Appl Biochem Biotechnol (2016) 179:697–714
DOI 10.1007/s12010-016-2025-8

Identifying a Carotenoid Cleavage Dioxygenase 4a Gene and Its Efficient *Agrobacterium*-Mediated Genetic Transformation in *Bixa orellana* L.

Mohan Sankari[1] · Hridya Hemachandran[1] · Amirtha Anantharaman[1] · Subramanian Babu[1] · Renata Rivera Madrid[2] · George Priya Doss C[1] · Devanand P. Fulzele[3] · Ramamoorthy Siva[1]

Received: 16 October 2015 / Accepted: 18 February 2016 /
Published online: 27 February 2016

Abstract Carotenoids are metabolized to apocarotenoids through the pathway catalysed by carotenoid cleavage oxygenases (*CCOs*). The apocarotenoids are economically important as it is known to have therapeutic as well as industrial applications. For instance, bixin from *Bixa orellana* and crocin from *Crocus sativus* are commercially used as a food colourant and cosmetics since prehistoric time. In our present study, CCD4a gene has been identified and isolated from leaves of *B. orellana* for the first time and named as *Bo*CCD4a; phylogenetic analysis was carried out using CLUSTAL W. From sequence analysis, *Bo*CCD4a contains two exons and one intron, which was compared with the selected *At*CCD4, *Rd*CCD4, *Gm*CCD4 and *Cm*CCD4a gene. Further, the *Bo*CCD4a gene was cloned into pCAMBIA 1301, transformed into *Agrobacterium tumefaciens* EHA105 strain and subsequently transferred into hypocotyledons and callus of *B. orellana* by agro-infection. Selection of stable transformation was screened on the basis of PCR detection by using GUS and *hptII* specific primer, which was followed by histochemical characterization. The percent transient GUS expression in hypocotyledons and callus was 84.4 and 80 %, respectively. The expression of *Bo*CCD4a gene in *B. orellana* was confirmed through RT-PCR analysis. From our results, the sequence analysis of *Bo*CCD4a gene of *B. orellana* was closely related to the *Cs*CCD4 gene of *C. sativus*, which suggests this gene may have a role in various processes such as fragrance, insect attractant and pollination.

✉ Ramamoorthy Siva
rsiva77in@rediffmail.com

[1] School of Bio Sciences and Technology, VIT University, Vellore 632014 Tamil Nadu, India

[2] Cenro de Investigacion Cientifica de Yucatan, A.C. Calle 43 No. 130, Col. Chuburnade Hidalgo, 97200 Merida, Yucatan, Mexico

[3] Plant Biotechnology and Secondary Metabolites Section, NA&BTD, Bhabha Atomic Research Centre, Mumbai, Maharashtra, India

Keywords *Bixa orellana* · Carotenoid cleavage dioxygenase · CCD4a · RT-PCR · Apocarotenoids · Agro-infection

Abbreviations

CCD	Carotenoid cleavage dioxygenase
NCED	9-*cis* epoxy carotenoid dioxygenase
CCO	Carotenoid cleavage oxygenase
BLAST	Basic Local Alignment Sequence Tool
NJ	Neighbour-joining method
CTAB	Cetyltrimethyl ammonium bromide
MS	Murashige and Skoog
LB	Luria broth
YEP	Yeast extract peptone
NAA	α-Naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
BA	Benzyladenine
35S CaMV	35S promoter of the cauliflower mosaic virus
GUS	β-Glucucauronidase
hptII	Hygromycin phosphotransferase II
OD600	Optical density at 600 nm
GFP	Green fluorescence protein
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase–polymerase chain reaction
X-Gluc	5-Bromo-4-chloro-3-indolyl-b-D-glucuronic acid

Introduction

Carotenoids are tetraterpenoid pigments synthesized in photosynthetic plants and non-photosynthetic organism such as bacteria and fungi. They reside in the plastids of plant to provide red, orange and yellow colours to many flowers and fruits. In addition, they play a vital role in photosynthesis, light harvesting, photoprotection [1]. Carotenoids are cleaved through oxidative tailoring to yield apocarotenoids by the enzyme called carotenoid cleavage dioxygenase [2, 3].

Apocarotenoids are isoprenoid compound which serve as an insect attractants, as well as contribute the flavour and aroma in flowers and fruits [4, 5]. Apocarotenoids are mainly responsible for the regulation of gene expression in both plants and animals. The biologically important apocarotenoids like abscisic acid (ABA) plays an essential role in seed development and environmental adaptation, such as biotic and abiotic stresses [6–11]. Apocarotenoids are also involve in the allelopathic interactions and plant defence mechanism [12]. A commercially valuable apocarotenoid like bixin and crocin gets its role as colourant in nutritional supplement, food and cosmetic industries [13, 14].

Bixin is a dicarboxylic monomethyl ester apocarotenoid pigment that confers orange-red colour in the *Bixa orellana* seeds. *Bixa orellana* Linn. (Bixaceae), widely called as annatto or achiote, is a small tree native to South America and cultivated in many tropical countries and southern parts of India [15]. The genetic improvement of this traditional dye-yielding plant is

hindered by limited genetic variability, long reproductive cycle and recalcitrant tissue culture regeneration [16]. To overcome this, *Agrobacterium*-mediated genetic transformation or direct DNA transfer method is widely used for transformation of the genes into the plant cells. In *B. orellana*, *Agrobacterium*-mediated genetic transformation was carried out in two varieties such as Peruana and Criolla in which the transient β-glucucauronidase (GUS) gene expression was carried out in hypocotyls and it showed 50 and 100 % of expression frequency, respectively [16]. Parimalan et al. studied the direct and callus-mediated somatic embryogenesis of *B. orellana* and it showed 86.7 % of transient GUS expression [17]. The advantage of genetic transformation is used to regulate the biosynthetic pathway to enhance the nutritional content, stress tolerance, productivity and pest resistance of the plant [18, 19]. *Agrobacterium*-mediated transformation has also been carried out in tomato to improve the quality of fruit and to produce disease-resistant plants [20], and carotenoid biosynthesis has been achieved by genetic engineering in rice [21, 22] and *Citrus paradise* [23].

Bixin is one of the oldest pigments used by humans in foods and cosmetics [24]. There was an increase in demand of bixin, because of the consumer banned on the chemically synthesized azo dyes. Among natural colour additives, bixin ranked as second in the various field applications [25]. Even though it has great economic importance, the molecular characterization of *B. orellana* remains poorly characterized. The biosynthetic pathway of bixin was elucidated by [26] and documented in a heterologous system [27, 28]. The three genes especially lycopene cleave dioxygenase (*Bo*LCD), bixin aldehyde dehydrogenase (*Bo*BADH) and norbixin carboxyl methyltransferase (*Bo*BMT), which catalyse the sequential conversion of lycopene into bixin. Carotenoid cleavage dioxygenase (CCD) are non-haem oxygenases that cleave carotene into apocarotenoids which synthesize hormones that regulate plant growth and flower development [2]. The well-known example is abscisic acid, which plays a major role in the regulation of drought tolerance, seed growth and sugar sensing. These enzymes provide unique colour, flavour and aroma to flowers and fruits. CCD genes and enzymes have been studied in a number of species such as *B. orellana* [5], *Crocus sativus* [29], *Chrysanthemum morifolium* [30] and potato [31].

Despite the increasing number of carotenoid cleavage dioxygenases characterized from plants over the last few years, relatively little is known about the function of the dioxygenases in *B. orellana*. Recently, Rodrı'guez-A vila et al. reported a new CCD1 gene member (*Bo*CCD1) in different plant tissues of *B. orellana* plantlets [5]. However, there is no report on CCD4 gene in *B. orellana* and knowledge remains to be clarified. Here in this paper, we report the identification of a new CCD4 gene member, namely *Bo*CCD4a. The phylogenetic analysis was performed, in order to determine the close relationship between CCD4 families; we cloned the CCD4a gene into binary expression vector pCAMBIA 1301 and successful evidence of transformation was carried out by *Agrobacterium*-mediated transformation.

Materials and Methods

Isolation of *Bo*CCD4a Gene

Genomic DNA was isolated from *B. orellana* leaves using cetyltrimethyl ammonium bromide (CTAB) method [32]. The isolated DNA was quantified and amplified with gene specific primers (CCD4a-F: 5′-CAATCTCAAGTATTAGCATTC-3′, CCD4a-R: 5′-CTGCTGTGACAGCAGCTCAGC-3′). Primers were designed based on the available

sequence of CCD4a from *C. sativus*. Polymerase chain reaction (PCR) was carried out in thermal cycler (Eppendorf, Germany). The PCR reaction was performed at initial denaturation at 95 °C for 5 min, denaturation (95 °C for 1 min), annealing (40.3 °C for 1.10 min) and extension (72 °C for 1.10 min) for 30 cycles, then a final extension at 72 °C for 7 min. The amplified fragment was cloned into the pGEM-T Easy vector (Promega, USA) at 16 °C for 12 h and then transformed into DH5α *Escherichia coli* competent cells. Selection of recombinants was done by blue-white screening method [33] and confirmed by colony PCR based on the size of the fragment. The PCR products were sequenced and the sequences were compared with GenBank nucleotide database using the Basic Local Alignment Sequence Tool (BLAST, National Center for Biotechnology Information). The sequence homology and phylogenetic analysis was performed by using the CLUSTAL W tool and neighbour-joining method respectively.

Phylogenetic and Gene Structure Analysis of *Bo*CCD4 Gene

The phylogenetic analysis was carried out for *Bo*CCD4 gene, and the homologous sequences of other plants CCD4 genes were retrieved from GenBank by using the BLASTP [34]. The nucleotide sequences were aligned using CLUSTAL W with default settings [35]. TrimAI tool was used for trimming the sequence that eliminates the spurious sequence and improve the quality of the phylogenetic tree [36]. For tree building analyses, the Akaike information criterion (AIC) was used in ModelTest v2.4 server [37] to estimate the most appropriate model of nucleotide substitution. The phylogenetic tree was constructed by using the neighbour-joining (NJ) method with 1000 bootstrap values through MEGA v6.0 software [38]. NJ analysis was executed by using the model *p*-distance with other parameters like gap/missing data was pairwise deletion and substitution was Transition + Transversion. The gene structure analysis was carried out in this study by using the available genomic sequence of CCD4. The gene sequences were retrieved from GenBank [39] using NCBI program. FGENESH the gene prediction software [40] was used to predict the gene structure of isolated *Bo*CCD4 gene from *B. orellana* and compared with already reported CCD4 gene structure.

Construction of Plant Expression Vector pCAMBIA1301: *Bo*CCD4a

The recombinant plasmid was named as pGEMT-*Bo*CCD4a. The *Bo*CCD4a gene was re-isolated using these primers (CCD4a-F: 5′-GCGGAATTCGCTCGGTTGTCATCCTCCTCCCTC-3′ and CC4a-R: 5′-GCGGGATCCGCCTACTGCTGTGACAGCAGCT-3′) from recombinant plasmid pGEMT-*Bo*CCD4a, and pCAMBIA 1301 was subjected to restriction digestion for transfer of *Bo*CCD4a gene from pGEMT-*Bo*CCD4a to pCAMBIA 1301. Both vectors were subjected to double digestion individually. The reaction mixture contained 5 μl of the DNA, 1 μl (20,000 U/ml) *Eco*RI enzyme, 5 μl of 10× NEB buffer and 9 μl of sterile water making up to a final volume of 20 μl. The reaction mixture was incubated at 37 °C for 4 h and the reaction was stopped by incubating the vials at 65 °C for 10 min. The product was further purified using Qiagen purification kit (Qiagen, Hilden, Germany). The restricted products were used for a second digestion. The reaction mixture contained 10 μl of the purified product, 1 μl *Bam*HI (20,000 U/ml), 5 μl of 10× NEB buffer and 4 μl of sterile water making up the total volume of 20 μl. The reaction mixture was incubated at 37 °C for 4 h. The mixture was spinned briefly and incubated at 16 h for 16 °C. The vials were chilled on ice and moved to −20 °C till further use.

Transformation of *E. coli* DH5α with pCAMBIA 1301: *Bo*CCD4a

All standard transformation methods were followed by [41]. Transformation of DH5α competent cells with the ligated product was performed using the heat shock method. One hundred microlitres of DH5α competent cells with 5 μl of ligated product was incubated for 30 min. Heat shock was given to the tube at 42 °C for 45 s in water bath and then kept in ice for 5 min. The Luria broth (LB) broth (800 μl) was added into the tube and kept for incubation at 37 °C for 2–3 h. The culture was centrifuged at 4000 rpm for 1 min to obtain the pellet to which 100 μl LB broth was added and spread over LB agar plates containing kanamycin (100 mg/l) and subsequently incubated overnight at 37 °C. Single colonies from the plates were inoculated in LB broth and incubated at 37 °C for overnight from which plasmid DNA was isolated using Qiagen plasmid isolation kit (Qiagen, Hilden, Germany) and confirmed by colony PCR based on the size of the fragment.

Transformation of *Agrobacterium* EHA105 with pCAMBIA 1301: *Bo*CCD4a

Transformation of *Agrobacterium tumefaciens* EHA105 was done by the freeze thaw method with modification [42]. Briefly, 5 μl of plasmid DNA was mixed with 100 μl of *Agrobacterium* competent cells, and the mixture was frozen in liquid nitrogen and thawed at room temperature and incubated in ice for 30 min. The yeast extract peptone (YEP) broth (800 μl) was added to the tubes and incubated at 28 °C for 2–4 h. The tubes were centrifuged at 4000 rpm for 1 min and the supernatant was discarded. The YEP broth (100 μl) was mixed with the pellet and plated on YEP medium containing kanamycin (50 mg/l) and rifampicin (20 mg/l). The plates were incubated at 28 °C for 2 days. Transformed colonies were confirmed by colony PCR.

Explants Preparation and Callus Induction

The seeds of *B. orellana* were collected from Shervaroy Hills, Eastern Ghats, and established in VIT University nursery, VIT University, Vellore, Tamil Nadu, India. It was thoroughly washed with running tap water for 20 min. The seeds were sterilized with 0.1 % mercuric chloride for 30 s and then rinsed with sterile distilled water followed by washed with 70 % (*v*/*v*) ethanol for 1 min. After 1 min of washing, seeds were carefully rinsed with sterile distilled water for five times and kept for in vitro germination for 10–15 days.

For callus induction, the nodal explant selected from the in vitro grown seedlings was sterilized and inoculated into Murashige and Skoog (MS) medium [43] supplemented with α-naphthaleneacetic acid (NAA; 1.07–2.14 μM) and BA (10.2 μM) and 2,4-dichlorophenoxyacetic acid (2,4-D; 0.45–0.90 μM) and BA (10.2 μM) hormones and 2.6 g/l gelrite [17]. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH and autoclaved at 121 °C for 20 min, and the culture was incubated in plant tissue culture room at 25 ± 2 °C and maintained with 16/8 h photo period for 3 weeks (Table 1). The 3-week-old callus cultures were sub-cultured onto the same medium for maintenance.

Agrobacterium Infection and Co-cultivation of Callus

Agrobacterium tumefaciens strain EHA105 harbouring the binary vector pCAMBIA 1301 plasmid (11.8) contained the selectable marker gene hygromycin phosphotransferase II (*hptII*),

Table 1 MS media composition used for this study

Name of media	Composition
Callus induction medium	MS media supplemented with vitamins, 0.1 % myoinositol, 3 % sucrose, 1.07-2.14 μM NAA, 10.2 μM BAP and 0.45-0.90 μM 2-4-D, 10.2 μM BAP, gelrite 2.6 g/L, pH 5.8
Callus sub-culture medium	MS media supplemented with vitamins, 0.1 % myoinositol, 3 % sucrose, 1.07–2.14 μM NAA, 10.2 μM BAP and 0.45–0.90 μM 2,4-D, 10.2 μM BAP, gelrite 2.6 g/L, pH 5.8
Co-cultivation medium	MS media supplemented with vitamins, 0.1 % myoinositol, 3 % sucrose, 100 μM acetosyringone, gelrite 2.6 g/L, pH 5.8
Selection medium	MS media supplemented with vitamins, 0.1 % myoinositol, 3 % sucrose, 10.2 μM BAP, 0.45 μM 2,4-D 10 mg/L hygromycin, kanamycin 50 mg/L, gelrite 2.6 g/L, pH 5.8

and uidA reporter gene GUS (X-glucuronidase) was used for transformation. A single colony of *A. tumefaciens* strain EHA105 was inoculated into 5 ml of YEP medium containing rifampicin (10 mg/l) and kanamycin (100 mg/l). The culture was incubated overnight at 28 °C at 150 rpm on shaker. An aliquot of the overnight culture (50 μl) was inoculated into 150 ml of fresh YEP broth containing same antibiotic and incubated with the same condition, when the culture reached an absorbance 1 at OD of 600 nm. Cells were pelleted by centrifugation at 4000 rpm for 10 min. The pellet was suspended in 50 ml of YEP medium supplemented with acetosyringone (10 μM). Callus was infected with the above suspension culture with 30–45 min intermittent shaking. After infection, the callus was cultured on the co-culture medium (Table 1) containing acetosyringone (100 μM) without any antibiotics. Cultures were incubated under dark for 2 days.

Selection of Callus and Molecular Confirmation

After co-cultivation, the callus was transferred into MS medium containing hormones with hygromycin (10 mg/l) and cefotaxime (250 mg/l) (Table 1). The cultures were sub-cultured for every 2 weeks on the same medium. For confirmation of transformants, molecular confirmation by PCR using GUS and *hptII* primers, following histochemical GUS assay using X-glcA, was also done to confirm the transformed callus [17].

Molecular Confirmation for the Selection of Transformed Callus

From transformed callus, genomic DNA was isolated by modified CTAB method [32]. For PCR analysis, forward and reverse primers specific to partial hygromycin phosphotransferase (*hptII*) (*hptII*-F: 5′GATGTTGGCGACCTCGTATT-3′, *hptII*-R: 5′-GTGTCACGTTGCAAGACCTG-3′) and glucuronidase (GUS) (GUS-F: 5′-CCGTCCCAAGCAAGTTACAAT-3′, GUS-R: 5′ TTCGGAATCTCCACGTTACC-3′) genes were used [37]. PCR was carried out in thermal cycler (Eppendorf, Germany). The PCR reaction was performed at 94 °C for 2 min, denaturation (94 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 30 s) for 30 cycles, then a final extension at 72 °C for 5 min and final hold at 4 °C for 10 min. The amplified products were analysed electrophoretically (Bio-Rad mini sub-cell GT, USA).

Histochemical GUS Assay

The co-cultivated callus was used for GUS assay by using established methods with some modifications [44]. For histochemical confirmation, the callus were incubated overnight in a solution containing 2 mM of histochemical substrate 5-bromo, 4-chloro, 3-indolyl glucuronide (X-Glc,), 50 mM potassium phosphate buffer (pH 7.0), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.1 % triton X-100 and sterile distilled water. After overnight incubation, the callus were washed with 70 % ethanol to remove the chlorophyll and blue colour callus were visualized. Percentage of transformants was calculated by the total number of blue colour explants divided by the number of explants inoculated [17].

Visualization of GFP Fluorescence

Transient and stable green fluorescence protein (GFP) expression was analysed using fluorescence microscope (Wesmox, India). The images were recorded under a blue filter, in a range of 420–495 nm and UV filter in a range of 340–380 nm in ×10 magnification. All explant transformed with GFP were tested for GFP expression.

*Bo*CCD4 Expression by Reverse Transcriptase-Mediated PCR

Total RNA was extracted from *B. orellana* callus using RaFlex RNA isolation kit protocol (Genei, Bangalore, India). The RNA was further treated with DNase-1, amplification grade (Sigma-Aldrich, USA). The first-strand complementary DNA (cDNA) was synthesized using 1 μg total RNA with the help of cDNA synthesis kit (AMV RT PCR kit, Genei, Bangalore) in a reaction volume of 20 μl, at 42 °C for 60 min followed by 10 min according to manufacturer's instructions. The first-strand cDNA was immediately subjected to PCR amplification with gene-specific primers. The gene-specific primers (CCD4-F: 5′-CCGCGCCATCACAATATTCA-3′, CCD4-R: 5′TAGGTAGAGGGTGAGTGCCT-3′) were designed from the *Bo*CCD4a gene sequence used in this study. PCR reaction mixture containing 1 μl of first-strand cDNA template, 1 μl of forward primer, 1 μl of reverse primer, 10 μl of PCR master mix (amplicon) and 7 μl of sterile water was carried out in Eppendorf Mastercycler under the following conditions: 95 °C at 5 min as an initial denaturation followed by 30 cycles of 1 min 94 °C, 1 min at 35 °C, 1 min at 72 °C and a final extension at 72 °C for 10 min. A parallel reaction with 28 cycles and specific primers (18S rRNA-F: 5′-CGCGTCCGGTCCTCG-3′, 18S rRNA-R: 5′-TTAGAAAAATAAAGTTGGGTGTCGG-3′) for the 18S rRNA gene was used as an expression control.

Results and Discussion

Cloning of Partial Genomic DNA Clone of CCD4a Gene

Bixin of *B. orellana* and crocin of *C. sativus* are apocarotenoids having a similar chemical structure [14]. Taking this advantage, the primers were designed from a sequence of *C. sativus* CCD4a gene. A partial CCD4a gene was obtained by using two gene-specific oligonucleotides. The obtained 1083-bp (Accession number: KT378217) product was cloned into pGEMT vector and selection of recombinant

was done by blue-white screening method, and recombinants were confirmed by the same fragment size obtained from colony PCR. The obtained nucleotide sequence showed similarity with *C. sativus*. The relationship between *B. orellana* CCD4a sequence with other plant CCD4 sequences was analysed by sequence comparison using CLUSTALW and by construction of a phylogenetic tree.

In biosynthetic pathway, the breakdown of carotenoids into apocarotenoids was catalysed by the enzyme carotenoid cleavage oxygenases (CCOs). The CCOs are categorized into 9-*cis* epoxy carotenoid dioxygenase (NCEDs) and carotenoid cleavage dioxygenase (CCDs). CCDs are further divided into four groups, viz, CCD1, CCD4, CCD7 and CCD8. The CCD1 enzyme cleaves numerous cyclic and linear all-*trans*-carotenoids (C5–C6, C7–C8, C9–C10 double bonds) to produce multiple apocarotenoid products. The CCD4 are involved in the production of plant volatile compounds like β-ionone and damascene and also reported to help during drought stress. The other two CCD enzymes, CCD7 and CCD8, are involved in shoot branching and strigolactone production. The CCD7 enzyme cleaves β-carotene to produce β-ionone and C27 10′-apo-β-carotenal. The CCD8 cleaves the C27 aldehyde at its C13–C14 double bond, which result in the formation of apocarotenoids [5, 45, 46]. In *B. orellana*, CCD1 gene was isolated by [5] and reported that *Bo*CCD1 enzymes cleaves the lycopene the double bonds 5–6/5′–6′ leading to the formation of bixin. It is also interesting to note that the production of bixin in different developmental stages of seeds of *B. orellana* and BoCCD1 plays an important role in bixin production. However, in several other plant species, the expression of CCD1 was controlled by the production of important apocarotenoids such as beta-ionone, a fragrance volatile in petunia flowers, or C13-norisoprenoids, considered flavour compounds in grapeberries [47, 48].

In the present study, a new CCD gene, *Bo*CCD4a, was isolated and its sequence showed high homology with CCD4a of *C. sativus*. The function of CCD4 gene has been reported in several plant species like *C. sativus* and *C. morifolium*. In *C. sativus*, the function of CCD4 expression pattern revealed *Cs*CCD4a and b emissions of high level of β carotene and emission of β ionone formation during stigma development [29]. In CCD4 enzymes belonging to the N-terminal domain for plastid targeting, *Cs*CCD4a and *Cs*CCD4b proteins are targeted to plastid plastoglobules in plant and the activity that was determined in this enzyme has a 9, 10 (9′, 10′) cleavage activity on β carotene [29]. The silencing of CCD4a gene in *C. morifolium* by RNA interference (RNAi) resulted in the change of petal colour from white to yellow. It occurs by the accumulation of carotenoid in *Chrysanthemum* petals by cleaving β-carotene, which finally confirms the CCD4a gene expression resulting in white colour petal formation [30]. Similar studies have been carried out in potato tubers, by using RNAi approach by downregulation of CCD4 gene in tubers resulted in high level of carotenoid formation [31].

Phylogenetic Tree Construction

Phylogenetic tree was constructed to explore the evolutionary relationship of isolated CCD4a gene (*Bo*CCD4a) using MEGA v6.0. The best-fit model suggested by ModelTest v2.4 was (GTR + I + G) for nucleotide substitution. The sequence alignment of *Bo*CCD4 gene shows the conserved region with other plant CCD4 genes. The phylogenetic tree shows relatively high sequence homology of *Bo*CCD4a gene with various plant CCD4 (Fig. 1). The different plant families included for comparing the phylogenetic tree construction are Rosaceae (*Malus domestica*, *Prunus persica*, *Rosa damascena*), Rutaceae (*Citrus clementine*, *Citrus unshiu*,), Asteraceae (*C. morifolium*),

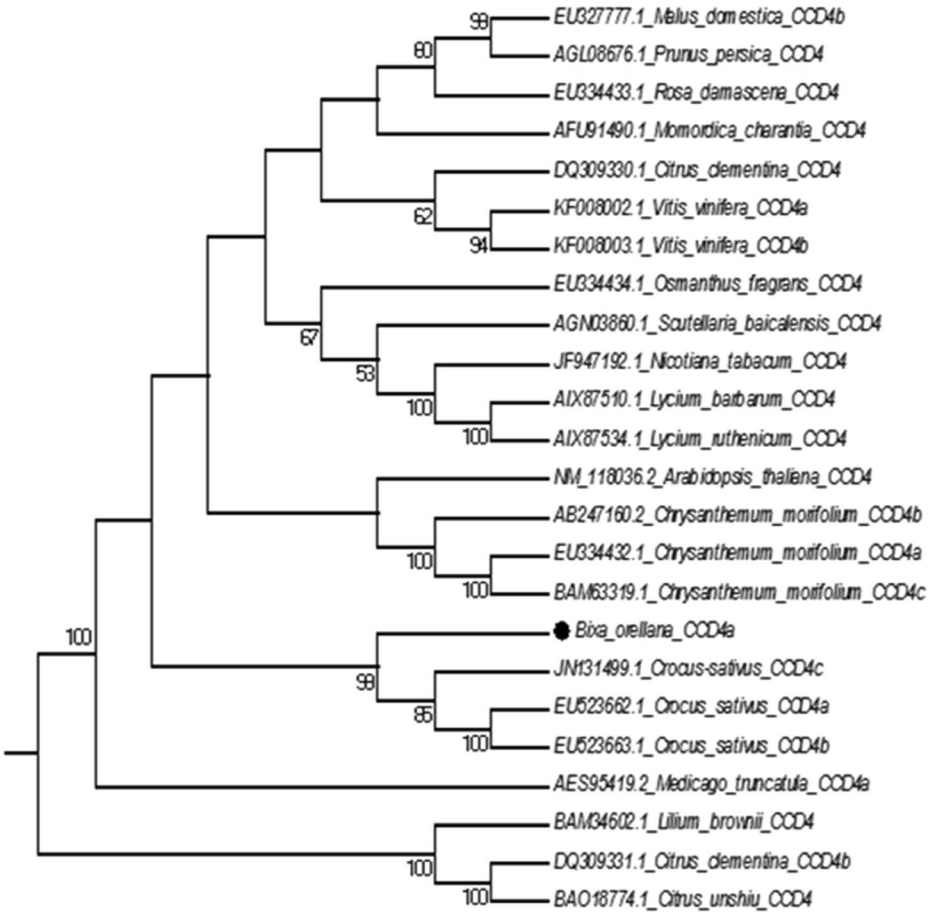

Fig. 1 Phylogenetic tree retrieve from the neighbour-joining analysis of the *B. orellana* CCD4a isolated. Bootstrapping values (1000 replicates) are shown on the tree. Evolutionary analyses were conducted in MEGA 6. Accession numbers are on the *left side*. *Black-coloured circle* indicates the gene under study

Oleaceae (*Osmanthus fragrans*), Iridaceae (*C. sativus*), Vitaceae (*Vitis vinifera*), Fabaceae (*Medicago truncatula*), Brassicaceae (*Arabidopsis thaliana*), Lamiaceae (*Scutellaria baicalensis*), Solanaceae (*Nicotiana tabacum*, *Lycium barbarum*, *Lycium ruthenium*), Liliaceae (*Lilium brownie*), Cucurbitaceae (*Momordica charantia*) and Bixaceae (*B. orellana*). The evolutionary relationship of CCD sub-class genes were well documented by [3]. The tree result suggested that the isolated *B. orellana* CCD4 gene was closely related to *C. sativus* CCD4 gene family. To study the structural variation of isolated CCD4 genes, the exon regions were predicted through FGENESH software and identified that two exons were present in our isolated *Bo*CCD4 genes from *B. orellana*. It has been evidenced by comparing the *B. orellana* CCD4 exon position with already reported CCD4 genes in the species of *At*CCD4, *Rd*CCD4, *Gm*CCD4a and *Cm*CCD4a (Fig. 2, Table 2). According to previous report, in *Glycine max* CCD4a gene sequence and *C. morifolium* CCD4a gene sequence showed the presence of two exons and one intron [3, 6]. However, in *A. thaliana* CCD4a and *Rosa domestica* CCD4a gene sequence showed the absence of introns [2].

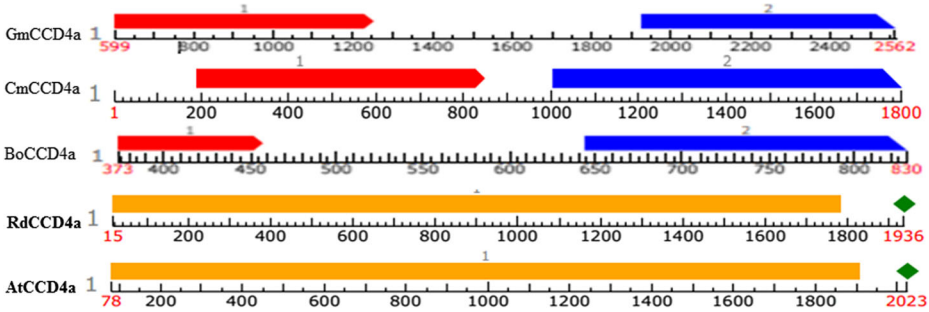

Fig. 2 Structural analysis of CCD genes to detect the exon and intron region. *Red*: first exon region in the sequence, *blue*: last exon region in the sequence, *yellow*: exon region

Expression of *Bo*CCD4a Gene in pCAMBIA 1301

The expression vector was generated and named as pCAMBIA 1301: *Bo*CCD4a (Fig. 4), in which the *Bo*CCD4a gene was under the control of 35S CaMV promoter. The regenerated expression vector pCAMBIA 1301: *Bo*CCD4a was then introduced into *Agrobacterium* strain EHA105, and the recombinant strain EHA105/pCAMBIA1301: *Bo*CCD4a was used for transformation experiments. The transformation of pCAMBIA1301: *Bo*CCD4a gene into *Agrobacterium* strain EHA105/pCAMBIA1301: *Bo*CCD4a was verified by colony PCR (Fig. 3a). Similar study was carried out in rice, where the gene was constructed into the pCAMBIA 1301 vector to study the expression of the *Agrobacterium*-mediated transformation in rice seedlings [49].

Optimization Conditions for Callus Induction

For callus initiation in *B. orellana*, different explants like cotyledon, hypocotyledon, leaves and nodal region were used. Here, we observed maximum growth of callus was obtained from the nodal explants. For callus induction study, we have followed [17] protocol and, accordingly, different concentrations and combinations of NAA, BA and 2,4-D were used. In brief,

Table 2 CCD4a gene structural information in selected plant species

Organism name	Chromosome location	Genomic sequence	Number of exons	Length of exons	
				Exon 1	Exon 2
Glycine max CCD4a	I	NC_016088.1	2	599-1250	1940-2562
Chrysanthemum morifolium CCD4a	–	–	2	190-840	1000-1800
Rosa domestica CCD4a	–	–	1	15-1790	
Arabidopsis thaliana CCD4a	IV	NC_003075.7	1	78-1900	
Bixa orellana CCD4a	–	–	2	373-455	645-830

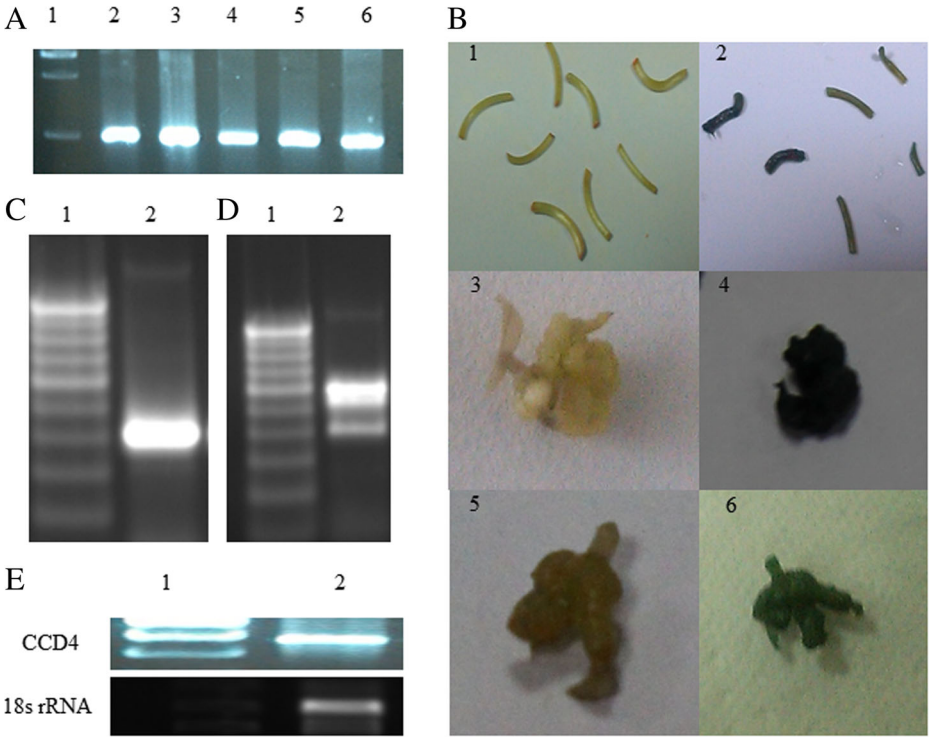

Fig. 3 **a** Confirmation by five randomly selected colonies after transformation. *Lane 1*: 1-kb ladder, *Lanes 2–6*: transformed colonies. **b** Transient GUS gene expression analysis. (*1*) Non-transformed explant and (*3*, *5*) non-transformed 3-week-old calli. (*2*) Stable expression of transformed explants and (*4*, *6*) stable expression of 3-week-old calli, showing GUS expression in following co-cultivated in presence of 100 μM acetosyringone. **c** PCR amplification of partial *hptII* gene. 1100-bp ladder, 2407 amplicon of partial *hptII* gene. **d** PCR amplification of GUS gene. *Lane 1*: 100-bp ladder, *Lane 2*: 589-bp amplicon of partial GUS gene. **e** *Bo*CCD4 expression profile obtained for *B. orellana* callus. RT-PCR analysis was carried out with CCD4 and 18S rRNA gene. *Lane 1*: 100-bp ladder, *Lane 2*: amplification of specific CCD4 gene and amplification of 18S rRNA

NAA (1.07–2.14 μM) with BA (10.2 μM) and 2,4-D (0.45 and 0.90 μM) with BA (10.2 μM) were supplemented with MS medium.

Among the different combinations, MS medium supplemented with NAA (1.07 μM) with BA (10.2 μM) showed good response (57 %). The combination with 2,4-D (0.45 μM) with BA (10.2 μM) does not show the proper growth (Table 3). Our result shows that most of the nodal region explant exhibits callus initiation after 2 weeks and developed compact, creamish white calli after 2 weeks of culture initiation. The highest growth of callus was observed in the lower concentration of hormones, while in higher concentration of hormones showed the browning of callus and also affected further proliferation in a sub-cultured medium. In order to study the *Agrobacterium*-mediated transformation, callus was further sub-cultured for every 2 weeks, and the high amount of gelling agent would reduce the water potential in medium and resulting in the formation of hard and fragile embryogenic calli that are supposed to be more responsive for transformation [50]. In the present study, we observed the nodal region was most suitable for the induction of callus as compared to any other explants. Only the fresh nodal region segments were totipotent and produced with the ability to regenerate the callus. In our observation, any callus that turned brown was found to be unsuitable for regeneration and transformation.

Table 3 MS medium with hormones for callus induction using nodal region

Medium	Plant growth hormone (μM)			No. of explants cultures	No. of callus formed	Percentage of explants inducing callus
	NAA	2,4-D	BAP			
MS	1.07		10.2	40	23	57.5
MS		0.45	10.2	40	19	47.5

Agrobacterium-Mediated Transformation and Co-cultivation, Selection of Callus

Three-week-old callus and explants were infected with *Agrobacterium* EHA105 strain for transformation study. The callus and explants were co-cultured on MS medium supplemented with 100 μM acetosyringone to enhance the transformation efficiency. Co-cultivation for 2 days in the presence of 100 μM acetosyringone was found to be most suitable for optimum transformation. The chemical acetosyringone produces natural phenolic compound that induces the virulence and enhances the genetic transformation in plant [51]. After co-cultivation, callus and explants were transferred to selection medium containing MS medium supplemented with 50 mg/l hygromycin and 250 mg/l cefotaxime. The antibiotic cefotaxime was widely used to avoid the overgrowth of *Agrobacterium*, and the selection marker will be useful in selection of transformed tissue without effecting the transformation. In this study, cefotaxime with a concentration of 250 mg/l was found to be more efficient for growth and differentiation of transformed callus. Higher concentration of antibiotics decreased the transformation efficiency. Apart from this, the selection could be based on the antibiotics like kanamycin or hygromycin. The plant cells regenerate only in the selective agent; the non-transformed cell dies in selectable marker genes. In this study, we used hygromycin as selectable marker gene for the efficient transformation; it is widely used to select the *Agrobacterium*-transformed explants or callus for further study [52]. Many researchers have worked in transformation of *B. orellana* using different strains of *Agrobacterium*. For instance, Zaldivar–Cruz et al. (2003) used hypocotyledons as explant for agroinfection using LBA4404 strain containing pBI.121 and pCAMBIA 2301 in PC-L2 medium [53] containing 200 μm of acetosyringone and 250 mg^{-1} of cefotaxime [16]. Parimalan et al. (2011) transformed somatic embryos with *A. tumefaciens* strain GV 3101 harbouring pCAMBIA 1305.2 vector in RBANGT medium containing 10 μm of acetosyringone, 10 mg/l of hygromycin and 250 mg/l of cefotaxime [17]. In loblolly pine, the effect of different antibiotics like carbenicillin, Claforan and Timentin was involved in the removal of *Agrobacterium* overgrowth through *Agrobacterium*-mediated transformation [54]. In *Agrobacterium* transformation, the co-cultivation time and density of *Agrobacterium* are important to affect T-DNA delivery and integration. The transformation efficiency was affected by several important factors such as MS medium with nutrient supplement, hormones, co-cultivation time, *Agrobacterium* vectors and selection [55]. The main problem in *Agrobacterium* transformation was necrotic response, because it decreases the transformation efficiency after co-cultivation and that leads to callus browning and tissue

shrinkage. It was caused by the cell death at the site where *Agrobacterium* was applied. In our observation, any callus that turned brown within 2 weeks was untransformed. Such kind of activity was recognized as hypersensitivity defence mechanisms of plants to *Agrobacterium* infection [56]. The callus that grow rapidly were found to be transformed and further investigated for the β-glucuronidase (GUS) assay. Other factors like culture medium, incubation condition, mode of injury for *Agrobacterium* infection to improve the T-DNA delivery in plant species and acetosyringone concentrations play a vital role in transformation efficiency [57]. In this study, we used *A. tumefaciens* strain EHA105 harbouring pCAMBIA 1301 for stable transformant study in *B. orellana*. The *Agrobacterium* transformation work was carried out in *Vitis vinefera* CCD1 [58]. The vector pCAMBIA 1301 was widely used in *Agrobacterium*-mediated transformation in rice [59], *C. paradise* [22] and *Arabidopsis thaliana* [60]. For GUS assays, pCAMBIA 1301 vector was used for stable transformation and it could be used for suitable reporter gene system.

Histochemical GUS Expression in Transformed Plants

To further confirm the transformed plants, GUS assay was used as one of the markers. GUS gene expression was extensively applied in many plant species like rice [61, 62], tomato [20], corn [63], yam [64] and maize [65]. The result has shown successful agro-infection and transient expression of GUS gene expression, which was indicated as blue colour staining in transformed explants and callus (Fig. 3b (2, 4, 6)). The frequency of transient GUS expression was 84.4 % in explants and 80 % in tested callus (Table 4). In GUS assay, acetosyringone plays a major role and it enhances the transformation efficiency [66]; the removal of acetosyringone from the co-cultivation medium and inoculation medium has decrease the GUS gene expression. The low level GUS expression in 100 mM concentration of acetosyringone was observed and showed significant result in GUS activity. The GUS gene expression was reported in two varieties of *B. orellana* and in hypocotyls of annatto seedlings [16]. A similar GUS transformation efficiency was reported in *B. orellana* by [17].

Visualization of GFP Fluorescence in Transformants

GFP fluorescence visualization is a useful tool for selecting transformed plants. It was widely used in gene transformation of many plant species such as yam [64], barley [67], alfalfa [68] and apple [69]. In this study, we have examined explants from the hypocotyledon, leaf and roots for GFP fluorescence. Fig. 4b, d, f, h shows GFP expression in transformed hypocotyledons, leaf and root. In control, GFP was not observed (Fig. 4a, c, e, g). Visualizing

Table 4 Transformation efficiency of *B. orellana* explants and callus

No. of explants infected	No. of callus infected	GUS-positive plants	GUS-positive callus	Percentage of transformation efficiency in explants	Percentage of transformation efficiency in explants	Mean ± S.E. of GUS positive explants	Mean ± S.E. of GUS positive callus
15	5	38	12	84.4	80	12.67 ± 0.47	4 ± 0.82

Values represent mean ± standard error (SE) of explant and callus (values are the mean of three replicates)

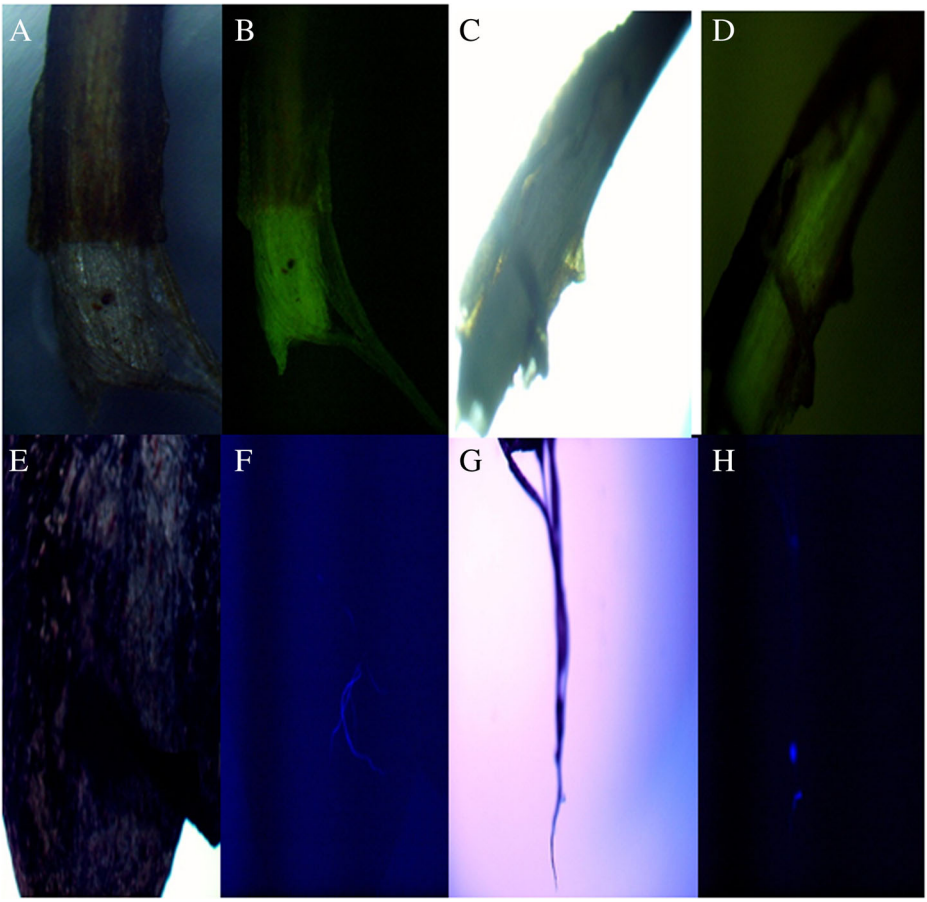

Fig. 4 Transient expression of GFP gene **a**, **c** without agro-infection hypocotyledons and **b**, **d** 2 days after agro-infection. **e** Leaf without agro-infection, **f** 2 days after agro-infection in leaf, **g** root without agro-infection and **h** root with 2 days after agro-infection

GFP expression is an advantage to select transformation in early stage, thus avoiding regeneration of non-transformants.

Molecular Analysis of Transformants

To confirm the occurrence of foreign genes into the genome of transformed callus, antibiotic-resistant plants were examined for PCR. PCR analysis was performed with transformed genomic DNA to confirm the presence of transformants. Like in previous report by [17], here also we obtained the expected result in the partial amplified product of about 589 bp (Fig. 3c, d) corresponding to the GUS gene that was observed from genomic DNA using GUS gene-specific primers. An amplified fragment of 407 bp (Fig. 3c, d) was also observed from transformed plants using *hptII*-specific primers that confirms the presence of *hptII* gene. The amplified products were observed in all transformants tested, confirming the presence of both transformed GUS and *hptII*. The molecular confirmation of the GUS and *hptII* was reported in many plants such as in yam [64], GUS gene in maize [56].

RT PCR Analysis

To investigate the CCD4a gene expression of transformed callus, reverse transcriptase PCR (RT-PCR) was carried out. The cDNA from the transformed callus was subjected to RT-PCR to analyse the expression level. The expected size of the band 390 bp (Fig. 3e) was observed in transformed callus. It is noticeable that the CCD4a gene was expressed in transformed callus. In parallel, the 18S rRNA was used as an mRNA expression as a control. In *Bo*CCD1, RT-PCR was evaluated to study the expression pattern in vegetative organs and reproductive tissues, and also the expression was carried out to study the *Bo*CCD1 expression level with bixin accumulation in mature seeds [5]. After *Agrobacterium* transformation, a study of the presence and expression level of the gene with control has been carried out in previous report such as in mint with glutathione synthetase gene [70] and potato [71].

Conclusion

In our study, for the first time, we have isolated CCD4a gene from *B. orellana* leaves. The phylogenetic tree result suggested that the CCD4a gene from *B. orellana* showed homology with the other plant CCD genes. Further, we investigated the expression of CCD4a gene in *B. orellana* using *Agrobacterium* transformation strain EHA105. The co-cultivation medium showed higher transformation activity and the expression of CCD4a was confirmed by RT-PCR analysis. It is necessary to analyse if the presence of CCD4a enzymes in *B. orellana* would cleave different carotenoid substrates at their 9, 10 (9′,10′ double bonds) by bacterial expression assays. Moreover, the characterization of CCD4a is required to reveal the functional role and enzymatic activity of apocarotenoid production in *B. orellana*.

Acknowledgments We express our heartfelt gratitude to Science and Engineering Research Board—Department of Science and Technology, New Delhi, India, for the support extended through the project [SR/FT/LS-75/2011]. We express our gratitude to Dr. K. Suthindhiran for the fluorescence microscope. The authors are thankful to the VIT University management for their constant support.

Authors' Contribution Statement RS conceived and designed the experiments. MS performed the experiments and wrote the manuscript. GC corrected the bioinformatics part of experiments. HH, DF and AA helped to correct the manuscript. SB and RM reviewed the manuscript. All authors have read and approved the manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

References

1. Lu, S., & Li, L. (2008). Carotenoid metabolism: biosynthesis, regulation, and beyond. *Journal of Integrative Plant Biology, 50*, 778–785.
2. Huang, F. C., Molnar, P., & Schwab, W. (2009). Cloning and functional characterization of carotenoid cleavage dioxygenase 4 genes. *Journal of Experimental Botany, 60*, 3011–3022.
3. Priya, R., & Siva, R. (2014). Phylogenetic analysis and evolutionary studies of plant carotenoid cleavage dioxygenase gene. *Gene, 548*, 223–233.
4. Schwartz, S. H., Qin, X., & Zeevaart, J. A. (2001). Characterization of a novel carotenoid cleavage dioxygenase from plants. *The Journal of Biological Chemistry, 276*, 25208–25211.

5. Rodrı'guez-A Vila, N. L., Narvaez-Zapata, J. A., Ramı'rez-Benı'tez, J. E., Aguilar-Espinosa, M. L., & Rivera-Madrid, R. (2011). Identification and expression pattern of a new carotenoid cleavage dioxygenase gene member from *Bixa orellana*. *Journal of Experimental Botany, 62*, 5385–5395.
6. Ahrazem, O., Trapero, A., Gómez, M. D., Rubio-Moraga, A., & Gómez-Gómez, L. (2010). Genomic analysis and gene structure of the plant carotenoid dioxygenase 4 family: a deeper study in *Crocus sativus* and its allies. *Genomics, 96*, 239–250.
7. Moise, A. R., von Lintig, J., & Palczewski, K. (2005). Related enzymes solve evolutionary recurrent problems in the metabolism of carotenoids. *Trends in Plant Science, 10*(4), 178–186.
8. Kato, M., Matsumoto, H., Ikoma, Y., Okuda, H., & Yano, M. (2006). The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. *Journal of Experimental Botany, 57*, 2153–2164.
9. Hirschberg, J. (2001). Biosynthesis in flowering plants. *Current Opinion in Plant Biology, 4*, 210–218.
10. Cazzonelli, C. I. (2011). Carotenoids in nature: insights from plants and beyond. *Functional Plant Biology, 3*, 833–847.
11. Priya, R., & Siva, R. (2015). Analysis of phylogenetic and functional diverge in plant nine-cis epoxycarotenoid dioxygenase gene family. *Journal of Plant Research, 128*, 519–534.
12. Bouvier, F., Isner, J. C., Dogbo, O., & Camara, B. (2005). Oxidative tailoring of carotenoids: a prospect towards novel functions in plants. *Trends Plant Science, 10*, 187–194.
13. Siva, R., Mathew, G. J., Venkat, A., & Dhawan, C. (2008). An alternative tracking dye for gel electrophoresis. *Current Science, 94*, 765–767.
14. Siva, R., Prabhu Doss, F., Kundu, K., Satyanarayana, V. S. V., & Kumar, V. (2010). Molecular characterization of bixin—an important industrial product. *Industrial Crops and Products, 32*, 48–53.
15. Srivastava, A., Shukla, Y., Jain, S., & Kumar, S. (1999). Chemistry pharmacology and uses of *Bixa orellana*—a review. *Journal of medicinal and Aromatic Plant Science, 21*, 1145–1154.
16. Zaldivar–Cruz, J. M., Ballina- Gomez, H., Guerrero- Rodriguez, C., Aviler- Berzunia, E., & Godoy-Hernandez, G. C. (2003). *Agrobacterium*-mediated transient transformation of annatto (*Bixa orellana*) hypocotyls with the GUS reporter gene. *Plant Cell Tissue Organ Culture, 73*, 281–284.
17. Parimalan, R., Venugopalan, A., Giridhar, P., & Ravishankar, G. A. (2011). Somatic embryogenesis and *Agrobacterium*-mediated transformation in *Bixa orellana* L. *Plant Cell Tissue Organ Culture, 105*, 317–328.
18. Parimalan, R., Giridhar, P., & Ravishanker, G. A. (2008). Mass multiplication of *Bixa orellana L*. through tissue culture for commercial propagation. *Industrial Crops and Products, 28*, 122–127.
19. Huang, J., Pray, C., & Rozelle, S. (2002). Enhancing the crops to feed the poor. *Nature, 418*, 678–684.
20. Sharma, M. K., Solanke, A. U., Jani, D., Singh, Y., & Sharma, A. K. (2009). A simple and efficient *Agrobacterium*-mediated procedure for transformation of tomato. *Journal of Bioscience, 34*, 423–433.
21. Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P., & Potrykus, I. (2000). Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice enosperm. *Science, 287*, 303–305.
22. Burkhardt, P. K., Beyer, P., Wunn, J., Kloti, A., Armstrong, G. A., Schledz, M., Von Lintig, J., & Potrykus, I. (1997). Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *The Plant Journal, 11*, 1071–1078.
23. Costa, M., Otoni, W., & Moore, G. (2002). An evaluation of factors affecting the efficiency of *Agrobacterium*-mediated transformation of *Citrus paradise* (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. *Plant Cell Reports, 4*, 365–373.
24. Siva, R. (2003). *Assessment of genetic variation in some dye-yielding plants using isoenzyme data* (Ph. D Thesis). Tiruchirapalli: Bharathidasan University.
25. Anantharaman, A., Hemachandran, H., Priya, R. R., Sankari, M., Gopalakrishnan, M., Palanisami, N., & Siva, R. (2016). Inhibitory effect of apocarotenoids on the activity of tyrosinase: multi-spectroscopic and docking studies. *Journal of Bioscience and Bioengineering, Journal of Bioscience and Bioengineering, 121*, 13–20.
26. Jako, C., Coutu, C., Roewer, I., Reed, D. W., Pelcher, L. E., & Covello, P. S. (2002). Probing carotenoid biosynthesis in developing seed coats of *Bixa orellana* (Bixaceae) through expressed sequence tag analysis. *Plant Science, 163*, 141–145.
27. Bouvier, F., Dogbo, O., & Camara, B. (2003). Biosynthesis of the food and cosmetic plant pigment bixin (annatto). *Science, 300*, 2089–2091.
28. Bouvier, F., Suirem, C., Mutterer, J., & Camara, B. (2003). Oxidative remodeling of chromoplast carotenoids: identification of the carotenoid dioxygenase *Cs*CCD and *Cs*ZCD genes involved in *Crocus* secondary metabolite biogenesis. *Plant Cell, 15*, 47–62.
29. Rubio, A., Rambla, J. L., Santaella, M., Gómez, M. D., Orzaez, D., Granell, A., & GómezGómez, L. (2008). Cytosolic and plastoglobule targeted carotenoid dioxygenases from *Crocus sativus* are both involved in β ionone release. *Journal of Bioliogical Chemistry, 283*, 24816–24825.

30. Ohmiya, A., Kishimoto, S., Aida, R., Yoshioka, S., & Sumitomo, K. (2006). Carotenoid cleavage dioxygenase (*Cm*CCD4a) contributes to white color formation in *Chrysanthemum* petals. *Plant Physiology, 142*, 1193–1201.
31. Campbell, R., Ducreux, L. J., Morris, W. L., Morris, J. A., Suttle, J. C., Ramsay, G., Bryan, G. J., Hedley, P. E., & Taylor, M. A. (2010). The metabolic and developmental roles of carotenoid cleavage dioxygenase4 from potato. *Plant Physiology, 154*, 656–664.
32. Murray, M. G., & Thompson, W. F. (1980). Rapid isolation of higher molecular weight plant DNA. *Nucleic Acids Research, 8*, 4321–4325.
33. Ullmann, A., Jacob, F., & Monod, J. (1967). Characterization by in vitro complementation of a peptide corresponding to an operator-proximal segment of the beta-galactosidase structural gene of *Escherichia coli. Journal of Molecular Biology, 24*, 339–343.
34. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25*, 3389–3402.2.
35. Thompson, J. D., Higgins, D. G., & Gidson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*, 4673–4680.
36. Capella- Gutierrez, S., Silla-Martinez, J. M., & Gabaldon, T. (2009). TtrimA1: a tool for automated alignment trimming in large trimming in large-scale phylogenetic analyses. *Bioinformatics, 25*, 1972–1973.
37. Posada, D. (2006). ModelTest Server: a web-based tool for the statistical selection of models of nucleotide substitution online. *Nucleic Acids Research, 1*, 34.
38. Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution, 24*, 1596–1599.
39. Benson, D. A., Karsch-Mizrachi, I., Clark, K., Lipman, D. J., Ostell, J., & Sayers, E. W. (2012). GenBank. *Nucleic Acids Research, 40*(D1), D48–D53.
40. Solovyev, V., Kosarev, P., Seledsov, I., & Vorobyev, D. (2006). Automatic annotation of eukaryotic genes, pseudogenes and promoters. *Genome Biology, 7*(1), 10.1–10.12.
41. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (2nd ed.). NY: Cold Spring Harbor Lab. Press, Plainview.
42. Holsters, M., De-Waele, D., Depicker, A., Messens, E., Montagu, V. M., & Schell, J. (1978). Transfection and transformation of *Agrobacterium* tumefaciens. *Molecular and General Genetics, 163*, 181–187.
43. Murashige, T., & Skooge, F. (1962). A revised medium or rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473–497.
44. Jefferson, R. A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter, 5*, 387–405.
45. Vallabhaneni, R., Bradbury, L. M. T., & Wurtzel, E. T. (2010). The carotenoid dioxygenase gene family in maize, sorghum and rice. *Archives of Biochemistry and Biophysics, 504*, 104–111.
46. Vogel, J. T., Tan, B. C., McCarty, D. R., & Klee, H. J. (2008). The carotenoid cleavage dioxygenase 1 enzyme has broad substrate specificity, cleaving multiple carotenoids at two different bond positions. *The Journal of Biological Chemistry, 283*, 11364–11373.
47. Simkin, A. J., Underwood, B. A., Auldridge, M., Loucas, H. M., Shibuva, K., Schmelz, E., Clark, D. G., & Klee, H. J. (2004). Cardian regulation of the *Ph*CCD1 carotenoid cleavage dioxygenase controls emission of beta-ionone, a fragrance volatile of *petunia* flowers. *Plant Physiology, 136*, 3504–3514.
48. Mathieu, S., Terrier, N., Procureur, J., Bigey, F., & Gunata, Z. (2005). A carotenoid cleavage dioxygenase from *Vitis vinifera* L.: functional characterization and expression during grape berry development in relation to C13-norisoprenoid accumulation. *Journal of Experimental Botany, 56*, 2721–2731.
49. Pooja, S., Sweta, K., Mohanapriya, A., Sudandiradoss, C., Siva, R., Gothandam, K. M., & Babu, S. (2015). Homotypic clustering of *Osmy*4 binding site motifs in promoters of the rice genome and cellular- level implications on shealth blight disease resistance. *Gene, 561*, 209–218.
50. Kumar, K. K., Maruthasalam, S., Loganathan, M., Sudhakar, D., & Balasubramanian, P. (2005). An improved *Agrobacterium*-mediated transformation protocol for recalcitrant elite *indica* rice cultivars. *Plant Molecular Biology Reporter, 23*, 67–73.
51. Mohanty, A., Sarma, N. P., & Tyagi, A. K. (1999). *Agrobacterium*-mediated high frequency transformation of an elite indica rice variety Pusa Basmati 1 and transmission of the transgenes to R2 progeny. *Plant Science, 147*, 127–137.
52. Ziemienowicz, A. (2014). Agrobacterium-mediated plant transformation: factors, applications and recent advances. *Biocatalysis and Agricultural Biotechnology, 4*, 95–102.
53. Phillips, G. C., & Collins, G. B. (1979). In vitro tissue culture of selected legumes and plant regeneration from callus culture of red clover. *Crop Science, 19*, 59–64.

54. Tang, W., Luo, H., & Ronald Newton, J. (2004). Effects of antibiotics on the elimination of *Agrobacterium* tumefaciens from loblolly pine (*Pinus taeda*) zygotic embryo explants and on transgenic plant regeneration. *Plant Cell Tissue Organ Culture, 70*, 71–81.
55. Katiyar, S. K., Chandel, G., Singh, P., & Pratibha, R. (1999). Genetic variation and effect of 2,4, D in in vitro plant regeneration in indica rice cultivars. *Oryza, 36*, 254–256.
56. Ombori, O., Muoma, J. V. O., & Machuka, J. (2013). *Agrobacterium*-mediated genetic transformation of selected tropical inbred and hybrid maize (*Zea mays L.*) lines. *Plant Cell Tissue Organ Culture, 113*, 11–23.
57. Aggarwal, D., Kumar, A., & Sudhakara Reddy, M. (2011). *Agrobacterium tumefaciens* mediated genetic transformation of selected elite clone(s) of *Eucalyptus tereticornis*. *Acta Physiologiae Plantarum, 33*, 1603–1611.
58. Lashbrooke, J. G., Young, P. R., Dockrall, S. J., Vasanth, K., & Vivier, M. A. (2013). Functional characterization of three members of the vitis vinifera L. carotenoid cleavage dioxygenase gene family. *BMC Plant Biology, 13*, 156.
59. Hiei, Y., Ohta, S., Komari, T., & Kumashiro, T. (1994). Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *The Plant Journal, 6*, 271–282.
60. Yan, J., Gan, L., Guo, Y., Du, L., Wang, F., Wang, Y., Zhen, L., Wang, Q., Zou, D., Chen, W., Yu, L., Li, H., & Li, X. (2015). Expression of biologically recombinant human acidic fibroblast growth factor in *Arabidopsis thaliana* seeds via oleosin fusion technology. *Gene, 566*, 89–94.
61. Karthikeyan, A., Pandian, S. K., & Ramesh, M. (2011). *Agrobacterium*-mediated transformation of leaf base derived callus tissues of popular indica rice (*Oryza sativa* L. sub sp. Indica cv. ADT 43). *Plant Science, 181*, 258–268.
62. Andrieu, A., Breitler, J. C., Sire, C., Meynard, D., Gantet, P., & Guiderdoni, E. (2012). An in planta, *Agrobacterium*-mediated transient gene expression method for inducing gene silencing in rice (*Oryza sativa* L.) leaves. *Rice, 5*(23), 12.
63. Cao, S. L., Masilamany, P., Lia, W. B., & Pauls, K. P. (2014). *Agrobacterium* tumefaciens-mediated transformation of corn (*Zea mays L.*) multiple shoots. *Biotechnology Biotechnological Equipment, 28*, 208–216.
64. Nyaboga, E., Tripathi, J. N., Manoharan, R., & Tripath, L. (2014). *Agrobacterium*-mediated genetic transformation of yam (*Dioscorea rotundata*): an important tool for functional study of genes and crop improvement. *Frontires in Plant Science, 5*, 463.
65. Frame, B. R., Shou, H., Chikwamba, R. K., Zhang, Z., Xiang, C., Fonger, T. M., Pegg, S. E., Li, B., Nettleton, D. S., Pei, D., & Wang, K. (2002). *Agrobacterium* tumefaciens-mediated transformation of maize embryos using a standard binary vector. *System Plant Physiology, 129*, 13–22.
66. Humara, J. M., Lopez, M., & Ordas, R. J. (1999). *Agrobacterium* tumefaciens mediated transformation of *Pinus pinea* L. cotyledons: an assessment of factors influencing the efficiency of uidA gene transfer. *Plant Cell Reports, 19*, 51–58.
67. Murray, F., Brettell, R., Matthews, P., Bishop, D., & Jacobsen, J. (2004). Comparison of *Agrobacterium*-mediated transformation of four barley cultivars using the GFP and GUS reporter genes. *Plant Cell Reporter, 22*, 397–402.
68. Duque, A. S., Araujo, S. S., Cordeiro, M. A., Santos, D. M., & Fevereiro, M. P. (2007). Use of fused GFP and GUS reporters for the recovery of transformed *Medicago truncatula* somatic embryos without selective pressure. *Plant Cell Tissue Organ Culture, 90*, 325–330.
69. Maximova, S., Dandekar, A. M., & Guiltinan, M. J. (1998). Investigation of *Agrobacterium* mediated transformation of apple using green fluorescent protein: high transient expression and low stable transformation suggest that factors other than TDNA transfer are rate-limiting. *Plant Molecular Biology, 37*, 549–559.
70. Kumar, A., Chakraborty, A., Ghanta, S., & Chattopadhyay, S. (2009). *Agrobacterium*-mediated genetic transformation of mint with *E. coli* glutathione synthetase gene. *Plant Cell Tissue Organ Culture, 96*, 117–126.
71. Bhaskar, P. B., Venkateshwaran, M., Wu, L., Ane, J. M., & Jiang, J. (2009). *Agrobacterium*-mediated transient gene expression and silencing: a rapid tool for functional gene assay in potato. *Plos One, 4*, e5812.