 gene (*4CL11*) in host plant cells, the recombinant binary expression vector comprising:

   a pRI101AN vector comprising an open reading frame (ORF) of a 4-coumarate-Co-enzyme A ligase gene isolated from *Ocimum kilimandscharicum* (pRI101AN-*Ok4CL11*-overexpression construct); and

   at least one transcriptional regulatory element,

   wherein the pRI101AN-*Ok4CL11*-overexpression construct comprises the ORF of the *Ok4CL11* gene ligated into a multiple cloning site (MCS) of the pRI101AN vector after restriction digestion of the pRI101AN vector with NdeI and EcoRI restriction enzyme sites.
2. The recombinant binary expression vector as claimed in claim 1, wherein the ORF of the *Ok4CL11* gene comprises a nucleotide sequence with at least 70% similarity to SEQ ID NO: 1.
3. The recombinant binary expression vector as claimed in claim 1, wherein the ORF of the *Ok4CL11* gene encodes a polypeptide having an amino acid sequence with at least 70% similarity to SEQ ID NO: 2.
4. The recombinant binary expression vector as claimed in claim 1, wherein the transcriptional regulatory element is a 35S CaMV promoter.
5. The recombinant binary expression vector as claimed in claim 1, wherein the transcriptional regulatory element is a 5′ untranslated region (UTR) of an alcohol dehydrogenase gene from *Arabidopsis thaliana.*
6. The recombinant binary expression vector as claimed in claim 1, wherein the transcriptional regulatory element is a nopaline synthase terminator.
7. A method for expressing a host *4CL11* gene in a plant, the method comprising:
   a. providing the pRI101AN-*Ok4CL11*-overexpression construct as claimed in claim 1;
   b. providing a plant cell, a calli tissue, or a leaf explant;
   c. stably transforming the plant cell, the calli tissue, or the leaf explant with the pRI101AN-*Ok4CL11*-overexpression construct to obtain a transformed plant cell, a transformed calli tissue, or a transformed leaf explant;

d. isolating and identifying the transformed plant cell, the transformed calli tissue, or the transformed leaf explant overexpressing the host *4CL11* gene; and
e. regenerating the transformed plant cell, the transformed calli tissue, or the transformed leaf explant overexpressing the host *4CL11* gene into the plant using a plant growth media.

8. The method as claimed in claim 7, wherein the plant cell derived from a leaf or stem tissue obtained from *Nicotiana benthamiana, Arabidopsis thaliana,* or *Solanum tuberosum* were used as explants for *Agrobacterium*-mediated stable transformation.
9. The method as claimed in claim 7, wherein the stable transformation is effected by an *Agrobacterium*-mediated leaf transformation method.
10. The method as claimed in claim 9, wherein the stable transformation is effected by an *Agrobacterium tumefaciens* strain (GV3101 strain) using a freeze and thaw transformation method.
11. The method as claimed in claim 7, wherein the pRI101AN-*Ok4CL11*-overexpression construct integrates into the genome of cells of *Nicotiana benthamiana, Arabidopsis thaliana*, or *Solanum tuberosum* by non-homologous recombination.
12. A transgenic plant is produced by the method as claimed in claim 7, wherein the transgenic plant overexpresses a host *4CL11* gene.

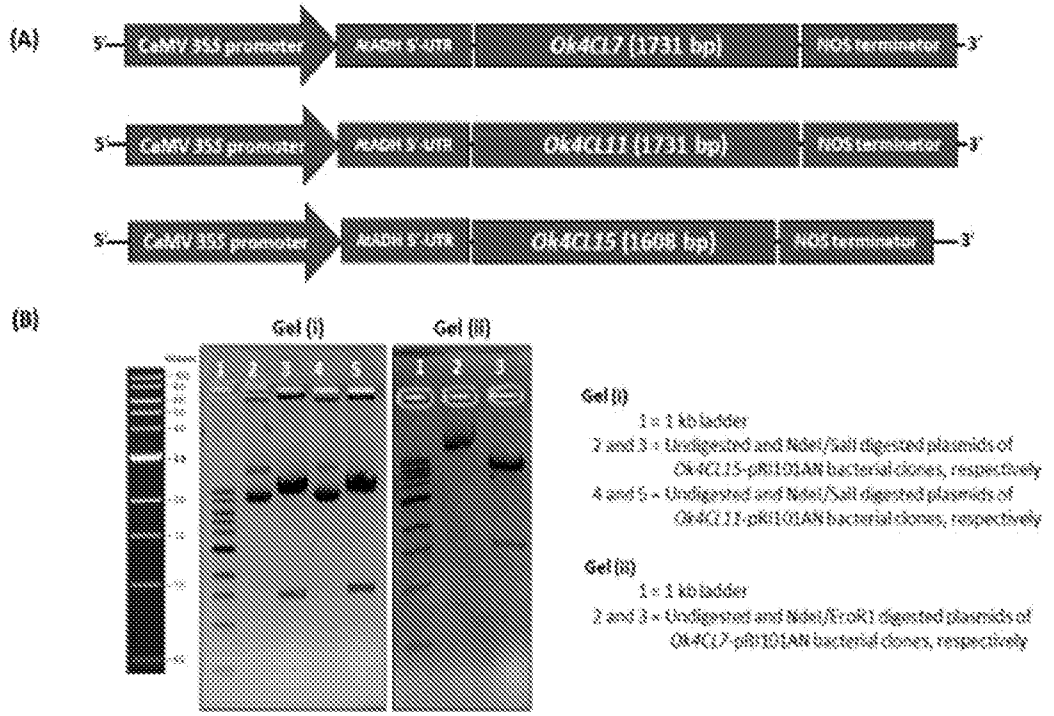


Figure 1

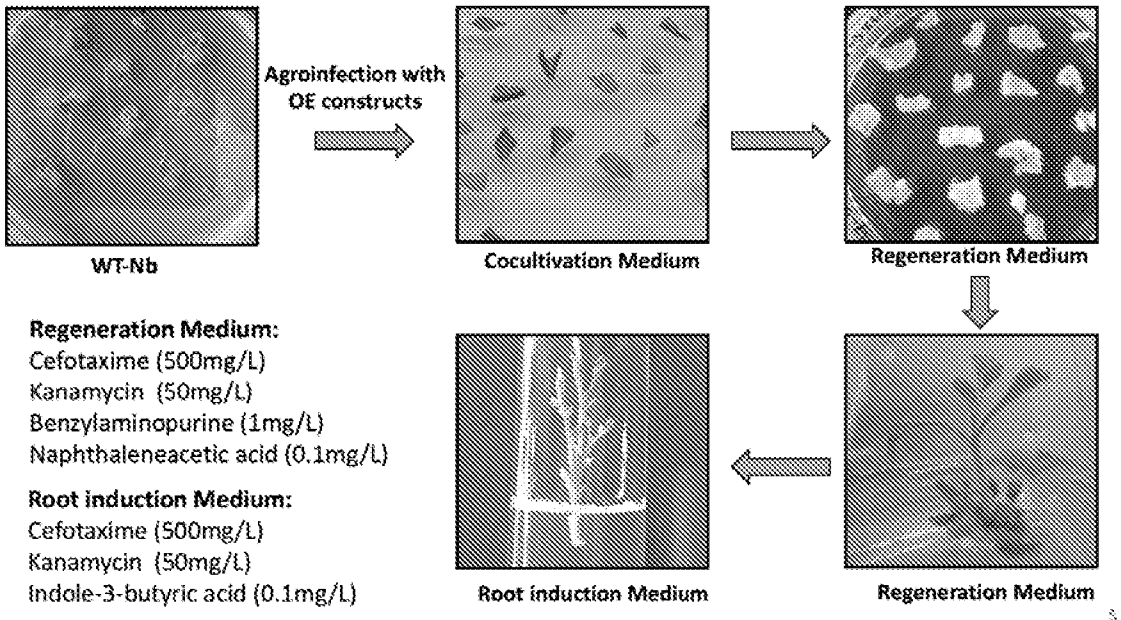


Figure 2

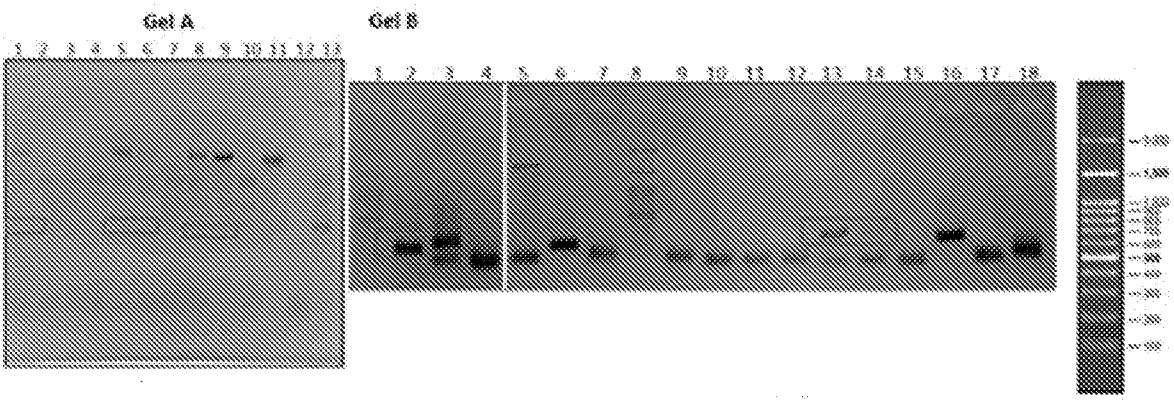


Figure 3

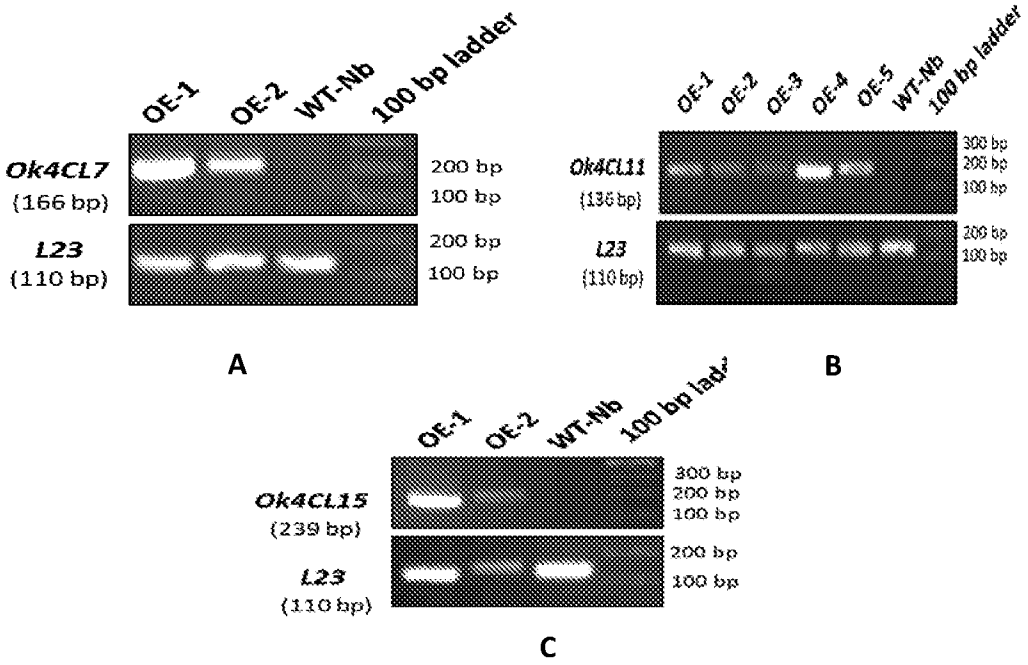


Figure 4

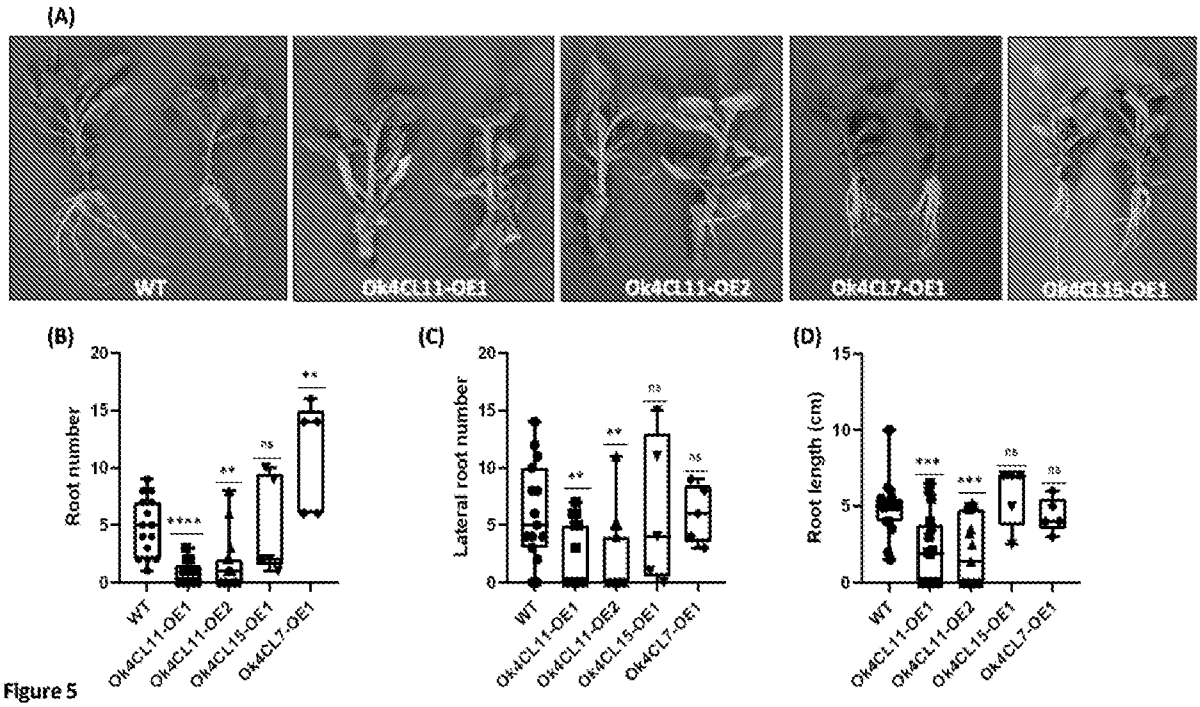


Figure 5

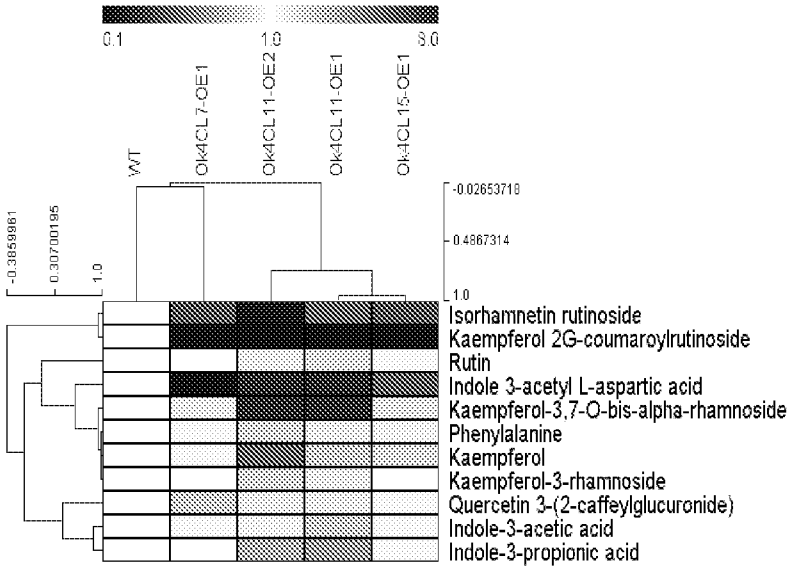


Figure 6A

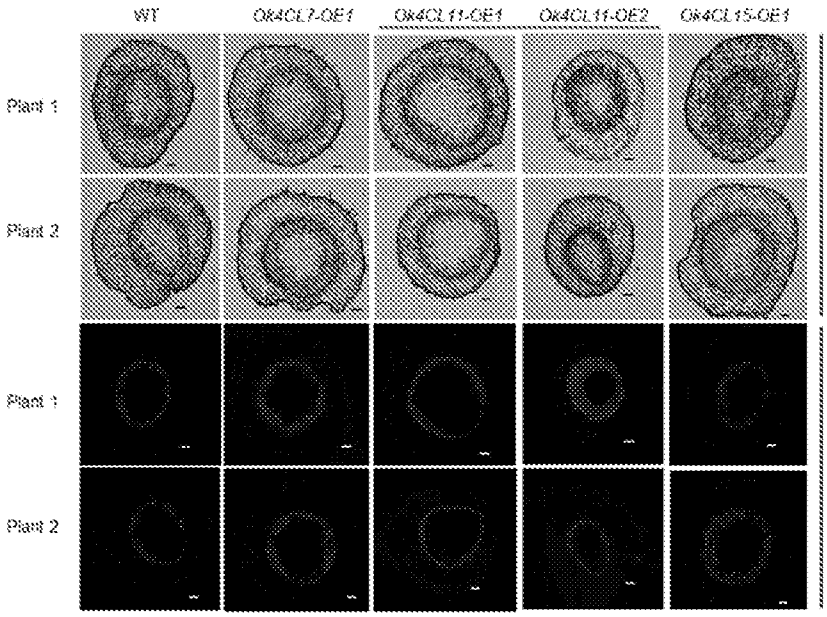


Figure 6B

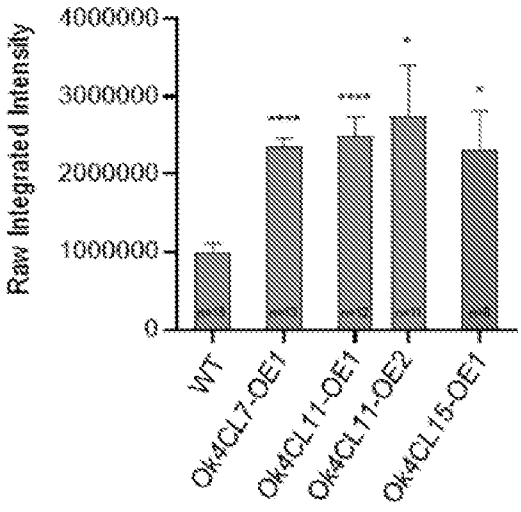


Figure 6C

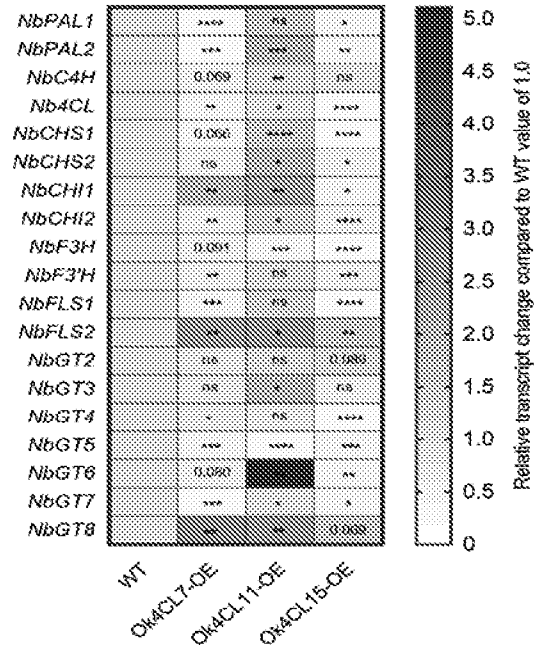


Figure 6D

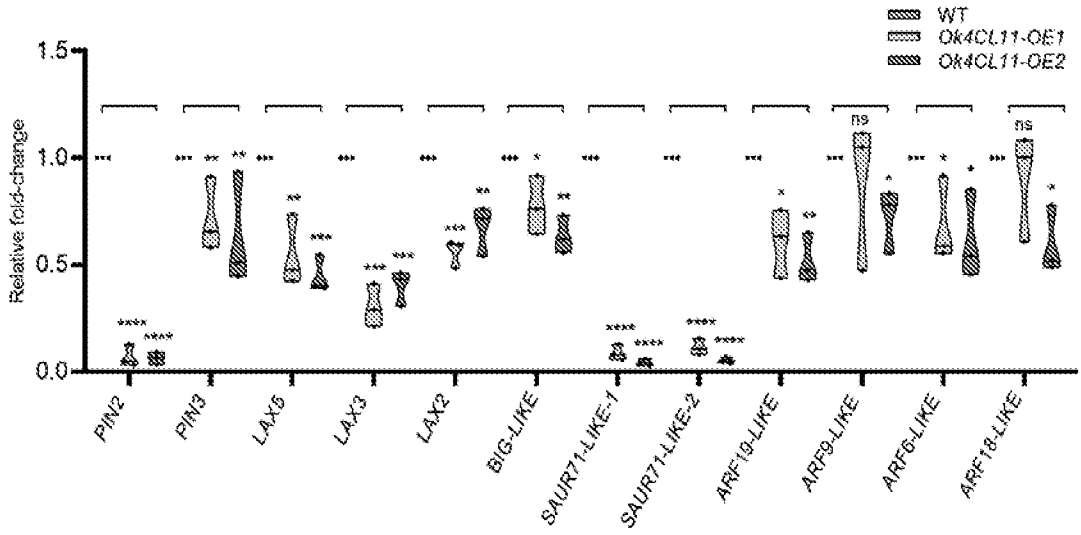


Figure 6E

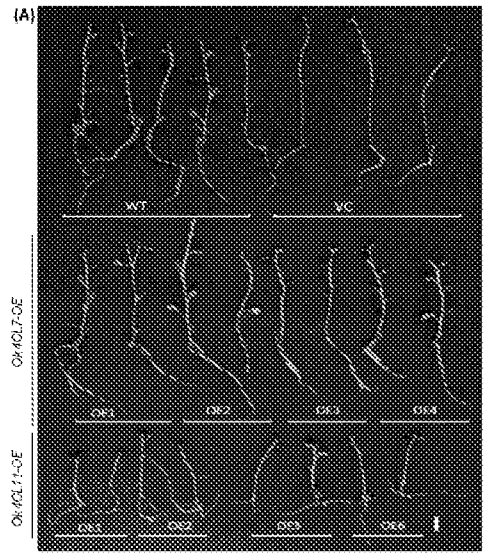


Figure 7A

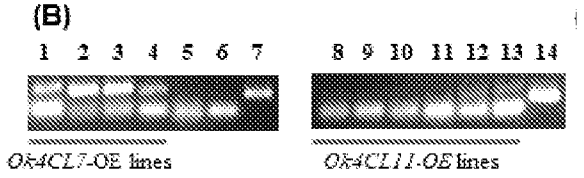


Figure 7B

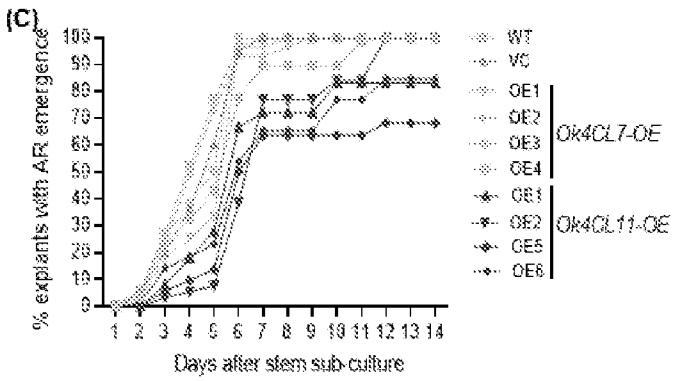


Figure 7C

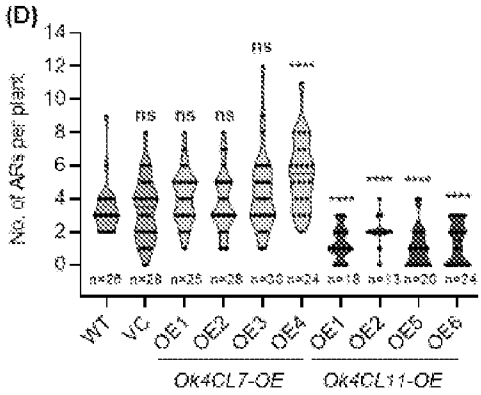


Figure 7D

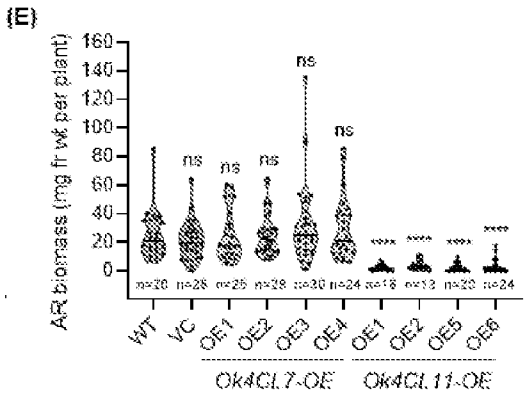


Figure 7E

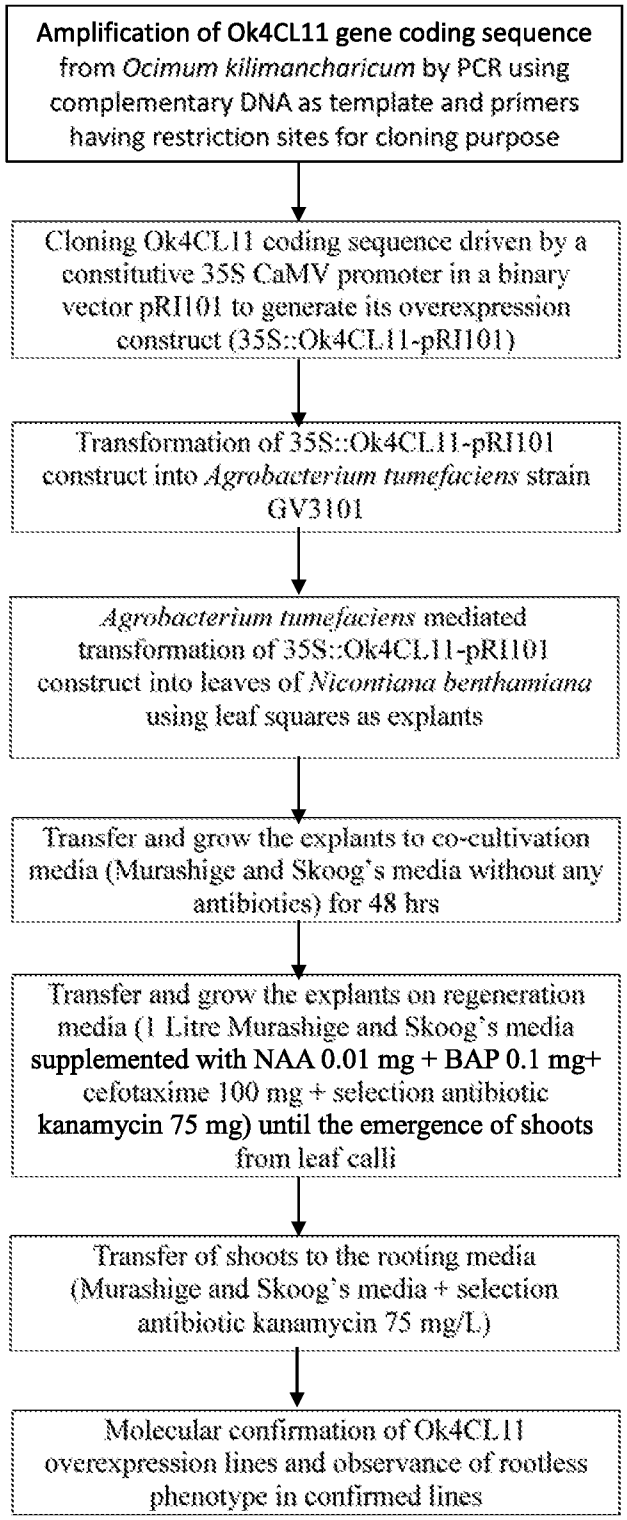

Amplification of Ok4CL11 gene coding sequence from *Ocimum kilimancharicum* by PCR using complementary DNA as template and primers having restriction sites for cloning purpose
Cloning Ok4CL11 coding sequence driven by a constitutive 35S CaMV promoter in a binary vector pRI101 to generate its overexpression construct (35S::Ok4CL11-pRI101)
Transformation of 35S::Ok4CL11-pRI101 construct into *Agrobacterium tumefaciens* strain GV3101
Agrobacterium tumefaciens mediated transformation of 35S::Ok4CL11-pRI101 construct into leaves of *Nicontiana benthamiana* using leaf squares as explants
Transfer and grow the explants to co-cultivation media (Murashige and Skoog's media without any antibiotics) for 48 hrs
Transfer and grow the explants on regeneration media (1 Litre Murashige and Skoog's media supplemented with NAA 0.01 mg + BAP 0.1 mg+ cefotaxime 100 mg + selection antibiotic kanamycin 75 mg) until the emergence of shoots from leaf calli
Transfer of shoots to the rooting media (Murashige and Skoog's media + selection antibiotic kanamycin 75 mg/L)
Molecular confirmation of Ok4CL11 overexpression lines and observance of rootless phenotype in confirmed lines

Figure 8

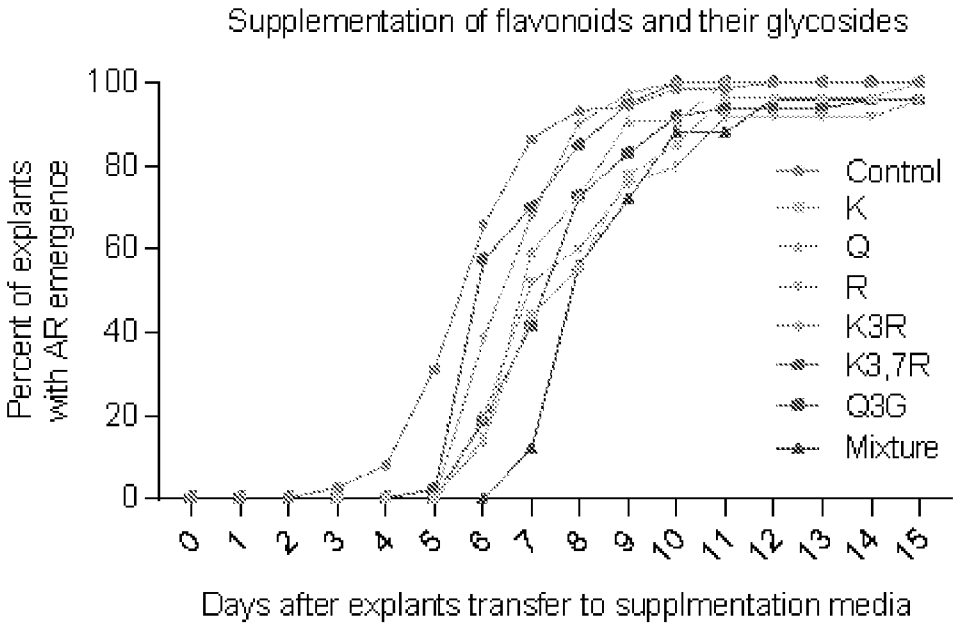


Figure 9A

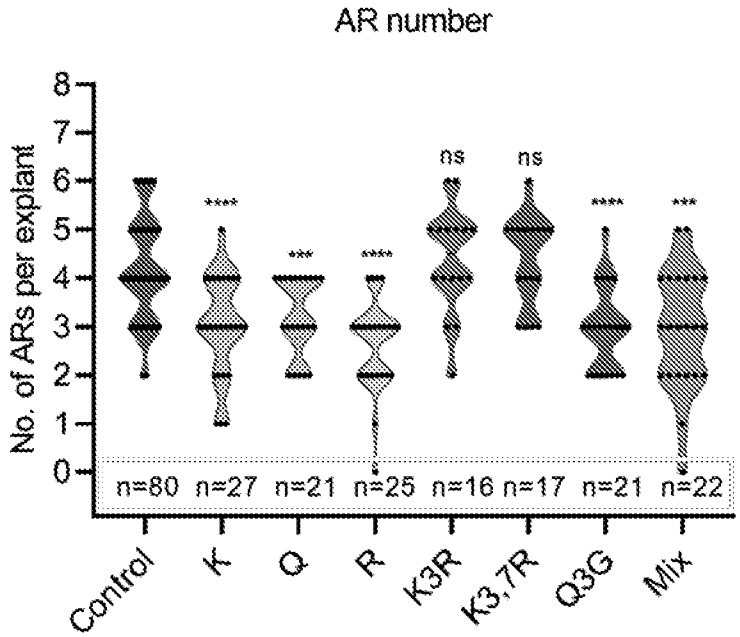


Figure 9B

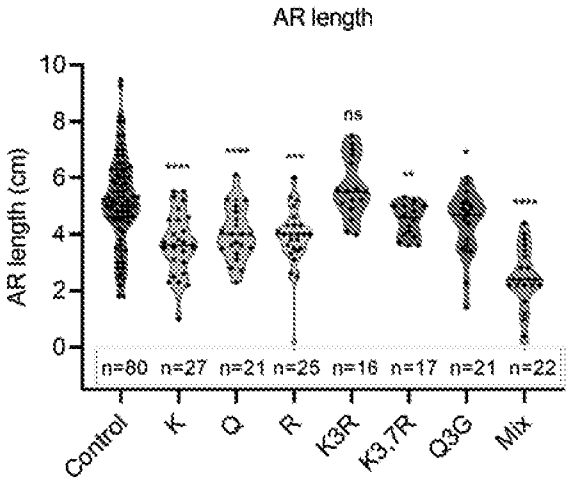


Figure 9C

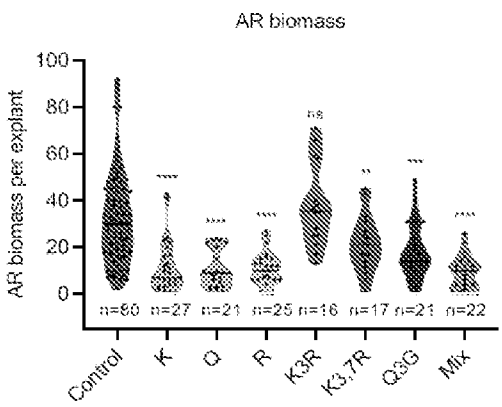


Figure 9D

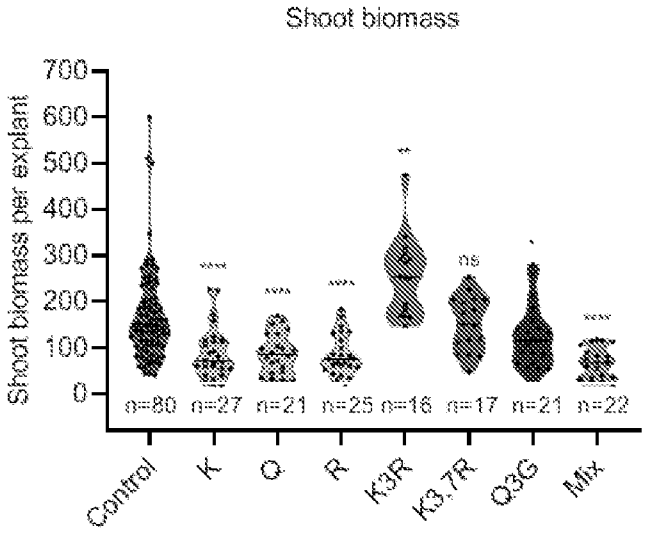


Figure 9E

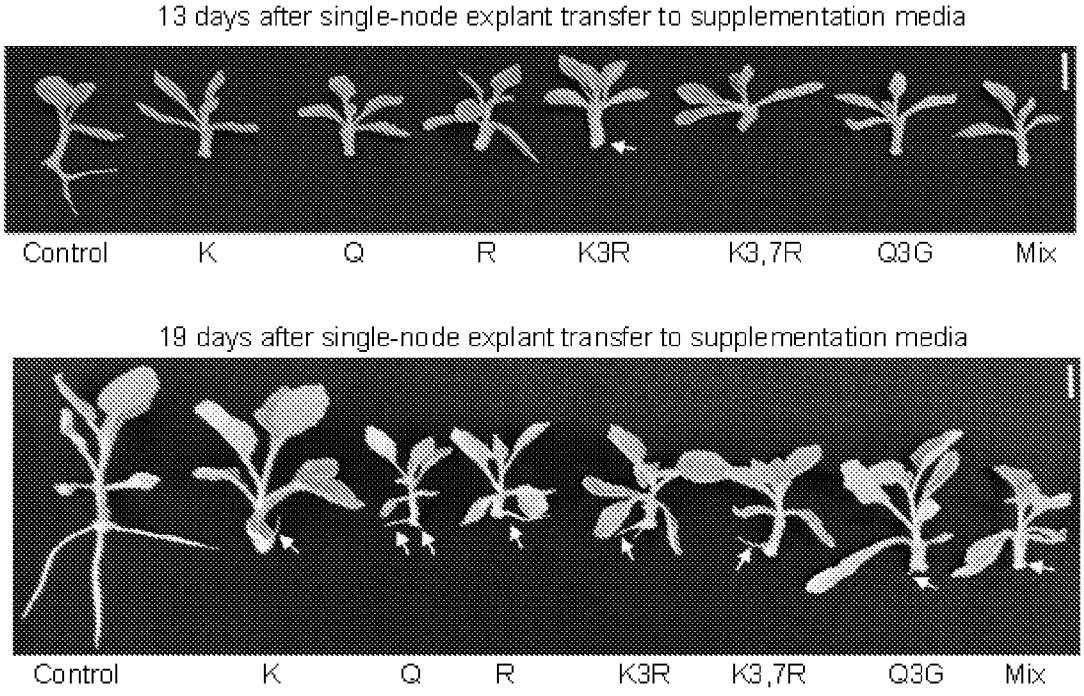


Figure 9F

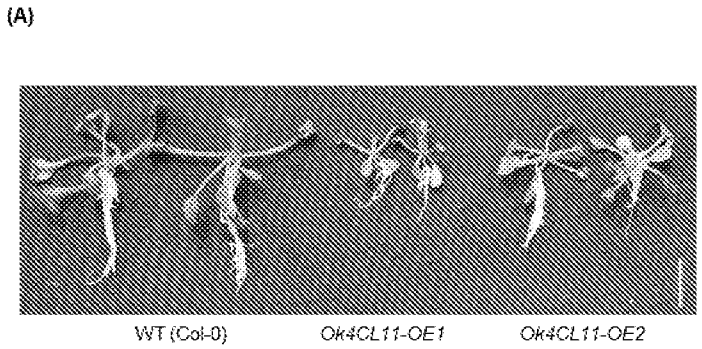


Figure 10A

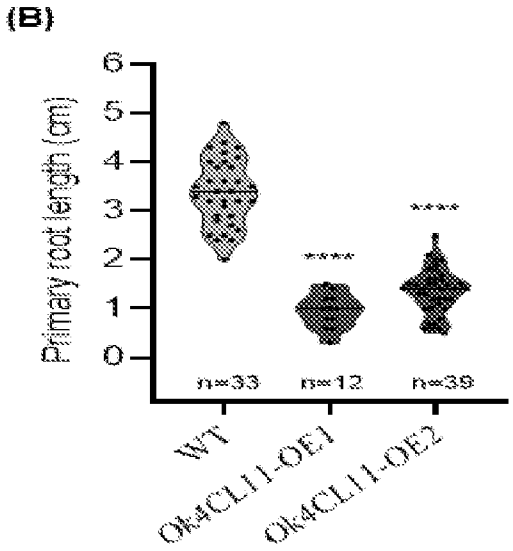


Figure 10B

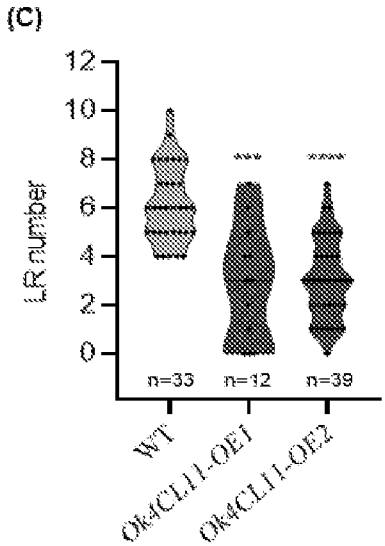


Figure 10C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2023/051211

A. CLASSIFICATION OF SUBJECT MATTER
C12N15/82,C12N15/84,C07K14/415 Version=2024.01

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)

C12N, C07K

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

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

PatSeer, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Lavhale Santosh Govind: "Functional characterization of 4 Coumarate CoA ligases involved in phenylpropanoid biosynthesis from Ocimum kilimandscharicum", Ph. D. Thesis, (CSIR-NCL, Pune) AcSIR, March 2022. Whole document	1-12
Y	Chen X et al: "Fraxinus mandshurica 4-coumarate-CoA ligase 2 enhances drought and osmotic stress tolerance of tobacco by increasing coniferyl alcohol content". Plant Physiol Biochem. 2020 Oct;155:697-708. Whole document	1-12
Y	Lavhale SG et al: "Functional insights into two Ocimum kilimandscharicum 4-coumarate-CoA ligases involved in phenylpropanoid biosynthesis". Int J Biol Macromol. 2021 Jun 30;181:202-210. Table 1, page 208, ln.8, 9	1-12
Y	Singh P et al: "Biosynthesis and tissue-specific partitioning of camphor and eugenol in Ocimum	

☒ 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
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25-04-2024	25-04-2024

Name and mailing address of the ISA/	Authorized officer
Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Ezhil Nilamani G Telephone No. +91-1125300200

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	kilimandscharicum. Phytochemistry". 2020 Sep;177:112451. fig. 3; fig.5; page 9, col.2, para 5.7.1	1-12
Y	Anand A: "Major Secondary Metabolites and Their Biosynthesis in Selected Ocimum sp", Ph. D. Thesis, CSIR-NCL, November 2016. Table 1, page 106	1-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2023/051211

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:

   a. ☒ forming part of the international application as filed.

   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments: