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ORIGINAL ARTICLE

Development of an improved *Agrobacterium*-mediated transformation and callus-based regeneration system for sugarbeet (*Beta vulgaris* L.)

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Abstract

Sugarbeet (*Beta vulgaris* L.), cultivated for its sucrose content, a staple sugar used globally, is affected by several diseases caused by many different pathogens. The application of biotechnological approaches for generating disease-resistance requires a reliable transformation and regeneration system. In this study, we first developed a straightforward regeneration protocol for hypocotyl explants by optimizing various conditions for callus induction to shoot formation. Subsequently, using hypocotyl-derived callus, we established an efficient *Agrobacterium*-mediated transformation system with the regeneration of whole plants. With the cotyledons, although regeneration is a concern, transient expression of RUBY, a visual marker, was detectable with 32.9% of transformed cotyledons. For the hypocotyl-derived transformed lines 11 out of 22 were molecularly RUBY positive. The frequency of callus induction was higher when the explants were treated with 0.4 optical density *Agrobacterium* culture, two days co-cultivation and incubation in the dark. The shoot regeneration of 36% was achieved for hypocotyl-derived calli by gradually increasing N^6-benzylaminopurine, kinetin, and α-napthaleneacetic acid. By optimizing hormone levels, root formation occurred within two weeks. Transgene integration in the regenerated lines was confirmed by sequencing of PCR amplicons. Further, the transgene expression in the lines was confirmed by reverse transcription PCR analysis. The transgenic lines were acclimatized and grown in soil under controlled conditions. The transformation and callus-based regeneration protocols developed in this study can facilitate the production of non-chimeric transgenic lines compared to direct *in planta* regeneration methods. This way, the first-generation transgenic lines are suitable for evaluating RNAi and gene-editing approaches to target important sugarbeet pathogens, particularly viruses.

Keywords *Beta vulgaris* · Sugarbeet · Transformation · Regeneration

Key message

Developed efficient callus-based regeneration and *Agrobacterium*-mediated transformation protocols by optimizing tissue-culture conditions for *Beta vulgaris*, which is useful for the introduction of new sources of disease resistance.

Abbreviations

BAP	N^6-Benzylaminopurine
CIM	Callus induction medium
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
NAA	α-Naphthaleneacetic acid
PPM	Plant preservative mixture
RIM	Root induction medium
SIM	Shoot induction medium

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Introduction

Sugarbeet (*Beta vulgaris* L.) is an important crop grown for its high sucrose content throughout the temperate longitudes of the world. It contributes more than 35% of the world's sucrose production (Wozniak et al. 1999; Bekheet et al. 2007). Sugarbeet is susceptible to several diseases caused by viruses, bacteria, and fungi (Rush et al. 2006; Harveson et al. 2009; Ramachandran et al. 2023; Chinnadurai et al. 2024), and crop improvement is essential to develop new varieties to cope with continuously evolving pathogens.

The development of new sugarbeet varieties through conventional breeding is limited due to high heterozygosity, a biennial reproductive cycle, and the presence of intrinsic genetic factors that complicate introducing new traits (Lindsey et al. 1991; Gurel et al. 2008; Mukherjee and Gantait 2023; Tehseen et al. 2023). On the other hand, breeding for new traits can be accelerated through genetic transformation using biotechnological approaches. Despite its economic importance, genetic transformation of sugarbeet remains challenging due to its recalcitrance to tissue culture and regeneration, as well as genotype dependency and a complex genome that hinder achieving stable integration and expression of the introduced transgenes (Snyder et al. 1999; Zhang et al. 2001; Ivic-Haymes and Smigocki 2005; Tehseen et al. 2024). To overcome these challenges, various genetic transformation methods have been explored in sugarbeet, including *Agrobacterium*-mediated transformation, biolistic particle bombardment, polyethylene glycol (PEG)-mediated transformation, electroporation, and somatic hybridization (Lindsey and Jones 1989; Hall et al. 1997, 1996; Gurel et al. 2002; Hisano et al. 2004; Yang et al. 2005; Jafari et al. 2009; De Marchis et al. 2009; Moazami-Goodarzi et al. 2020; Zhang et al. 2023). Among these methods, *Agrobacterium*-mediated transformation was the most widely used due to its simplicity, cost effectiveness, high efficiency, and reliability for plant transformation.

The success of stable transformation essentially depends on the efficiency of regeneration into whole plant. In sugarbeet, different explants have been used for *Agrobacterium*-mediated transformation including leaf discs, cotyledons, hypocotyls (Jacq et al. 1993; Wozniak and Owens 1994), leaf blades (Jafari et al. 2009), petioles (De Marchis et al. 2009), leaf lamina, shoot bases (Lindsey and Gallois 1990), blade-petiole transition zones (Zhang et al. 2023), and bud tips (Yang et al. 2005). However, regeneration was shown only via direct shoot formation from explants without undergoing callus formation (Yang et al. 2005; Zhang et al. 2023). In a typical regeneration process, callus induction offers a homogenous population of transformed cells potentially leading to consistent transgene integration, which is yet to be established for sugarbeet.

In this study, we established a simple and efficient *Agrobacterium*-mediated transformation protocol using hypocotyl-derived explants. While transformation of sugarbeet using hypocotyls and cotyledons has been previously reported (Jacq et al. 1993), those studies were limited to short-term GUS assays after 14 days on selection medium at callus stage. In essence, previous studies have either focused on factors affecting *Agrobacterium*-mediated transformation efficiency up to callus stage or evaluate different types of explants for direct-regeneration. Our research reports the optimization of a complete process that includes callus induction from hypocotyl explants, *Agrobacterium*-mediated transformation, and the indirect regeneration of whole-plants. In vitro regeneration from callus culture offers less chimerism and somaclonal variations and thus can generate transgenic lines that are suitable for evaluating RNAi and gene editing approaches to target important sugarbeet pathogens, particularly viruses.

Materials and methods

Plant materials, seed sterilization and culture medium

Seeds of *B. vulgaris* used in this study was a gift from KWS, Einbeck, Germany. Seeds were soaked in water and incubated at 4 °C overnight. Seeds were surface sterilized with 70% ethanol for 2 minutes, after rinsing in sterile water, the seeds were treated with 1x Fungigone (Plantmedia, OH, USA) for 5 min, 1% H_2O_2 for 30 minutes and then with 5% sodium hypochlorite for 20 minutes with gentle shaking (Chu et al. 2022). Finally, seeds were washed with autoclaved water for 4 minutes for five times. Seeds were dampdried on autoclaved tissue paper and transferred to 50 ml falcon tubes containing Murashige and Skoog (MS) basal solid medium pH 5.8 with 3% sucrose and 0.8% agar (PhytoTech Labs, KS, USA) and 0.1% Plant Preservative Mixture (PPM; Plant Cell Technology, Washington DC, USA) and kept at 24 ± 2 °C in the dark for 5 days (Table 1). Subsequently, the falcon tubes were incubated at 24 ± 2 °C with a 16-hour photoperiod.

Kanamycin lethality and PPM sensitivity testing

To establish an appropriate kanamycin concentration for selecting transformed explants, hypocotyls of *B. vulgaris* were cultivated on callus induction medium (CIM) containing MS medium pH 5.8, supplemented with 2.0 mg l^{-1} N6-benzylaminopurine (BAP), 3% sucrose and 0.8% agar (PhytoTech Labs, KS, USA) with different concentration of kanamycin (0, 25, 50, 75, 100, 125, 150, 175 and

Table 1 Details of culture of medium for sugarbeet transformation and regeneration

Medium	Phytohormones (mg l^{-1})				Antibiotics (mg l^{-1})			PPM™[1] (%)	Acetosyringone (µM)	Sucrose (g l^{-1})	Agar (g l^{-1})
	BAP[2]	Kinetin	NAA[3]	IBA[4]	Kan[5]	Cefotaxime	Timentin				
Seed germination								0.1		30	8
Bacterial infection									100	30	
Co-cultivation										30	8
Callus induction	2				50	250	300	0.1		30	8
Shoot induction-I	0.25			0.1		250	300	0.1		30	8
Shoot induction-II	1		0.5			250	300	0.1		30	8
Shoot induction-III	1	1	0.25			250	300	0.1		30	8
Shoot induction-IV	2	2	0.5			250	300	0.1		30	8
Root induction-I			1.5	1.5		125		0.1		30	8
Root induction-II			2	2			150	0.1		30	8
Root induction-III			3	3				0.1		30	8

[1] PPM: Plant preservative mixture

[2] BAP: N6-benzylaminopurine

[3] NAA: α-Naphthaleneacetic acid

[4] IBA: Indole-3-butyric acid

[5] Kan: Kanamycin

200 mg l^{-1}) and kept at 24 ± 2 °C in the dark (Table 1). Fifteen explants from 10 day-old in vitro germinated sugarbeet seedlings were placed onto each Petri plate (100 ×15 mm) containing 25 ml CIM. A total of three Petri plates, representing three replicates across various kanamycin concentrations were used, and the experiment was repeated three times. The survival rate of explants was evaluated after 21 days of cultivation. Similarly, the PPM sensitivity test was performed using explants from 10-day-old seedlings of *B. vulgaris*. Explants were cultured on CIM supplemented with varying concentration of PPM (0, 0.05%, 0.1%, 0.2%, 0.5% and 1.0% v/v). Cultures were maintained at 24 ± 2 °C in the dark.

Optimization of B. vulgaris regeneration

Hypocotyls of 10 to 14-day-old sugarbeet seedlings were used for regeneration optimization. Approximately 1 cm size hypocotyls were cut from seedlings and transferred to CIM and incubated at 24 ± 2 °C in dark and sub-cultured every three weeks. The calli developed were further transferred on to shoot induction medium (SIM-I through III), containing MS medium supplemented with various concentrations of BAP, kinetin, and α-Naphthaleneacetic acid (NAA), having 0.1% PPM, 3% sucrose, 0.8% agar with pH 5.8 and incubated at 24 ± 2 °C, 16 hours photoperiod (Table 1). The developed shoots were cultured on SIM-IV containing MS medium pH 5.8 supplemented with 2 mg l^{-1} BAP, 2 mg l^{-1} kinetin, 0.5 mg l^{-1} NAA, 0.1% PPM, 3% sucrose, 0.8% agar and incubated at 24 ± 2 °C with 16 hours photoperiod (Table 1).

Mobilization of pCBL101-RUBY vector into Agrobacterium tumefaciens

The pCBL101-RUBY vector was obtained from Addgene (Lee et al. 2023). The plasmid carries RUBY, a visual reporter that comprises of betalain biosynthesis genes viz. *CYP76AD1*, *DODA*, and *Glucosyl transferase* under the control of CaMV 35S promoter, a *neomycin phosphotransferase II* (NPT II) gene driven by maize ubiquitin promoter for plant selection, and a spectinomycin resistance gene for bacterial selection. The pCBL101-RUBY vector was mobilized into *Agrobacterium tumefaciens* GV3101 competent cells (GoldBio, MO, USA) using freeze thaw method (Holsters et al. 1978).

Agrobacterium-mediated transformation of B. vulgaris

A single colony of *Agrobacterium tumefaciens* GV3101 harboring the pCBL101-RUBY vector was inoculated into 5.0 ml Luria-Bertani (LB) medium containing 50 µg ml^{-1} rifampicin (PhytoTech Labs, KS, USA) and 50 µg ml^{-1} spectinomycin (Sigma-Aldrich, MO, USA) and incubated at 28 °C with shaking at 200 rpm. Twenty µl 0.1% (v/v) of primary *Agrobacterium* culture was inoculated into 20.0 ml LB medium supplemented with the same antibiotics and 100 µM acetosyringone (PhytoTech Labs, KS, USA) and incubated under the same conditions for 16 hours. Once the bacterial culture reached an optical density at 600 nm (OD_{600}) of 0.4 to 0.5, the bacterial cells were pelleted by centrifugation at 4000 rpm for 5 minutes at 22 °C. The pellet was resuspended in MS liquid medium containing 100 µM

acetosyringone and incubated in the dark with gentle shaking at 40 rpm for 2 hours.

Ten to 14-day-old sugarbeet seedlings germinated on 1X MS basal medium were used for transformation. Hypocotyl and cotyledons were excised into 1.0 cm segments and immersed in the *Agrobacterium* suspension (infection medium) for 15 mins with gentle mixing at 10 rpm. The explants were then blotted damp-dry on sterile tissue paper and transferred to 1X MS basal medium pH 5.8 containing 3% sucrose, 0.8% agar followed by 48 hours of co-cultivation in dark at 24 ± 2 °C (Table 1).

Post co-cultivation, explants were washed first with autoclaved water and then with 500 mg l^{-1} cefotaxime for 40 min to eliminate excess *Agrobacterium*. The explants were damp-dried on autoclaved tissue paper and transferred to CIM containing 50 mg l^{-1} kanamycin (PhytoTech Labs, KS, USA) and incubated in dark at 24 ± 2 °C. Emerging callus from transformed explants were subsequently transferred to SIM having varying concentration of BAP, kinetin, NAA and IBA (PhytoTech Labs, KS, USA) and incubated initially one week in dark and then moved to 16 hours photoperiod (Table 1). Regenerated shoots were moved to root induction medium (RIM) containing MS medium pH 5.8 with varying concentrations (1.5 to 3.0 mg l^{-1}) of NAA and IBA, 0.1% PPM, 0.8% agar and incubated at 24 ± 2 °C with 16 hours photoperiod (Table 1). Finally, rooted sugarbeet plantlets were acclimatized in sterile sand: soil (60:40) mixture and maintained in a growth chamber at 24 ± 2 °C under a 16 hour photoperiod. The *B. vulgaris* transformation experiment was repeated with three biological replicates and each replicate with three technical replicates.

Molecular analysis of putative transformed plants

Total genomic DNA was extracted from regenerated transformed sugarbeet plants using the DNeasy® Plant Mini Kit following the manufacturer's instructions (Qiagen, MD, USA). DNA quality and quantity were assessed by using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, MA, USA). PCR was carried out in a 20 µl reaction volume containing 1X Phusion Hot Start Flex Buffer, 200 µM dNTPs, 625 µM $MgCl_2$, 0.5 µM each of forward and reverse primers (Supplementary Table S1), 100 ng of genomic DNA and 0.5 U of Phusion Hot Start Flex DNA Polymerase (New England Biolabs, MA, USA). PCR conditions for amplifying the *RUBY* with specific primers (He et al. 2020) include an initial denaturation at 98 °C for 30 s, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 57.6 °C for 30 s, and extension at 72 °C 30 s, with a final extension at 72 °C for 10 min (Supplementary Table S1). Subsequently, *NAD* FP and *NAD* RP primers were used to amplify mitochondrial *NAD5* gene as an internal control (Menzel et al. 2002). PCR amplicons were resolved on 1.2% agarose gel stained with Safe DNA Gel Stain (APExBIO, TX, USA) and visualized using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, CA, USA). The expected DNA bands were excised from the gel, and DNA was eluted using the QIAquick® Gel Extraction Kit (Qiagen, MD, USA), according to the manufacturer's instructions. The gel purified DNA was then subjected to Sanger sequencing (Eurofins Genomics, KY, USA).

Reverse transcription-PCR

Total RNA was extracted from the leaf tissues of regenerated transgenic sugarbeet plants using the RNeasy® Plant Mini Kit following the manufacturer's instructions (Qiagen, MD, USA). RNA concentration was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, MA, USA). First-strand cDNA was synthesized from 1 µg of total RNA with oligo dT primer using the Verso cDNA Synthesis Kit according to the manufacturer's protocol (ThermoFisher Scientific, MA, USA). The synthesized cDNA was used as a template for PCR amplification using 0.5 µM each of forward and reverse RUBY specific primers (Supplementary Table S1), following the same PCR conditions described in the section above. Subsequently, 0.5 µM each of 18S-F and 18S-R primers (Supplementary Table S1) were used to amplify the housekeeping gene as an internal control (Chen and Gilbertson 2009). PCR products were resolved on 1.2% agarose gel, stained with Safe DNA Gel Stain (APExBIO, TX, USA), and visualized using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, CA, USA).

Random amplified polymorphic DNA (RAPD) to identify somaclonal variation in transformed and regenerated B. vulgaris lines

Random amplified polymorphic DNA (RAPD) was performed to assess genetic fidelity/stability in transgenic *B. vulgaris* lines. A total of eight transgenic lines and four non-transformed *B. vulgaris* plants were analyzed using 4 RAPD primers. PCR reaction was carried out in 25 µl reaction mixture containing 1X ThermoPol Reaction Buffer, 200 µM dNTPs, 2 mM $MgCl_2$, 10 µM RAPD primer (Supplementary Table S1) with 45 ng genomic DNA and 1.25 U *Taq* DNA Polymerase (New England Biolabs, MA, USA). The PCR conditions are an initial denaturation 95 °C for 30 s, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 35 °C for 1 min, and extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min. PCR amplicons were resolved on 1.5% agarose gel stained with Safe DNA Gel Stain (APExBIO, TX, USA) and visualized using the

ChemiDocTM MP Imaging System (Bio-Rad Laboratories, CA, USA).

Statistical analysis

The data on *B. vulgaris* transformation and regeneration were analyzed to determine the number of explants resistant to kanamycin, those showing transient RUBY expression, and the number of regenerated plants. A one-way analysis of variance (ANOVA) followed by a post-hoc Tukey HSD test was performed to identify statistical differences across and among the three transformation experiments, each conducted with three biological replicates, using an Online Web Statistical Calculator (https://astatsa.com/OneWay_Anova_with_TukeyHSD/). The bar graph was generated using Microsoft Excel 365.

Results

Optimization of kanamycin concentration for the selection of transformed explants of B. vulgaris

The sensitivity of *B. vulgaris* explants to kanamycin was evaluated to determine the optimal concertation required to effectively inhibit tissue growth of untransformed explants. No explants survival was observed when cultured on CIM supplemented with kanamycin at concentration on or above 50 mg l^{-1} (Fig. 1). In contrast, 91% explants were survived when cultured on CIM without kanamycin. A significant reduction (49%) in explant survival was observed at 25 mg l^{-1} kanamycin (Fig. 1A, B, and C). All the explants turned brown and died after 21 days on CIM containing 50 mg l^{-1} and/or above kanamycin concertation (Fig. 1A and C). Based on these results, a concentration of 50 mg l^{-1} kanamycin was chosen for explant selection in *B. vulgaris* transformation experiments.

Additionally, PPM sensitivity test on *B. vulgaris* explants showed that it effectively suppressed microbial contamination at ≥0.1% and tolerated up to 0.2% without growth inhibition or browning in explants (Supplementary Figure S1A). An optimal concentration of 0.1% PPM was found to balance explant viability and contamination control. On the other hand, *B. vulgaris* explants grown on CIM without PPM or with 0.05% PPM showed microbial contamination (Supplementary Figure S1B).

Establishment of in vitro regeneration for B. vulgaris

To establish an effective and reproducible in vitro regeneration system for *B. vulgaris*, hypocotyls were found to be highly effective responding to callus induction. Notably, callus-derived from hypocotyls exhibited excellent shoot

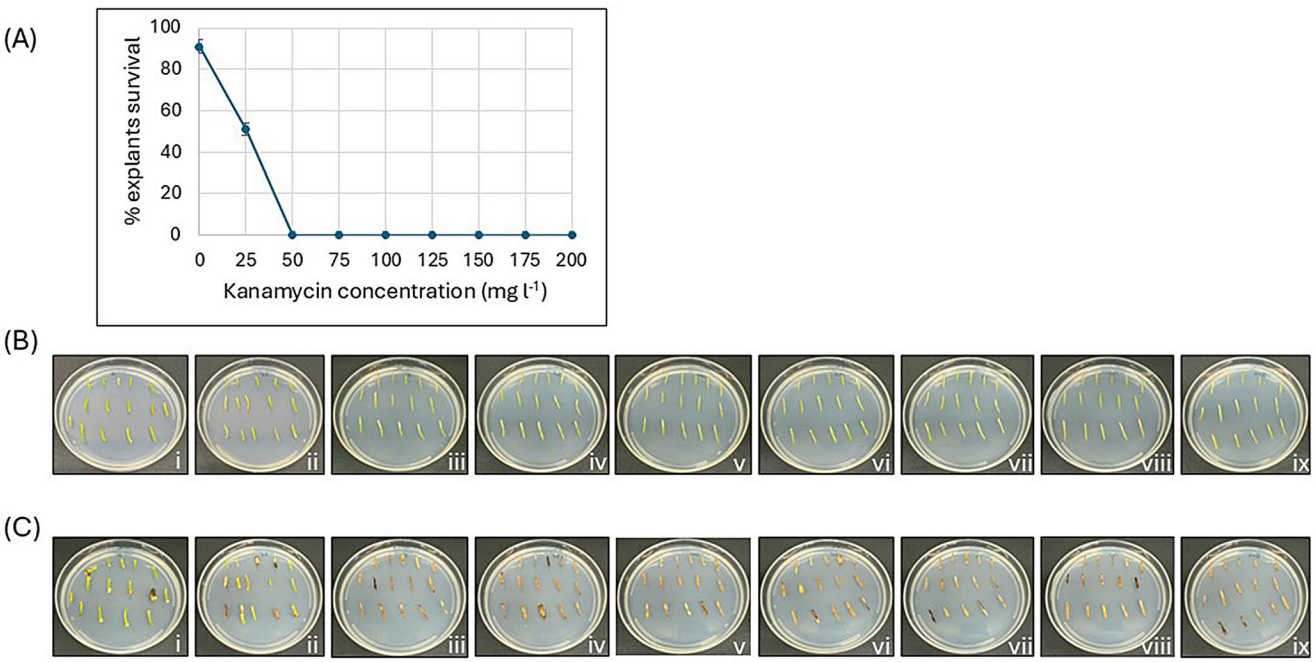


Fig. 1 Effect of kanamycin on the survival of hypocotyl explants of *B. vulgaris* after three weeks of culture on callus induction medium (CIM). (**A**) data represents means ± standard deviation of three experiments, each consisting of 15 hypocotyl explants. The error bars represent standard deviation. (**B**) hypocotyl explants from 10-day-old *B. vulgaris* seedlings cultured on CIM containing varying concentrations of kanamycin (**i**) 0 mg l^{-1}, (ii) 25 mg l^{-1}, (iii) 50 mg l^{-1}, (iv) 75 mg l^{-1}, (**v**) 100 mg l^{-1}, (vi) 125 mg l^{-1}, (vii) 150 mg l^{-1}, (viii) 175 mg l^{-1}, and (ix) 200 mg l^{-1} on day 0. (**C**) effect of kanamycin on the survival of hypocotyl explants of *B. vulgaris* after 3 weeks of culturing on CIM

regeneration (Fig. 2). At 21 days post-transfer (DPT) to CIM, callus formation was observed from hypocotyl incubated in the dark at 24 °C. Shoots were induced at 35 DPT on SIM-III from hypocotyl-derived calli. Additionally, multiple shoots of *B. vulgaris* were regenerated at 21 DPT on SIM-IV (Fig. 2).

Optimization of Agrobacterium-mediated genetic transformation of B. vulgaris

Explants generated from the cotyledon and hypocotyl of 10 to 14-day-old sterile seedlings were used for *Agrobacterium*-mediated transformation. The streamlined *Agrobacterium*-mediated transformation and regeneration protocol for *B. vulgaris* is outlined in Fig. 3. We optimized several parameters using RUBY as a visual reporter (Fig. 4A) to obtain embryonically viable callus for regeneration since callus provides a homogenous population of cells. To determine the density of *Agrobacterium* culture, explants derived from both cotyledons and hypocotyls were treated with different concentration of *Agrobacterium* and 0.4 to 0.5 optical density was found optimal to induce callus formation. The *Agrobacterium* culture of 0.4 optical density with 15 mins infection period yielded 32.9% transient RUBY expression observed in cotyledons two days after co-cultivation (Fig. 4C). Transformants did not survive when the *Agrobacterium* culture density was greater than 0.6 optical density with a slight increase in the infection time to 20 mins and three days co-cultivation due to excessive *Agrobacterium* infection, despite multiple washes with cefotaxime. Upon incubation on CIM, the transformed cotyledon explants developed callus at three to four weeks, and the expression of RUBY was observed in the callus (Fig. 4C). For the hypocotyl-derived explants, similar conditions were found

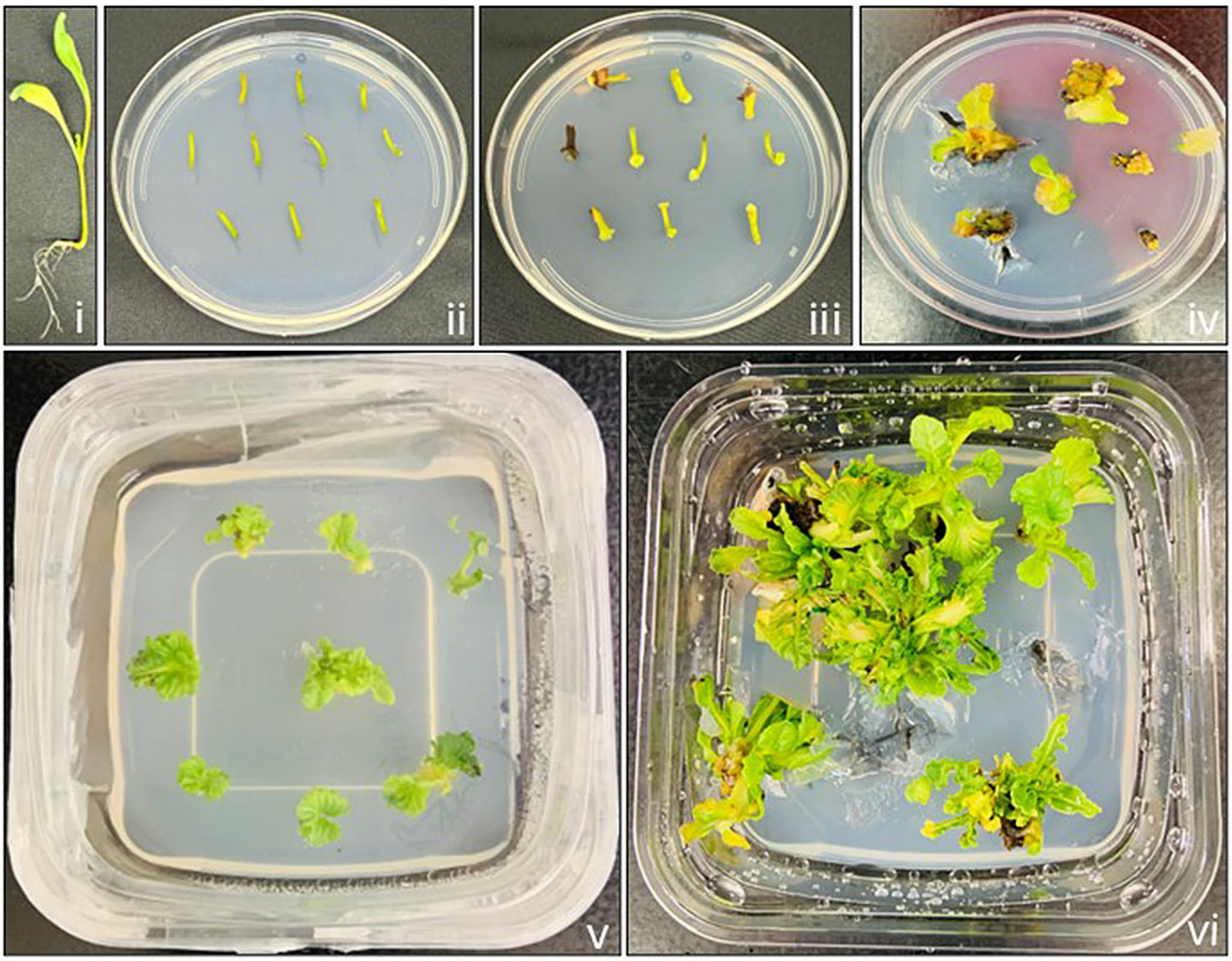


Fig. 2 Optimization of regeneration protocol of *B. vulgaris* from hypocotyl. (**i**) Representation of 10-day-old *B. vulgaris* seedling, (ii) hypocotyl explants on callus induction medium (CIM), (iii) callus induction after 21 days transfer on CIM, (iv) shoot induction from callus on shoot induction medium-III (SIM-III), (**v**) shoot culture on SIM-IV, and (vi) shoot regeneration after 21 days culturing on SIM-IV

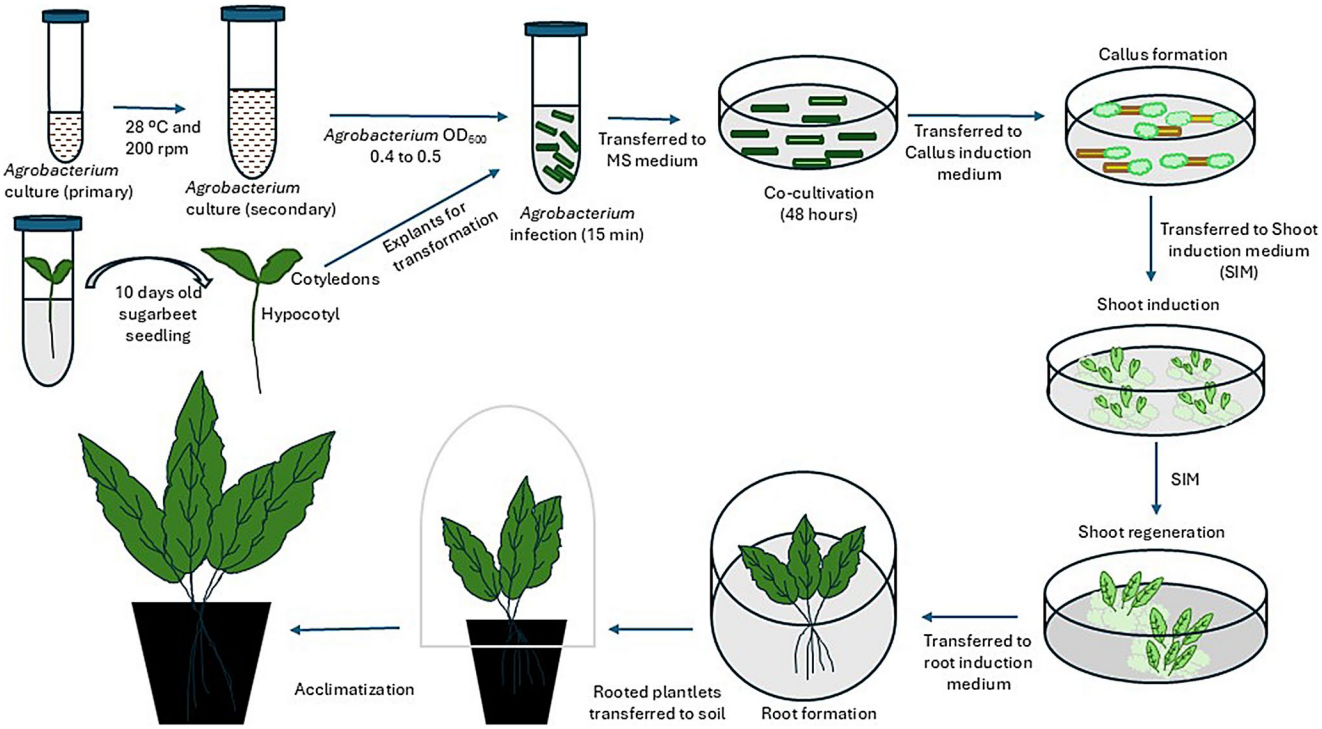


Fig. 3 Schematic representation of the procedure developed for transformation and regeneration system of *B. vulgaris*

suitable for optimal transformation; however, the appearance of red color was not detectable. Development of callus from the transformed hypocotyl explants was observed at 21 days on CIM in dark (Fig. 4B). Stable RUBY expression was detected in 9.8% of callus derived from cotyledons after 25 days on CIM (Table 2), and this expression persisted for up to two months (Fig. 4C). After two to three consecutive subcultures on CIM, the percentage of kanamycin-resistant explants was 24.8% for cotyledons and 13.4% for hypocotyls (Table 2). One-way ANOVA revealed the number of cotyledon explants used for transformation, and the number of transient RUBY expression were significant with $p<0.01$ and $p<0.05$, respectively (Fig. 5A). Callus formation from either hypocotyls or cotyledons was observed only when the explants were incubated in dark (Fig. 4B and C), while the transformed explants turned brown when incubated in light (data not shown). For seed germination, we introduced a 4 °C incubation step and that increased the germination rate, and we used 10 to 14 days old seedlings for explant preparation instead of six to twelve weeks old seedlings.

Regeneration of transformed callus of B. vulgaris

To induce shoot regeneration from the kanamycin-resistant callus, four different SIM were tested, each containing different combinations of auxins and cytokinin (Table 1). No shoots were induced from the callus developed from hypocotyl or cotyledons at 21 days post transfer on SIM-I either incubated in light or in dark. Shoot formation occurred from hypocotyl derived callus between three and four weeks when the callus were transferred onto SIM-II and SIM-III (Fig. 4B). Further, shoots appeared on SIM-II and SIM-III were regenerated on SIM-IV at equal efficiency in three weeks (Fig. 4B). The occurrence of shoot regeneration was 36% for hypocotyl-derived callus at 3 weeks on SIM-IV, which contained 2.0 mg l^{-1} each BAP and kinetin and 0.5 mg l^{-1} NAA (Table 1). No shoot formation occurred from the cotyledon-derived callus after eight weeks of culturing on SIM-II, SIM-III and SIM-IV (Fig. 4C). Using one-way ANOVA, the number of hypocotyls used for transformation and the number of hypocotyls resistant to kanamycin, selectable marker, were found to be significant with $p<0.01$ (Fig. 5B)

The regenerated shoots were transferred to RIM containing various combinations of auxins, ranging from 1.5 mg l^{-1} to 3.0 mg l^{-1} of NAA and IBA (Table 1). No root formation occurred even after four weeks on RIM-I (1.5 mg l^{-1} NAA, 1.5 mg l^{-1} IBA, 125 mg l^{-1} cefotaxime and 0.1% PPM). However, root formation was initiated 25 days post-transfer on RIM-II, which contained 2.0 mg l^{-1} NAA, 2.0 mg l^{-1} IBA, 150 mg l^{-1} timentin and 0.1% PPM. Rooting occurred more rapidly within two weeks on RIM-III that was supplemented with higher concentrations of NAA and IBA to 3.0 mg l^{-1}, and 0.1% PPM (Fig. 4B). By increasing the hormone concentrations, BAP and Kinetin in the SIM, and NAA and IBA in the RIM the duration of shoot-formation and root-induction were accelerated. To control

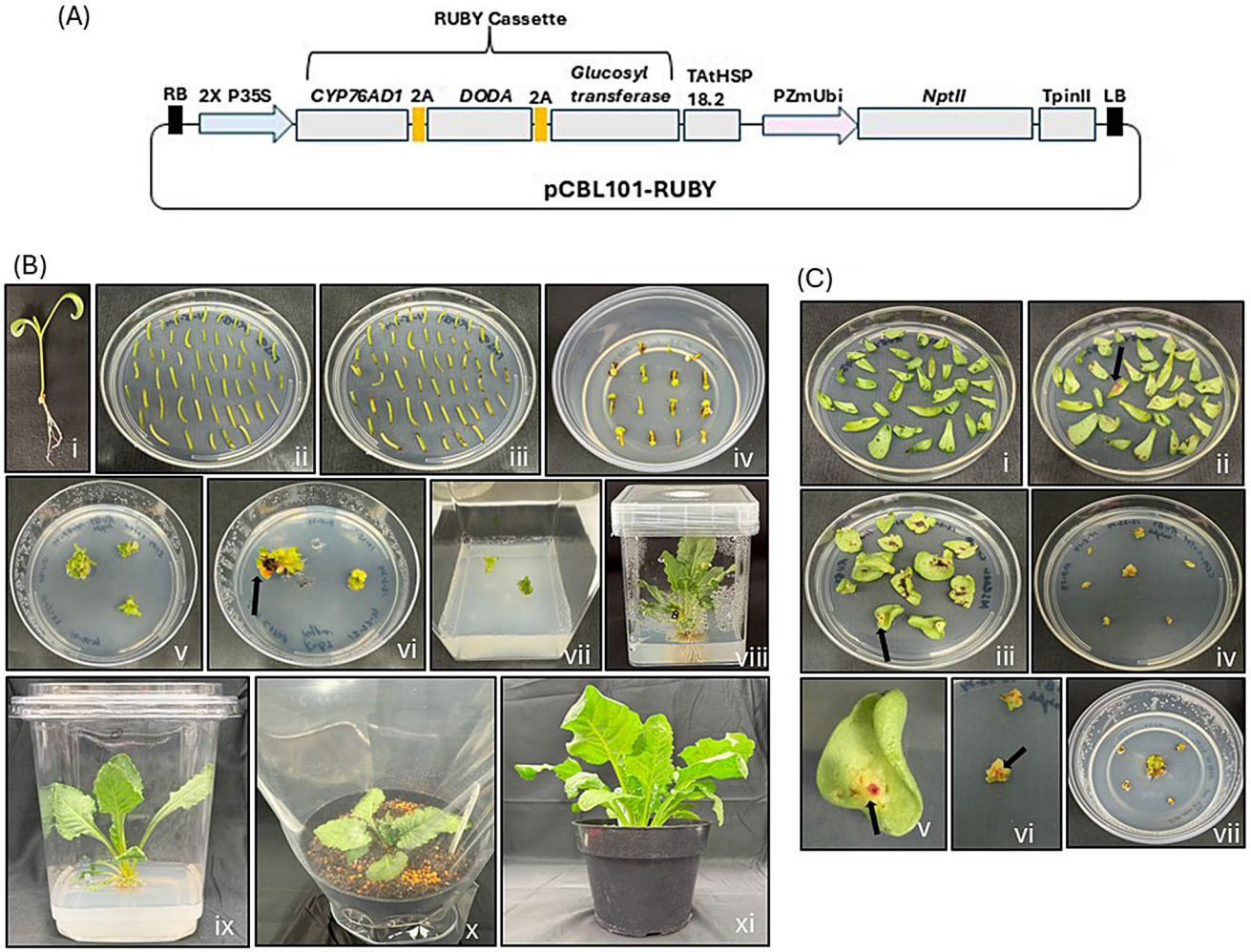


Fig. 4 Transformation and regeneration of *B. vulgaris*. (**A**) schematic representation of pCBL101-RUBY vector. (**B**) *agrobacterium*-mediated transformation of *B. vulgaris* using hypocotyl, (i) 14-day-old sugarbeet seedling, (ii) transformed hypocotyls with *agrobacterium* harboring pCBL101-RUBY, (iii) transformed hypocotyls after 2 days of co-cultivation, (iv) transformed hypocotyls on callus induction medium 21 days after transfer, (v) and (vi) shoot induction and regeneration of sugarbeet plantlets from hypocotyl-derived callus on shoot induction medium (SIM-II and SIM-III), respectively, (vii) shoots sub-cultured on SIM-IV, (viii) 21 days cultured shoots on root induction medium III (RIM-III), (ix) rooted plantlets transferred on bigger tissue culture container having RIM-III, (**x**) putative transgenic plantlets acclimatized in soil and covered with polybag to maintain humidity, and (xi) transgenic sugarbeet plant after 21 days transfer in soil. (**C**) *agrobacterium*-mediated transformation of *B. vulgaris* using cotyledons, (i) transformed cotyledons taken from B-i, (ii) transient RUBY expression in transformed cotyledons after 2 days of co-cultivation, (iii) transformed cotyledons on callus induction medium 21 days after transfer, (iv) callus with RUBY expression on SIM-III, (v) and (vi) enlarged images of iv and v, respectively showing RUBY expression, and (vii) callus after 6 weeks culture on SIM-IV. Black arrows indicate RUBY expression in transformed explants

Agrobacterium overgrowth after co-cultivation, cefotaxime and timentin were used at concentrations of 250 mg l^{-1} and 300 mg l^{-1}, respectively, throughout the callus induction and regeneration duration. Cefotaxime was reduced to 125 mg l^{-1} in RIM-I, and timentin was reduced to 150 mg l^{-1} in RIM-II (Table 1). Neither of the antibiotics were used in RIM -III due to their detrimental effects on root formation.

Further, the rooted plantlets were acclimatized in soil and covered with polybags for one week to maintain humidity-controlled growth chamber. The plants were slowly exposed to light by opening the top corners of polybags for four days followed by top cutting to expose plants to direct light. After five days, polybags were removed. All the transformed plants that were acclimatized survived in soil. The transgenic plants exhibited normal growth and morphology without any abnormalities (Fig. 4B).

Molecular analysis of transgenic plants

The putative transgenic lines of sugarbeet were confirmed by PCR amplification of *RUBY* gene. Eleven sugarbeet plantlets showed 400 bp *RUBY* gene amplification while no amplification was observed in non-transformed sugarbeet plant (Fig. 6A). Further, sequencing of the transgene

Table 2 Transformation and regeneration frequency of sugarbeet cotyledon and hypocotyl explants

Experiments	No. of explants transformed		No. of explants showed Transient expression of RUBY		No. of callus showed RUBY expression		No. of explants resistant to Kan[1]		No. of regenerated plants		No. of PCR positive plants (RUBY)/no. of tested plants	
	Cot[2]	Hyp[3]	Cot	Hyp	Cot	Hyp	Cot	Hyp	Cot	Hyp	Cot	Hyp
1	90	61	38	NV[4]	4	NV	18	8	NR[5]	2	NT[6]	1/1
	90	60	42	NV	5	NV	22	9	NR	3	NT	1/1
	106	63	49	NV	8	NV	31	9	NR	4	NT	2/2
2	70	50	16	NV	8	NV	13	3	NR	3	NT	1/1
	70	50	15	NV	7	NV	14	3	NR	2	NT	1/1
	63	51	11	NV	5	NV	8	3	NR	2	NT	1/1
3	40	40	9	NV	7	NV	15	10	NR	2	NT	1/1
	40	40	11	NV	7	NV	15	9	NR	2	NT	2/2
	51	39	13	NV	10	NV	18	7	NR	2	NT	1/1

[1] Kan: Kanamycin

[2] Cot: Cotyledons

[3] Hyp: Hypocotyl

[4] NV: Not visible

[5] NR: No regeneration

[6] NT: Not tested

Experiments 1, 2, and 3 refer to three biological replicates and each experiment with three technical replicates.

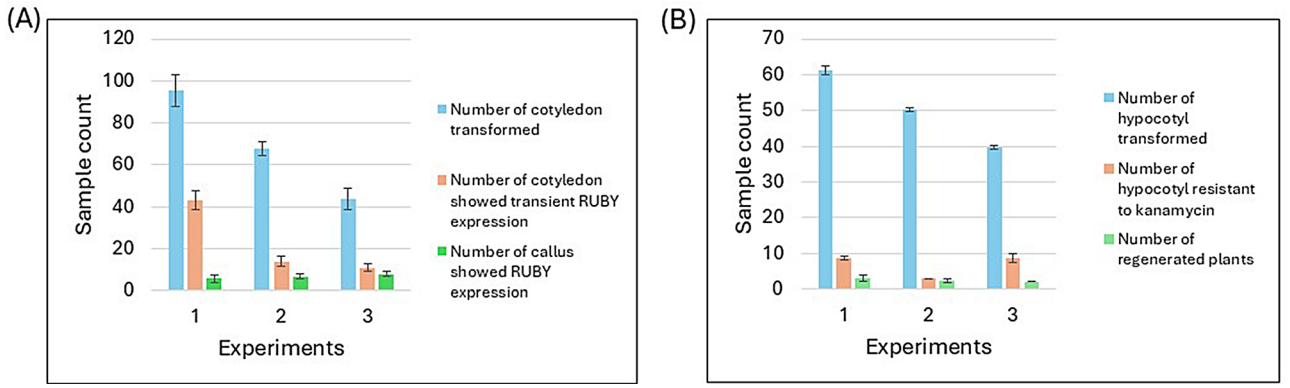


Fig. 5 Statistical analysis of *B. vulgaris* transformation. (**A**) transformation of *B. vulgaris* using cotyledon explants and RUBY expression in transformed cotyledons. (**B**) transformation and regeneration of *B. vulgaris* using hypocotyl explants. Data represents means ± standard deviation of three biological replicates each with three technical replicates

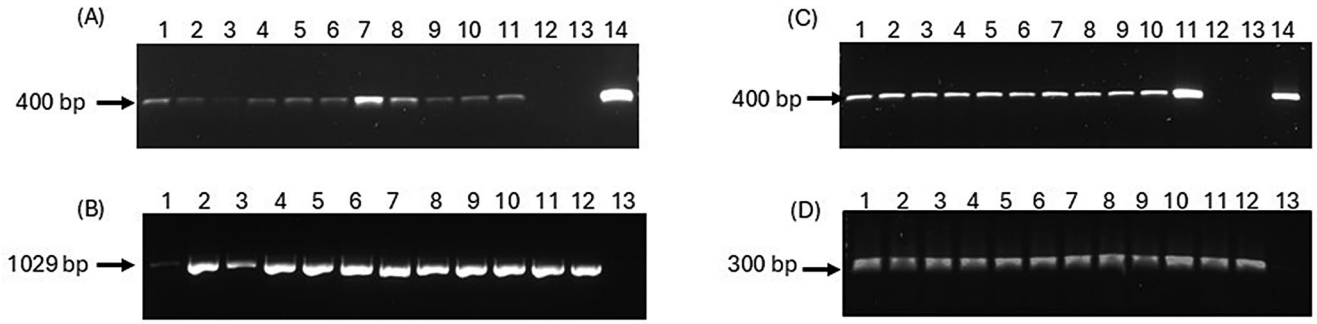


Fig. 6 Molecular analysis of transgenes in transformed *B. vulgaris* lines. (**A**) transgene confirmation using PCR amplification of RUBY with specific primers (oligo 32/Oligo 33) resulted 394 bp DNA fragment. (**B**) PCR amplification of *NAD5* gene as internal control (1029 bp). (**C**) transgene expression analysis using RT-PCR with RUBY specific primers (oligo 32/Oligo 33). (**D**) RT-PCR amplification of internal control using 18S rDNA specific primers resulted in 300 bp amplicon. Lanes 1–11: transformed sugarbeet lines; lane 12: non-transformed sugarbeet; lane 13: negative control without template; lane 14: positive control pCBL101-RUBY plasmid

amplicons from 11 putative transgenic sugarbeet plants showed 97 to 100% nucleotide identity with the introduced RUBY reporter sequence confirming transgene integration to the genome of transformed sugarbeet. Additionally, internal control *NAD5* gene was successfully amplified in 11 transformed sugarbeet lines as well as in non-transformed sugarbeet plant (Fig. 6B). As summarized in Table 2, 50% of the regenerated sugarbeet plants were tested for transgene presence by PCR, and all tested positive for transgene integration. Although all eleven transformed lines were PCR positive, the red color phenotype was not detectable. To understand the expression status of *RUBY* gene in the transformed sugarbeet plants, RT-PCR was performed to detect the transcripts using the total RNA extracted from individual transgenic plants. The results confirmed the presence of *RUBY* transcripts in all eleven transformed lines tested (Fig. 6C).

RAPD analysis

To assess somaclonal variations in transformed plants, four arbitrary decamer RAPD primers, previously used for identifying genetic diversity in sugarbeet, were screened in this study to assess the genetic makeup of in vitro regenerated transgenic sugarbeet lines (Srivastava et al. 2007; Izzatullayeva et al. 2014; Abekova et al. 2022). RAPD primer A05 yielded a similar pattern with a total of about 17 amplified fragments in the in vitro regenerated independent transgenic lines and non-transformed sugarbeet plants, ranging from 300 bp to 2500 bp (Fig. 7). RAPD analysis confirmed reproducible DNA banding patterns in regenerated transgenic lines and non-transformed sugarbeet plants with no detectable genetic variations.

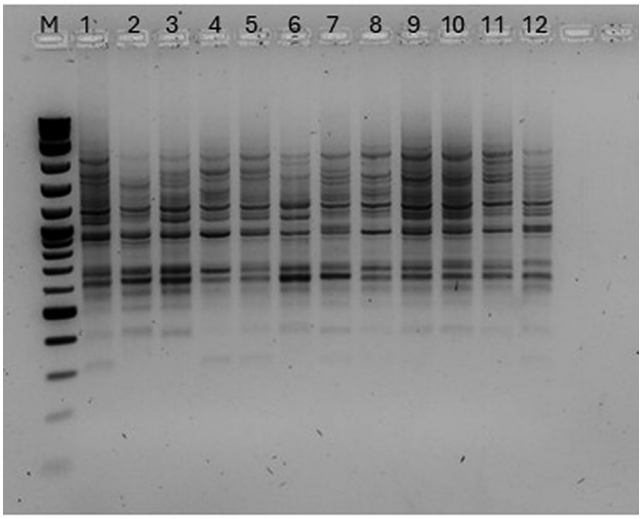


Fig. 7 Random amplified polymorphic DNA (RAPD) profile of eight in vitro regenerated independent transgenic *B. vulgaris* lines and four non-transformed *B. vulgaris* plants using A05 RAPD primer. Lane M1: 1 kb plus DNA ladder; lane 1–8: independent transgenic *B. vulgaris* lines; lane 9–12: non-transformed *B. vulgaris* plants

Discussion

There are several reports on genetic transformation of sugarbeet; however, the challenge is regeneration, which is difficult due to its low dedifferentiation nature (Lindsey and Gallois 1990; D'Halluin et al. 1992). *Agrobacterium*-mediated transformation method offers the most efficient way to introduce genes into plants (St Schell 1987; Hooykaas 1989). Establishing a simple and efficient transformation and regeneration system is essential for the development of transgenic sugarbeet plants. In this study, we have optimized the conditions and developed a reproducible and reliable transformation and regeneration pipeline for *B. vulgaris* with a reduced timeline. As the first requirement for setting up any plant transformation (Aggarwal et al. 2024), we determined kanamycin-sensitivity for hypocotyl-derived callus to ensure tight selection essential for eliminating non-transformed events. We obtained callus from *B. vulgaris* hypocotyls on MS medium containing 2.0 mg l^{-1} BAP, consistent with the findings by Dovzhenko and Koop (2003). We successfully regenerated *B. vulgaris* with shoots emerging from hypocotyl-derived calli by 35 DPT on SIM-III. Within three weeks, multiple shoots were regenerated from hypocotyl explants on SIM-IV.

Previous research indicated that callus induction and the shoot formation response depend on the sources of explants (Krens and 1989). Investigation on the efficiency of T-DNA transfer revealed that the addition of acetosyringone improved transformation in both cotyledons and hypocotyl explants based on GUS assay on 14-day old callus culture (Jacq et al. 1993). To increase the efficiency of transformation and callus formation in the cotyledon and hypocotyl explants, in addition to acetosyringone, we optimized several other conditions that include the *Agrobacterium* density 0.4 to 0.5 OD and duration of infection, 15 mins, co-cultivation not more than 48 hours along with BAP 2.0 mg l^{-1} in the callus-induction medium. Further, callus formation from hypocotyl was only observed when the transformed explants were incubated in dark. Thus, the generated callus can provide a more homogenous population of transformed cells from cotyledons and hypocotyls, potentially leading to more consistent transgene integration for subsequent regeneration. For selecting transformed explants, the pCBL101-RUBY vector uses kanamycin resistance under a maize ubiquitin promoter that is typically used in monocots. However, previous studies have demonstrated its effectiveness in dicot and gymnosperm as well, showing GUS expression in tobacco and GFP expression in Chinese fir, respectively (Schledzewski and Mendel 1994; Ye et al. 2023).

Direct shoot regeneration without a callus phase has been reported for several explants from sugarbeet namely *de novo* generation of adventitious shoots was noted using cotyledonary node region explants (Krens et al. 1996). Similarly, *Agrobacterium*-mediated sugarbeet transformation and direct shoot formation demonstrated for the below mentioned explants as shoot-base (Hisano et al. 2004), leaves (Norouzi et al. 2005), bud tips (Yang et al. 2005), leaf blade with attached shoot bases (Jafari et al. 2009), adventitious bud excised leaflets (Moazami-Goodarzi et al. 2020), and blade-petiole transition zone (Zhang et al. 2023). However, direct regeneration is often associated with reduced transformation efficiency, as bypassing callus formation can compromise uniform transgene integration. In the current study, we standardized several conditions in the culture medium that promoted the development of multiple shoots enabling the production of several transformed plants from a single hypocotyl-derived callus. Subsequently, conditions were established to induce roots in the regenerated shoots within two weeks on RIM-III. There could be many reasons in delaying root induction while using RIM-I and RIM-II, one could be cefotaxime that inhibits rooting in plants (Nauerby et al. 1997; Estopà et al. 2001) and other factors could be lower concentration of auxins used in RIM-I and RIM-II as compared to RIM-III. One hundred percent of the rooted plantlets survived when transferred to pots containing sterile sand and soil.

In recent years, RUBY has been used as a selection reporter for plant transformation due to the feasibility of readily visible red color at whole plant level, for example *Nicotiana benthamiana* (Yu et al. 2023). Traditional selection reporters like green fluorescent protein require special equipment to visualize transgenic events. About 10%

of cotyledons-derived callus was observed with visible color for RUBY expression, which persisted for up to two months. Various factors may underlie the lack of visible color in hypocotyl-derived callus and in the regenerated whole plantlets. The presence of *RUBY* gene was confirmed by sequencing the PCR amplicons in the transformed and regenerated sugarbeet plants. In addition, the expression of *RUBY* was confirmed with detecting the transcripts in RT-PCR in transformed lines. It is possible that in sugarbeet the lack of RUBY phenotype could be due to several factors including host specific differences, cellular mechanisms like gene silencing, transgene copy numbers, and other molecular mechanisms yet to be uncovered (Vaucheret et al. 2001; Vanitharani et al. 2003; Betti et al. 2021; Forestier et al. 2021). The possible somaclonal variation in the transformed sugarbeet lines was assessed using genetic markers as described previously (Srivastava et al. 2007; Izzatullayeva et al. 2014; Abekova et al. 2022). No polymorphism was detected based on the RAPD analysis of in vitro regenerated transgenic *B. vulgaris* lines with the non-transformed *B. vulgaris* plants. This confirmed that the uniformity of the regenerated plants was maintained, indicating high genetic stability among the transgenic *B. vulgaris* lines.

The successful production of stably transformed sugarbeet plants from hypocotyl explants reported here was achieved by optimizing explant selection, callus formation, *Agrobacterium* culture density, infection time, and co-cultivation duration and hormonal concentrations and acclimatization important for next generation analysis.

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Author contributions V. R: project conceptualization and design, data analysis, and manuscript writing and revision. Z. K: performed experiments, conducted data analysis, and prepared the manuscript draft. T. H: performed experiments. C. C and N. W: data analysis and manuscript revision. M. B: data analysis, provided resources, and revised the manuscript. All authors have read and approved the final manuscript for submission.

Data availability All the data supporting the findings of this study are available within the paper and its supplementary information file.

Declarations

Ethical approval Not applicable.

Conflict of interest The authors declare no conflict of interest.

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