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ORIGINAL ARTICLE

Low titre of agroinoculum with prolonged incubation period and low auxin concentration in the regeneration media are the key to high frequency of transformation in climate-resilient *Aus*-type rice genotype Nagina 22

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Abstract

Developing an efficient and reproducible regeneration protocol holds paramount significance for advancing genetic transformation technologies in rice, facilitating their utilisation in crop improvement. Nagina 22 (N22), a climate-resilient *Aus*-type rice genotype known for its tolerance against multiple stresses, lacks a standardised transformation protocol, limiting its utilisation as a background for genetic transformation. This study reports, for the first time, a highly efficient transformation and regeneration protocol for N22 using a CRISPR/Cas9 vector. Mature seeds were used to induce embryogenic calli on CHU(N6)-based callus induction media (CIM) with varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). The highest callus induction efficiency (~94%) was achieved using 3 mgL^{-1} 2,4-D. For regeneration, calli were transferred to different regeneration media-I (RM-Ia to RM-Ie), where a combination of 5 mgL^{-1} 6-benzylaminopurine (BAP) and 0.02 mgL^{-1} naphthalene acetic acid (NAA) resulted in ~44% regeneration frequency. Subsequent optimisation of regeneration media-II (RM-II) with low NAA concentration enhanced shoot elongation and root development. Furthermore, reducing basal salt concentration in the resuspension media significantly enhanced transformation efficiency to 44%, achieved, by only using sterile distilled water (SDW) with 150 mM acetosyringone for calli infection. The optimised protocol was successfully validated using CRISPR/Cas9 vector, facilitating targeted gene knockouts for functional genomic studies. This approach addresses a critical gap in N22 genetic transformation, providing a reliable protocol for advancing rice improvement through gene editing. It offers valuable insights for future research and practical applications in genetic transformation of this elite rice genotype for various agronomic and scientific purposes.

Keywords N22 · Genetic transformation · Rice · Plant regeneration · Stress resilience

Introduction

Rice (*Oryza sativa* L.) serves as a genomic model crop species within the monocot group and serves as the primary staple food for over half of the global population (Wang and Han 2022), which is cultivated in more than a hundred countries. Climate change and environmental degradation, consequences of human activities, pose a significant threat to food security. In such cases, the genotypes which are known for climate resilience and tolerance to multiple stresses, even if their yield is not very high, are extremely important because such genotypes can be used for studying the genes and their functional validation. Improvement

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of rice is a continuous process, where conventional crop enhancement and marker-assisted breeding approaches have been routinely utilised to achieve desirable agronomic traits (Ashkani et al. 2015). However, genetic engineering integrated with plant tissue culture has emerged as an alternative technique to provide a more precise and time-efficient method for crop improvement today (Hasnain et al. 2022). While there have been many studies on rice tissue culture, they are mostly on *japonica* rice cultivars, and the dependence on genotype still poses a challenge for the in vitro regeneration of *indica* rice (Kumria et al. 2000). There are many important rice genotypes, which continue to pose challenges for genetic modifications owing to their limited efficiency for regeneration (Sahoo et al. 2011). Therefore, it is crucial to develop a reliable and efficient protocol for consistently achieving a high frequency of plant transformation and regeneration using readily accessible explants, such as mature seed embryos. Though there are several reports on *indica* rice transformation with varied degrees of efficiency (Sahoo et al. 2011; Karthikeyan et al. 2012; Mostafiz and Wagiran 2018; Behera et al. 2019; Liang et al. 2021; Greenwood and Glaus 2022; Jayaraman et al. 2021; Yadav et al. 2023), there are no reports of regeneration and transformation in Nagina 22 (N22), an *Aus*-type rice genotype. Hence, our objective was to develop high-frequency regeneration and transformation in N22.

N22 is an important semi-dwarf, upland rice variety with a short bold grain having a brief cycle of only 85–102 days, renowned for its resilience to adverse environmental conditions like heat and drought (Poli et al. 2013; Sevanthi et al. 2018), which also possesses resistance to pests and diseases (Mohapatra et al. 2014). It is also utilised as a donor parent for drought-resistant breeding programmes globally, making it valuable germplasm for developing new rice cultivars against global warming. Beyond its agricultural utility, the genetic basis underlying its exceptional characteristics becomes a subject of scientific curiosity. Identification and isolation of the genetic factors responsible for its resilience is extremely important, which can be achieved through the strategic introduction of loss-of-function mutations affecting key traits, as well as gain-of-function mutations in genes pivotal to both, developmental processes and agronomic significance (Mohapatra et al. 2014). A large number of novel stress-tolerant genes have been identified during last 1 decade from this elite *Aus*-type genotype N22 (Reddy et al. 2002; Kulkarni et al. 2013; Poli et al. 2013; Panigrahy et al. 2014; Sevanthi et al. 2021). The gain of function of many of these genes from N22 have already been established in other rice genotypes (Araus et al. 2016; Wang et al. 2020; Jayaraman et al. 2021, 2023) as well as in heterologous system (Araus et al. 2016; Kumar et al. 2018, 2022). However, studying the loss of function of these novel genes is equally important, which could be achieved by knocking out these genes in N22 rice genotype. Hence, our primary objective was to optimise a high-efficiency regeneration and transformation protocol for *Aus*-type rice genotype N22. Since our major emphasis was on gene editing, standardisation of transformation protocol in N22 genotype with gene editing vector was very important, and hence, we used pRGEB32-carrying Cas9 gene for our study. Ultimately our goal is to optimize a transformation and regeneration protocol for gene editing as well as stable transgenic line development in the N22 background.

Optimising the media composition in the protocol is pivotal to bridge the gap and enhancing the success rate of callus proliferation, regeneration and transformation of the plants. During in vitro culture, achieving embryogenic potential involves careful manipulation of several factors, including explant source, medium composition, plant genotype and incubation conditions (Hesami et al. 2018). Several studies documented the application of different types of media, including Murashige and Skoog (MS), CHU (N6), and Linsmaier and Skoog (LS), along with varying concentrations of macro and micro salts (Lin and Zhang 2005; Yookongkaew et al. 2007; Karthikeyan et al. 2012). Among these, the CHU (N6) medium has been frequently utilised as a basal medium and has shown excellent callus induction efficiency (CIE) in rice (Herath et al. 2007; Behera et al. 2019). In addition, growth regulators commonly used in tissue culture, such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP), can be adjusted at different concentrations in the culture environment to improve callus induction and subsequent plant regeneration (Hesami et al. 2018). Furthermore, various concentrations of gelling agents have been utilised to desiccate the calli in regeneration media (Kumar et al. 2005). Nevertheless, these procedures present several bottlenecks in terms of transformation and regeneration efficiency, and hence there is a need for a reliable and reproducible approach for transformation and in vitro plant regeneration employing callus derived from mature seeds. In the present study, an attempt was made to establish a streamlined and enhanced in vitro regeneration process, offering significant utility for transformation in *Aus*-type rice genotype, N22.

Methods

Explants and embryogenic callus induction

Mature and healthy seeds of the N22 were used as explants. Seeds were dehusked, cleansed and then treated with 1% (w/v) Bavistin, followed by ethanol (70% v/v) for sterilisation. Subsequently, they underwent a 1% (v/v) sodium hypochlorite (NaClO) treatment for 1 h, followed by rinsing with sterile distilled water (SDW) for five times. After

drying the seeds on autoclaved Whatman paper no. 3, 15–20 seeds were placed in each Petriplate on the callus induction media (CIM) and kept in darkness at 28 ± 1 °C with a relative humidity of 60–85%. The details pertaining to specific media composition utilised in the study are provided in Supplementary file; Table S2. The pH of all the media was adjusted to 5.7 and 0.4% phytagel was added before autoclaving it for 20 min at 121ºC. Stock solutions of the plant growth regulators (PGRs) and other chemical components utilised in the study are provided in the Supplementary file; Table S1. To examine the callus induction efficiency (CIE), five CIM were prepared by augmenting varying concentrations of the 2,4-D (0–4 mgL^{-1}), and CIE was calculated using the formula given below:

$$\text{Callus induction efficiency}\,(\%) = [\text{Number of seeds with callus/Total number of seeds}] \times 100$$

After 14-day incubation period in darkness, morphological differences between embryogenic and non-embryogenic calli were recorded. Non-embryogenic calli, characterised as compact and non-friable with root-like structures, were removed, leaving only embryogenic calli for further processing. These embryogenic calli were then separated from the seeds and roots (if present), sub-cultured on fresh CIM, and were kept in dark at 28 ± 1 °C with a relative humidity of 60–85% for next 14 days to promote callus proliferation. Data regarding CIE were recorded after 4 weeks to identify the optimal combination of media suited for the N22 genotype. Subsequently, the most effective media combination was employed to examine the regeneration frequency in N22. The callus growth and morphology were examined using a stereo microscope (RSMR-8, Radical Trinocular Microscope, India).

Shoot regeneration

After 4 weeks, uniformly proliferated embryogenic calli (approx. size 5 mm x 5 mm x 5 mm) were first transferred onto regeneration media-I (RM-I) for 14 days at 28 ± 1 °C, with a relative humidity of 60–85% and 16 h light/ 8 h darkness (Sahoo et al. 2011). Only granular embryogenic calli were sub-cultured onto fresh RM-I media for the next 14 days under the same conditions, and then the calli were transferred to regeneration media-II (RM-II) for next 14 days. To optimise the RM-I media compositions, five different RM-I (RM-Ia to RM-Ie), consisting of BAP (2–6 mgL^{-1}) along with a very low concentration of NAA (0.02 mgL^{-1}), were used. Data regarding regeneration frequency (RF) were recorded after 4 weeks to identify the optimal combination of RM-I suited for the N22 genotype. For further development, regenerated shoots were transferred to a modified RM-II media, consisting of MS media with 5 mgL^{-1} BAP and a very low concentration (0.02 mgL^{-1}) of NAA (Supplementary file; Table S2) for shoot elongation. Regeneration frequency (RF) was calculated by the formula given below:

$$\text{RF}(\%) = [\text{Number of microcalli regenerated shoots /Number of microcalli incubated}] \times 100$$

The effect of various media formulations on embryogenic calli formation and regeneration was evaluated by testing different concentrations of 2,4-D in CIM and BAP in RM-I. Parameters such as embryogenic callus induction and shoot regeneration were recorded to determine the optimal media composition.

Rooting media and plant hardening

Regenerated shoots transferred to culture bottles containing rooting media (RooM) as described by Toppo et al. (2014) and maintained at 28 ± 1 °C in the light (16 h light/ 8 h darkness) for 7–14 days (Supplementary file; Table S2). Healthy plantlets with well-established root systems were transferred into pots containing a mixture of autoclaved soilrite for primary hardening. The healthy acclimatised plants were then transferred into larger pots (10`` diameter) filled with soil for growing them till maturity.

Vector and gene cloning

pRGEB32 vector (#63142; Xie et al. 2015) was used as the transformation vector. The Green Fluorescent Protein (GFP) gene was cloned into this vector as a reporter gene with minor modification as it contains an internal BsaI site. The GFP gene was PCR amplified from pCAMBIA1302 (ab275760) using the following primers; forward: 5'ATGGTCTCTTGCAGC AATTGAGACTTTTCAAC 3'; reverse: 5' ATGGTCTCC AAACGATCTAGTAACATAGATGACACCGC 3'. Cloning of gene of interest was usually done at BsaI site of the vector. As the gene for GFP contains an internal BsaI site (at 1022 position), it was altered by site directed mutagenesis (SDM) employing the following primers, forward: 5' TACGTCTCA AGATCACATGGTCCTTCTTGAGTTTG 3'; reverse: 5' TACGTCTCAATCTCTCTTTTCGTTGGGATCTTTCG 3' by altering G to T in forward and C to A in reverse primer (changes are shown by underline).

Transformation and selection of transformed calli

The calli were sub-cultured onto fresh CIM and kept in the dark (28 ± 1 °C) for 3 days before the transformation with *Agrobacterium tumefaciens* as described by Sahoo et al. (2011). *A. tumefaciens* strain EHA105 was transformed with the binary vector pRGEB32. This vector comprises the hygromycin phosphotransferase (*hptII*) and kanamycin (*kan*) genes as plant and bacterial selectable markers, respectively, along with the modified reporter gene GFP, under the control of CaMV35S promoter (Fig. 1). Primary culture for *Agrobacterium* was prepared by inoculating a single colony from the mother plate into 5 mL of luria broth (LB) medium supplemented with 50 mgL^{-1} kanamycin, 25 mgL^{-1} rifampicin. The culture was incubated in the dark at 28 °C on a rotatory shaker (Kuhner, Switzerland) operating for 48 h. For preparing the secondary culture, 500 µL of the primary culture was transferred into 20 mL of fresh LB medium containing the same antibiotic concentrations and incubated under identical conditions as mentioned earlier for the primary culture. However, $O.D_{600}$ of secondary culture (having *Agrobacterium*) was maintained at 0.4–0.6, which was pelleted by centrifugation at 5000 rpm for 10 min at 4 °C, and suspended in three different resuspension media (RsM) with a dilution factor of 1:10 for transforming the calli. For optimising the suitable RsM, three different RsM were used in the study (Supplementary file; Table S2). The bacterial suspension was incubated at 28 °C with gentle shaking. Meanwhile, the embryogenic calli were washed with SDW and prepared for inoculation. They were immersed in the *Agrobacterium* culture for 1 h with occasional gentle shaking (120 rpm) at 28 °C, followed by air-drying the calli on sterile Whatman no. 3 filter paper. The dried calli were then transferred to a co-cultivation media (CCM) containing acetosyringone and incubated for approximately 72 h at 28 ± 1 °C with a relative humidity of 60–85% in the dark (Supplementary file; Table S2). After 3 days of co-cultivation, the calli were rinsed twice with SDW, followed by a 30 min washing with SDW supplemented with 300 mgL^{-1} cefotaxime and 150 mgL^{-1} timentin with occasional gentle shaking at 120 rpm and incubation at 28 °C. Subsequently, the calli were washed thoroughly several times with SDW, dried on sterile Whatman no. 3 filter paper and transferred to selection media-I (SM-I) for 14 days incubation period at 28 ± 1 °C in the darkness (Supplementary file; Table S2). Following the first selection, dead or brown calli were removed, leaving only the healthy ones to be transferred to the selection media-II (SM-II) (Supplementary file; Table S2), maintained at 28 ± 1 °C in the darkness for the next 14 days.

Visualisation of GFP in the transformed calli

The expression of GFP in the successfully transformed calli was observed under a stereo microscope (RSMR-8, Radical Trinocular Microscope, India) with a GFP2 filter system (excitation filter 480/40 nm, emission filter 535/50 nm) after 3 weeks of infection.

Regeneration and development of transgenic plants

Shoot regeneration from the transformed calli was carried as described earlier using RM-I (Sect. "Shoot Regeneration"). However, agroinoculated calli with three RsM were transferred to five different RM-I media (3 RsM × 5 RM-I = 15 different combinations) to select the optimum media combination. Rooting, plantlet development and hardening were carried out as described earlier (Sect. "Rooting media and plant hardening"). A flowchart summarising the essential steps for transgenic plant development has been given in Fig. 2. Transformation efficiency (TE) was calculated by the formula given below:

$$TE(\%) = \left[\text{Number of confirmed transgenic plants} / \text{Number of calli inoculated with } \textit{Agrobacterium}\right] \times 100$$

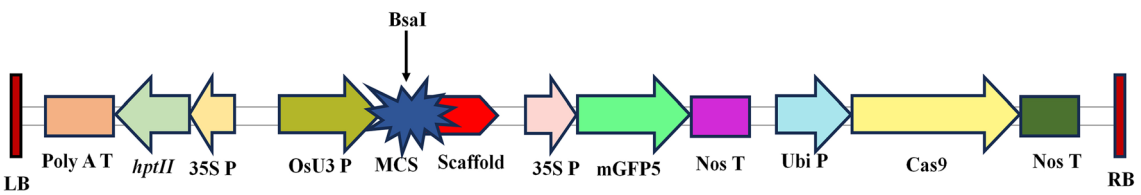

Fig. 1 Schematic representation of the gene construct used for *Agrobacterium*-mediated transformation of N22 callus

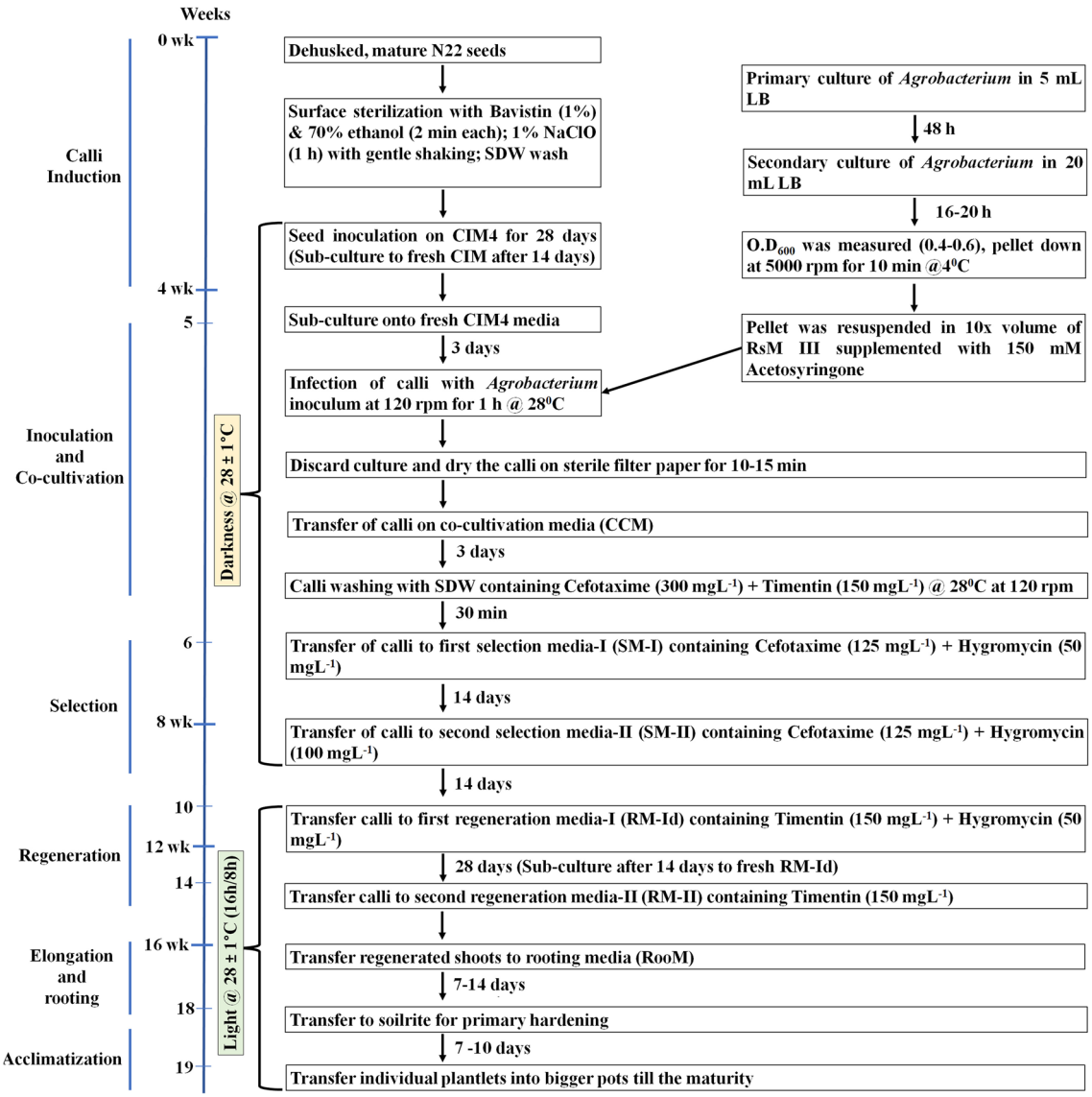

Fig. 2 Schematic workflow of *Agrobacterium*-mediated transformation protocol in Nagina 22 (N22) using embryogenic callus derived from mature seeds as source material

Molecular confirmation of putative transgenic plants

PCR analysis

Putative T_0 plants were confirmed by standard PCR method (Jayaraman et al. 2021) by randomly using DNA from the five putative transgenic plants, where the presence of hygromycin transgene was assessed using specific primers for *hptII*. To confirm the integration of T-DNA region, gene-specific PCR was also carried out for *Cas9* (primer details are provided in Supplementary file; Table S3). The PCR products were analysed on 1% (w/v) agarose gel containing ethidium bromide and visualised under the gel documentation system.

Southern blot hybridisation analysis of transgenic events

DNA sample from the same T_0 plants which were used for PCR confirmation was subjected to Southern blot analysis using *hptII* gene-specific probe. For this analysis, 10 µg genomic DNA was isolated from WT plants, which served as negative control, as well as from the PCR-positive plants. As a positive control, 25 pg genomic DNA from the binary vector pRGEB32 harbouring the hygromycin gene was used. The DNA was digested with the HindIII-HF restriction

enzyme (20 U/µl) (New England Biolabs, United Kingdom). The digested DNA fragments were resolved on 0.8% (w/v) agarose gel using 1X TAE buffer. The separated DNA was then transferred to the positively charged Nylon membrane (GE healthcare, Illinois, United States) according to the protocol outlined by Singh et al. (2023). A 968 bp gene-specific probe was prepared by labelling with digoxigenin (DIG) via probe labelling kit (Roche, Switzerland), and gene-specific primers. The labelled probe was diluted to 1–4 µg/ml in the ULTRAhybTM Ultrasensitive hybridisation buffer (Thermo Scientific, Waltham, MA, USA) and applied to the nylon membrane for hybridisation at 42 °C for 18 h. After the hybridisation, the membrane was washed, blocked, and incubated with anti-DIG AP-Alkaline phosphatase conjugated antibody and other reagents by following the standard protocol (Singh et al. 2023). The membrane was then exposed to X-ray film (Kodak Carestream, United States) for 2 min and developed.

Assessment of genetic fidelity

The clonal fidelity of 11 regenerated plants was compared with the wild-type (WT) mother plant using inter-simple sequence repeats (ISSRs) markers. In this study, a total of 9 primers (Supplementary file; Table S4) with clear bands were selected for amplification after initial screening of available 100 ISSRs primers from University of British Columbia (UBC 801 to UBC 900). PCR amplification was carried out as per the method of Shingote et al. (2019). The PCR products were then separated on 1.5% (w/v) agarose gel and the gel pictures were documented using the gel documentation system. The PCR amplification and gel electrophoresis were repeated twice with each selected primer.

Statistical analysis

All the experiments were repeated thrice with proper biological and technical replicates. The data analysed were subjected to analysis of variance (ANOVA) with Duncan's multiple range test (DMRT) and least significant difference (LSD) test at a significance level of $p \leq 0.05$ using the agricolae library of R package to observe significance differences among the studied variables. All the tables and figures were presented as mean of replicates ± standard error of means (SEM).

Results

In the present study, we initially optimised the media composition for embryogenic callus formation, followed by regeneration media optimisation using the callus derived from mature seeds of N22 genotype. Simultaneously, we have transformed the embryogenic calli using three RsM and used all the five RM-I (RM-Ia to RM-Ie) for studying the combinatorial effect of RsM x RM-I on transformation efficiency.

Optimisation of embryogenic callus induction in N22

In vitro callus induction was performed using dehusked mature seeds employing CHU(N6) media supplemented with varying concentrations of 2,4-D (1–4 mgL^{-1}), along with a control group where 2,4-D was omitted to evaluate the influence of different concentrations of 2,4-D on CIE. The distinct morphological features of embryogenic and non-embryogenic calli were observed under the Stereo microscope (Fig. 3). Calli derived from media supplemented with 3 mgL^{-1} 2,4-D (CIM4) appeared whitish, fragile, compact, nodular, and exhibited embryogenic characteristics. Conversely, media containing 4 mgL^{-1} 2,4-D (CIM5)

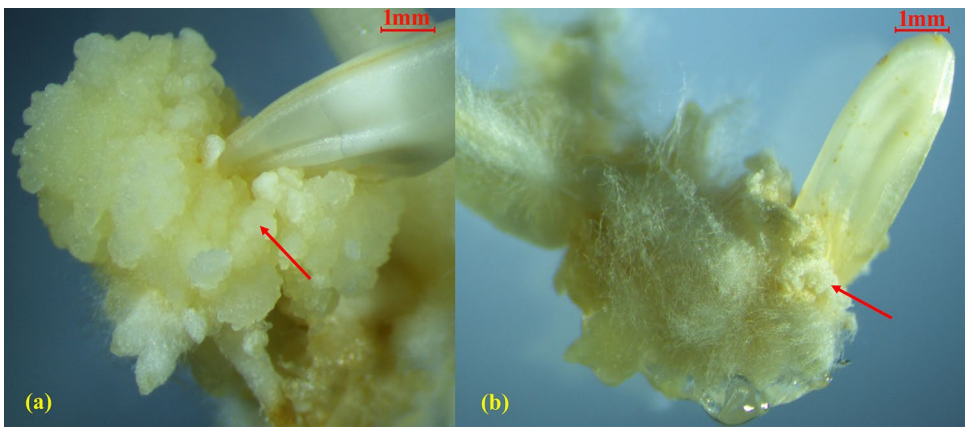

Fig. 3 Morphological differences between embryogenic and non-embryogenic N22 rice calli. **a** Embryogenic callus: yellowish-white, compact and nodular; **b** non-embryogenic callus: hard, necrotic, and hairy. (Bar = 1 mm)

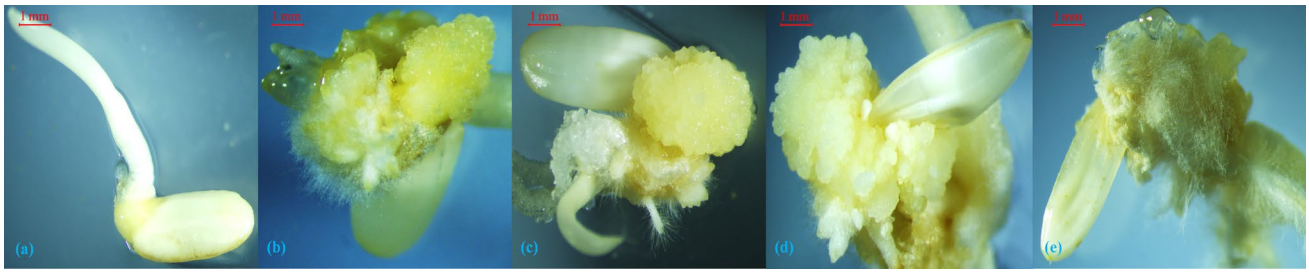

Fig. 4 The morphology of N22 calli grown on different formulations of callus induction media (CIM) with varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) was observed after 14 days of culturing. Distinct morphological responses to 2,4-D concentrations were as follows: **a** calli cultured on CIM1, which contains 0 mgL^{-1} of 2,4-D, show minimal/no callus induction; **b** CIM2, with 1 mgL^{-1} 2,4-D, induces moderate callus formation; **c** CIM3, containing 2 mgL^{-1} 2,4-D, promotes further callus proliferation; **d** CIM4, with 3 mgL^{-1} 2,4-D, shows optimal callus induction, yielding dense, compact calli; and **e** CIM5, at the highest concentration of 4 $mgL.^{-1}$ 2,4-D, induces substantial callus formation with a more nodular appearance. (Bar = 1 mm)

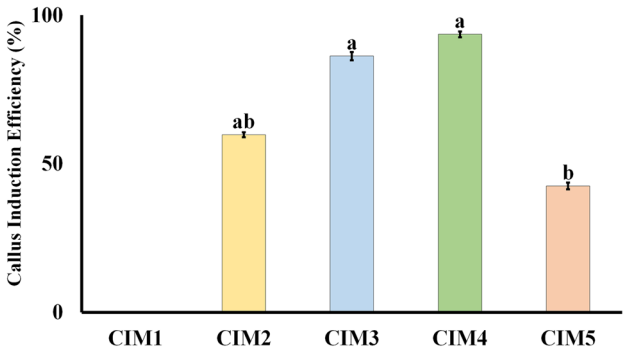

Fig. 5 Comparison of embryogenic callus induction efficiency from mature N22 seeds cultured on different callus induction media (CIM). The data represent the mean callus induction efficiency across various CIM formulations. CIM: callus induction media. Mean values marked with same letter do not differ significantly ($p \leq 0.05$) in DMRT test. Error bars represent ± SEM (n = 3)

depicted hard, necrotic, and hairy features, indicative of a non-embryogenic nature. No callus formation was observed in the control group (CIM1) lacking 2,4-D (Fig. 4).

The response of callus induction using different CIM was investigated 4–5 days after plating, and callus initiation was observed from individual CIM formulations. The results revealed a significant dependence of CIE on the concentration of 2,4-D in the media. A gradual increase in CIE was recorded from 1 mgL^{-1} to 3 mgL^{-1} of 2,4-D in the media, and above these concentrations (4 mgL^{-1} of 2,4-D), CIE declined significantly. We found the CIE ranged between 40–94% among the different media used for callus induction (Fig. 5) and no callus induction was found on the CIM1 which was used as a control. CIM4 supplemented with 3 mgL^{-1} 2,4-D was found to be optimum for N22 rice genotype with a CIE of ~ 94%.

Optimisation of media for shoot regeneration

For studying the regeneration frequency (RF) of proliferated embryogenic calli, CHU(N6) media were supplemented with a varying concentration of BAP (2–6 mgL^{-1}) along with a fixed concentration of NAA (0.02 mgL^{-1}). The greening of the calli on RM-I was observed after 18–20 days of sub-culturing (Fig. 6).

The RF of the non-transformed calli observed across the various regeneration media used in the study ranged from 29 to ~ 92% for the regenerated plants (RP) (Fig. 7). Different BAP concentration in the RM-I media showed significantly different results. A gradual increase in RF was observed from 2 mgL^{-1} to 5 mgL^{-1} BAP concentration in the media and above these concentrations (6 mgL^{-1} of BAP), RF declined significantly. Since a low concentration of NAA (0.02 mgL^{-1}) was kept constant, RM-Id media supplemented with 5 mgL^{-1} BAP and 0.02 mgL^{-1} NAA was found to be the optimum, which showed a RF up to 92% in case of RP. Most of the shoot buds were developed into multiple shoots after 3–4 weeks. After obtaining a large number of regenerated shoots, these were subsequently shifted to the rooting media (RooM) which led to the development of roots.

Optimisation of resuspension media for *Agrobacterium*-mediated transformation

For agroinoculating the calli, three different RsM were used. Agroinoculated calli were incubated in the selection media, and then few of them were observed after 3 weeks of infection (WAI) under the fluorescence stereo microscope for the visualisation of reporter gene expression. We observed green fluorescence due to GFP expression in the transformed calli, whereas no fluorescence was observed in control (WT) calli (Fig. 8a and b), which confirmed our construct is successfully integrated and the calli being transformed.

Fig. 6 Different stages of rice (N22) regeneration. **a** Induced embryogenic callus from N22 mature seed on callus induction media 4 (CIM4) after 14 days of incubation in dark, **b** excised embryogenic callus sub-cultured on fresh CIM4 for callus proliferation, **c** and **d** morphology of calli, after the first and second selection cycles in presence of hygromycin (indicated by red arrows), **e** and **f** greening and initiation of shoot development (indicated by red arrows) from embryogenic calli after first phase of regeneration on RM-Id, **g** plantlet development during the second phase of regeneration on RM-II, **h** plantlets transferred to rooting media (RooM), **i** transgenic plants grown and maintained in the phenotyping facility. (Bar = 1 mm)

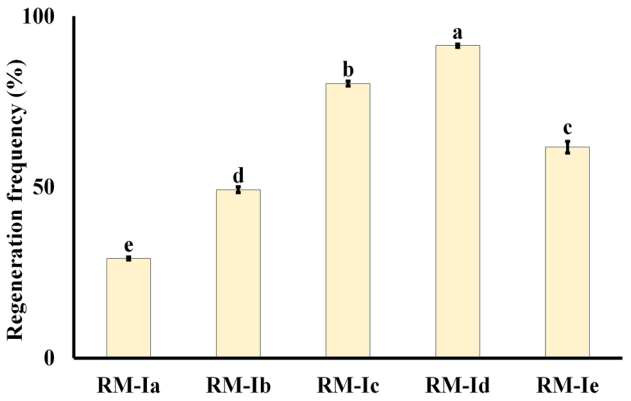

Fig. 7 A comparative analysis of regeneration frequency (%) for regenerated plants (RP) derived through embryogenic calli using different regeneration media is shown. The regeneration media are labelled as RM-Ia to RM-Ie, corresponding to different formulations of regeneration media -I, with their detailed compositions provided in the Supplementary File (Table S2). The RP represents the regenerated plants that were derived from the callus without *Agrobacterium*-mediated transformation (i.e., non-transformed calli). Mean values marked with same letter do not differ significantly ($p \leq 0.05$) in LSD test. Error bars represent ± SEM ($n = 3$)

We found that RM-Id is the optimum regeneration media for non-transformed calli. However, to confirm the same for transgenic calli, we have used all the five RM-I (RM-Ia to RM-Ie) for regenerating the transformed calli. All the three differently agroinoculated calli were transferred to 5 different RM-I, making it 15 different combinations. During this phase, we also observed few calli for GFP expression after 5 weeks and 7 weeks of *Agro*infection, and found the grown calli with higher fluorescence (Fig. 8c and d).

Over all, RF ranged between 1.5–47%, whereas TE after PCR analysis ranged between 1.5- 44% (Fig. 9). We found that the RsM III is the best resuspension media with all the five combinations of RM-I, and this confirms a novel resuspension media without having any basal salt. Interestingly, RM-Id was found to be the best among five RM-I with all the three combinations of RsM. This reconfirms the best regeneration is RM-Id. Out of 15 different combinations of RsM x RM-I, we found the highest number of transformations was with RsM III x RM-Id, and hence this is the most optimum media.

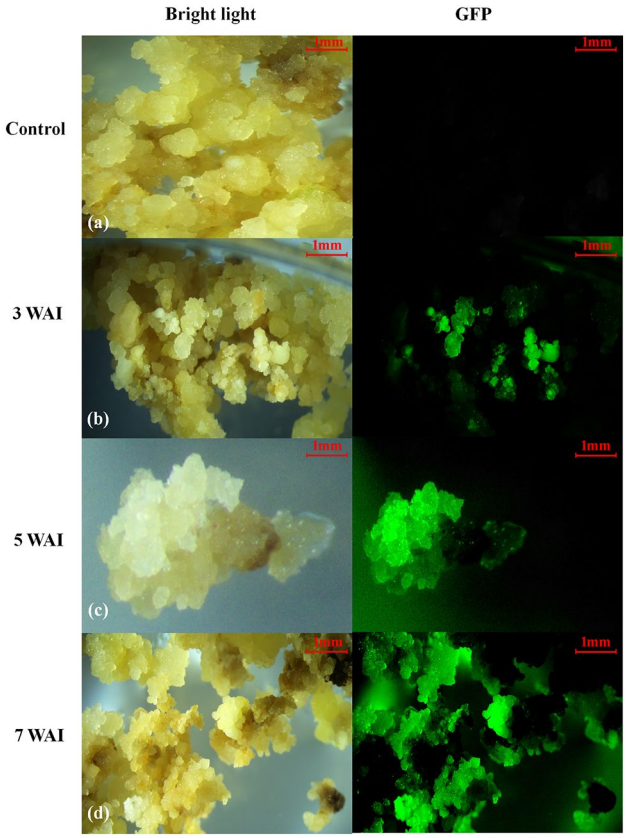

Fig. 8 A representative photograph of GFP expression in the transformed calli infected with *Agrobacterium* using RsM III observed under bright and fluorescence light. **a** Wild-type (WT) callus serves as a negative control, showing no GFP expression under fluorescence light, **b** GFP expression is visible in transgenic callus 3 WAI, demonstrating successful transformation, **c** GFP expression in transgenic callus 5 WAI, **d** GFP expression in transgenic callus 7 WAI, showing sustained fluorescence as the regeneration progresses. WT: wild type; WAI: weeks after infection. (Bar = 1 mm)

Molecular analysis

Confirmation of the T_0 putative transgenic plants (PTP) was carried out by PCR analysis using two set of primers specific for hygromycin (*hptII*) and *Cas9* genes. Agarose gel electrophoresis of the PCR products revealed the presence of a single band corresponding to 968 bp for *hptII* and 1499 bp for *Cas9* in the genomic DNA of only four PTP. No bands were observed in one PTP (sample N) for both gene (*hptII* and *Cas9*), indicating that the regenerated plant was likely a false positive and thus considered non-transgenic. No such amplicons were detected in the PCR products from the wild-type (WT) plant as well (Fig. 10a and b).

Transgene integration analysis using Southern blot in T_0 transgenic plants

Further assessment of the T-DNA integration pattern in T_0 PTP was conducted through Southern blotting. We analysed the same five plants taken earlier for PCR analysis, among which four were PCR positive (transgenic plants) and one was PCR negative (non-transgenic plant) along with control (wild-type). Transgene integration was re-confirmed by Southern hybridisation results and we found single as well as double copy inserts in the genomic DNA (Fig. 11).

Genetic fidelity testing of regenerated plants

Genetic fidelity testing of 11 regenerated plants concerning the mother plant (WT) revealed a total of 78 bands using 9 amplified primers. An average of 8.66 bands were produced per primer. Among the amplified primers, UBC 855 and UBC 864 resulted in a maximum of 13 bands followed by UBC 826 with 12 bands whereas UBC 857 produced a minimum of 5 bands (Fig. 12). The amplicon size ranges from 300 to 4500 bp. Amplification of mother plant (WT) along with the regenerated plants using ISSR markers showed uniformity among the plant samples.

Discussion

N22 rice genotype is known for its multiple abiotic and biotic stress tolerance (Mohapatra et al. 2014; Sevanthi et al. 2018), and several genes offering resistance to such stresses are present in this genotype. These genes can be exploited for conferring stress resistance to other varieties of rice and also to other crops by marker-assisted selection, transgenic approach or by gene editing (Adlak et al. 2019; Kumar et al. 2024), once they are functionally validated. Function of many genes from this genotype needs to be proved through loss or gain of function (Mohapatra et al. 2014), and the N22 genotype will be the best background for that. As there is no report on transformation of this genotype, our primary objective was to develop a robust transformation protocol and develop transgenic N22 genotype. There are several major components of regeneration followed by transformation, and we addressed every step and their optimisation.

For the optimisation of regeneration protocol from mature seeds, obtaining the embryogenic calli is a prerequisite. In rice, when mature seeds are used as explants, two distinct morphological phenotypes of calli are typically observed, which are critical for determining their further utility in tissue culture applications. Earlier studies (Sahoo et al. 2011; Behera et al. 2019; Greenwood and Glaus 2022; Yadav et al. 2023) have emphasised the

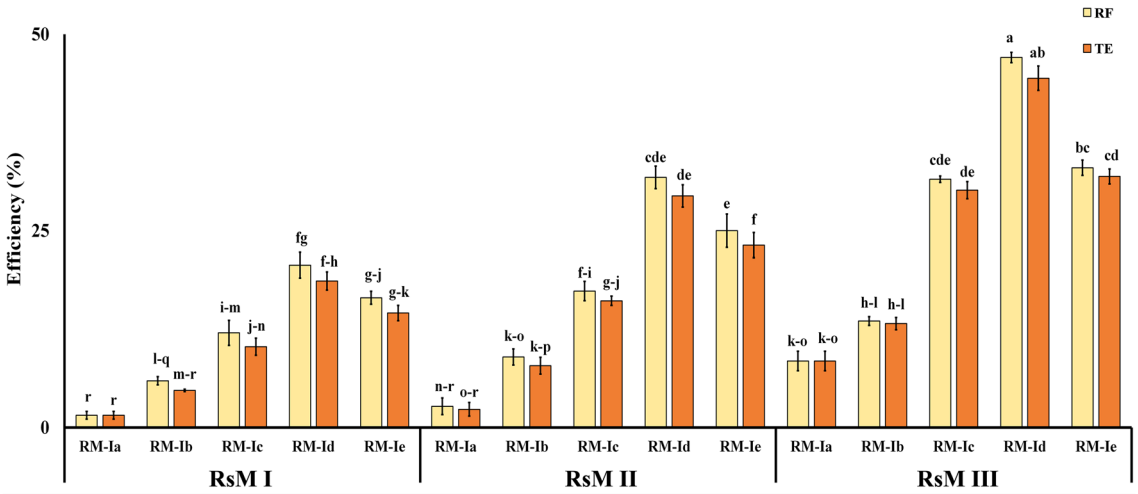

Fig. 9 Comparison of regeneration frequency (RF) and transformation efficiency (TE) of N22 rice calli in various RsM in combination with different RM-I formulations (RM-Ia to RM-Ie). RsM: resuspension media; RsM I: full strength CHU(N6); RsM II: ½ strength CHU(N6); RsM III: sterile distilled water [without CHU(N6)]; RM-I: regeneration media I. Mean values marked with same letter do not differ significantly ($p \leq 0.05$) in DMRT test. Error bars represent ± SEM ($n = 3$)

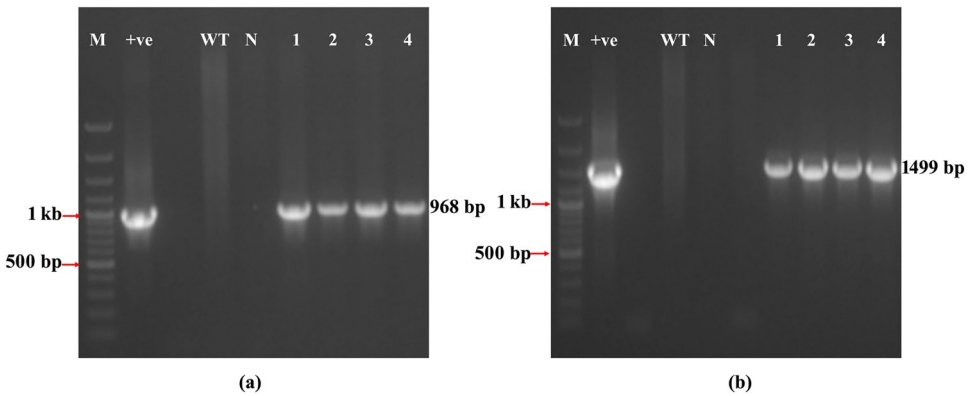

Fig. 10 Molecular confirmation of putative transgenic rice plants via *hptII* and *Cas9* gene-specific primers. **a** PCR amplification with hygromycin (*hptII*) primers showing an amplicon size of 968 bp, confirming the presence of the hygromycin phosphotransferase gene in transgenic plants, **b** PCR amplification with *Cas9* primers showing an amplicon size of 1499 bp, confirming the presence of the CRISPR/Cas9 gene editing construct in the transgenic plants. + ve: positive control (plasmid); WT: wild-type N22; 1 to 4: PCR positive (transgenic plants); N: PCR negative (non-transgenic plant); M: marker

importance of selecting embryogenic calli as a critical factor. These embryogenic calli were further utilised for the optimisation of regeneration protocol without focussing on the optimisation of somatic embryogenesis. The optimisation of embryogenic callus induction and further regeneration of these calli was the first part of our study, which is an essential pre-requisite for genetic transformation. The potentiality of callus induction and regeneration frequency depends on culture conditions like media composition, genotype and source of explants (Ali et al. 2023). Various studies have explored the development of embryogenic calli using different types of explants; among these, callus derived from mature seeds are particularly favoured due to their year-round availability and suitability for long-term storage (Saika and Toki 2010; Karthikeyan et al. 2012; Chen et al. 2023). In this study, dehusked mature seeds of the N22 *Aus*-type rice genotype was utilised as explants to de-differentiate into calli.

In our study, we optimised the CIM, and observed the development of compact, embryogenic calli with a creamish-white, globular appearance from mature seeds when cultured on CIM4 media. Similar morphological characteristics were also documented for embryogenic calli within 14 days, after inoculation of explant onto CIM in a few *indica* rice varieties such as IR64, Pusa Basmati 1, CSR10, and Swarna (Sahoo et al. 2011). Susanto et al.

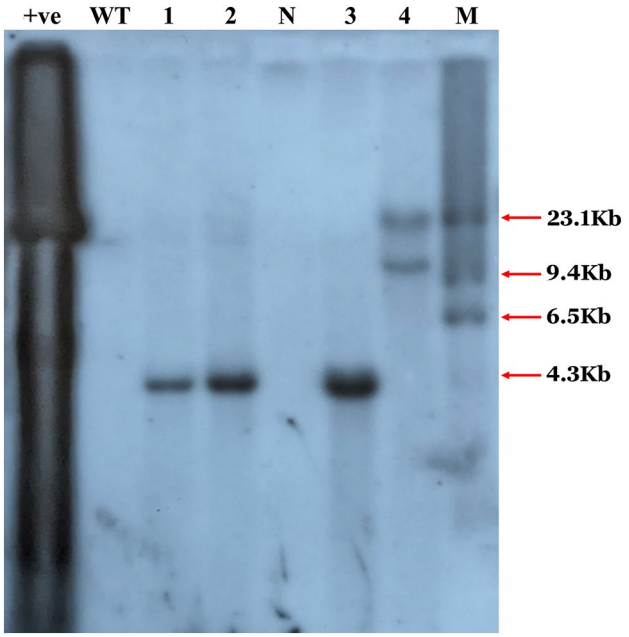

Fig. 11 Southern blot assessment of T_0 generation from *Agrobacterium*-mediated transformation of Nagina 22. The blot shows the hybridisation pattern of genomic DNA from transgenic and non-transgenic plants. The presence of distinct hybridisation signals in the transgenic lines (Plants 1–4) confirms the integration of the transgene(s) in the N22 genome, while the wild-type and non-transgenic plants show no signal. + ve: positive control (plasmid); WT: DNA of wild-type N22; Plant 1, 2, 3, and 4: independent transgenic lines of N22; N: non-transgenic plant (PCR negative); M: DIG-labelled marker

(2020) reported the CHU(N6) media supplemented with vitamins and 3% sucrose as the most effective culture media for rice callus induction; but in our study, we have added myo-inositol (100 mgL^{-1}) to CIM as it is known for supporting growth, imparting antioxidant properties, and forming the structural basis of numerous signalling molecules that aid in coping with various stresses (Al-Mushhin et al. 2021). Optimum concentration of auxin in the media is known to be primary contributor for the callus formation (Naik et al. 2017). There are reports on application of combination of auxin (2–4-D) and cytokinin (BAP) in the CIM, where gradually the concentration of cytokinin is increased and auxin is reduced during sub-culturing for shooting and regeneration (Sahoo et al. 2011; Behera et al. 2019; Liang et al. 2021; Yadav et al. 2023). There are also reports where only auxin is used in the CIM (Vega et al. 2009; Pawar et al. 2015; Mostafiz and Wagiran 2018; Greenwood and Glaus 2022). One such report mentioned 2 mgL^{-1} of auxin (2,4-D) was the optimum, and more than that was inhibiting the callus induction and proliferation in *indica* rice genotypes, viz. Pawana, Jaya, Indrayani and Ambemohar (Pawar et al. 2015). We experimented with five different concentrations (0–4 mgL^{-1}) of 2,4-D in the CIM and found CIM4 with 3 mgL^{-1} was the best, resulting in approximately 94% CIE for N22. So far, callus induction efficiency in *indica* rice is reported up to 97% in CSR10 genotype (Sahoo et al. 2011) but mostly ranged between 75–90% (Mostafiz and Wagiran 2018; Behera et al. 2019; Greenwood and Glaus 2022).

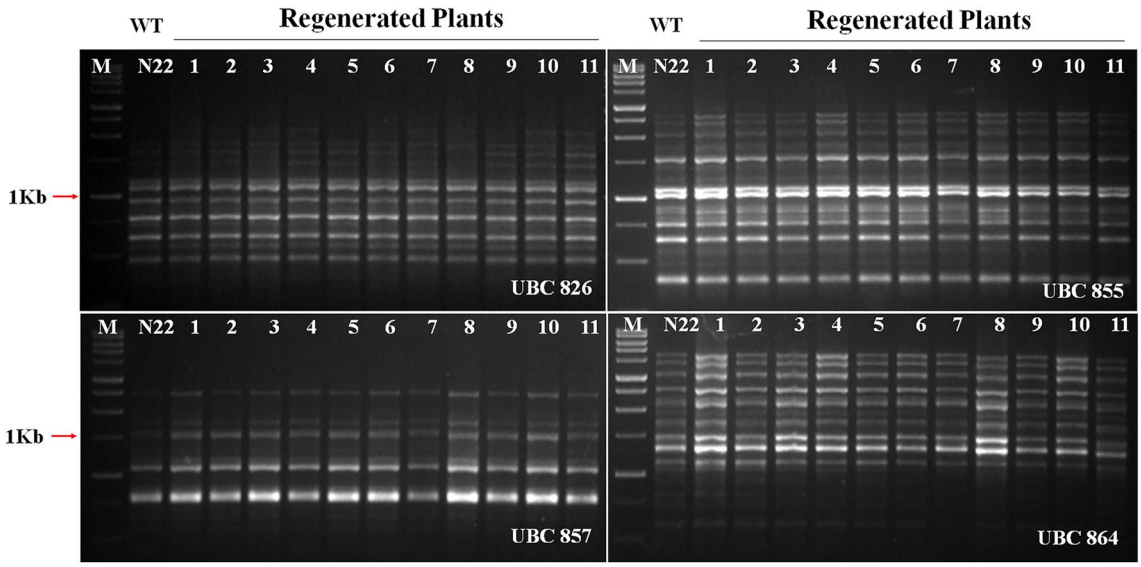

Fig. 12 ISSR amplification profiles of four representative ISSR primers (UBC 826, UBC 855, UBC 857 and UBC 864) for genetic fidelity testing in N22 genotype of rice. The figure shows the uniformity of amplification patterns across regenerated plants confirming that the regenerated plants maintain genetic stability, with no deviation from the wild-type pattern. WT: wild-type N22 plant and sample 1 to 11: regenerated N22 plants showed uniform amplification in all regenerated plants; M: marker

Our protocol exhibited a very high CIE (~ 94%), specifically for N22 genotype.

The subsequent phase involves developing a reliable media composition to facilitate the regeneration of derived embryogenic callus into shoot and root growth. There are reports of using different basal media, namely MS (Murashige and Skoog 1962), CHU(N6) (Chu et al. 1975) and LS (Linsmaier and Skoog 1965) for regeneration (RM-I), which are supplemented with different concentrations of cytokinin and a low concentration of auxin. A low auxin–cytokinin ratio has a considerable effect on induced callus regeneration (Alam et al. 2012; Mostafiz and Wagiran 2018), which significantly impacts cell growth signalling (Wang and Ruan 2013). Of these, CHU(N6) was reported to give a better regeneration frequency (Herath et al. 2007; Tariq et al. 2008; Sundararajan et al. 2017). We used CHU(N6) as our basal media where we experimented with different concentrations of cytokinin (2–6 mgL^{-1} of BAP), and a fixed concentration of auxin (0.02 mgL^{-1}). Other than PGRs, mostly 3% sucrose is used as main source of carbohydrate for successful regeneration of rice callus (Lee et al. 2002). However, efficiency of regeneration increases with induction of desiccation (Chand and Sahrawat 2001; Saharan et al. 2004), and therefore, either mannitol or sorbitol is used as a desiccating carbohydrate in addition to sucrose (Jain et al. 1996; Huang and Liu 2002; Feng et al. 2011; Lee and Huang 2014). Sorbitol is reported to be better than mannitol in many cases (Jain et al. 1997, 2022; Repalli et al. 2017), and hence we used 3% sorbitol in all the five RM-I media. Finally, we found RM-Id was the optimum regeneration media comprising CHU(N6) basal, 5 mgL^{-1} BAP, 0.02 mgL^{-1} NAA along with sorbitol as desiccant carbohydrate, and sucrose as carbon source. Though 5 mgL^{-1} BAP concentration in RM-I media is reported to be optimum (Noor et al. 2022), no report is found where as low as 0.02 mgL^{-1} of NAA is used in RM-I before our study. During second phase of regeneration, either MS or CHU(N6) is reported as basal media in RM-II for rice regeneration (Paramasivam and Ann 2020). We used MS as basal in the RM-II media, as this was reported as superior over CHU(N6) in case of *indica* rice regeneration (Mostafiz and Wagiran 2018; Behera et al. 2019).

Optimising a robust transformation protocol is imperial for developing transgenic plants. Our objective was to streamline the key attributes for successful rice transformation and develop a simplified and efficient protocol for N22 rice genotype. Among the available transformation methods, *Agrobacterium*-mediated transformation is widely preferred due to the ability of *Agrobacterium* to produce a large number of independent transformation events. Previous studies have highlighted that factor such as explant types, *Agrobacterium* cell density, inoculation period, vector size, and co-cultivation conditions have impact on T-DNA delivery efficiency (Amoah et al. 2001; Wu et al. 2003). A very important step in this context is optimising the RsM employed in *Agrobacterium*-mediated transformation and the cell density in the RsM. Usually, cell density of 0.6–0.8 in the secondary cell culture is pelleted down and then suspended with same volume (1x) of RsM for callus transformation (Karthikeyan et al. 2012; Islam and Md 2015; Behera et al. 2019). However, usually, when the cells are diluted, the inoculation period is also increased (Niedbala et al. 2021; Jain et al. 2022). We have diluted the cell density to great extent, where *Agrobacterium* cells with $O.D_{600}$ of 0.4–0.6 in secondary culture were pelleted and suspended in 10 × volume of RsM. Since, the reported inoculation time is 20–30 min (Behera et al. 2019; Greenwood and Glaus 2022) with the cell density of 0.6–0.8 suspended in 1 × RsM, we further increased the inoculation time to 1 h because the cell density in the RsM was much less. This finding suggests that transformation with much lower cell density is enough compared to those earlier reported studies (Nishimura et al. 2006; Sahoo et al. 2011; Greenwood and Glaus 2022).

Mostly, the same basal media is used in RsM, which was used in CIM (Sahoo et al. 2011; Karthikeyan et al. 2012; Greenwood and Glaus 2022; Yadav et al. 2023). Since, we have used CHU(N6) in the CIM, we had used the same basal media in RsM also. Reports are available on use of full strength, ½ strength and ¼ strength basal media in the RsM (Karthikeyan et al. 2012; Yadav et al. 2023). Notably, reducing strength of basal media in the RsM significantly increases the transformation efficiency as elevated salt concentrations can induce osmotic stress in bacteria, leading to dehydration and reduced survival. The elevated salt concentration also influences *Agrobacterium* attachment efficiency to the plant surface (Cheng et al. 1997; Karthikeyan et al. 2012; Yadav et al. 2023). On the other hand, lower salt concentration enhances *vir* gene expression in *Agrobacterium*, boosting T-DNA transfer efficiency and integration into the plant genome (Anand et al. 2008; Subramoni et al. 2014; Yadav et al. 2023). Furthermore, lower salt concentration in RsM mimics the plant cell environment, ensuring optimal transformation rates and emphasising its importance in experimental design for efficient genetic modification of plants (Gelvin 2003, 2010). So far, ½ strength MS media in the RsM showed highest transformation efficiency in *indica* rice genotype (Yadav et al. 2023). We tried to optimise the strength of CHU(N6) as basal media in the RsM for transformation and used full strength (RsM I), ½ strength (RsM II) and only sterile distilled water (RsM III) [without CHU(N6)] as basal media. As reported earlier, ½ strength MS basal showed significantly higher transformation efficiency of calli (44%). But to our surprise, the transformation efficiency was found significantly higher than ½ strength basal media (~ 30%) when only sterile distilled water was used (44%).

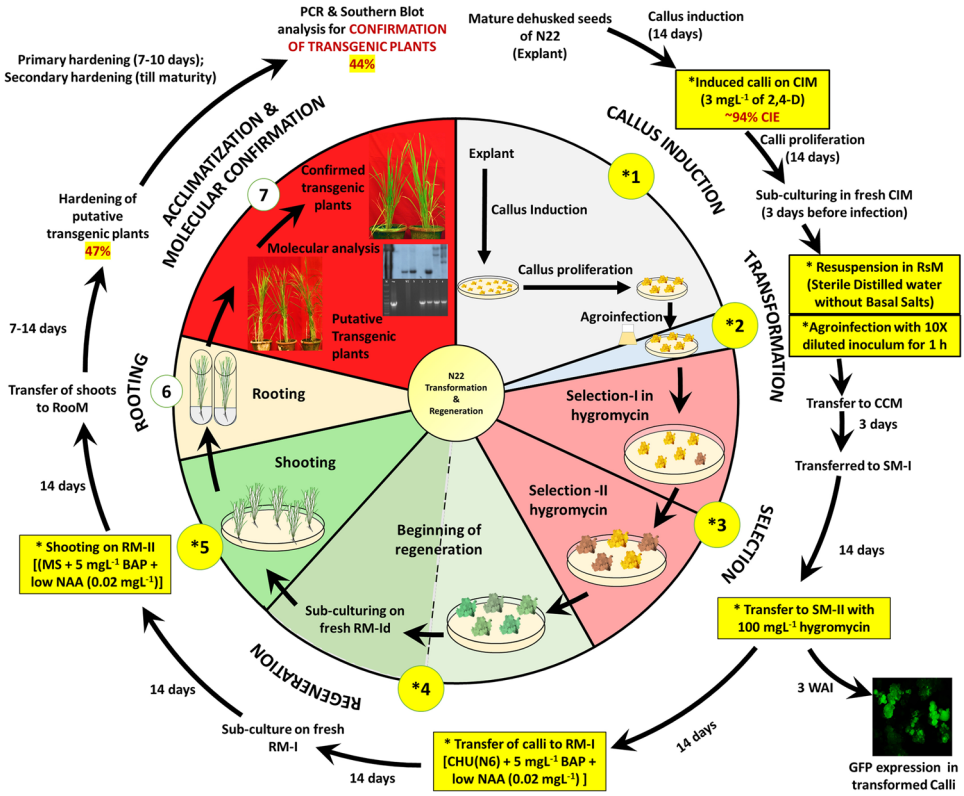

Fig. 13 A schematic illustration of highly efficient transformation and regeneration steps using the embryogenic calli derived through mature seeds of Nagina 22 (N22). (1) Callus induction media (CIM) optimisation on CHU(N6) with varying 2,4-D concentrations (0–4 mgL^{-1}), highlighting the optimal concentration (3 mgL^{-1}) which resulted in ~ 94% callus induction efficiency (CIE); (2) high transformation efficiency (TE) was achieved by reducing salt concentration in resuspension media (RsMIII; sterile distilled water without basal salts), agroinfection with 10 × diluted inoculum and longer inoculation period (1 h) for infection, are the key parameters, leading to a notable 44% TE; (3) higher hygromycin concentration (100 mgL^{-1}) in SM-II is found to be the optimum for the selection of transformed calli; (4) regeneration media-I (RM-I) optimisation testing different BAP (2–6 mgL^{-1}) and a very low NAA (0.02 mgL^{-1}) concentrations, with the ideal combination of 5 mgL^{-1} BAP and 0.02 mgL^{-1} NAA achieving 47% regeneration frequency from infected calli; (5) regeneration media -II (RM-II) was modified using MS media along with low NAA (0.02 mgL^{-1}); (6) regenerated shoots were transferred to rooting media (RooM); (7) healthy plantlets with well-developed roots and shoots were transferred to bigger pots for acclimatisation. Molecular analysis of the putative transgenic plants (PTP) was carried out through PCR and Southern blotting. Yellow highlights (*) indicate the steps where media have been optimised or modified

In our optimised protocol, we have used higher concentration of hygromycin in the selection media (100 mgL^{-1} in SM-II), whereas most of the earlier reports mentioned the use of 50 mgL^{-1} hygromycin in SM-I and SM-II, respectively (Sahoo et al. 2011; Sundararajan et al. 2017; Greenwood and Glaus 2022; Yadav et al. 2023). We also started with the lower concentration (50 mgL^{-1}), but most of the calli survived, and hence, we increased the selection pressure (100 mgL^{-1}), which led to very high percentage of transgenic plants (> 96%) from the total number of putatively regenerated transgenic plants. In our study, we used GFP as the reporter gene, which showed the integration of transgene in the callus as first level of confirmation.

As we mentioned earlier, several stress tolerance genes in this unique genotype N22 have been identified (Reddy et al. 2002; Poli et al. 2013; Kulkarni et al. 2013, 2014; Panigrahy et al. 2014; Sevanthi et al. 2021). For their functional validation by loss of function, we wanted to go ahead with CRISPR/Cas9-mediated gene editing, which required a high-frequency transformation protocol. Since no transformation protocol was available for N22 *Aus*-type rice genotype, we standardised it in the present study. But instead of developing a simple transformation protocol for N22 *Aus*-type rice, we wanted to develop a transformation protocol with gene editing vector which having Cas9 gene. That was the main reason of using pRGEB32 as transformation vector, instead of routine plant transformation vector like pCAMBIA. Interference of Cas9 protein in the transformation efficiency was not a matter of concern in our standardised protocol, because after optimising different components, the transformation efficiency we obtained was quite high (44%), which was either on par or better than the reported high

efficiency transformation in *Japonica* as well as *Indica* rice (Sahoo et al. 2011; Behera et al. 2019; Liang et al. 2021; Yadav et al. 2023). This makes our protocol more robust, and can be used easily for gene editing as well as for developing GMO from N22.

Since, rice tissue culture is not used for micropropagation, and mainly used for genetic transformation, no reports on genetic fidelity testing for the regenerated plants have been reported. But we found this aspect is important because somaclonal variation and genetic change due to tissue culture may interfere with the functionality of the transgene. Hence, even for transgenic development, a protocol should be such which is genetically true to type and does not cause any change in the regenerated plants. To rule out the possibility of genetic variation caused due to chromosomal breakage, deletions, mutations, chromosomal and gene rearrangements, and activation of transposable elements using varying culture growth conditions, chemicals and hormones in the media (Larkin and Scowcroft 1981; Liu et al. 2004), genetic fidelity test was also carried out using 11 regenerated plants along with the parent. We found our optimised protocol for transformation, especially meant for N22 genotype, did not cause any genetic variation.

Conclusion

This study aimed to develop a high-efficiency transformation and regeneration protocol for the important stress-tolerant rice genotype N22. To optimise the protocol, we substantially modified CIM, RsM and RM-I media, and achieved a very high efficiency of regeneration (92%) and transformation (44%). Here, we wanted to establish the transformation protocol using a complex CRISPR/Cas9 vector, ensuring the successful integration of the Cas9 gene into the plant genome. Since it is only standardisation of the protocol for transformation and regeneration, we did not incorporate any gRNA in this study. Moreover, our protocol is not only robust, but also, we found that the genetic fidelity is not disturbed and the regenerated plants developed are true to parent-type N22. After establishing the successful transformation and regeneration protocol using CRISPR/Cas9 vector, our future work plan is to proceed with gene editing in N22 using specific gRNA for studying the functional validation of the genes. This protocol will serve as a valuable source for conducting functional genomics studies to characterise various genes in rice, especially in N22 genotype. The summary of the entire protocol with all the crucial steps and modifications is illustrated in Fig. 13.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13205-025-04210-y.

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Author contributions PKM conceptualised the work, designed the experiment, did the fund acquisition, supervised the study, edited critically and finalised the manuscript. AK, P, JK, MK, EM, BS, AUS performed the experiments. AK performed molecular cloning, plant tissue culture, compilation of data, figures and wrote the first draft. P performed plant tissue culture and southern blot analysis. JK performed southern blot analysis. MK performed the statistical analysis. EM performed molecular cloning. BS and AUS performed ISSR analysis. MK, MS, JR, SP, JM, AMS, AUS, PK reviewed and edited the manuscript.

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Data availability The data supporting the findings of this study are provided in the main text and supplementary file.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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