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PLANT TISSUE CULTURE

Meta-topolin enhances regeneration and *Agrobacterium*-mediated genetic transformation in radish (*Raphanus sativus* L.)

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Abstract

Meta-topolin (*m*T) is a novel aromatic cytokinin that stimulates morphogenesis and is an alternative source of cytokinins frequently employed in regeneration systems. Hence, the present research explored the prospect of *m*T for improving regeneration and genetic transformation efficiency. Cotyledonary node explants were cultured on optimum plant growth regulator medium incorporated with *m*T for enhanced shoot induction (93.6%, 2.0 mg l^{-1} BA and 0.9 mg l^{-1} *m*T), elongation (89.3%, 2.0 mg l^{-1} GA_3 and 0.9 mg l^{-1} *m*T), and rooting (90.3%, 0.9 mg l^{-1} IBA and 0.9 mg l^{-1} *m*T) respectively. The plant transformation study was carried out through *Agrobacterium*-mediated transformation using the pCAMBIA1301 vector containing the LB4404 strain for standardizing transformation strategies. Transformed shoots and rooting were determined in two stages using 10 and 6 mg l^{-1} hygromycin B for exterminating chimeric explants. After co-cultivation, explants were cultured at the optimal concentration of 0.9 mg l^{-1} *m*T, 2.0 mg l^{-1} BA, 2.0 mg l^{-1} GA_3, and 0.9 mg l^{-1} IBA for enhanced transformation efficiency (27.3%), corresponding to transformed regeneration without *m*T (18.6%) occurring with less efficiency. The existence of transgenes in the radish genome was ascertained by the GUS assay, PCR, RT-PCR, and qRT-PCR. Overall, our investigation demonstrated that including *m*T increases regeneration and enhances transformation efficiency in radish. Therefore, diverse radish varieties could use a designed transformation strategy to acquire essential traits.

Keywords *Raphanus sativus* · Cotyledonary node · *Meta*-topolin · pCAMBIA1301 · *Agrobacterium tumefaciens* · Hygromycin B

Introduction

Radish (*Raphanus sativus* L., $2n = 18$), associated with the Brassicaceae family (Gómez-Campo 1980), is an annual or biennial, commonly significant vegetable crop cultivated in tropical and temperate regions. It has been developed globally on 70,773 ha, predominantly cultivated in Japan, China, and Korea (Kurina *et al*. 2021). Each year, Japan produces 3.7 million tons of radish daikon and imports another 0.9 million tons from various countries. In contrast, China produced 1.2 million ha and occupied 6% of the cultivated area for vegetable crops (Kurina *et al*. 2021). It contains high levels of vitamin C (18%), potassium (5%), calcium (3%), iron (3%), and protein (1%) (USDA 2020). The significant edible portion of the juicy taproot in radish includes high nutritional content and health benefits (Yu *et al*. 2016; Pervitasari *et al*. 2022). Aside from the roots, the leaves and sprouts also have nutritious and therapeutic values (Takaya *et al*. 2003; Manivannan *et al*. 2019). Both round and extended radishes are consumed raw or pickled, preserved later, or boiled in various oriental cuisines (Park *et al*. 2005). It has a good source of antioxidants, including pyrogallol, vanillic acid, coumaric acid, catechin, and other phenolic compounds, which assist in preventing diabetes, neurological disorders, cancer, Parkinson's, and cardiovascular diseases (Manivannan *et al*. 2019).

Conventional breeding is vital for designing superior radish varieties. Nevertheless, breeding success rates are hindered by sexual incompatibility and difficulty finding viable progenies (Elayaraja *et al*. 2019). Furthermore,

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developing a unique variety of breeding practices requires an extended period, which could be solved by genetic transformation strategies (Elayaraja *et al*. 2019). On the other hand, radish is recalcitrant to regeneration and demands a specific transformation strategy. Consequently, efficient regeneration and genetic transformation methods are necessary for essential vegetable crops like radish. The available regeneration and few reports of genetic transformation methods on radish have been observed employing hypocotyl and cotyledon explants that exhibit a low frequency of shoot regeneration, a limited range of plant growth regulators (PGRs), and less transformation efficiency (Paek *et al*. 1987; Pua *et al*. 1996; Curtis *et al*. 2004; Cho *et al*. 2008; Manawadu and Dahanayake 2015; Muto *et al*. 2021). These concerns would be solved by optimizing regeneration, including PGR conditions, and standardizing regeneration protocol to enhance transformation efficiency. A broad range of auxins and cytokinins have been previously used separately or combined with diverse vegetable crops (Elayaraja *et al*. 2019; Kapildev *et al*. 2020). Further, cytokinins recreated an essential role in shoot proliferation and were successfully adopted in radish (Jeong *et al*. 1995; Manawadu and Dahanayake 2015; Kozar *et al*. 2021). Consequently, *m*T is a natural aromatic cytokinin initially extracted from poplar leaves (Horgan *et al*. 1975) that plays an essential role in enhancing the growth of shoots and roots, increasing photosynthetic pigments, delaying senescence, and modulating the activities of antioxidant enzymes (Werbrouck *et al*. 1996; Aremu *et al*. 2012). *In vitro m*T regeneration has been successfully employed in several plants, including *Aloe polyphylla* (Bairu *et al*. 2007), *Dierama erectum* (Koetle *et al*. 2010), *Merwilla plumbea* (Baskaran *et al*. 2012), *Drimia robusta* (Baskaran *et al*. 2013), *Sesamum indicum* (Elayaraja *et al*. 2019), and *Vigna mungo* (Kapildev *et al*. 2020). To date, there is no report to describe the impact of *m*T on radish regeneration and genetic transformation efficiency. Hence, the present investigation aimed to understand the implications of *m*T with diverse PGRs for enhancing regeneration and genetic transformation efficiency in radish using the pCAMBIA1301 (Fig. 1) vector.

Materials and methods

Plant material We have collected radish Pusa Chetki variety (var.) seeds (Fig. 2*a*) from Murugan Agricultural Distributors, Tiruchirappalli, Tamil Nadu, India, to regularize regeneration and transformation studies. This variety has been developed by the Indian Agricultural Research Institute (IARI), New Delhi, India, *via* selection from Denmark germplasm. It is remarkably tolerant of high temperatures. Roots are medium sized, mildly spicy, pure white, slim, soft with a blunt end, and harvested 40–45 d after planting.

Surface sterilization, *in vitro* seed germination, and preparation of explants Surface sterilization of radish seeds was performed as described by Balasubramanian *et al*. (2018). After sterilization, the seeds were shifted into a 150 ml Erlenmeyer flask comprising 40 ml of sterile distilled water and incubated at 25 ± 2 °C in a dark room for 1 d in a rotary shaker at 125 rpm. After 1 d, seeds were germinated on MS basal medium (Murashige and Skoog 1962) for 7 d at 27 ± 2 °C under a 16-h photoperiod. Seven-day-old seedlings (Fig. 2*b*) develop cotyledonary node explants (Fig. 2*c*) (~ 4 mm), which were collected by excluding hypocotyls, primary shoots, and cotyledons.

Optimization of PGRs and acclimatization Cotyledonary node explants were inoculated on a shoot induction medium (SIM) containing various concentrations (1.0–3.0 mg l^{-1}) of N^6-benzyladenine (BA), kinetin (K), and zeatin riboside (Z). After 6 wk, the proliferated shoots were shifted into a shoot elongation medium (SEM) having different concentrations (1.0–3.0 mg l^{-1}) of indole-3-acetic acid (IAA) and gibberellic acid (GA_3). Subsequently, after 4 wk on SEM, they were sub-cultured for 4 wk on a rooting medium (RM) comprising various concentrations (0.3–1.5 mg l^{-1}) of indole-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA). The control explants were cultured on hormone-free MS basal medium for shoot induction, elongation, and rooting stages. All chemicals were procured from Hi-Media, Mumbai, India. All cultures were incubated at 27 ± 2 °C for 16 h with a photoperiod of 50 µmol m^{-2} s^{-1}. The well-rooted

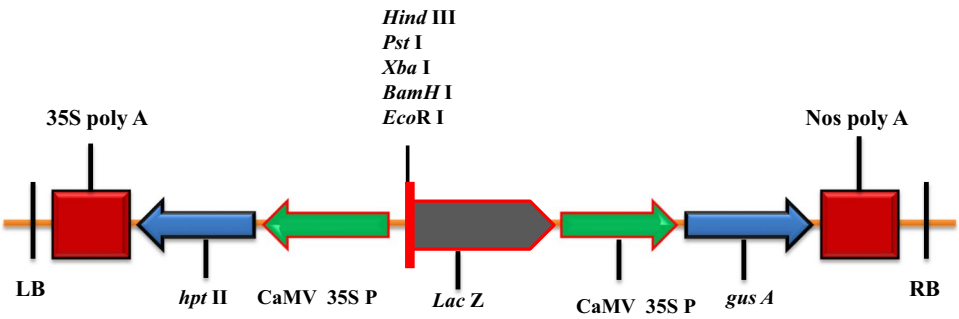

Figure 1. A linear map of the pCAMBIA1301 vector was used for transformation studies. CaMV 35S promoter-driven *via* hygromycin *phosphotransferase* II (*hpt* II) as a plant selection marker gene, whereas β-glucuronidase (*gus* A) as a reporter gene inside the T-DNA section.

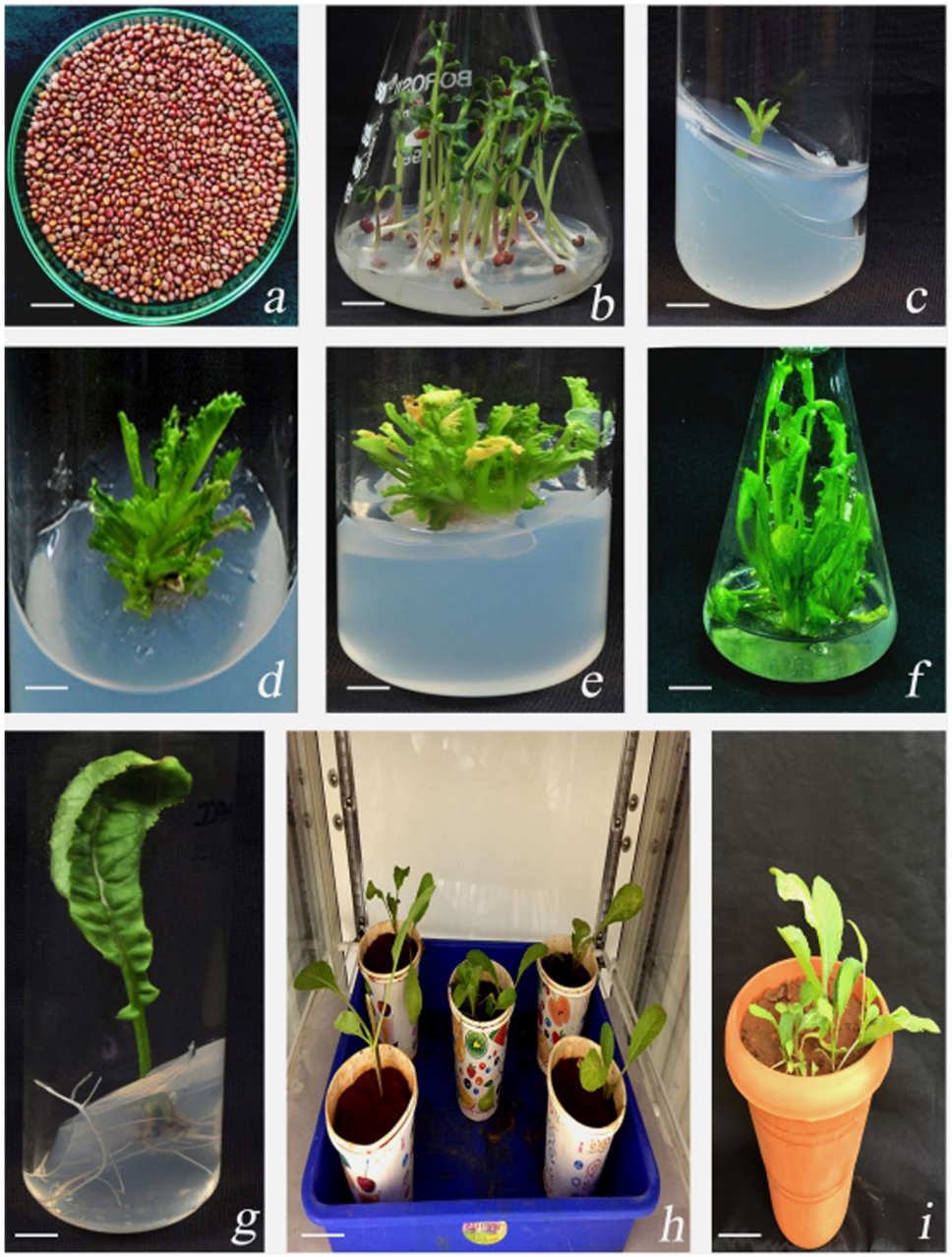

Figure 2. Impact of *m*T on the regeneration of radish var. Pusa Chetki. (*a*) Mature radish seeds. (*b*) 7-d-old seedlings. (*c*) Cotyledonary node explant inoculated on SIM supplemented with BA (2.0 mg l^{-1}) and *m*T (0.9 mg l^{-1}) after 3 d of initial culture. (*d*) and (*e*) Shoot multiplication on SIM containing BA (2.0 mg l^{-1}) and *m*T (0.9 mg l^{-1}) after 3 and 3 wk of culture. (*f*) Elongated shoots on SEM including GA_3 (2.0 mg l^{-1}) and *m*T (0.9 mg l^{-1}) after 4 wk of culture. (*g*) Rooted shoot on RM containing IBA (0.9 mg l^{-1}) and *m*T (0.9 mg l^{-1}) after 4 wk of culture. (*h*) Plants grown in a paper cup for 2 wk are maintained in a growth chamber. (*i*) After 2 wk, the plant hardened into greenhouse condition. *Bars* (*a* to *i*—1.0 cm).

plantlets were rinsed under running tap water and shifted into paper cups filled with a mixture of soil, sand, and vermiculite (1:1:1 v/v/v) and hardened for 2 wk in a growth chamber (Sanyo, Osaka, Japan) at 27 ± 2 °C, 60–80% relative humidity (RH). In the subsequent 2 wk, the plants were moved into earthen pots containing the above soil mixture and grown to maturity in greenhouse conditions. Furthermore, it would determine the optimized regeneration systems by employing *m*T.

Influence of *m*T on regeneration stages We evaluated the impact of *m*T on regeneration stages for culturing explants on MS basal medium augmented with different concentrations (0–1.5 mg l^{-1}) of *m*T (Hi-Media, Mumbai, India) along with optimum concentrations of SIM [MS, 3% sucrose, 2.0 mg l^{-1} BA, 0.8% agar (pH 5.6)], SEM [MS, 3% sucrose, 2.0 mg l^{-1} GA_3, 0.8% agar (pH 5.6)], and RM [MS, 3% sucrose, 0.9 mg l^{-1} IBA, 0.8% agar (pH 5.6)] respectively. *m*T was filter sterilized using a 0.22-µm syringe-driven filter added after autoclaving in the medium noted overhead. The control explants were cultured on a separate medium comprising optimal PGRs without *m*T. All regenerated plants were hardened in similar conditions as described to optimize PGRs. Further, *m*T-supplemented radish regeneration utilizes transformation studies.

Sensitivity of the cotyledonary node and rooting explants to hygromycin B We determined the sensitivity concentration of hygromycin B (Hi-Media) to the cotyledonary node. Initially, the explant was inoculated on SIM for 15 d devoid of hygromycin B. Explants were sub-cultured twice at 15 d intervals into fresh SIM, including various hygromycin B (0, 2, 4, 6, 8, and 10 mg l^{-1}) concentrations for 30 d. After 45 d, explants were moved into SEM containing hygromycin B (0, 2, 4, 6, 8, and 10 mg l^{-1}) concentrations for 30 d. Following 30 d, explants were transferred into RM supplemented with hygromycin B (0, 2, 4, 6, 8, and 10 mg l^{-1}) concentrations for 30 d. Hygromycin B sensitivity concentration evaluates individual regeneration stages for lessening getaway. The control explant was grown in a hygromycin B-free medium. All explants were incubated at 27 ± 2 °C for a 16-h photoperiod at 50 µmol m^{-2} s^{-1}.

Binary vector, *Agrobacterium* strain, and preparation of *Agrobacterium* suspension The present investigation employed binary vector pCAMBIA1301 containing the *Agrobacterium tumefaciens* strain LB4404. The strain specifications and T-DNA portion of the binary vector pCAMBIA1301 were previously defined by Karthik *et al.* (2021). It has been cultured in *Agrobacterium* minimal (AB) agar medium comprising 10 mg l^{-1} rifampicin (Hi-Media) and 50 mg l^{-1} kanamycin (Hi-Media). A single colony was picked and inoculated with 10 ml of Luria–Bertani (LB) broth of seed culture, containing appropriate antibiotics as mentioned above, on a rotary shaker at 180 rpm, 28 °C for 48 h. From this seed culture, 300 µl was moved into 35 ml of LB broth with similar antibiotics and maintained at 180 rpm at 28 °C till it reached 0.8 at OD 600 nm. Bacterial cells were re-suspended in 35 ml of liquid infiltration medium [(LIM: MS, 3% sucrose, 3 mM 2-(N-morpholino ethanesulfonic acid) (MES), 2.0 mg l^{-1} BA (pH 5.4)] comprising 100 µM acetosyringone (AS) (Hi-Media) after centrifugation at 5000 rpm for 5 min. Subsequently, *Agrobacterium* suspensions were incubated at 28 °C at 130 rpm for 1 h before transforming.

Influence of *m*T on radish transformation The cotyledonary node explants were gently pricked two times in the axillary and apical meristematic regions by a sterile hypodermic needle, 24-in. gauge (Hindustan Syringes & Medical Devices Ltd, New Delhi, India) and then transferred into the *Agrobacterium* suspensions for 30 min at room temperature with a rotary shaker at 90 rpm. Following infection, explants were dried on sterile tissue paper and shifted to co-cultivation medium [CCM: MS, 3% sucrose, 3 mM MES, 2.0 mg l^{-1} BA, 0.9 mg l^{-1} *m*T, 100 µM AS, 0.8% agar (pH 5.4)] for 3 d, maintained in the dark. After 3 d, explants were exhaustively washed twice with 350 mg l^{-1} cefotaxime, dried, and transferred to SIM containing 0.9 mg l^{-1} *m*T, 350 mg l^{-1} cefotaxime for 15 d, except for hygromycin B. After that, explants were moved into SIM with 0.9 mg l^{-1} *m*T, 350 mg l^{-1} cefotaxime, and 10 mg l^{-1} hygromycin B for 30 d. Following 45 d, explants were cultured on SEM with 0.9 mg l^{-1} *m*T, 350 mg l^{-1} cefotaxime, and 10 mg l^{-1} hygromycin B for 30 d. Finally, explants were inoculated with RM comprising 0.9 mg l^{-1} *m*T, 350 mg l^{-1} cefotaxime, and 6 mg l^{-1} hygromycin B for 30 d. All cultures were incubated at 27 ± 2 °C for 16 h with a photoperiod of 50 µmol m^{-2} s^{-1}. Rooted plants were hardened in a similar condition as described to optimize PGRs. An individual study was performed without *m*T after infection in the respective medium mentioned overhead to explore transformation efficiency.

GUS assay and polymerase chain reaction (PCR) analysis GUS expression in transformed and non-transformed (NT) explants was analyzed following Jefferson *et al.* (1987).Further, three randomly selected gus-positive 1-mo-old plants (anointed R1, R2, and R3) were chosen to verify *hpt* II (FP: 5′-ATGAAAAGCCTGAACTCACCGCGA CG-3′; RP: 5′-CTATTTCTTGCCCTCGGACGAGTGCT-3′) and *gus* A (FP: 5′-ACTCGACGGCCTGTGGGCATTCAG TCTG-3′; RP: 5′-CACTGACCGGATGCCGACGCGAAG

Table 1. Impact of *m*T on shoot multiplication from cotyledonary node explants of radish on SIM after 6 wk of culture

Treatments		Percentage (%) of explant responding for shoot multiplication	Mean number of shoots per explant (after 3 wk) 1st subculture	Mean number of shoots per explant (after 3 wk) 2nd subculture
BA (mg l^{-1})	*m*T (mg l^{-1})			
2.0	0.0	78.6 ± 0.40^{e}	14.3 ± 0.52^{d}	20.6 ± 0.38^{f}
2.0	0.3	84.0 ± 0.43^{d}	17.6 ± 0.37^{c}	24.0 ± 0.42^{e}
2.0	0.6	89.3 ± 0.45^{b}	21.3 ± 0.32^{b}	28.3 ± 0.26^{c}
2.0	**0.9**	**93.6 ± 0.42^{a}**	**24.0 ± 0.42^{a}**	**32.6 ± 0.43^{a}**
2.0	1.2	88.6 ± 0.56^{b}	20.3 ± 0.49^{b}	29.6 ± 0.34^{b}
2.0	1.5	86.3 ± 0.47^{c}	17.6 ± 0.39^{c}	26.3 ± 0.39^{d}

Control: Treatment without *m*T. Mean values of triplicated experiments (±) with standard errors. As per DMRT at a 5% level, values inside *columns* denoted by different *letters* are significantly different

Table 2. Impact of *m*T on shoot elongation from cotyledonary node explants of radish on SEM after 4 wk of culture

Treatments		Percentage (%) of explant responding for shoot elongation	Mean number of elongated shoots per explant (after 4 wk)	Mean shoot length (cm)
GA_3 (mg l^{-1})	*m*T (mg l^{-1})			
2.0	0.0	81.0 ± 0.47^{e}	13.3 ± 0.39^{d}	5.4 ± 0.35^{c}
2.0	0.3	84.3 ± 0.47^{d}	16.6 ± 0.31^{c}	6.9 ± 0.32^{b}
2.0	0.6	86.6 ± 0.49^{bc}	19.3 ± 0.49^{b}	$7.5. \pm 0.32^{ab}$
2.0	**0.9**	$\mathbf{89.3 \pm 0.45^{a}}$	$\mathbf{23.6 \pm 0.41^{a}}$	$\mathbf{8.3 \pm 0.40^{a}}$
2.0	1.2	87.0 ± 0.42^{b}	20.0 ± 0.39^{b}	7.8 ± 0.30^{ab}
2.0	1.5	85.6 ± 0.29^{c}	17.3 ± 0.26^{c}	7.1 ± 0.45^{b}

Control: Treatment without *m*T. Mean values of triplicated experiments (±) with standard errors. As per DMRT at a 5% level, values inside *columns* denoted by different *letters* are significantly different

Table 3. Impact of *m*T on rooting from cotyledonary node explants of radish on RM after 4 wk of culture

Treatments		Percentage (%) of explant responding for rooting	Mean number of roots per explant (after 4 wk)	Mean root length (cm)
IBA (mg l^{-1})	*m*T (mg l^{-1})			
0.9	0.0	83.6 ± 0.47^{d}	7.3 ± 0.38^{e}	9.4 ± 0.31^{d}
0.9	0.3	86.3 ± 0.47^{c}	10.6 ± 0.28^{d}	11.2 ± 0.47^{c}
0.9	0.6	88.6 ± 0.56^{b}	14.0 ± 0.37^{c}	12.7 ± 0.48^{b}
0.9	**0.9**	$\mathbf{90.3 \pm 0.56^{a}}$	$\mathbf{17.3 \pm 0.26^{a}}$	$\mathbf{14.3 \pm 0.40^{a}}$
0.9	1.2	87.0 ± 0.42^{c}	15.6 ± 0.45^{b}	$11.9 \pm 0.30b^{c}$
0.9	1.5	84.3 ± 0.47^{d}	13.3 ± 0.39^{c}	9.8 ± 0.31^{d}

Control: Treatment without *m*T. Mean values of triplicated experiments (±) with standard errors. As per DMRT at a 5% level, values inside *columns* denoted by different *letters* are significantly different

-3′) genes by PCR. The isolation of genomic DNA and PCR program strategies were followed as described by Karthik *et al*. (2018). Genomic DNA isolated from the radish NT plant and pCAMBIA1301 acted as negative and positive controls, respectively. PCR products were checked on a 1.0% agarose gel (w/v) (Hi-Media) and visualized under a Gel Documentation system (UVITEC, Cambridge, UK).

Reverse transcriptase-PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) Total RNA was extracted from three *hpt* II gene PCR positive events of transformed and NT

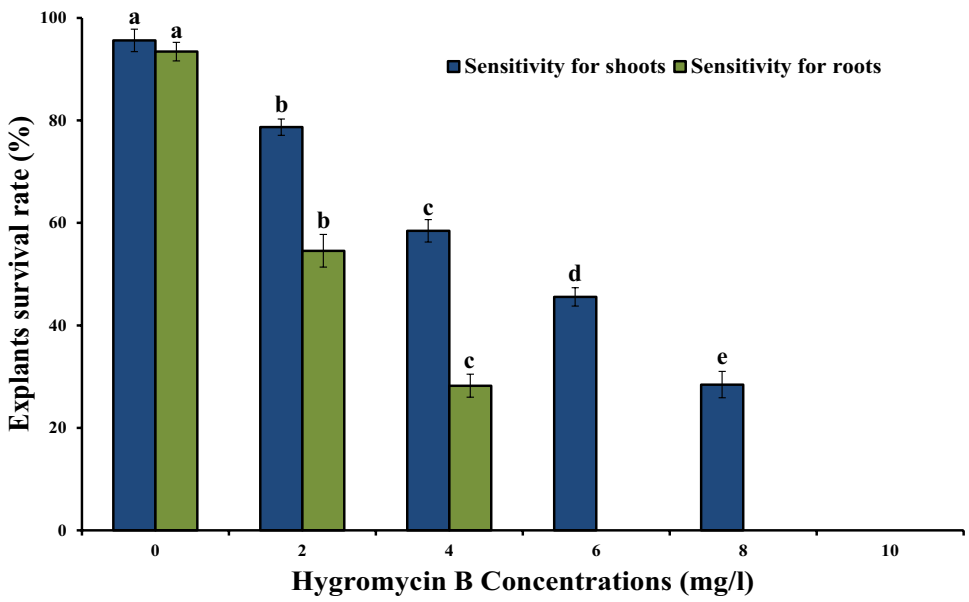

Figure 3. Sensitivity analysis of the cotyledonary node and rooting explants to hygromycin B.

Table 4. Influence of *m*T on *A. tumefaciens*–mediated genetic transformation efficiency in radish

Experiment plan	Total no. of explants infected	Total no. of explants responded	Total no. of plants regenerated	Total no. of plants survived (after Hygromycin B)	Total no. of GUS-positive plants	Transformation efficiency (%)[x]
***m*T (−)[z]**	100	62.3 ± 0.43[b]	302.0 ± 0.39[b]	92.6 ± 0.46[b]	18.6 ± 0.35[b]	18.6 ± 0.35[b]
***m*T (+)[y]**	**100**	**82.6 ± 0.52[a]**	**456.3 ± 0.43[a]**	**148.3 ± 0.38[a]**	**27.3 ± 0.47[a]**	**27.3 ± 0.47[a]**

Mean values of triplicated experiments (±) with standard errors. As per DMRT at a 5% level, values inside *columns* denoted by different *letters* are significantly different

[z]After co-cultivation, explants regenerated in SIM with 2.0 mg l^{-1} BA and RM containing 0.9 mg l^{-1} IBA

[y]After co-cultivation, explants regenerated in SIM with 2.0 mg l^{-1} BA, 0.9 mg l^{-1} *m*T, and RM containing 0.9 mg l^{-1} IBA and 0.9 mg l^{-1} *m*T

[x]Transformation efficiency = Total no. of GUS-positive plants/Total no. of explants infected × 100

plants using the RNAqueousTM kit (Ambion Inc., Austin, TX). RT-PCR was executed per the kit instructions to operate one-step RT-PCR equipment (Qiagen, Germantown, MD). The *hpt* II gene was amplified from first-strand cDNA using the above primer, and ACTIN (FP: 5′-GCTTTTCCT TGATGTCTCTC-3′; RP: 5′-TCCTGCCATGTATGTTGC TA-3′) was employed as a reference gene (Pervitasari *et al*. 2022). Expected RT-PCR products were checked on a 1.0% agarose gel and visualized over a Gel Documentation System (UVITEC, Cambridge, UK). Furthermore, qRT-PCR was performed by the Light Cycler® 480 Real-time PCR system (Roche, Mannheim, Germany). Three *hpt* II gene RT-PCR positives and NT plants were examined by the Prime Script™ RT Reagent Kit (Takara Bio Inc., Shiga, Japan) to quantify the *hpt* II (FP: 5′-TATCCACGCCCTCCT ACAT-3′; RP: 5′-CGACGTCTGTCGAGAAGTTT-3′) gene in triplicates. As per the kit recommendation, the reaction mixture is up to 50 µl, including water. The cycling parameters were described by Wang *et al*. (2019). Relative *hpt* II gene expression in transgenic radish was assessed by a cycle threshold (C_T) value employing the $2^{-\Delta\Delta}C_T$ method (Livak and Schmittgen 2001).

Statistical analysis A hundred explants in triplicates were employed to statistically analyze regeneration and transformation strategies. Duncan's multiple range test (DMRT) examined the data using one-way analysis of variance (ANOVA). At a 5% level, SPSS® 16.0 was used to calculate the significance level.

Results and discussion

Plant material Cotyledonary node explants emanating from 7-d-old seedlings were employed in this investigation. Superficial *m*T has been revealed to significantly enhance regeneration in diverse crops (Baskaran *et al*. 2013; Elayaraja *et al*. 2019; Kapildev *et al*. 2020). Nevertheless, to date, there has been no report on the role of *m*T in enhancing

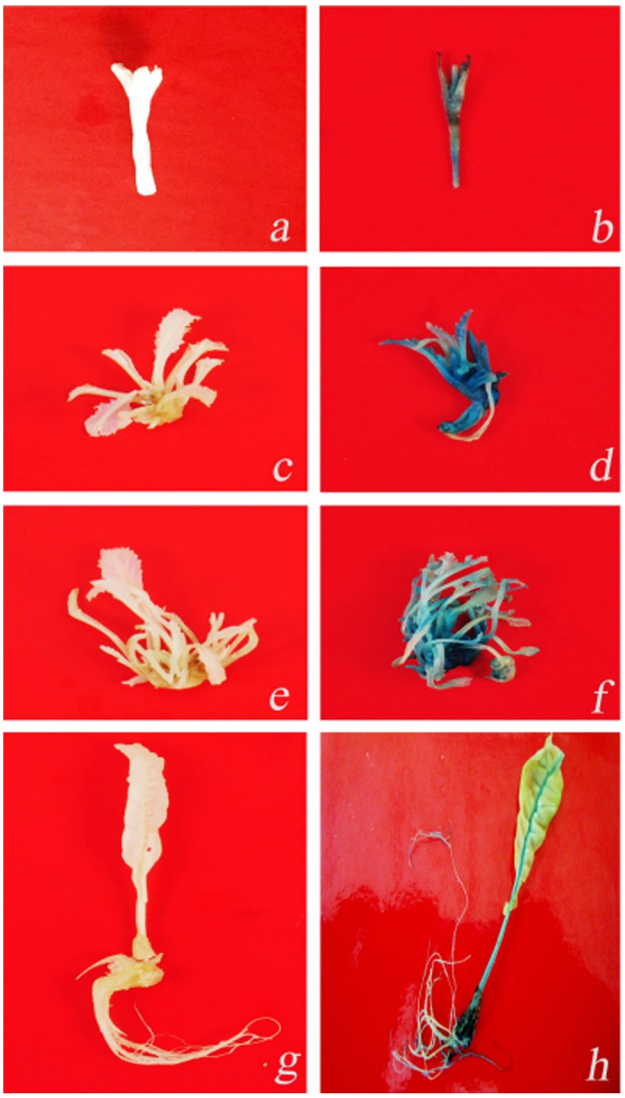

Figure 4. Stable expression of the *gus* A gene in radish is induced by various regeneration stages of explant. (*a*) NT cotyledonary node. (*b*) Transformed cotyledonary node. (*c*) NT shoot induction. (*d*) Transformed shoot induction. (*e*) NT shoot elongation. (*f*) Transformed shoot elongation. (*g*) NT plantlet. (*h*) Transformed plantlet.

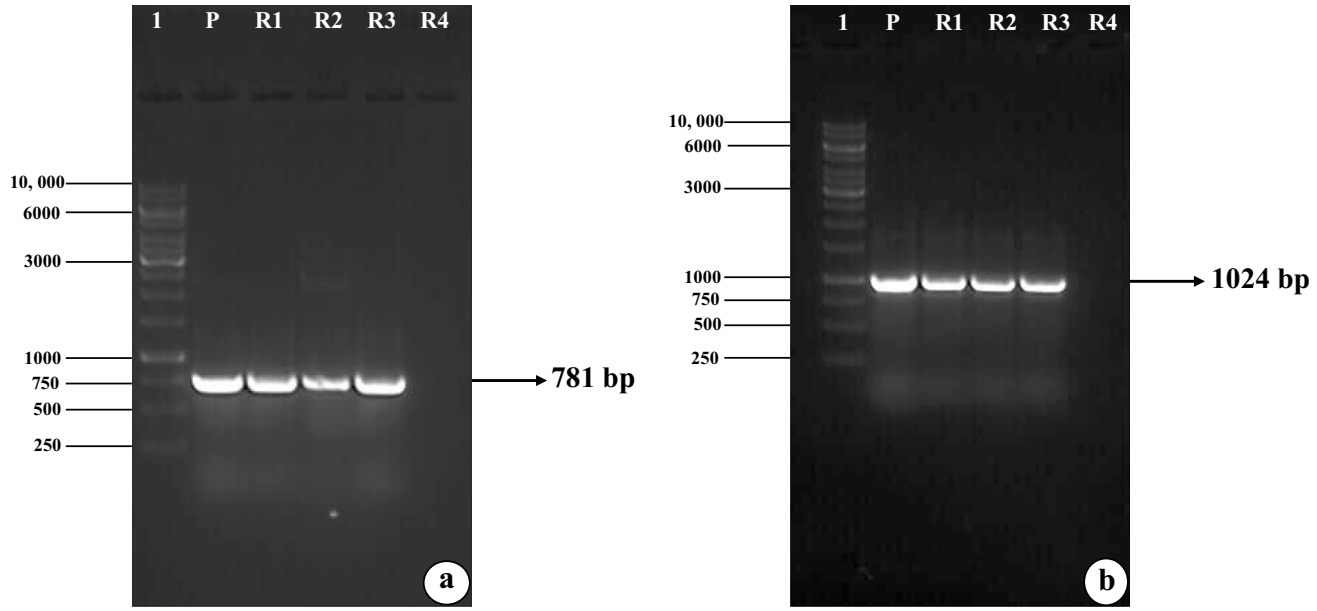

Figure 5. Detection of *gus* A and *hpt* II genes integration in transformed radish plants. (*a*) and (*b*) PCR analysis. *Lane 1*—Gene Ruler-1 kb DNA ladder (#SM0313). *Lane P*—pCAMBIA1301; *Lanes R1, R2*, and *R3*—transformed plants. *Lane R4*—NT plant.

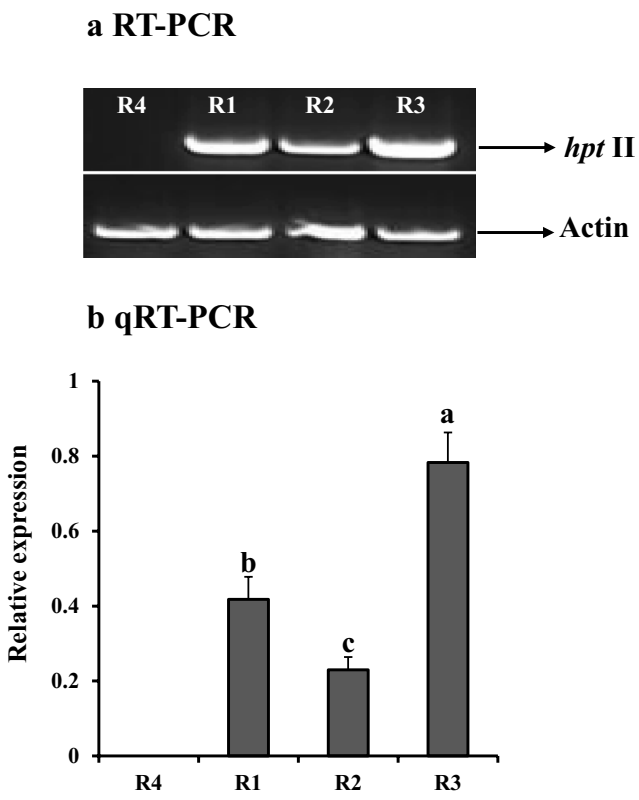

Figure 6. Expression and quantification level of the *hpt* II gene in transformed radish plants. (*a*) RT-PCR analysis. *Lane R4*—NT plant RNA as a negative control. *Lanes R1, R2*, and *R3*—transformed plant RNA samples. (*b*) qRT-PCR analysis. The relative expression of three transformed lines (R1, R12, and R3) and R4 acting as NT plants was examined by the $2^{-\Delta\Delta}$Ct method.

plant genetic transformation efficiency. Consequently, the present investigation aimed to understand the impact of *m*T on improving direct regeneration and genetic transformation efficiency in radish.

Optimization of PGRs and influence of *m*T on regeneration stages PGRs are an essential process for *in vitro* regeneration of plants. Explants cultured on SIM containing BA (2.0 mg l^{-1}), SEM supplemented GA_3 (2.0 mg l^{-1}), and RM including IBA (0.9 mg l^{-1}) showed the highest response in shoot multiplication (Table 1), elongation (Table 2), and rooting (Table 3) compared with other PGRs examined (data not shown). The control explants cultured on hormone-free MS basal medium exhibited the lowest response (data not shown). *In vitro* regeneration has been significantly enhanced, including *m*T (Elayaraja *et al*. 2019). In our examinations, a maximum number of shoots (32.6) was achieved when 0.9 mg l^{-1} *m*T was combined with BA 2.0 mg l^{-1} on SIM (Fig. 2*d* and *e*, Table 1). Kapildev *et al*. (2020) reported cotyledonary node explants cultured on SIM containing BA 0.5 mg l^{-1} and *m*T 1.5 mg l^{-1}, resulting in maximum shoot regeneration in black gram. Elongated shoots (23.6) were highest on SEM containing 2.0 mg l^{-1} GA_3 and 0.9 mg l^{-1} *m*T (Fig. 2*f*) as compared to SEM with GA_3 alone (Table 2). Elayaraja *et al*. (2019) proved that adding *m*T to SEM substantially influenced the elongation of shoots in *Sesamum indicum*. Rooting (17.3) was highest when 0.9 mg l^{-1} *m*T was combined with 0.9 mg l^{-1} IBA on RM (Fig. 2*g*, Table 3). Similarly, *m*T has been successfully implemented in *Corylus colurna* (Gentile *et al*. 2017) and

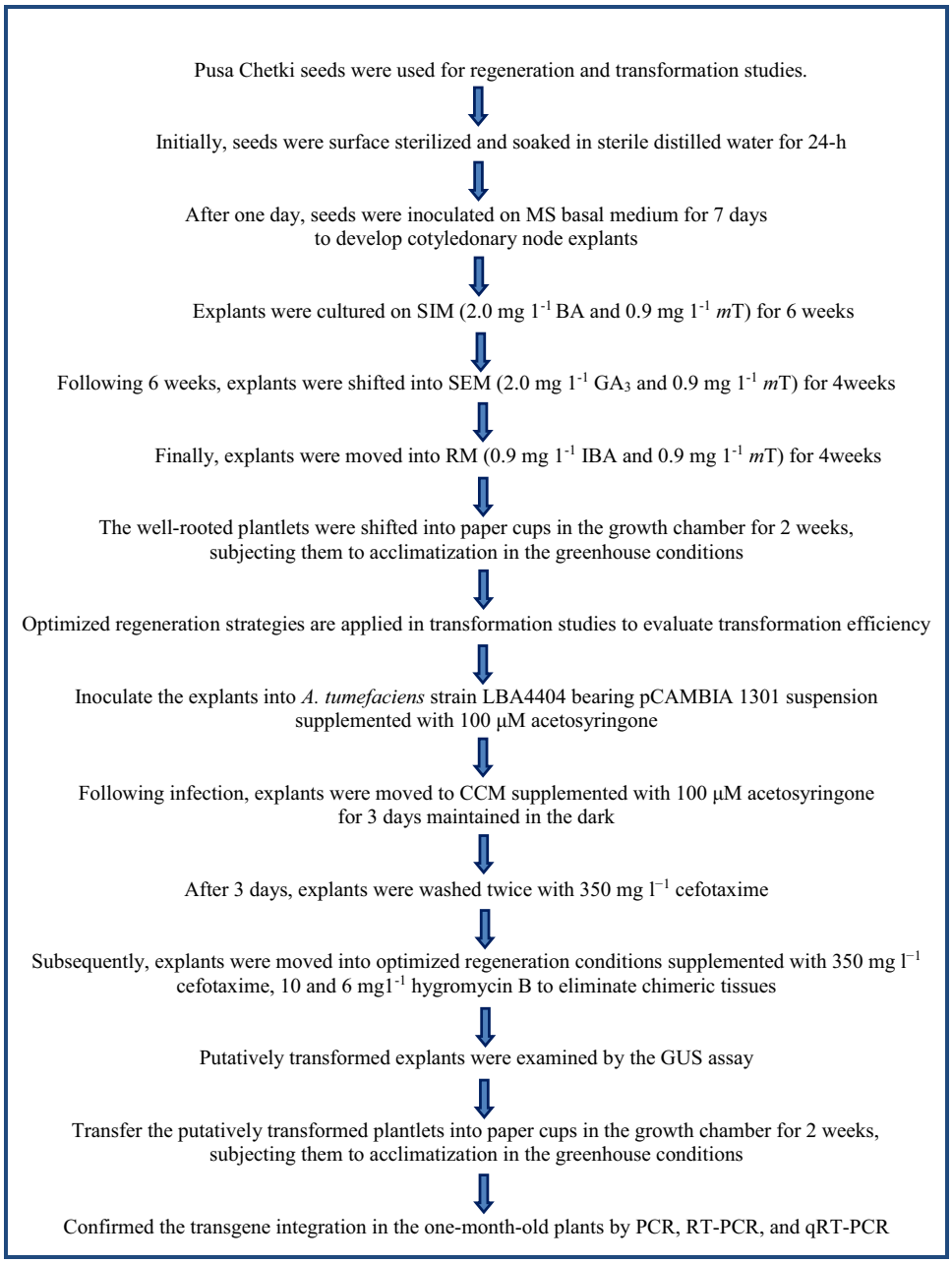

Figure 7. A flowchart demonstrated the regeneration and genetic transformation steps of radish var. Pusa Chetki.

Actinidia chinensis (Saeiahagh *et al*. 2019) for root development. The well-rooted plantlet was hardened in a paper cup (Fig. 2*h*) in the growth chamber. After 2 wk, the hardened plants were shifted to an earthen pot and grown to maturity in the greenhouse (Fig. 2*i*), where 85% of the plants were established successfully.

Sensitivity of the cotyledonary node and rooting explants to hygromycin B A small portion of the target tissue or explant could be genetically transformed during the transformation strategy, and the remnant would be non-transformed. Hence, the selection agent should strictly recognize transformed cells and address them by employing a selection agent in a medium that includes hygromycin B. It is an antibiotic that interferes with protein synthesis by intercepting polypeptide elongation, and the demise of non-transformed tissue or plants assists in selecting transformed plants (Olhoft *et al*. 2003). We evaluated diverse hygromycin B concentrations and found that 10 mg l^{-1} hygromycin eventually hindered shoot induction and elongation from cotyledonary node explants, whereas 6 mg l^{-1} hygromycin B inhibited root growth from elongated shoots. Therefore, 10 and 6 mg l^{-1} (Fig. 3) hygromycin B concentrations were employed to select the transformed plants. Muto *et al*. (2021) revealed hypocotyl explants screened on SIM containing 10 mg l^{-1} hygromycin B in radish. Hence, hygromycin B serves as a

promising selection agent that has successfully been introduced into diverse crops such as soybean (Arun *et al*. 2015), wheat (Zale *et al*. 2009), sorghum (Yellisetty *et al*. 2015), and bitter gourd (Karthik *et al*. 2021).

Influence of *m*T on radish transformation Plant genetic transformation necessitates increasing regeneration and transformation efficiency (Karthik *et al*. 2020). Following co-cultivation, explants were washed and shifted onto SIM containing 2.0 mg l^{-1} BA and 0.9 mg l^{-1} *m*T, which had a higher response to explants responded (82.6%), resulting in 456.3 plants regenerated (Table 4). After that, 148.3 transformed plants were established for hardening (Table 4). Conversely, explants were cultured on SIM with only 2.0 mg l^{-1} BA, which responded to 62.3% of explants and produced 302 regenerated plants (Table 4). Eventually, 92.6 transformed plants were designated as hardened. After screening for hygromycin B, the Gus assay was performed for all transformed plants. The respective regeneration medium augmented with *m*T had maximum transformation efficiency (27.3%) (Table 4), while the regeneration system without *m*T had a lower transformation efficiency (18.6%) (Table 4). Our findings reveal that the combination of *m*T substantially enhanced transformation efficiency in radish *via* an optimized regeneration system. Furthermore, there has been no report on the association of *m*T with improving transformation efficiency in other plants.

GUS assay and molecular confirmation of transformed radish A distinct blue color was noticed in the transformed explants, including a cotyledonary node (Fig. 4*b*), shoot induction (Fig. 4*d*), shoot elongation (Fig. 4*f*), and rooting (Fig. 4*h*) that showed the *gus* A gene was successfully expressed in transformed tissues. The blue color was not found in the NT cotyledonary node (Fig. 4*a*), shoot induction (Fig. 4*c*), shoot elongation (Fig. 4*e*), and rooting explants (Fig. 4*g*). Previously, the *gus* A gene was successfully used to establish transformed radish as a reported gene (Cho *et al*. 2008; Muto *et al*. 2021). PCR was performed on three gus-positive plants to confirm the presence of the *gus* A (Fig. 5*a*, lanes R1–R3) and *hpt* II (Fig. 5*b*, lanes R1–R3) genes at about 781 and 1024 bps in the transformed plants and plasmid DNA (Fig. 5*a* and *b*, lane P). Nevertheless, amplification was not noticed in the NT plants (Fig. 5*a* and 5*b*, lane R4). In RT-PCR, a 1024-bp amplicon was significantly amplified by *hpt* II gene–specific primers in three PCR-positive plants (Fig. 6*a*, lanes R1–R3), which confirmed that *hpt* II is successfully expressed in radish plants. There was no expression in the NT plant (Fig. 6*a*, lane R4). Furthermore, *hpt* II expression has been validated by qRT-PCR in three RT-PCR-positive plants (Fig. 6*b*). In comparison, the NT plant exhibited no expression (Fig. 6*b*). Our study revealed the maximum level of *hpt* II expression detected in R3 (Fig. 6*b*). The lowest level was noticed in R2 (Fig. 6*b*). Similarly, *hpt* II is effectively expressed in radish using hypocotyl explants (Muto *et al*. 2021) as well as a variety of crops such as *Arachis hypogaea* (Karthik *et al*. 2018), *Kalanchoe laxiflora* (Wang *et al*. 2019), and *Momordica charantia* (Karthik *et al*. 2021).

Conclusion

This would probably be the first study to demonstrate the impact of *m*T on radish regeneration (Fig. 7) and genetic transformation strategies (Fig. 7). Consequently, genetic engineering must establish a solid connection between regeneration and transformation efficiency to raise improved crop varieties for sustainable agriculture. Regeneration efficiency is enhanced by *m*T incorporated into PGRs. Furthermore, transformation efficiency increased (27.3%) when transformed cotyledonary nodes regenerated on a medium comprising an optimal concentration of 0.9 mg l^{-1} *m*T, 2.0 mg l^{-1} BA, 2.0 mg l^{-1} GA_3, and 0.9 mg l^{-1} IBA, corresponding to regeneration without *m*T (18.6%) efficiency. Hence, these strategies could assist in developing essential traits in different radish varieties.

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Declarations

Conflict of interest The authors declare no competing interests.

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