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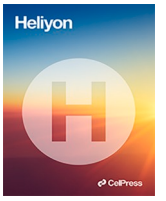

Research article

Overexpression of *AtMYB12* transcription factor simultaneously enhances quercetin-dependent metabolites in radish callus

Muthusamy Balasubramanian [a,b], Shanmugam Girija [a,*]

[a] *Metabolic Engineering Laboratory, Department of Biotechnology, Bharathiar University, Coimbatore, 641 046, Tamil Nadu, India*
[b] *Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, 52900, Israel*

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ABSTRACT

The study aimed to enhance quercetin production in radish by optimizing *Agrobacterium tumefaciens*-mediated *in-planta* transformation. This protocol involved infecting radish seed embryo axis with *A. tumefaciens* EHA105 strain carrying the *35S::AtMYB12*. Radish seeds were infected with the *Agrobacterium* suspension (0.8 OD_{600}) for 30 min, followed by sonication for 60 s and vacuum infiltration for 90 s at 100 mm Hg. A 3-day co-cultivation in Murashige and Skoog medium with 150 μM acetosyringone yielded a transformation efficiency of 59.6% and a transgenic callus induction rate of 32.3%. Transgenic plant and callus lines were confirmed by GUS histochemical assay, PCR, and qRT-PCR. The transgenic lines showed an increased expression of flavonoid pathway genes (*AtMYB12, CHS, F3H,* and *FLS*) and antioxidant genes (*GPX, APX, CAT,* and *SOD*) compared to WT plants. Overexpression of *AtMYB12* in transgenic callus increased enzyme activity of phenylalanine ammonia lyase, catalase, and ascorbate peroxidase. In half-strength MS medium with 116.8 mM sucrose, the highest growth index (7.63) was achieved after 20 days. In *AtMYB12* overexpressed callus lines, phenolic content (357.31 mg g^{-1} dry weight), flavonoid content (463 mg g^{-1} dry weight), and quercetin content (48.24 mg g^{-1} dry weight) increased significantly by 9.41-fold. Micro-wounding, sonication, and vacuum infiltration improved *in-planta* transformation in radishes. These high-quercetin-content transgenic callus lines hold promise as valuable sources of flavonoids.

1. Introduction

Radish (*Raphanus sativus* L.) is a globally consumed root vegetable known for its richness in anthocyanin [1]. While some genotypes exhibit anthocyanins production in the leaves, stems, and epidermis, the concentration in the taproot varies [2,3]. Anthocyanin, a type of flavonoid, plays a role in coloring fruits and attracting seed dispersers [4,5]. In model plant species, pivotal transcription factors that play a crucial role in regulating flavonoid synthesis and accumulation encompass BHLH (basic helix-loop-helix), MYB (myeloblastosis), and WD40 [5]. Recently, metabolic engineering has been employed to enhance secondary metabolic biosynthesis in plants [6]. Notably, some secondary metabolite production genes have been altered by endogenous or exogenous overexpression of a single transcription factor [6–8]. For instance, the *AtMYB12* gene from *Arabidopsis thaliana* has been found to enhance flavonoid content in tomato and upregulate flavonoid-specific genes, including *phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4 Coumarate: CoA ligase (*4CL*), chalcone synthase (CHS), flavonol synthase (FLS)* [9–11]. When *AtMYB12* was overexpressed to enhance

* Corresponding author.
E-mail address: girijabiotech@yahoo.co.in (S. Girija).

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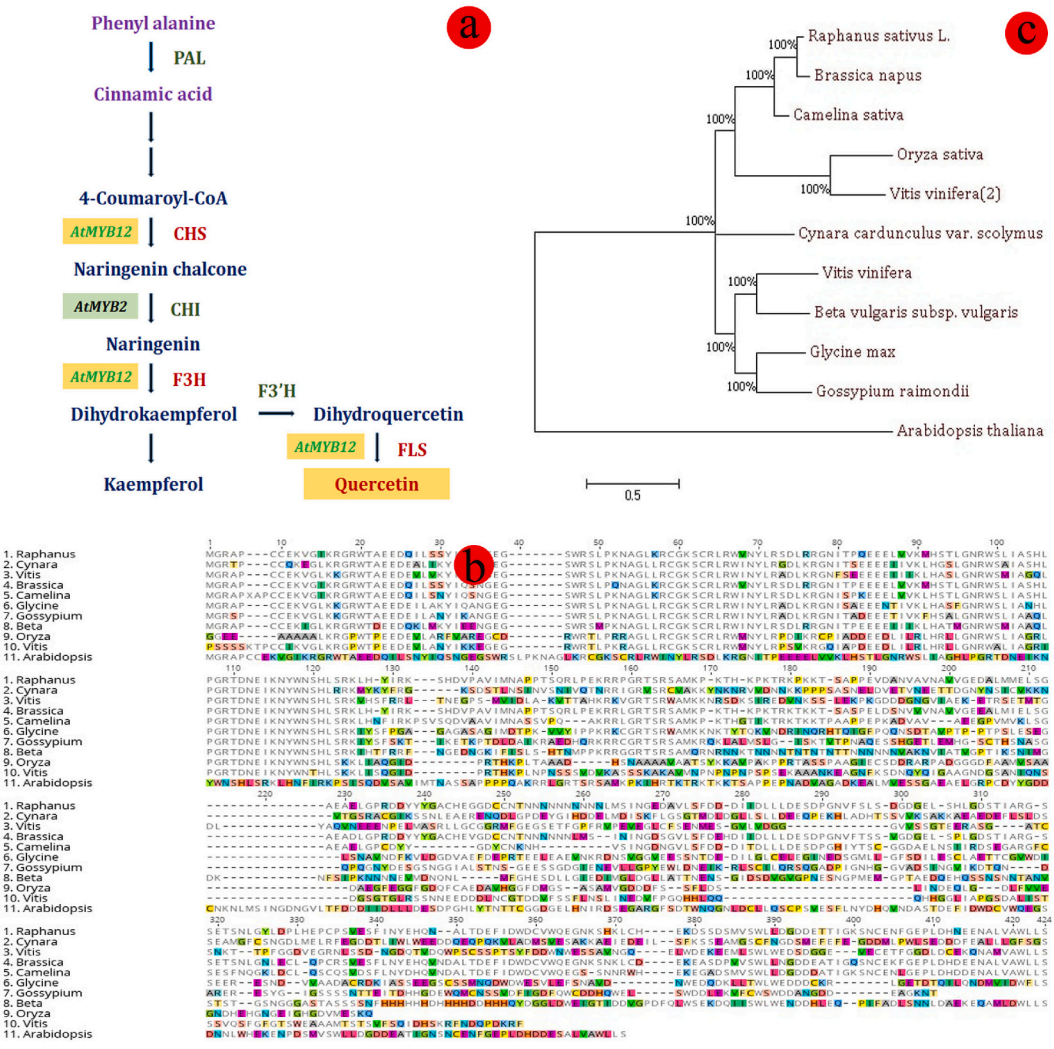

Fig. 1. The flavonol biosynthetic pathway, sequence and phylogenetic tree analysis (A) Schematic of flavonol biosynthesis (PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3 hydroxylase; FLS, flavonol synthase). Grey boxes beside target enzymes show transcription factor *AtMYB12*. **(B)** *ATMYB12* amino acid sequence protein alignment. **(C)** Phylogenetic analysis.

insect resistance, it led to increased flavonol content in tomato and tobacco [11,12]. Furthermore, the overexpression of *AtMYB12* in tobacco callus culture resulted in increased rutin and quercetin production [13,14], as observed in hairy root buckwheat cultures as well [13]. Remarkably, the biosynthesis of heterologous resveratrol and flavonoids has been achieved through the metabolic modification of grapevine callus cell cultures [15,16].

The phenylpropanoid pathway relies on PAL enzyme (Fig. 1A). Downstream enzymatic actions produce various aglycons. Many flavonoids, such as quercetin, kaempferol, and rutin, play a role in regulating auxin transport and protecting plants from UV radiation [17]. Quercetin can be found in several sources, including red wine, onions, green tea, apples, berries, and Ginkgo biloba. Notably, tartary buckwheat, with quercetin contents of up to 3% in seeds and 1.7%. Plants serve as the primary source of quercetin [18]. Due to rising agricultural and industrial demand, there is a need to enhance quercetin yield [19]. Quercetin levels in tomato and tobacco have been observed to increase through heterologous expression of the *AtMYB12*, as demonstrated in previous studies [11,12]. Additionally, research has demonstrated that *AtMYB12*-expressing tobacco calluses exhibit higher rutin levels [13]. Furthermore, the overexpression of *AtMYB11*, a homolog of *AtMYB12*, has been shown to enhance quercetin levels in tobacco leaves and tomatoes [20].

The efficiency of *A. tumefaciens*-mediated transformation (ATMT) and regeneration is influenced by factors such as plant age and explant type. *In-planta* transformation primarily targets the close-apical meristem cell for T-DNA transfection, resulting in the efficient production of transgenic lines. However, it has been found that ethylene inhibits shoot growth in radish explants during the regeneration process [21–23]. To counteract the inhibitory effects of ethylene, silver nitrate and L-a-2-aminoethoxyvinylglycine (AVG) has been employed to inhibit ethylene production in regeneration media. Additionally, the combination of 2 mg L^{-1} 6-benzyl adenine (BA)

and 1 mg L^{-1} α-naphthaleneacetic acid (NAA) significantly enhances hypocotyl regeneration in the Chinese radish cv [21] and red coat reported [21–23]. Silver nitrate treatment also leads to a remarkable 60% increase in seedling radish cv. Jinju daepyung shoot recovery compared to control treatments [22]. However, it's important to note that both hypocotyl and cotyledon explants yield shoots after undergoing antibiotic screening [24]. Poor shoot regeneration from embryogenic calli and microspores further complicates ATMT [25,26]. Recent studies have demonstrated that radish transformation is more successful using cotyledon with pCAMBIA1301 and pPTN290 vectors, with transformation frequencies of 0.26% and 0.18%, respectively [27]. Earlier research achieved a 22% radish seed transformation efficiency using ultrasonic and vacuum approaches [28]. The highest transformation efficiency, at 13.3%, was observed with radish hypocotyl explants [29]. Recent findings also indicate that radish seedling apical meristems can significantly increase transformation efficiency by up to 53% [21]. However, due to the challenges posed by recalcitrant cells and limited tissue regeneration in radish, these studies suggest that *in-planta* transformation may be a more suitable approach. In the present study, we optimized several key factors, including wounding, co-cultivation duration, sonication, and vacuum infiltration, to achieve high-efficiency *in-planta* transformation. This optimization resulted in the successful overexpression of *AtMYB12* in radish transgenic plants. Notably, the expression of quercetin, flavonoid biosynthesis, and antioxidant genes was found to be significantly higher in *AtMYB12*-overexpressing transgenic radish callus compared to non-transformed plants.

2. Materials and methods

2.1. Plant material

Three different varieties of radish (Co1, Pusa chetki, and Pusa desi) seeds were procured from the Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The surface sterilization method was performed as described [30]. Subsequently, the surface sterilized seeds were incubated at 24 °C in the dark in a 250 ml Erlenmeyer, each containing 100 ml of sterile double-distilled water (DDH_2O). For *in-planta* transformation, single sprouting seeds were aseptically decoated to expose the embryo axis (approximately 4 mm in length).

2.2. Sequence analysis of AtMYB12

The amino acid sequences of *AtMYB12* and its homologs from a diverse range of plant species were aligned using DNAMAN 9.0 software (LynnonBiosoft, Quebec, Canada, 2022) to perform a multiple sequence alignment analysis. For the investigation of molecular evolutionary genetics, we conducted a phylogenetic analysis using MEGA 4.0 (http://www.megasoftware.net/, accessed on May 7, 2007).

2.3. Vector construction

Arabidopsis *MYB12* (Accession No. AY142067, AT2G47460) cDNA clone (1116 bp) in the pUNI51 plasmid was procured from The Arabidopsis Biological Resource Centre (ABRC, USA). The forward and reverse primers were manually designed to amplify the full-length cDNA of *AtMYB12* present in plasmid pUNI51. The forward primer 5'- AGT TAT CTG GAA TTC GGC CG-3' was designed with the *Eco*RI restriction site (5' -GAA TTC) and the reverse primer 5 '- GAC GGC CAG TGC CAA GCT TTC ATGA CA GAA GCC AAG CG-3' was designed with the *Hin*d III restriction site (5'- AAG CTT). To subclone the *AtMYB12* cDNA into the pCAMBIA2301 vector, the amplified PCR product of *AtMYB12* was purified using the QIAquick PCR purification kit (Qiagen, Germany). Then the PCR product and pCAMBIA2301 vector were subjected to restriction digestion with the restriction enzymes *Eco*RI and *Hin*dIII (NEB, England). The restriction-digested PCR product of *AtMYB12*, and the digested pCAMBIA2301 were mixed in the 3:1 ratio, and ligated. Subsequently, using the $CaCl_2$ technique, the ligation product was transformed into *E. coli* DH5α competent cells. The kanamycin resistant colonies formed on the selection agar plate were screened for the presence of *35S::AtMYB12* clone using the colony PCR. The integration of *AtMYB12* in the pCAMBIA2301 vector was confirmed by DNA sequencing. The mobilization of the construct into *A. tumefaciens* strain EHA105 was achieved through the modified electroporation method. Luria Bertani (LB) agar containing 50 mg kanamycin L^{-1} and 25 mg rifampicin L^{-1} (Himedia, India) was used for culturing *Agrobacterium*.

2.4. Minimum inhibitory concentration of kanamycin on seed explants

The gene cassette carrying the selection marker neomycin phosphotransferase (NPTII) was tested for its minimum inhibitory concentration (MIC) in kanamycin, ranging from 25 to 150 mg L^{-1}, with respect to seed germination. The seeds were subjected to various doses (0–150 mg L^{-1}) and placed on MS medium with 0.8% Agar (Himedia, Mumbai, India) [31]. Seeds were stored in the dark 25 °C under a photoperiod cycle of 16 h of light and 8 h of darkness, with an intensity of 120 μmol m-2 s^{-1}, for a total of 25 days. This experiment was repeated five times, each time using 100 pre-treated seedlings for each condition. The untreated seeds served as a positive control.

2.4.1. Preparation of agrobacterium preculture

A single colony was inoculated into 30 ml of LB broth containing 25 mg rifampicin L^{-1} and 50 mg kanamycin L^{-1}. The culture was allowed to grow at 28 °C until the optical density at OD_{600} 1.0 ($\sim 5 \times 10^8$ cells/ml). This growth occurred in total darkness at 180 rpm in an orbital shaker. For each density, 30 ml of *Agrobacterium* culture was centrifuged at 8000 rpm for 10 min and the bacterial cell

pellet was resuspended in 40 ml of MS liquid infection medium (LIM; B_5 vitamins, and 3% sucrose). Acetosyringone (100 μM) from Himedia, Mumbai, India) was added to the LIM containing the *Agrobacterium* cell suspension.

2.5. Acetosyringone and co-cultivation transformation factors

Sprouted-seed explants were randomly punctured or microwounded (5 times) on the surface using a sterile hypodermic needle (27G1/1) (Dispovan, Mumbai, India) before *Agrobacterium* infection. The micro-wounded-explants were immersed in 20 ml *Agrobacterium* suspension and incubated at 28 °C for 20 min with occasional gentle agitation. After the infection, the explants were gently dried on sterile Whatman No. 1 filter paper (Whatman, Mumbai, India) for 5 min to remove any excess *Agrobacterium* cells. Following this, the sprouted seed explants underwent different infection durations, spanning from 10 to 80 min, and were subsequently co-cultured on co-cultivation medium (CCM) for varying time periods, ranging from 1 to 5 days (d). During the co-cultivation time, the GUS expression transformation efficiency was assessed. Infected seeds were co-cultivated on CCM medium with various acetosyringone dosages (ranging from 50 to 300 μM) at 25 °C under diffused light/dark conditions for 16/8 h (120 μmol m^{-2} s^{-1}) following standardization of a 3 days co-cultivation period. After two rinses with sterile DDH_2O, the explants were transferred to liquid MS medium containing 100 mg Timentin L^{-1} and 200 mg Cefotaxime L^{-1}. After 3 days of culture, the transient GUS expression and transformation efficiency in response to acetosyringone administration was examined.

2.6. Sonication combined vacuum infiltration effects on transformation efficiency

Co-cultivation time (3 d) and acetosyringone concentration (150 μM) were used to enhance transformation efficiency. Sprouted-seed explants were subjected to sonication using water bath sonicator (50 KHz; Branson Ultrasonic, Japan). The explants were sonicated for 30–180 s in a 35 ml *Agrobacterium* culture. Following sonication optimization, the sprouted-seed explants were sonicated for 60 s, to a fresh *Agrobacterium* suspension containing150 μM acetosyringone, and subjected to vacuum infiltration. Vacuum infiltration was carried out in a desiccator (Tarsons, Mumbai, India) connected to an Indian high vacuum pump for varying durations and vacuum pressures ranging from 100 to 750 s. Subsequently, the infected explants were dried as previously described, co-cultivated for three days, and then washed with antibiotics. After 3 days of culture, we calculated the percentage of transient GUS expression and transformation efficiency resulting from the acetosyringone treatment.

2.7. Selection pressure on transformants recovery

To remove any residual *Agrobacterium* cells, explants underwent a triple wash in DDH_2O and 1/2 MS liquid medium containing 300 mg of cefotaxime L^{-1} and 100 mg of timentin L^{-1} (Himedia, India). Following pre-treatment MS medium with 100 mg of Timentin L^{-1}, 200 mg of cefotaxime L^{-1}, and 150 mg of kanamycin L^{-1}, the explants were subsequently dried on sterile filter paper. The cultures were maintained at 25 °C with a 16-h photoperiod (120 μmol m^{-2} s^{-1}) for a duration of 25 days. Regenerated plants, derived from explants that survived sprouted seeds employed for transgene screening using *35S::AtMYB12*. CIM was supplemented with varying kanamycin concentrations (ranging from 10 to 50 mg L^{-1}), to determine the optimal concentration for selecting transformed callus, as indicated by necrosis in plant tissues. The selected callus cultures were subsequently transferred to an antibiotics containing medium and maintained for 5 weeks at 25 °C with a 16-h photoperiod (120-μmol m^{-2} s^{-1}, Phillips, India). Young leaves from 25 days old transgenic lines were cut into 1 cm^2 pieces and infected on petri plates using CIM medium with GB5 vitamins (modified CI-MS medium) containing 0.5 mg 2,4-D L^{-1}. Antibiotics (40 mg kanamycin L^{-1}) were used to induce callus induction in transgenic lines, which were incubated in a culture room at 25 °C for 16 h of light and 8 h of darkness cycle. At 2-weeks intervals, both WT (wild type) and transgenic radish induced calluses were subcultured in CIM. The calluses that survived the screening procedure were allowed to develop further and were subsequently tested for presence of transgenes (*35S::AtMYB12*). Transgenic callus induction lines were evaluated after 2 months.

2.8. Histochemical assay for GUS and PCR analysis

The β-glucuronidase (GUS) assay was performed as described in Ref. [32]. To confirm the presence of the target gene (*AtMYB12*), we extracted total DNA from nine feasible transformants, WT plant, transgenic callus, and WT callus, as outlined in Ref. [33]**.** PCR was used to amplify 1116 bp (1.116 kb) amplicon using *AtMYB12* gene-specific primers (FP: 5-AGT TAT CTG GAA TTC GGC CG-3 and RP: 5-GAC GGC CAG TGC CAA GCT TTC ATG ACA GAA GCC AAG CG-3). The amplification was performed in a thermal cycler (Bio-Rad, USA) with an initial DNA denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, concluding with a final extension at 72 °C for 10 min. Positive and negative controls were included, using plasmid DNA from pCAMBIA2301-*AtMYB12* as the positive control and genomic DNA from non-transformed calluses as the negative control. The amplified products were separated by electrophoresis on a 1% agarose gel (w/v).

2.9. RNA extraction and gene expression analysis

Semi-quantitative and quantitative real time polymerase chain reaction (qRT-PCR) were used to investigate the expression of the genes involved in flavonoid production and ROS scavenging. Total RNA was isolated from 20-days old transgenic and WT calluses using TRIzol reagent (Invitrogen). Subsequently, cDNA was synthesized from 2000 ng of total RNA (Invitrogen). Conventional semi-

quantitative RT-PCR was used to amplify several of the genes understudy. The relative expression of each gene was determined by normalizing it to a reference gene, (*ACTIN*). The qRT-PCR assay was performed as described in Ref. [13] and the primer sequences are provided in **Supp. Table S1** Takara Bio Inc. (Japan) SYBR Green PCR Master Mix was used for PCR amplifications on an ABI PRISM 7500 (7500 Fast RT-PCR Systems, USA). The quantification of the gene expression was validated using the method reported in Ref. [34].

2.10. Quantification PAL and antioxidative enzyme activity by spectrometric assay

2.10.1. PAL enzyme extraction and PAL activity assay

The transgenic and control callus, each weighing 100 mg, were frozen in liquid nitrogen, and subsequently ground using a mortar and pestle. The resulting fine powder from the callus was then subjected to extraction with a 50 mM Tris-HCl buffer (Sigma-Aldrich, Steinheim, Germany) at pH 8.8, supplemented with 10 mM 2-β-mercaptoethanol, 1 mM EDTA, and 2.5% polyvinylpyrrolidone-40 (PVP-40 from Sigma-Aldrich, Germany). After centrifuging at 21,180×*g* for 20 min, the resulting mixture was desalted in aliquots using an Amicon Ultra-15 Centrifugal Filter Unit (Merck Millipore, Tullagreen Carrigtwohill, Ireland) and subsequently analyzed for PAL activity under standard conditions.

Protein estimation was performed using the method previously described in Ref. [35], with crystalline bovine albumin (BSA) serving as the standard. PAL was isolated from fresh 20-day-old transgenic and wild-type (WT) callus cells using borate buffer (pH 8.8). To obtain a solid-free extract, the cells were ground in the buffer (0.15 g ml) for 2 min on ice and then centrifuged at 10,000 rpm and 4 °C for 20 min. PAL activity was determined by measuring the conversion of L-phenylalanine to cinnamic acid, as described in Ref. [36].

2.11. Antioxidant enzyme extraction

Callus samples (DW 100 mg) were first weighed, then frozen in liquid nitrogen, and subsequently homogenized in 2.0 mL of extraction buffer containing 100 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, and 10 mM ascorbic acid. The homogenate was centrifuged at 13,000 g for 15 min at 4 °C, and the resulting supernatant was analyzed for CAT and APX activity under standard conditions., Aliquots were stored at −20 °C before use.

2.12. Catalase (CAT) and ascorbate peroxidase (APX) antioxidant enzyme assay

The initial rate of disappearance of H_2O_2 in the CAT (EC 1.11.1.6) enzyme extract was measured according to the method described in Ref. [37]. The APX (EC 1.11.1.11) enzyme assay was performed following the procedure detailed in Ref. [38]. To determine protein content, an aliquot of the extract was used, with BSA serving as the reference protein, as specified in Ref. [35]. Each enzyme assay was repeated in triplicate and produced consistent results, utilizing a UV–visible spectrophotometer (Thermo Scientific, evolution201 series, USA).

2.13. Callus growth kinetics study

For sub-culturing and growth kinetic investigations, calluses from 20 days old wild and transgenic callus samples were used. Calluses weighing (FW 0.2 g) were inoculated in 250 ml erlenmeyer flasks containing 40 ml of 1/2 - strength MS liquid callus proliferation medium. At 5-days intervals from 20 to 25th days, calluses were removed and weighed (n = 18). The growth index was calculated using the formula Fw - Iw/Iw, where represents the initial inoculation weight (Iw) and Fw is the final callus mass (weight). Calluses were stored at −80 °C in liquid nitrogen until further use.

2.14. Transgenic callus extract preparation, total phenolic, flavonoid, and quercetin content quantification

Transgenic and wild callus (control) were rinsed with sterile water and air-dried. The callus was then pulverized into a fine powder using a mortar and pestle in liquid nitrogen. Subsequently, 1 g of the dried weight (DW) fine callus powder was extracted with 10 ml of 99.9% methanol in glass tubes. The samples were macerated on an orbital shaker at 150 rpm for 3 days at 22 °C. The resulting extracts were dried using Cyber Lab's rotary vacuum evaporator. To prepare the callus extract for analysis, 1 mg of the dried methanolic callus extract powder was diluted in 1 ml of 99.9% HPLC-grade methanol and filtered through a 0.45 μm polyvinylidene fluoride syringe filter [13]. The preparation of the callus extract and the quantification of total phenols, flavonoids, and quercetin followed a previously established method [30]. Total phenolic compounds were quantified as described in Ref. [39]. The total flavonoid content was determined following the procedure outlined in Ref. [40]. Quercetin was quantified using liquid chromatography (HPLC, Waters 2998, MA), as detailed in Ref. [30].

2.15. Statistical analysis

All of the data were assessed using one-way ANOVA and the Duncan Multiple Range Test (DMRT). Student's *t*-test was employed to examine the data in a two-tailed study, with statistical significance defined as $p < 0.05$ or $p < 0.01$. The trials were repeated five times and to determine significance, we used the software SPSS 17.0 (SPSS Inc., USA).

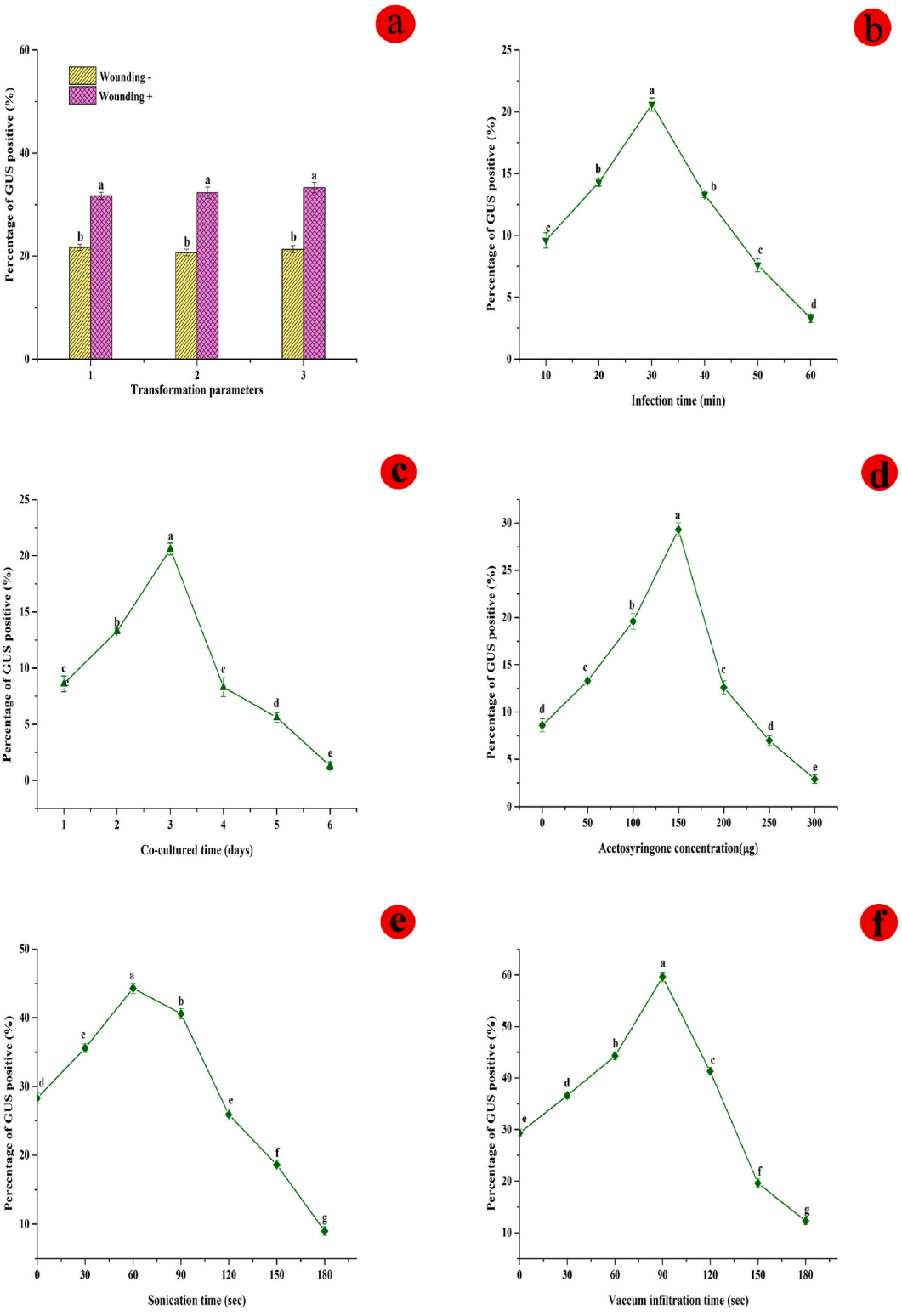

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Fig. 2. Effect of different transformation parameters on *in planta* transformation efficiency of radish cv. Co 1. (A) Influence wounding (±) on transformation efficiency. **(B)** Infection time. **(C)** Co-cultivation period. **(D)** Acetosyringone concentration. **(E)** Sonication time. **(F)** Vacuum time. Each treatment used 100 radish seeds and was repeated five times. Seeds were under vacuum for 0.8 OD Agrobacterium culture, sonicated for 90 s, infected for 20 min, and co-cultivated for 3 days on ½-strength MS medium with 150 μM ACS concentrations. Each treatment used 100 radish seeds and was repeated five times. Values represent the mean ± standard error of five replications (n = 5). Mean values followed by the same letters within a bar are not significantly different according to Duncan's multiple range test at 5 % level.

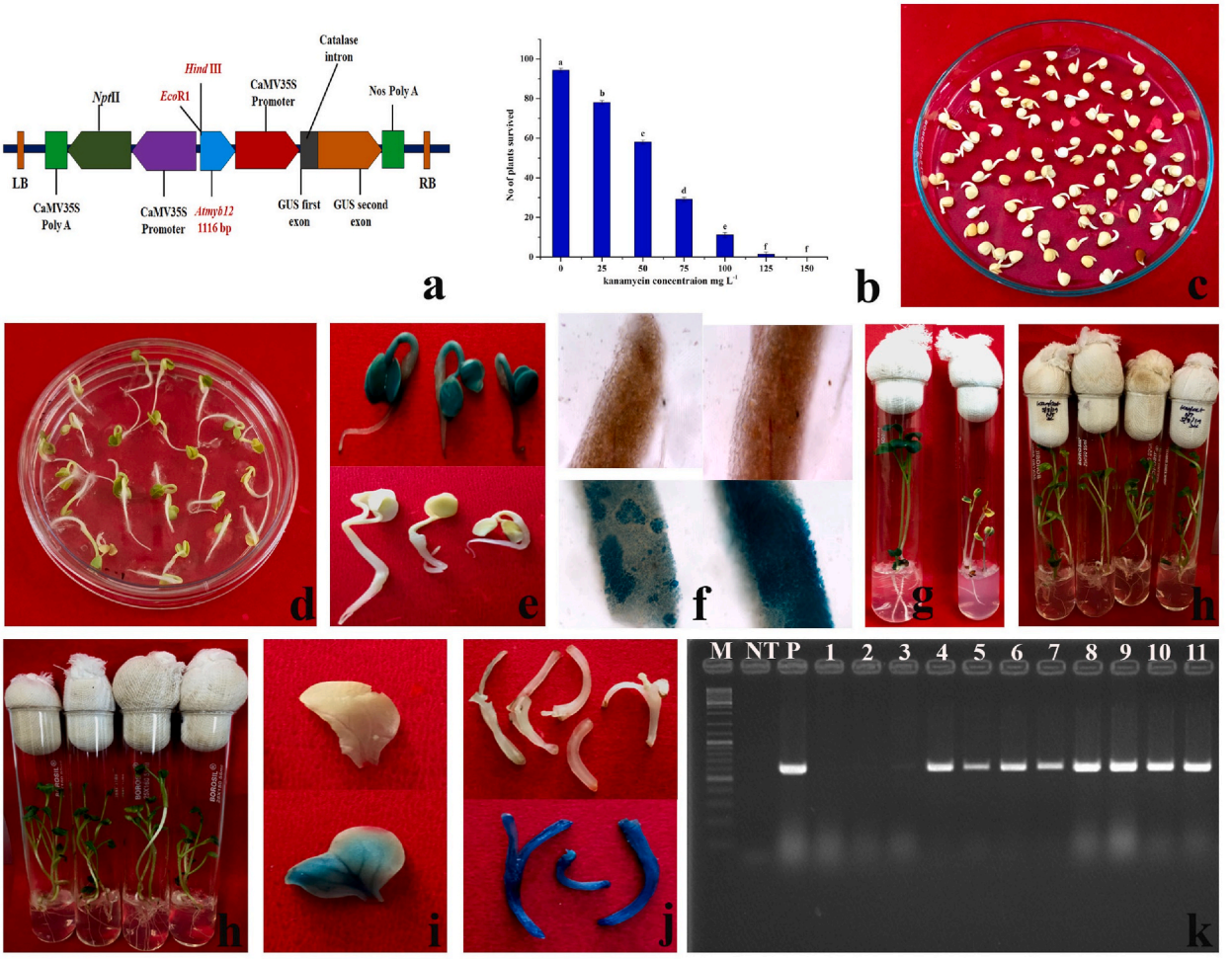

Fig. 3. *Agrobacterium* mediated *in-planta* transformation of full seed explant of radish co 1. **(A)** The binary vector map pCAMBIA2301-*AtMYB12* used to transform the radish contains a linear map of the T-DNA region. **(B)** Radish cv. CO1 half-seed explants' sensitivity to kanamycin-containing MS media after 25 days. **(C)** De-coated microwounded radish seed explants. **(D)** Co-cultivation medium seedlings after 5 days **(E)** WT control seedling and transient GUS expression. **(F)** WT control and transient seed radicle cross sections showing GUS expression (10X). **(G)** WT control and transient seed hypocotyl cross sections revealing GUS expression (10X). **(H)** Transgenic and WT non-transformed radish plants in MS medium with 150 mg L^{-1} kanamycin. 15-day-old transgenic radish plants in MS media with 150 mg L^{-1} kanamycin and WT non-transformed plants. (I) GUS-expressing leaf with WT control. (J) GUS-expressing stem with WT control. **(K)** Molecular confirmation of transgenic plants of radish cv. CO1 (PCR: Lane M DNA ladder (10 Kb); Lane 1 DNA sample from non-transformed leaves (WT negative control), Lane 2 Plasmid pCAMBIA 2301:35S:*AtMYB12* harbouring *AtMYB12* gene (positive control), Lane 3–11 DNA sample from GUS positive transformed leaves. Amplicon size-1116 bp (*AtMYB12* gene).

3. Results

3.1. Pre transformation parameters

3.1.1. Sequence analysis of AtMYB12 and kanamycin sensitivity of seed explants

The recombinant *AtMYB12* protein was found to be 100% identical to the wild-type protein, as indicated by BLAST analysis.

Further investigation revealed that *AtMYB12*'s amino acid sequence shared a 83.6% similarity with *Camelina sativa's* anticipated protein products (XP_010507890), and it also exhibited similarities to other species, such as *Raphanus sativus* (Rs365720, 73.6%), *Brassica napus* (XP_013747536, 73.1%), *Vitis vinifera* (42%), Beta vulgaris (39%), and *Gossypium raimondii* (39%). In addition, it showed some degree of similarity to *Cynara cardunculus* var. scolymus (AXF92691, 37.2%), *Oryza sativa* (BAA23340, 29.1%), and *Glycine* max (XP_006574536, 38.9%) (Fig. 1**B**). Phylogenetic analysis indicated that *AtMYB12* was closely related to the predicted protein products of *Camelina sativa* and *Raphanus sativus* (Fig. 1**C**) The construct includes, the pCAMBIA2301-*AtMYB12* with NptII marker gene (Fig. 3**A**), making it essential to determine the Minimum Inhibitory Concentration (MIC) of kanamycin for effective selection of transformants. After 25 days of culture, it was observed that a kanamycin concentrations of 150 mg L^{-1}completely inhibited seed germination (Fig. 3**B**). Therefore, this concentration was chosen as the MIC for the selection of transformants.

3.2. Optimized conditions for improved transformation efficiency

3.2.1. Effect of micro wounding on transformation efficiency.

Agrobacterium plasmid virulence (vir) genes are typically transcriptionally activated in response to micro-wounding of explants. In our efforts to enhance transformation efficiency, we performed micro-wounding on de-coated radish seeds with embryo axis explants using a sterile hypodermic needle (Fig. 3**C**) and subsequently infected them with an *Agrobacterium*. The results revealed that micro wounded radish seed cotyledon with embryo axis explants showed a significant increase in GUS expression, reaching a transformation efficiency of 33.3% when compared to non-wounded explants (Fig. 2**A**).

3.3. Infection duration influences transformation efficacy

To improve transformation efficacy in pre-cultured seed explants, we conducted experiments testing infection times ranging from 10 to 80 min, as shown in Fig. 2**B**. Notably, infecting pre-cultured seed explants with the *Agrobacterium* culture for 30 min achieved in the highest GUS expression percentage, reaching 20.6% in terms of transformation efficiency. However, it's important to highlight that the transformation efficiency of the *Agrobacterium* gradually declined when the infection time exceeded or fell below the 30-min. Additionally, it's worth noting that the survival rate of the explants decreased when the infection period extended beyond 30 min, as depicted in Fig. 2**B**.

3.4. Co-cultivation duration improves transformation efficiency

The percentage of transformation efficiency in seed explants treated with *Agrobacterium* (Fig. 3**D**) varied significantly for different time durations (1–6 days), as depicted in Fig. 2**C**. The transformation efficiency (%) improved as co-cultivation developed, rising at 3 days and then gradually declining. Among the seeds infected with *Agrobacterium*, 20.7% showed the highest GUS expression, percentage of transformation efficiency in radish at this optimal co-cultivation time (3 d).

3.5. Optimum acetosyringone concentration improves transformation efficiency

Plant tissue-specific phenolic compounds, which induce *Agro vir* genes expression, play a crucial role in gene transfer when the plant tissue is wounded. Another significant factor is the influence of acetosyringone on transformation efficiency. In the absence of acetosyringone, seed explants co-cultivated with *Agrobacterium* exhibited a reduced transformation efficiency, as shown in Fig. 2**D**. The highest transient GUS expression efficiency was (29.2%) observed in seeds infected with *Agrobacterium* culture and maintained on MS media supplemented with 150 μM acetosyringone. Treatment with acetosyringone increased transformation efficiency by 3.5-fold compared to plants without acetosyringone (Fig. 2**D**).

3.6. Sonication duration positively influences transformation efficiency

Transgenic plants must originate from the cells of the meristematic region. The primary objective of *Agrobacterium*-mediated transformation is to target meristematic cells within plant tissues. Sonication facilitates *Agrobacterium* entry into meristematic cells by creating micro-wounds. Due to the deep location of meristematic cells within the tissue, the probability of *Agrobacterium* infection is limited, resulting in a low transformation efficiency. In this study, we investigated sonication times ranging from 30 to 180 s at a frequency of 50 KHz to optimize the transformation process. The goal was to enhance the transformation efficiency in sprouted radish seeds by generating micro-wounds on the explant tissue. The results showed that during longer sonication periods, which extended up to 60 s, the transformation efficiency, as evaluated by GUS expression, reached 44.8% (Fig. 2**E**; Fig. 3**E**).

3.7. Vacuum infiltration duration improves transformation efficiency

After optimizing the sonication time, we employed vacuum infiltration for seed explants of different durations to further enhance the transformation efficiency. Vacuum infiltration is known to improve transformation efficiency by allowing gases to pass through the seeds, potentially wounding certain parts of the seed. When the vacuum is released and pressure rapidly rises, micro-wounded seeds can be exposed to an *Agrobacterium* suspension to replace the gases. In this investigation, vacuum infiltration was conducted for 30–180 s in radish seed explants to improve transformation efficiency. In 1/2-strength MS medium with the optimal sonication period,

Table 1
Competency of in planta transformation methodology on different radish cultivars.

Cultivars used for Transformation	No. of seeds infected	Mean no. of seeds germinated	Mean no. of GUS positive explants	Transformation efficiency (%)
Pusa chetki	100	61.7 ± 1.57^{c}	41.3 ± 0.25^{c}	41.3
Pusa Desi	100	64.3 ± 2.43^{b}	47.6 ± 0.23^{b}	47.6
Co 1	100	73.0 ± 2.40^{a}	59.6 ± 0.24^{a}	59.6

Seeds were vacuumed for different time interval at OD 0.8 of Agrobacterium culture, sonicated for 60 s and subjected to vacuum infiltration for 90 s. They were then, infected for 20 min and co-cultivated for 3 days on ½-strength MS medium containing 150 μM concentrations of ACS. Transformation efficiency was calculated as (number of GUS-positive explants/total number of radish seeds infected) × 100. One hundred radish seeds were used per treatment, and each treatment was repeated five times. Values represent the mean ± standard error of five replications (n = 5). Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at a 5% level.

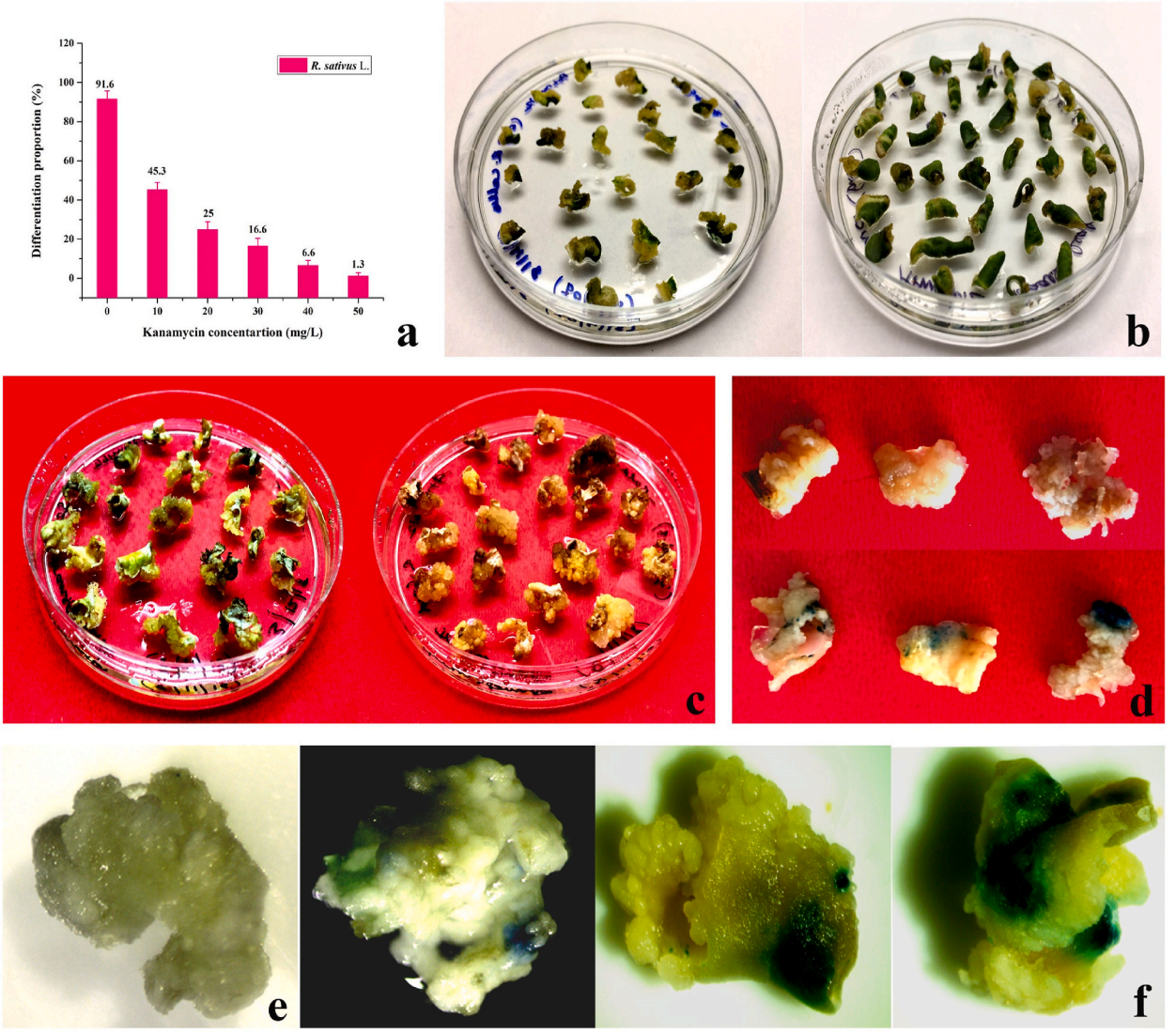

Fig. 4. Establishment and growth of transgenic and Wild Type radish calluses. **(A)** Calculated leaf disc differentiation with different kanamycin doses. **(B)** Formation of putative transformed radish leaf callus after 15 days in callus induction medium (MS media with Kanamycin 40 mg L^{-1} + 2,4 -D 0.5 mg L^{-1} + timentin 150 mg L^{-1}) and WT control (Non transformed). **(C)** Formation of transgenic radish leaf callus after 30 days in callus induction medium (MS media with Kanamycin 40 mg L^{-1} + 2,4-D 0.5 mg L^{-1} + timentin 150 mg L^{-1}) and WT control. **(D)** Transgenic callus showing GUS expression along with WT control (WT-Non transformed). **(E)** WT control callus (Stereo zoom microscope). **(F)** Transgenic callus showing GUS expression (Stereo zoom microscope).

Table 2
Selection pressure on recovery of *AtMYB12* transgenic callus produced from radish.

Experiment	No. of leaves transformed with *AtMYB12*	No. of leaf produced callus in selection medium after 30 days of culture.	No. of GUS positive callus	Transformation efficiency (%)[a]
WT	100	0.00	0.00	0.00
Transgenic				
1	100	51.0 ± 0.80^{d}	31.6 ± 1.00^{b}	31.6
2	100	52.3 ± 0.66^{b}	29.3 ± 1.50^{d}	29.3
3	100	51.6 ± 0.80^{c}	31.6 ± 1.00^{b}	31.6
4	100	53.3 ± 0.66^{a}	32.3 ± 0.55^{a}	32.3
5	100	50.6 ± 1.20^{e}	29.6 ± 1.50^{c}	29.6

WT: Wild type (control); Putative transformed radish leaf callus formation after 20 days of initial culture in the callus induction medium (MS medium containing Kanamycin 40 mg L^{-1} + 2,4-D - 0.5 mg L^{-1} + timentin 150 mg L^{-1}). Values represent the mean ± standard error of five replications (n = 5). Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5 % level.

a 90 s vacuum infiltration achieved in 59.6% increase in transient GUS expression, indicating higher transformation efficiency (**Fig. 2F**).

3.8. Selection of transgenic radish overexpressing AtMYB12

The selection strategy plays a crucial role in choosing transgenic plants with the *AtMYB12* gene while ensuring the elimination of chimeras and escapes. To achieve this, an optimized kanamycin concentration of 150 mg L^{-1} in MS media was used for transgenic plant selection. Non-transgenic showed tissue growth inhibition followed by chlorosis, necrosis and eventual death (**Fig. 3G**). In contrast, the putatively transformed explants developed into seedlings with robust roots and shoots within 25 days, ultimately maturing into plantlets (**Fig. 3H**). After allowing the putatively transformed plants develop, the transformation efficiency was achieved to be 59.6% (**Table 1**).

3.9. GUS histochemical analysis

The GUS assay was employed to assess the transformation efficiency of the transformed plants under optimized conditions. Blue staining indicating GUS expression was observed in the cross sections of radish roots and hypocotyls (**Fig. 3E** F) as well as in leaf and stem explants (**Fig. 3I** J). Conversely, no blue staining was observed in non-transformed seedlings and plantlets (**Fig. 3I** J).

3.10. Genotype effect on transformation efficacy

The improved *in-planta* transformation method for radish was applied to two other popular radish cultivars. With a transformation efficiency exceeding 40%, all tested cultivars performed exceptionally well (**Table 1**). Among the three evaluated cultivars, radish cv. Co 1. showed 59.6% higher transformation efficiency (**Table 1**). The results indicate that the *in-planta* transformation technique developed in this study can be effectively applied to a variety of radish cultivars, independent of their specific genotype. It is important to note that the efficacy of *Agrobacterium*-mediated *in-planta* transformation may vary depending on the genotype.

3.11. AtMYB12 transgenic callus recovery under selection pressure

The selection method is a crucial parameter for choosing kanamycin-resistant callus while eliminating chimeras and escapes. Leaf segments were grown at 25 °C in the dark for 15 days on petri dishes with a medium containing 10, 20, 30, or 40 mg L^{-1} of kanamycin to determine to optimal dosage for selecting transgenic calli. Leaf explants from both WT and transgenic plants expressing *AtMYB12* were incubated in a medium containing 2,4-D (0.5 mg L^{-1}) and kanamycin (40 mg L^{-1}) for callus induction media (**Fig. 4A** B). Callus cultures were developed after 25 days of incubation from the cultured radish leaf explants. Transgenic plant calluses exhibited a soft and friable texture. At 30-days intervals, these calluses were subcultured on the same medium. Browning was observed in all transgenic radish calluses during subculture and exposure to air (**Fig. 4C**). The highest GUS positive callus and transformation efficiency, reaching 32.3%, was achieved for transgenic callus from radish (**Table 2**). In comparison to wild-type (WT) leaf explants, those infected with *Agrobacterium* showed rapidly developing meristematic cells at both ends of the *Agrobacterium* attachment and T-DNA delivery target regions (**Fig. 4D** E F). For molecular validation of the transgene, the incorporation of the resistant transgenic callus survived kanamycin selection pressure.

3.12. Establishment of AtMYB12 transgenic callus cell lines

To improve the growth and color texture of kanamycin resistant calli, we investigated the impact of 2,4-D. We found that a concentration of 0.5 mg of 2,4-D per liter not only promoted callus growth but also induced differentiation into yellow, green, brown, and yellowish-green calli. This medium, referred to as the CS medium in the materials, was employed to sustain the callus and select nine distinct Callus Lines (CL1-9) These callus lines were established after approximately 55 days of sub-culturing on the CS medium

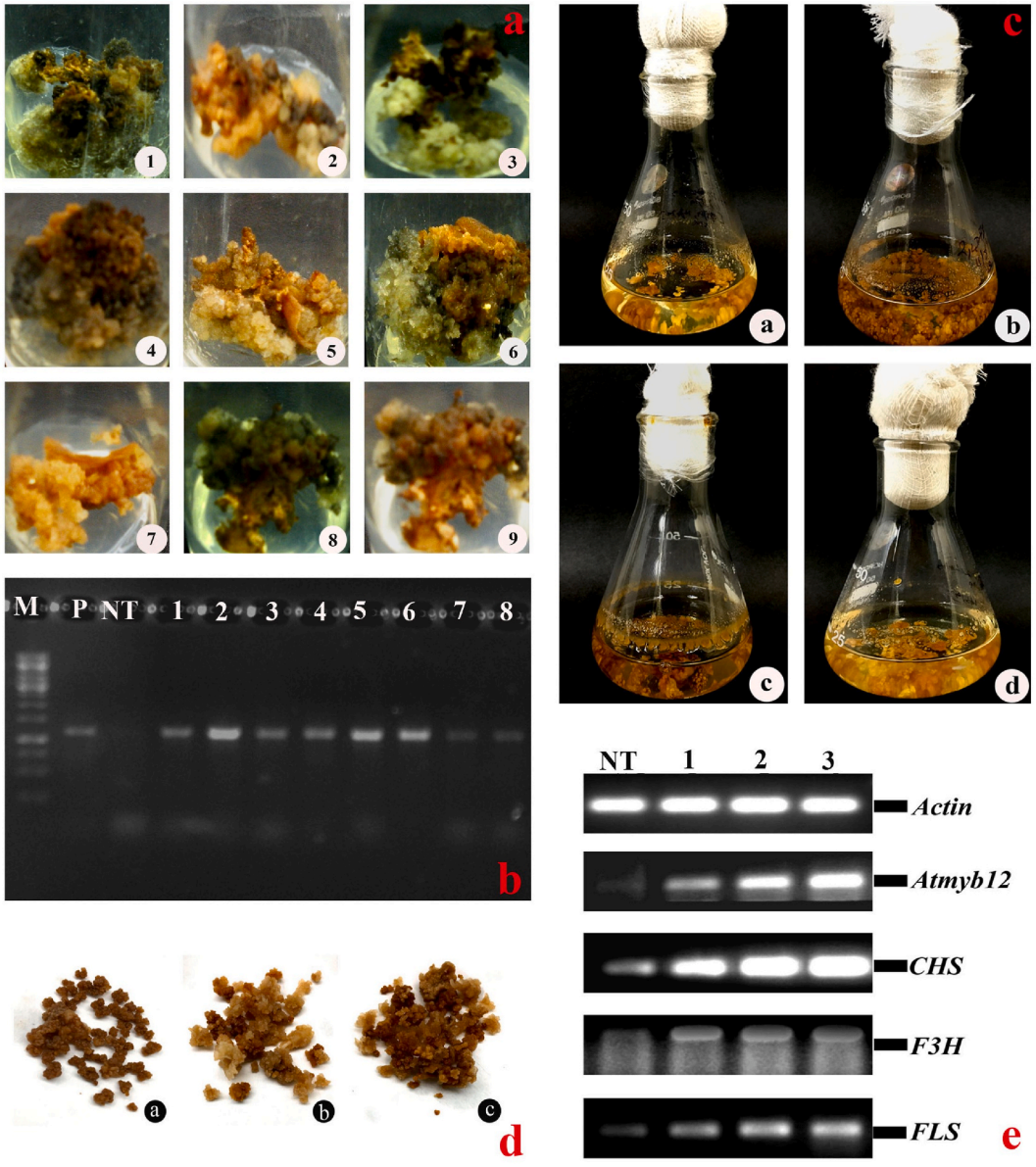

Fig. 5. Establishment, growth and gene expression in transgenic and WT callus radish. **(A)** Transgenic callus culture lines after 30 days on propagation medium under selective conditions. **(B)** Radish cv. Co 1 transgenic calluses were confirmed by PCR using Lane M DNA ladder (10 Kb) and Lane 2 Plasmid *pCAMBIA 2301:35S.AtMYB12* gene-containing callus, Lane 2 WT DNA sample, and Lane 4–11 DNA sample from GUS-positive transgenic calluses were included. *AtMYB12* gene showed a 1116 bp amplicon (Fig. S1). **(C)** Transgenic callus cultures were grown in half-MS liquid media, and the WT control flask callus suspension culture was observed after 25 days. **b, c & d** Flask-culture of transgenic suspension callus for quercetin production and growth kinetics. Callus lines 4, 5, & 6 after 25 days. (**D-E)** Culturing transgenic callus in flasks for quercetin production and growth was performed. The selection was based on RT-PCR-validated transgenic expression in *AtMYB12* callus (WT Non Transformed callus of control; Fig. S2).

(**Fig. 5A**). In this study, **Fig. 5A** serves to characterize the biosynthesis of flavonoids when compared to the WT. Furthermore, cell suspension cultures derived from six of the calli lines exhibited higher amount of phenolic and flavonoid content, when compared to other calli. Specifically, suspension cultures from CL4, CL5, CL6, and CL7 demonstrated higher biomass and greater phenolic and flavonoid content in comparison to the WT (**Table 3**). These selected callus lines were used for further study.

3.13. Molecular confirmation

Randomly selected 25 days old GUS positive transgenic line, resistant to kanamycin and their respective WT were PCR confirmation

Table 3
Establishment of *AtMYB12* transgenic callus cell lines from radish.

Callus Lines (CL)	Fresh weight of callus (mg)*	Dry weight of Callus (mg)*	Phenolic content (mg/g)[a]	Flavonoid content (mg/g)[b]
CL1	9.123 ± 1.0^{i}	1.012 ± 0.01^{f}	32.07 ± 2.7^{h}	43.03 ± 1.7^{h}
CL2	11.302 ± 1.1^{g}	1.054 ± 0.37^{d}	117.56 ± 22.5^{g}	203 ± 35.5^{g}
CL 3	11.368 ± 2.7^{c}	1.053 ± 0.41^{e}	125.07 ± 32.7^{f}	220 ± 45.9^{f}
CL 4	11.332 ± 2.5^{e}	1.054 ± 0.17^{d}	221.36 ± 12.3^{c}	348 ± 35.3^{b}
CL 5	12.487 ± 2.5^{a}	1.065 ± 0.46^{a}	262.06 ± 42.0^{a}	367 ± 51.6^{a}
CL 6	11.379 ± 1.4^{b}	1.061 ± 0.25^{b}	237.56 ± 32.3^{b}	334 ± 46.9^{c}
CL 7	11.334 ± 1.6^{d}	1.059 ± 0.23^{c}	137.56 ± 32.7^{d}	307 ± 43.9^{d}
CL 8	11.314 ± 1.2^{f}	1.055 ± 0.13^{d}	132.56 ± 12.3^{e}	243 ± 33.9^{e}

CL1: Wild callus (control); The cultures were initiated with 0.2g of inoculum mass (FW) in 40 ml 1/2 strength MS medium.*The data's were tabulated after 30 days of culture in the respective medium. 2, 4-D (0.5 mg L^{-1}) with Sucrose (3 mg L^{-1}; with 40 mg kanamycin L^{-1}) induced callus from leaves of transgenic radish. [a] mg gallic acid equivalent (GAE) g^{-1} DW. [b] mg quercetin equivalent g^{-1} DW. Each treatment contained five replicates (n = 5) with at least 30-inoculum mass per replicate. Data are expressed, as mean ± SE within columns from Duncan's multiple range test (DMRT) are significantly different at a 5 % level.

using *AtMYB12* gene specific primer. Similarly, 30-day-old GUS-positive transgenic callus lines, resistant to Kanamycin, and their respective WT were also PCR-confirmed with *AtMYB12* gene-specific primers. The PCR amplification of DNA from transgenic plants, callus and plasmid DNA using *AtMYB12* gene specific primer showed DNA bands within the expected size at 1.16 kb (**Fig. 3K**; 5B (**Fig. S1**)) originating from the coding region of *AtMYB12* [(**Fig. 3K**; 5B); (Lane-3-12; Lane 3–8) and (Lane-1)]. The specific *AtMYB12* gene product was not amplified in non-transgenic plants [(**Fig. 3K**); (Lane-2)]. These results demonstrate the successful transfer of the *AtMYB12* gene fragment into the transgenic plants and callus.

3.14. *AtMYB12 enhances the overexpression of genes involved in flavonol production in radish*

The *AtMYB12* enhances flavonoid biosynthesis, and it upregulates the expression of antioxidant-encoding genes in the leaf tissues of transgenic lines (**Fig. 6A**). The findings suggested that *AtMYB12* is involved in a variety of regulatory pathways. MYB transcription factors play a crucial role in the production of tissue-specific flavonoids and exhibit functional redundancy. To assess the expression of genes involved in flavonol production in *vitro* transgenic callus lines, semi-quantitative PCR was employed. The results revealed higher expression levels of *AtMYB12, CHS, F3H,* and *FLS* genes in the transgenic callus line compared to the control WT callus line (**Fig. 5E** (**Fig. S2**)). The expression of the *AtMYB12* transgene was verified in three transgenic callus lines through kanamycin GUS staining and quantitative RT-PCR. In transgenic callus lines, the transcription level of *AtMYB12* gene and biosynthetic genes were assessed using gene-specific qRT-PCR primers. The results showed that *AtMYB12* exhibited a substantial up to 6.42-fold increase in expression in the transgenic callus compared to the WT callus. Furthermore, the expression of various genes encoding enzymes such as CHS, F3Hand FLS was significantly upregulated in several folds in the transgenic callus lines (**Fig. 6b** I-L). The most pronounced improvement in expression was observed in the gene encoding *FLS*, followed by *CHS* and *F3H*, in *AtMYB12*-expressing transgenic callus lines. Notably, the expression of the gene encoding F3H was also enhanced by up to a remarkable 24.66-fold increase in the transgenic callus compared to the WT callus.

3.15. *AtMYB12 overexpression increases upregulation of ROS scavenging genes in radish*

The qRT-PCR has been validated to analyze the expression profiles of several differentially regulated genes that are involved in pathways other than flavonoid production. In the transgenic callus lines, we measured the expression of genes associated with flavonol production using qRT-PCR. Our experiments revealed that the expression of four well-known genes encoding GPX, APX, CAT, and SOD, which are involved in ROS scavenging, was upregulated in the transgenic callus lines (**Fig. 6B** M − P). These findings suggested that *AtMYB12* may be involved in multiple regulatory pathways. The results showed that the CL5 transgenic callus lines exhibited higher gene expression levels compared to the other lines (CL4, CL6, and CL7), as well as the control (CL1). As a result, we selected the CL5 transgenic callus line for further optimization studies. The overexpression of *AtMYB12* in transgenic callus lines consistently upregulated ROS scavenging genes, such as *GPX, APX, CAT,* and *SOD*, as depicted in **Fig. 6B** M-P. This indicates that the *AtMYB12* gene stimulates the ROS scavenging system, leading to increased expression of ROS-scavenging genes.

3.16. *AtMYB12 overexpression enhances PAL and antioxidative enzyme in radish*

In the transgenic callus overexpressing *AtMYB12*, we determined the activities of three essential enzymes: PAL, CAT, and APX. Our study reveals that the upregulation of the *AtMYB12* gene led to a significant increase in PAL, CAT, and APX activities, persisting at notably higher levels than those observed in the control group even after 20 days of callus culture, as illustrated in **Fig. 7**.

3.17. *Callus growth and dynamic phenolic, flavonoid, and quercetin quantities*

In this study, we investigated the effects of MS media and sucrose concentration on the growth and flavonoid biosynthesis in both

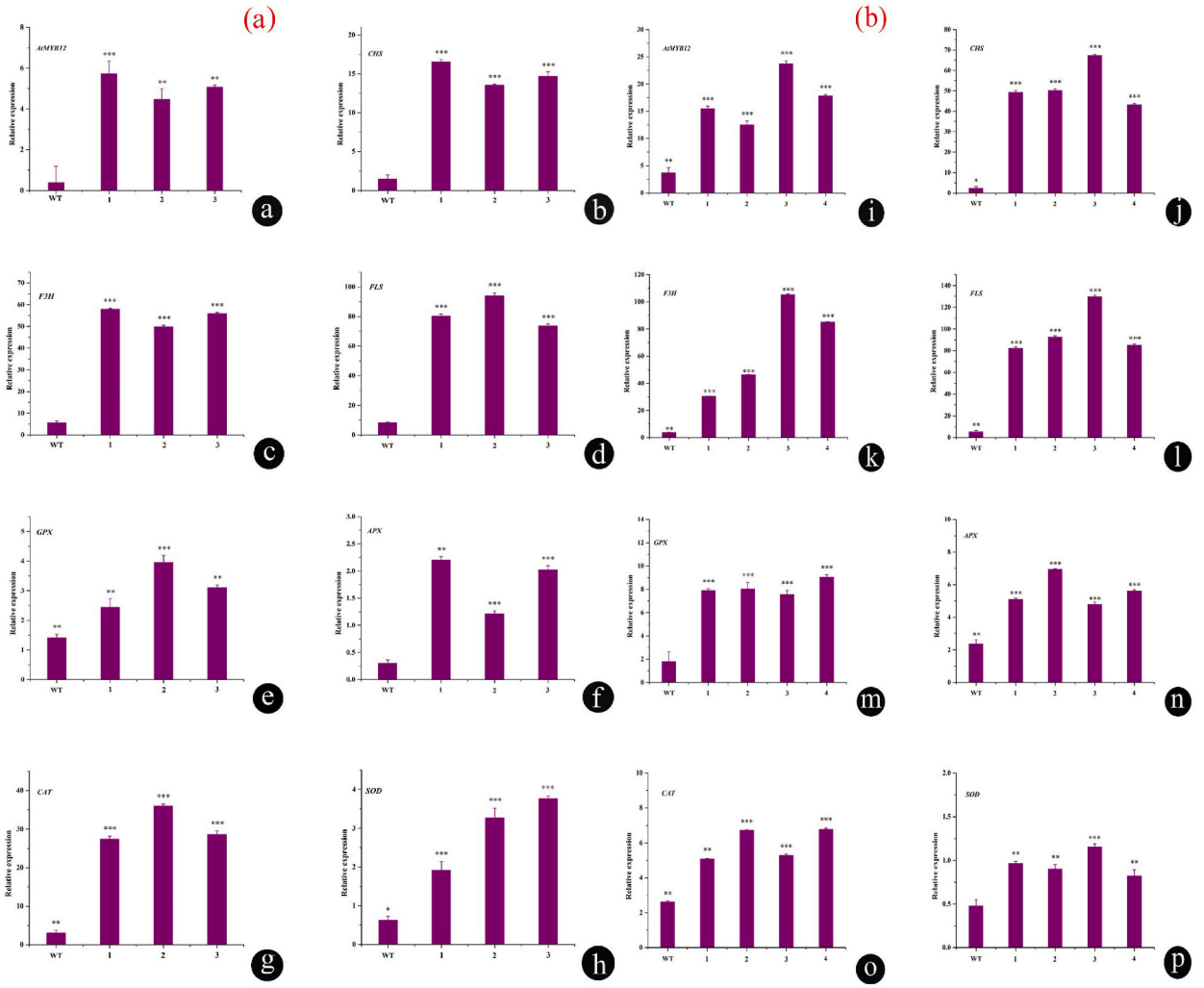

Fig. 6. The RT-PCR analysis on the overexpression of the *AtMYB12* gene. **(A B)** qRT-PCR analysis was conducted on selected flavonoid biosynthesis and antioxidant genes in transgenic plant and WT callus culture. **(A-D and I-L)** RT-PCR of total RNA from the transgenic plants and calluses assessed flavonoid biosynthesis structural genes (*AtMYB12*, CHS, F3H, and FLS) and *AtMYB12*. **M-P and E-H** RT-PCR of total RNA from transgenic plants and calluses was used to evaluate antioxidant-related gene expression (CAT, APX, GPX, and SOD) to antioxidant levels. The internal control gene was Radish actin. The results were compared to the Wild type (WT-Non-transformed plants and callus of control), with the reference sample set at 1.0. The data is presented as mean ± SE (n = 3), with radish calluses without transgenes as the baseline. Student's t-test was applied to evaluate three biological and three technical replicates (*P < 0.05, **P < 0.01, ***P < 0.001), and mean standard error bars were used.

wild-type (WT, CL1) and *AtMYB12* transgenic (CL5) callus lines. The experiments were conducted over a 25-day culture period, using 30-day-old calli as the inoculum **(Supp. Table S2; Fig. 5C)**. The growth patterns of WT and transgenic CL5 calluses followed a similar trajectory. Initially, both callus types exhibited slow growth for the first 10 days, followed by a rapid growth phase lasting up to 20 days, culminating in a 25-day period of apparent stagnation. Consequently, both WT and transgenic CL5 calluses appeared to exhibit sigmoid growth curves. The phenolic, flavonoid, and quercetin levels in transgenic CL5 were measured at various time intervals (on Day 0, 5, 10, 15, 20, and 25) and compared to WT calli. Over a 20-day period, transgenic CL5 cultured on hormone-free 1/2 MS media with a sucrose concentration of 116.8 mM displayed the highest growth index (11.53) and significantly increased phenolic content (357.31 mg g^{-1} DW), flavonoid content (463 mg g^{-1} DW), and quercetin content (48.23 mg g^{-1} DW) compared to WT callus (Fig. 9).

These results have significant implications for research on *AtMYB12* overexpression in transgenic radish callus, as they indicate an enhanced flavonoid level and the expression of flavonoid genes. Moreover, with the increased amount of extracts used for the assay and the addition of potent inhibitors, the antioxidant activity and reducing power of the transgenic CL5 extract were further enhanced (Supp. Table S3).

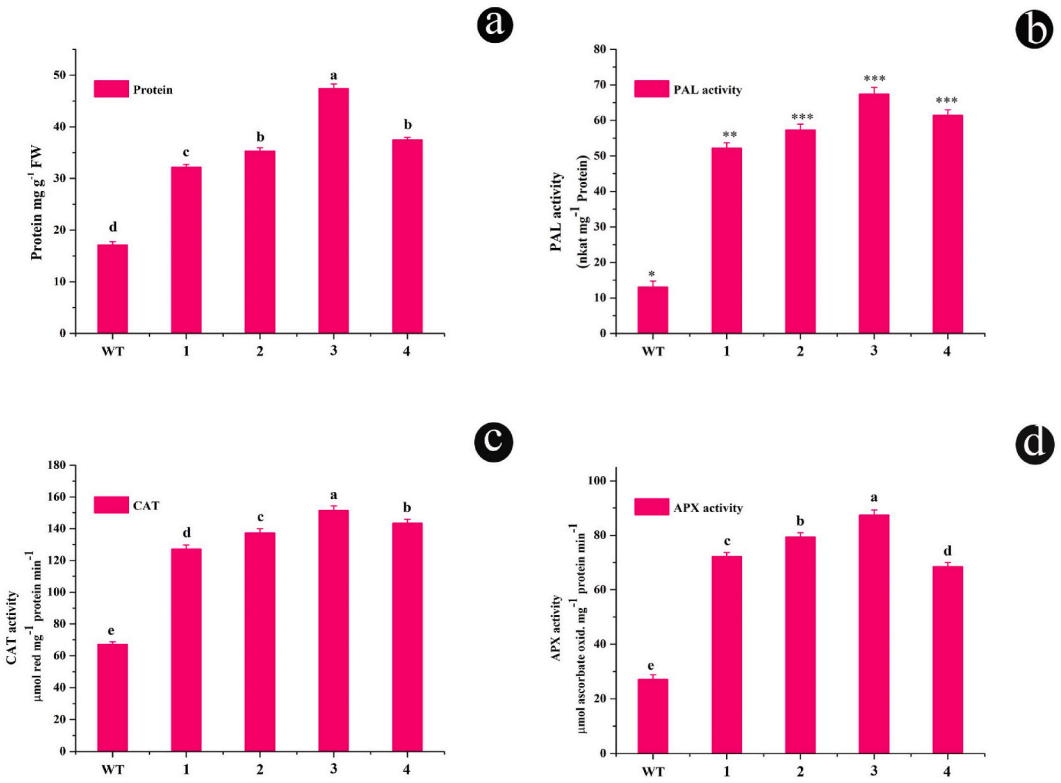

Fig. 7. Analysis of antioxidant enzyme assay in *AtMYB12* transgenic and WT callus extract. **(A)** Analysis of total soluble protein in control (WT Non-transformed) and *AtMYB12* transgenic radish leaf callus. **(B)** PAL activity comparison in WT and *AtMYB12* transgenic radish leaf callus. **(C)** CAT activity assessment in WT and *AtMYB12* transgenic radish leaf callus. **(D)** APX activity in WT and *AtMYB12* transgenic radish leaf callus lines. Data are shown as mean ± SE (n = 3), with non-transgenic radish callus as controls. The experiment included three biological and three technical replicates, analyzed using Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001). Mean bars represent standard error.

4. Discussion

The *A. tumefaciens*-mediated transformation (ATMT) is a commonly used method for transferring genes of interest into plant genomes. This article presents an *in-planta* transformation strategy that enhanced flavonoid content in radish using the *AtMYB12* transcription factor. *AtMYB12* transcription factor has been demonstrated to induce the expression of additional target genes in tobacco, leading to elevated flavonol levels. When produced tissue-specifically in tomato, *AtMYB12* promotes the biosynthesis of caffeoyl quinic acid and flavonols, possibly in a manner similar to the tomato orthologous MYB12-like protein [4,10,13–15].

In our current study, we successfully demonstrated the suitability of Indian radish cultivars (Co 1, Pusa Chetki, and Pusa Desi) for transformation, using full seeds as explants (**Fig. 3C**). This approach offers several advantages, including year-round accessibility, ease of explant handling, improved screening, and simple plantlet regeneration. We conducted an evaluation of the transformation experiment using 25-day-old radish seedlings in a kanamycin medium with a concentration of 150 mg L^{-1}. In this study, after 25 days of culture, it was observed that Kanamycin concentration of 150 mg L^{-1} completely inhibited seed germination (**Fig. 3B**). Additionally, two-stage selection process was used to eliminate non-transformed plant development in *A. thaliana,* [41]. Tissue wounds provide *Agrobacterium* with an entry penetrate into the host plant, and the presence of micro-wounds is crucial for plant cell competence, facilitating gene transfer [42]. This, in turn, enhances the delivery of bacterial T-DNA [43]. In our experiments, we observed that micro-wound radish seed cotyledons with embryo axis explants expressed 33.3% more GUS than those without wounding, which only showed 21.7% expression (**Fig. 2A**). The timing of infection varies significantly depending on the type of explant and plant. The absorbance of the *Agrobacterium* culture absorbance (OD_{600}) is essential as it allows the bacterial cells to adhere to explants and transport the T-DNA [42,43]. In our study, we found that *A. tumefaciens*-infected seed explants exhibited the highest GUS expression percentage at 20.6% compared to the control at 8.6%, but a longer *Agrobacterium* infection time led to lower transformation efficiency ((**Fig. 2B**). Previous research on soybeans showed that 30 min of infection time maximized transformation efficiency for seed explants [44]. In our optimization experiments, which involved co-cultivation periods of 1–6 days, we observed that a 3-day period resulted in an increase in transformation efficiency (%), followed by decrease beyond 3-days. Radish seeds infected with *Agrobacterium* showed the highest GUS expression percentage of transformation efficiency at 20.7% (**Fig. 2C**). This finding aligns with recent reports on 3-day co-cultivation for radish transformation efficiency [21,28]. Similarly, *Agrobacterium*-infected black gram and cowpea seed explants with micro wounds also exhibited improved transformation efficiency [44,45]. Consistent with earlier studies, the exhibited improved

transformation efficiency after 2–3 days of co-cultivation [46,47]. Deviating from this optimal co-cultivation time, either by reducing or increasing it, resulted in decreased transformation efficiency, while prolonged incubation caused bacterial leaching. Acetosyringone is known to induce the expression of the phenolic vir gene, which possesses an unsaturated lateral chain that enhances both virulence and transformation efficiency [48]. In our study, we observed that 150 μM of acetosyringone resulted in a remarkable 3.5-fold increase in GUS expression and transformation efficiency (29.3%) compared to the control (8.6%) without acetosyringone (**Fig. 2D**). This enhancement could be attributed to acetosyringone activation of the vir gene, facilitating transgenic incorporation through the wound at the explant's location. Previous research demonstrated that 100 μM acetosyringone was optimal for radish plant transformation efficiency [28]. More recently, it was found that a concentration of 125 μM acetosyringone further improved radish transformation efficiency [29]. Previous studies have indicated that acetosyringone concentrations below 100 μM did not influence bacterial pathogenicity, while those exceeding 500 μM were detrimental to both bacteria and explant growth [46]. In addition, many monocotyledon and dicotyledonous plants have improved genetic transformation efficiency using sonication [46]. Sonication was employed to create microcavities across the explant [47–49]. As a result, GUS expression percentage of transformation efficiency increased to 44.8% after 60 s of sonication, surpassing the control group's 28.3% (**Fig. 2E**). This demonstrates that sonication enhances GUS expression in the transformation process. In previous study, the transformation efficiency of the radish target gene reached 11% after 5 after 5 min of sonication [28]. Previous research has indicated that longer sonication durations can hinder cell lysis, RNA suppression, and protein production [50]. Sonication led to 31.3% yield of *Panicum virgatum* seeds in 1 min [51]. It has also proven effective in the transformation of banana and *Catharanthus roseus* [50,51]. Transformation stability improves with vacuum infiltration, as negative air pressure caused by vacuum infiltration led *Agrobacterium* to meristematic cells within the explants [52]. In previous research, we discovered that, black gram, watermelon and *Hybanthus enneaspermus* all exhibited two-stage kanamycin-resistant genes [45,53,54]. The highest percentage of *in-planta* transformation efficiency reached 59.6%, a significant improvement compared to the control at 29.3% (**Fig. 2F**; **Table 1**). In contrast, radish hypocotyl explants displayed a transformation efficiency of only 13.3% in a previous study [28]. More recently, transformation efficiency was enhanced by 53% through the utilization of apical meristems and an incubation period [29]. To overcome this limitation, we provided developing plants with low temperatures and light to induce robust, stumpy development. In trials, the temperature (22 °C) treatment resulted in a shorter and stumpier hypocotyl compared to the high-temperature (28 °C) treatment. Additionally, the active physiological and biochemical status of seedlings aids in rapid recovery after transformation. Most researchers employ 5- or 10-day-old seedlings for in-planta transformation [55–57]. However, seedling transformation is limited. For instance, it is typically done on seedlings with a thin, green stem (2–3 cm) [58,59]. Additionally, we created tiny physical wounds with a needle, sonication and vacuum to enhance bacterial distribution to plant tissues. A pierced hypocotyl side was used to prevent mechanical injury to the apical meristem. The epicotyl plumule, located above the cotyledonary node, produces the shoot apex and germ cells under the husk [60,61]. Apical meristems produce germ cells (egg and pollen) during maturation, with each germ cell carrying a single haploid copy of genomic DNA. A fertilized cell from an egg and sperm contributes to the full body of cells in each transformants in the next generation. These findings suggest the potential application of these techniques for a broader range of radish cultivars. GUS and PCR-positive transgenic radish plants were subjected to callus culture. The transgenic radish callus exhibited the highest GUS positivity and transformation efficiency at 32.3%. No blue coloration was observed in non-transformed callus (**Table 2**). For the establishment of stable transformed callus cell lines and Agro-infiltration transient transformation systems, grapevine leaves were employed [62]. Similarly, the expression of *AtMYB12* in transgenic tobacco leaf callus culture resulted in the production of 2,4-D calluses at a concentration of 0.5 mg L^{-1} [13]. The same concentration of *Hibiscus sabdariffa* callus culture promoted cell proliferation and development [63]. In **Table 3**, suspension cultures (CL4, CL5, CL6, CL7) exhibited higher biomass, phenolic, and flavonoid content compared to the wild callus control (non-transformed). Transgenic tomato and tobacco plants expressing *AtMYB12* in leaf callus produced a higher quantity of flavonoids [13,14]. Analysis revealed cell-dependent anthocyanin coloring of PAP1 transgenic plant leaves [64]. MYB transcription factors *AtMYB12*, *AtMYB111,* and *AtMYB11* were found to activate flavonol biosynthesis structural genes [4,10,13,65]. The expression of *AtMYB12* was gently increased by the HY5 transcription factor, and flavonoid synthesis was shown to be light-dependent [65]. Transgenic callus lines expressed F3H at a level 24.66-fold greater than the WT callus control (non-transformed) (**Fig. 6A** A-D). Studies demonstrated the increase of flavonol and anthocyanin levels in Arabidopsis [4] and tobacco [66]. Other research reported the coloring of petal, leaf, and fruit in *AtMYB12*-expressing transgenic Arabidopsis [4], tobacco [12], and tomato [11]. Cytoplasmic flavonoids are produced by endoplasmic reticulum membrane-bound multi-enzymatic complexes [67]. Previous research indicated that overexpression of *AtMYB12* in arabidopsis increased the expression of ROS-scavenging genes [4,10,15]. Genes such as *GPX, APX, CAT,* and *SOD* were consistently upregulated in *AtMYB12*-overexpressing transgenic callus lines compared to the wild callus control (**Fig. 6B** I-L). Reports suggest that flavonoids may have a role in linking the antioxidant system at both transcriptional and post-transcriptional levels [68]. Previous *in vivo* studies showed that upregulating FLS in tobacco plants increased ROS scavenging and the expression of antioxidant genes [69]. In Arabidopsis, boosting antioxidant enzyme activities such as APX, GPX, SOD, POD, and CAT improved resistance to oxidative stress [4,69]. The accumulation of secondary metabolites has often been found to affect enzyme activity [70]. In our study, the *AtMYB12* gene in callus cultures increased PAL, CAT, and APX activity after 20 days and maintained these levels above those of the wild callus control (Fig. 6B M – P). Previous Research suggests that activating key biosynthesis enzymes and up-regulating critical phenylpropanoid genes like PAL and CHS can lead to increased anthocyanin and flavonoid accumulation in plant leaves [70]. Similar findings have been reported in Glycine max suspension cell culture, where ROS (CAT and APX) increased secondary metabolite accumulation and phytoalexin synthesis [71,72].

The *AtMYB12* gene was found to increase flavonol levels and decrease anthocyanin content in tobacco, tomato, and kale [10,13]. Overexpressing *AtMYB12* in tobacco resulted in improved insect resistance, attributed to the high flavonol levels [12]. Transgenic tobacco callus and buckwheat hairy root cultures overexpressing *AtMYB12* demonstrated an enhanced production of rutin [73]. To

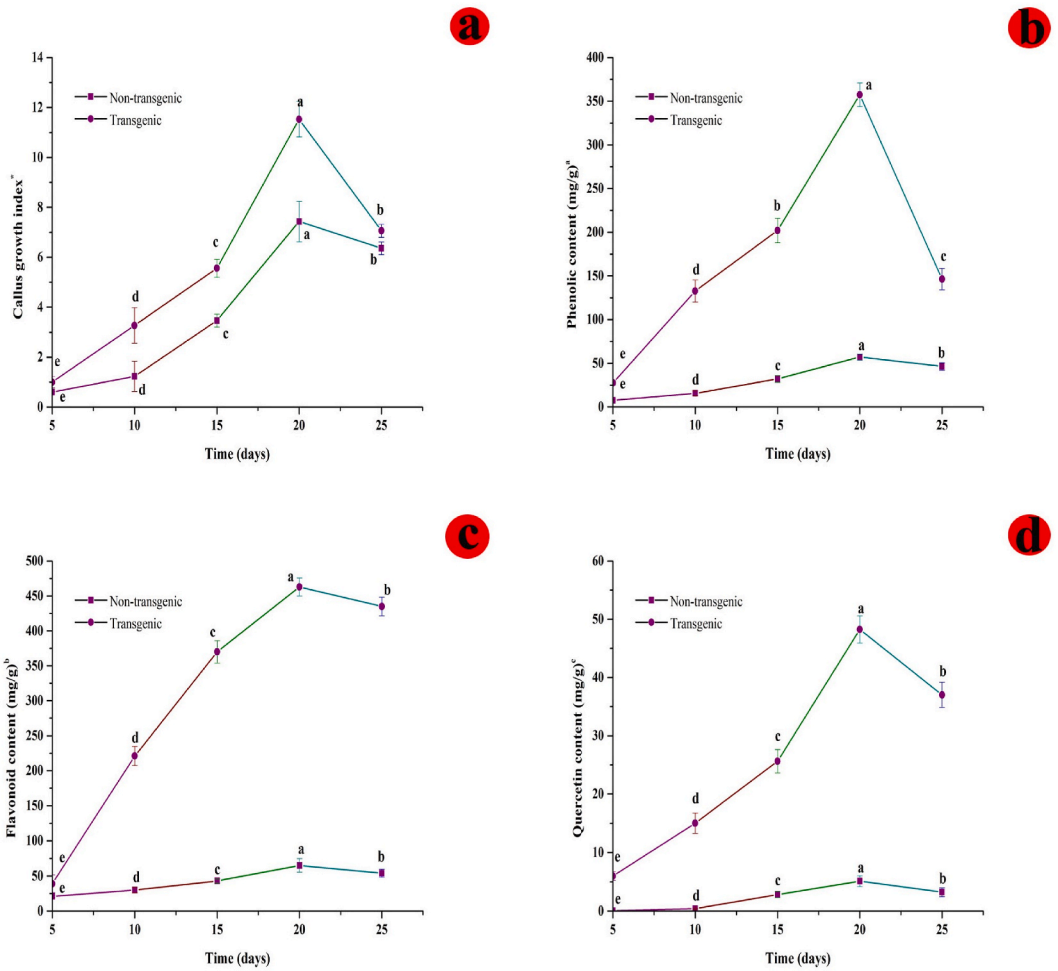

Fig. 8. Establishment and growth of CL5 *AtMYB12* transgenic and CL1 line wild radish callus. (A) Callus growth index. **(B)** Phenolic content. **(C)** Flavonoid content. **(D)** Quercetin content. Gallic acid equivalent (GAE) mg g^{-1} DW. Quercetin equivalent mg g^{-1} DW were measured. The cultures were initiated with 0.2g of inoculum mass (FW) in 40 ml ½ strength MS medium. The data's were tabulated after 30 days of culture in respective medium, with each treatment comprising five replicates (n = 5) and at least 30-inoculum mass per replicate. Data's are expressed as mean ± SE, and Duncan's multiple range test (DMRT) revealed significant differences at a 5% level within columns.

compare transgenic CL5 calli to the wild type, we assessed phenolic, flavonoid, and quercetin levels at various time intervals. Notably, transgenic CL5 exhibited the highest phenolic, flavonoid, and quercetin content among the control group and displayed the best growth index when cultivated in hormone-free 1/2 MS medium containing 116.8 mM sucrose for a duration of 20 days (Fig. 8 9). Previous research had reported rutin content ranging from 0.8% to 1.0% in buckwheat and tobacco by dry weight [74,75]. In buckwheat hairy root cultures, the rutin content ranged from 0.8 to 1.2 DW mg g^{-1} [76]. These findings have significant implications for advancing the study of *AtMYB12* overexpression in transgenic radish callus, with the aim of enhancing flavonoid content and gene transcription.

5. Conclusion

Transgenic bioengineering is essential for producing economically valuable compounds from natural sources. This study introduces a simple and effective *Agrobacterium*-mediated transformation method for creating transgenic radish plants utilizing the *AtMYB12* gene. The method includes needle-micro wounding of the seed cotyledon with the embryo axis, 30-min pre-incubation period, 3 days of co-cultivation, 150 μM acetosyringone concentration, 60s of sonication, and 90s of vacuum infiltration. Overexpression of *AtMYB12* in radish transgenic callus lines showed increased production of flavonoids. In this investigation, we confirmed the genetic and biochemical stability of *AtMYB12* in transgenic radish plants and callus lines (CL5) through PCR analysis of the *AtMYB12* gene, qRT-PCR analysis of flavonoid genes (*AtMYB12, CHS, F3H,* and *FLS*), and antioxidant genes (*GPX, APX, CAT,* and *SOD*). These analyses demonstrated an increased production of phenolic compounds, flavonoids, and quercetin after 45 days from the date of culture establishment. The total phenol, flavonoid, and quercetin content in the extracts of transgenic callus lines (CL5) were significantly

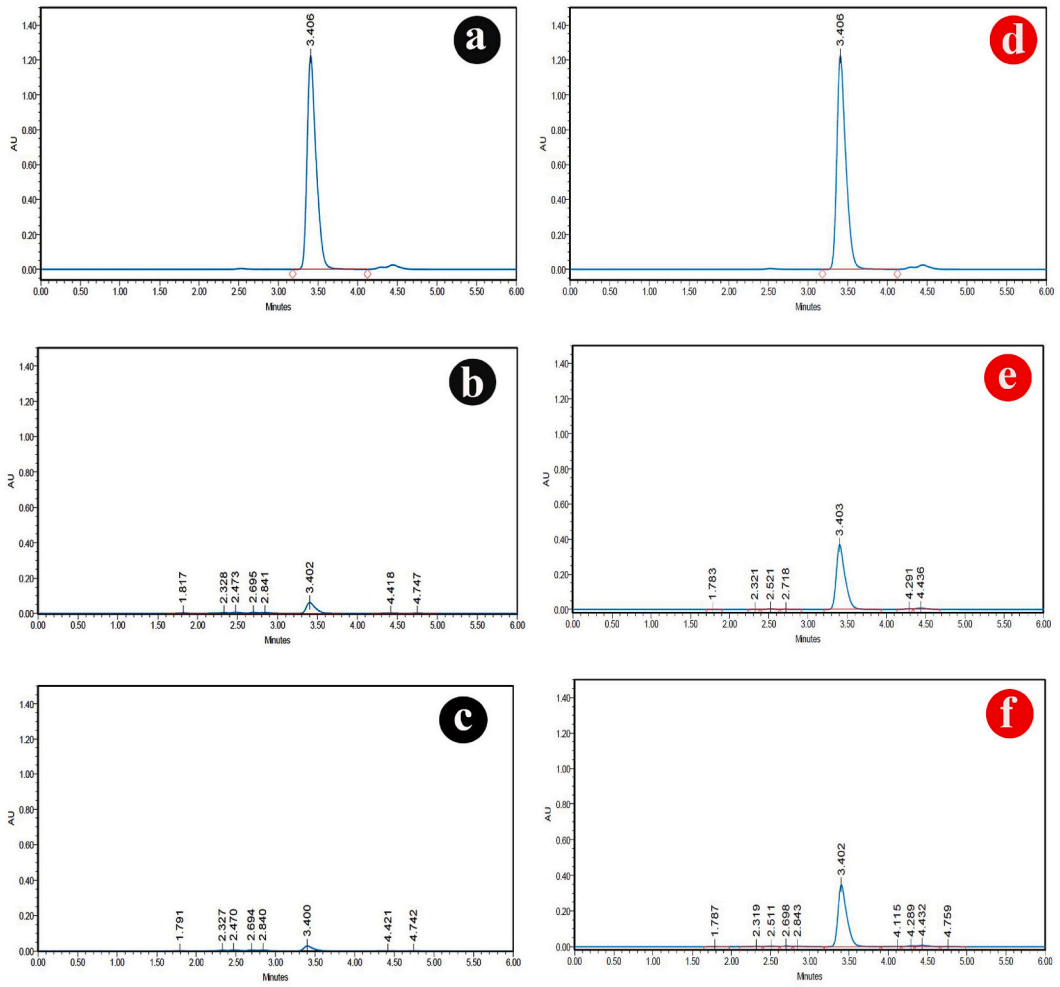

Fig. 9. HPLC chromatograms of quercetin in the WT and transgenic callus of callus suspension culture extracts. (A and D) Standard of quercetin. **(B)** Methanolic extract of callus from WT non-transformed radish after 20 days of culture. **(C)** Methanolic extract of callus from WT non-transformed radish after 25 days of culture. **(E)** Methanolic extract of callus from transgenic radish after 20 days of culture. **f:** Methanolic extract of callus from transgenic radish after 25 days of culture.

higher compared to the non-transformed control. However, this protocol does pose a few limitations. First, it is important to note that transformation efficiency may vary depending on the genotype. Second, the selection agent must be capable of prevent non-transformed cells/tissues from further development and its concentration need a standardization. To the best of our knowledge, this is the first research report on enhancing *AtMYB12* transgenic callus cultures in radish to increase the overall phenol, flavonoid, and quercetin content. Transgenic callus in suspension culture with increased flavonoids may offer an alternative source of quercetin with biopharmaceutical potential. This protocol could be utilized for large-scale production of antioxidants and health-beneficial bioactive compounds through radish callus culture.

CRediT authorship contribution statement

Muthusamy Balasubramanian: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Data curation, Conceptualization. **Shanmugam Girija:** Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Shanmugam Girija reports financial support was provided by Bharathiar University Tamil Nadu, India. Shanmugam Girija reports a relationship with Bharathiar University that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27053.

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