Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Improved method for regeneration and *Agrobacterium*-mediated transformation of Indian short-day onion (*Allium cepa* L.)

TUSHAR KASHINATH MANAPE
Directorate of Onion and Garlic Research, Rajgurunagar, Pune, Maharashtra 410 505, India
Viswanathan Satheesh
Shanghai Center for Plant Stress Biology and Center of Excellence in Molecular Plant Sciences, Chinese Academy of Sciences, Shanghai, 200032, China
Shweta Singh
Biosystems and Integrative Sciences Institute (BioISI), Plant Functional Biology Center, University of Minho, Campus de Gualtar, 4710057, Braga, Portugal
Major Singh
Directorate of Onion and Garlic Research, Rajgurungar, Pune, Maharashtra 410 505 India
Sivalingam Anandhan (anandhans@gmail.com)
Directorate of Onion and Garlic Research, Rajgurunagar, Pune, Maharashtra, 410 505 India

Research Article

Keywords: Agrobacterium, callus induction, onion, seedling radicle, transformation

Posted Date: May 7th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-316361/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on September 1st, 2021. See the published version at https://doi.org/10.1007/s11240-021-02161-9.

Abstract

A high-auxin medium, usually used for callus induction, was not effective for Indian short-day onion cv. Bhima super. In this study, we found that the onion seedling radicle was a better explant than shoot tip for embryogenic callus induction, and induction efficiency up to 85.33% along with high embryogenic calli weight was obtained in routinely used medium containing 1.0 mg/L 2,4-D, but specifically supplemented with 0.5 mg/L kinetin. MS medium supplemented with 1.5 mg/L kinetin and 0.125 mg/L ABA showed 73.15% shoot regeneration efficiency from the calli induced from seedling radicle. Geneticin and hygromycin B at 50 mg/L showed optimal selection pressure for 8-week-old onion calli. *Agrobacterium*-mediated transformation of 8-week-old friable embryogenic calli induced from seedling radicle resulted in phenotypically normal transgenic plants with 1% transformation efficiency. In this study, regeneration and transformation protocols were developed for a widely used Indian short-day onion cultivar, which is instrumental for the development of stable transgenics in this crop.

Introduction

Stable genetic transformation of onion (*Allium cepa* L.) has been reported in various genotypes with variable transformation efficiencies. The transformation efficiencies in onion cultivars Atlas and Kunning were different due to genotype-specific response of the calli induced from zygotic mature embryo (Zheng et al. 2001). Variable transformation efficiencies have been reported in other genotypes but the variation could be due to the type of explant and culture medium used in these studies. Eady et al. (2000, 2003) reported 0.9 to 2.7% transformation efficiency in onion cultivar Canterbury Longkeeper using immature embryos as an explant cultured on picloram-containing medium. Aswath et al. (2006) reported 25% average transformation efficiency in cultivar KU-31 from the calli induced from seedling radicle on 2,4-D-containing culture medium. In a study comparable to ours using an Indian variety Indian marshal, a transformation efficiency of 2.5% was reported from the calli induced from seedling radicle on 2,4-D-containing culture medium. Taken together, these results indicated that transformation efficiency of onion was dependent on genotypes, culture medium and explant types used in the transformation studies. Therefore, it is imperative to develop a genotype-specific transformation protocol using an appropriate explant and culture medium.

Transformation studies conducted during the initial stages of onion transformation protocol development (before year 2006), immature and mature zygotic embryos were used as explants for transformation (Eady et al. 2000, 2003; Zheng et al. 2001, 2005). In later studies, seedling radicle was the preferred explant probably due to frequent contamination in immature embryos, difficulty in isolation of mature zygotic embryos and, most importantly, high callus induction efficiency of seedling radicle/root tip (83.6%, Khar et al. 2005) as compared to immature embryos (40%, Eady et al. 1998) and zygotic mature embryos (58%, Zheng et al. 1998). Simultaneously, high transformation efficiency (2.5 to 25%) has been reported in seedling radicle (Aswath et al. 2006; Kamata et al. 2011; Naini et al. 2019) as compared to immature embryos (0.9 to 2.7%) (Eady et al. 2000, 2003) and zygotic mature embryos (0.15 to 3.68%)

(Zheng et al. 2001, 2005). Hence, the selection of appropriate explants plays an important role in determining the labour-intensiveness and efficiency of onion transformation protocol.

The selection of an appropriate selection agent is also an important factor for producing transgenics. The selection agent plays a major role in selecting transformed-calli and hence affects transformation efficiency. For the selection of transgenic onion-calli, different antibiotics such as kanamycin, hygromycin B, geneticin (Eady and Lister 1998, Eady et al. 2000, 2003; Zheng et al. 2001, 2005; Naini et al. 2019) and a non-antibiotic selection marker such as phosphomannose isomerase (PMI) (Aswath et al. 2006), was used. It was reported that kanamycin was ineffective for the selection of onion transgenic-calli obtained from an immature embryo (Eady and Lister 1998). Explants were found to be highly sensitive to mannose concentration and ceased to form shoots at high concentrations (Joersbo et al. 1998; Degenhardt et al. 2006). Therefore an elaborate preliminary study is required to calibrate the concentration of mannose in every selection stage before using PMI as a selection agent (Stoykova and Stoeva-Popova, 2011), which is a cumbersome process. However, stable transgenic onion plants were obtained using 50 mg/L hygromycin B (Zheng et al. 2001, 2005; Kamata et al. 2011; Naini et al. 2019) and 20-50 mg/L geneticin B (Eady et al. 2000, 2003) with relatively less effort.

For the successful transformation of any crop, callus induction from explants is an important first step and a critical parameter for transformation. The type, physical appearance and embryogenic potential of calli used for the transformation affect the overall transformation efficiency. In onion, picloram- and 2,4-D-containing culture medium have been used for callus induction from different explants during transformation of calli induced from different onion explants. MS medium supplemented with 1.0 mg/L 2,4-D have been used for callus induction from mature zygotic embryos (Zheng et al. 2001, 2005) and seedling radicle (Aswath et al. 2006; Naini et al. 2019) while producing transgenics. But, in our in-house experiment, this routinely used callus induction medium failed to induce significant number of calli from both shoot tip and seedling radicle explants. Hence there was a need to significantly improve the regeneration and transformation protocols.

In the present study, we compared two explants; shoot tips and seedling radicle of onion for callus induction and regeneration studies. We studied the effect of auxins and cytokinin on callus induction as well as cytokinin and abscisic acid (ABA) on shoot regeneration. We also optimized the selection agent for selection of transformed-calli. *Agrobacterium*-mediated transformation was carried out with the callus induced from seedling radicle and transgenic plants were regenerated. Finally, we report an efficient regeneration and *Agrobacterium*-mediated transformation protocol for the Indian short-day cultivar, Bhima super using seedling radicle as an explant.

Materials And Methods

Plant material and growth conditions

Genetically pure seeds of *Allium cepa* cv. Bhima super were used to get explants. Surface-sterilized seeds were germinated on B5 medium at 26 ˚C in dark for 72 h (Fig. 1a) and 120 h (Fig. 1d). Seedling radicle

and shoot tip excised from the germinated seeds were used as explants for *in vitro* regeneration.

In vitro regeneration and hardening of plantlets

Seedling radicle and shoot tips of 3-4 mm length (Fig. 1b and 1e) were excised from the dark-grown onion seedlings after 72 and 120 h of germination, respectively. Explants were placed in Murashige and Skoog (MS) medium containing 3% sucrose, 7 g/L agar and supplemented with different concentrations of 2,4-D or picloram (0.5, 1.0 and 2.0 mg/L) alone or in combination with kinetin (0.5, 1.0, 2.0 and 3.0 mg/L), grown in dark condition for callus induction (Supplementary Table 1 and 2). Grown calli mass was chopped out after 6 weeks and sub-cultured for another 2 weeks in the fresh medium with same media composition and maintained under similar conditions. Growth of 6-week- and 8-week-old calli was characterized in terms of colour, toughness, number and weight of callus. The experiment was carried out with 3 replicates for each treatment with 25 numbers of explants in each replicate (Fig. 1c and 1f). The best medium for callus induction and growth is designated as CI medium (shown by bold letters in supplementary Table 1).

About 8-week-old calli, obtained in CI medium, were transferred into MS medium supplemented with different concentrations of kinetin (1.0, 2.0 and 3.0 mg/L) alone or in combination with different concentrations of abscisic acid (ABA) (0.125, 0.25, 0.5 and 1.0 mg/L) for shoot regeneration (Table 1). The culture was maintained in 16 h light/ 8 h dark photoperiod at 23 ˚C temperature. The experiment was carried out with 3 replicates of each treatment with 8 number of calli in each replication. The best medium for shoot induction is designated as SIM (shown by bold letters in Table 1). Regenerated shoots were transferred to half-strength MS medium containing 3% sucrose and 7 g/L agar (rooting medium) to obtain roots.

The roots of regenerated plantlets were dipped into 0.1% bavistin solution for 5 min and transferred to the pot containing an autoclaved mixture of soil, sand and vermicompost in 3:1:1 ratio. Later, it was allowed to form a bulb in the green house at 25 ˚C in 16 h light / 8 h dark period.

Comparison of selection agents: hygromycin and geneticin

Eight-week-old embryonic calli (3-4 mm^2) induced from seedling radicle in CI medium were cultured in CI medium supplemented with different concentrations of hygromycin B (25, 50, 75 and 100 mg/L) and geneticin (25, 50, 75, 100, 125 and 150 mg/L) separately, at room temperature in the dark. The control treatment was also maintained without supplementing any selective agent. After 2 weeks, the calli were sub-cultured in the fresh medium with the same composition following removal of browned/bleached portion and maintained under similar conditions. The plates were observed for browning or bleaching of calli and data on survival were recorded after 4 weeks. The experiment was carried out with 3 replicates of each treatment with 10 numbers of embryonic calli in each replication.

Agrobacterium strain and plasmid

The plasmid pCAMBIA 1305.1 was mobilized into *A. tumefaciens* strain LBA4404 by freeze–thaw method as described by (Holsters et al. 1978). A single colony of *Agrobacterium* strain was inoculated in Luria-Bertani broth (LB) medium containing 50 mg/L kanamycin and 20 mg/L rifampicin and incubated at 28 ˚C for 48 h at 220 rpm. An aliquot (200 µL) of grown culture was freshly inoculated in 50 ml LB broth with appropriate antibiotics and incubated at 28 ˚C till the OD_{600} reached 0.6-0.8. The bacterial cells were collected by centrifugation (4000 rpm, 10 min) and re-suspended in infection medium (MS basal broth + 100 µM acetosyringone, pH 5.8).

Agrobacterium-mediated genetic transformation of onion

About 8-week-old embryonic calli obtained from the seedling radicle explants were chopped into 3-4 mm^2 sized pieces and immersed in *Agrobacterium* infection medium, agitated constantly for half an hour at room temperature and blot dried on sterile filter paper for 2 h. Dried calli were transferred to MS basal medium (supplemented with 1.0 mg/L 2,4-D, 100 µM acetosyringone and 7 g/L agar, pH 5.8) and incubated at room temperature for 3 days in dark condition.

After 3 days of co-cultivation, calli were transferred to CI medium supplemented with 250 mg/L cefotaxime (Resting medium) and incubated for 2 weeks at room temperature in the dark. Later these calli were transferred to selection medium (CI medium supplemented with 250 mg/L cefotaxime and 50 mg/L hygromycin B) and incubated at room temperature in dark condition. After 2 weeks, browned/bleached portions (dead cells) were removed from calli and surviving calli were again sub-cultured in the fresh medium with the same composition under similar conditions. The non-infected calli were also maintained as an experimental control. Six independent batches were co-cultivated with an average 202 calli in each batch.

After 2 weeks of sub-culture, dead cells were removed and surviving calli from 3 batches were used for histochemical analysis (experiment 1) whereas those from the other 3 batches were advanced for shoot regeneration studies (experiment 2). Selected calli of experiment 2 were transferred to SIM (supplemented with 250 mg/L cefotaxime and 30 mg/L hygromycin B) and incubated at 23 ˚C under 16 h/8 h light/dark condition for 3 weeks. The surviving calli were sub-cultured on the same fresh medium with the same composition under similar conditions. After 3-4 weeks of sub-culture, the shoot inducing calli were sub-cultured to SIM devoid of hygromycin for 3-4 weeks. The non-infected experimental control calli were also maintained under similar conditions. Regenerated shoots were transferred to rooting medium (half MS basal medium containing 3% sucrose and 7 g/L agar) and allowed to grow for a month. Multiple plantlets regenerated from the same calli were labeled as same event. The roots of well-developed plantlets were dipped into 0.1% bavistin solution for 5 min and transferred to pots containing an autoclaved mixture of soil, sand and vermicompost and allowed to form a bulb under green house conditions (at 25 ˚C with 16 h/8h light-dark cycle).

Histochemical *GUS* assay

To confirm the transformation, the expression of *β-Glucuronidase* in the transformed calli was assayed. Surviving infected calli from experiment 1 and control calli (8-week-old non-infected embryogenic calli) were incubated in GUS solution of pH 7.0 (Anbu and Arul 2013) at 37 °C for 12 h in dark and later washed thrice in 70% ethanol at 15-minute-intervals. The colour developed was visualized using a bright-field stereo microscope (Leica, Model No. 10450028) at low magnification and the image was captured with a digital camera. The tender leaf bits of T_0 and wild type plants were permeabilized in chilled 90% acetone (v/v), incubated at -20 °C for 2 h (Hemerely et al. 1993) to remove chlorophyll and washed twice with 100 mM phosphate buffer pH 7.0 for 5 min. Later leaf bits were analyzed for *GUS* assay as described above.

PCR analysis of putative transgenic plants

For PCR analysis, genomic DNA was extracted from tender leaves of putative T_0 transgenic plants and wild-type plants using a DNA extraction kit (Qiagen). Genomic DNA (50 ng) was used as a template for PCR amplification of *GUSplus* and *hpt*II genes using 1x Dream Taq mastermix (ThermoScientific) in presence of 0.5 µM of each primer, as per conditions described in Table 2. The plasmid DNA (pCAMBIA 1305.1) and wild-type onion DNA were used as positive and negative control, respectively. The amplified PCR products were resolved on 1% (w/v) agarose gel.

For RT-PCR analysis, total RNA was extracted from tender leaves of PCR positive T_0 and wild-type plants using RNeasy® Plant Mini kit (Qiagen). 1 µg of total RNA was treated with DNase using DNase I, RNase free kit (ThermoScientific) and first strand of cDNA was synthesized using RevertAid First Strand cDNA synthesis kit (ThermoScientific). First strand of cDNA was diluted in nuclease free water with 1:5 dilution factor and 1 µl aliquot of diluted cDNA was used as a template for PCR amplification of *GUSplus* as per conditions described in Table 2. The plasmid DNA (pCAMBIA 1305.1) and wild type onion cDNA were used as positive control and negative control, respectively. The amplified PCR products were resolved on 1% (w/v) agarose gel.

Data analysis

Experiments were set up in a completely randomized design. The callus induction efficiency was calculated in percentage by counting the number of induced calli from total number of cultured explants. The shoot induction efficiency was calculated in percentage by counting the number of shoot regenerating calli from total number of cultured embryogenic calli. Data were subjected to analysis of variance (ANOVA) by using SAS program (Release 9.2, SAS Institute, NC, USA). Differences between the mean values were assessed with Duncan's multiple range test at $p<0.05$. The transformation efficiency was calculated in percentage by counting number of transgenic events generated from total number of co-cultivated calli.

Results

Regeneration of *Allium cepa* cv. Bhima super via indirect organogenesis

An efficient regeneration protocol, DNA delivery system and a selection system are required for successful genetic transformation of any plant species. Previously reported regeneration protocols for *Allium cepa* showed the dependence of callus induction on cultivars, explants and media compositions (van der Valk et al. 1992; Eady 1995; Saker 1997; Eady et al. 1998; Zheng et al. 1998; Khar et al. 2005; Ramakrishnan et al. 2013; Sivanesan et al. 2015). In this study, we standardized the regeneration protocol for an Indian cultivar, Bhima super, which is one of the commercially important varieties of onion. We used seedling radicle and shoot tip from the dark-grown seedlings of onion as an explants for callus induction with varying concentration of two types of auxins *viz*., 2,4-D and picloram in combination with different concentrations of kinetin (Supplementary Table 1 and 2). Number of induced calli, weight of calli, its colour and physical appearance was observed after 6 and 8 weeks. Friable and yellowish calli were the best for the formation of shoots. Callus induction efficiency was better in seedling radicle explants compared to shoot tip explants along with high calli weight and physical appearance.

We also compared the effect of 2,4-D and picloram on/for callus induction from both shoot tip and seedling radicle explants. In the case of seedling radicle explants, 1.0 mg/L concentration of 2,4-D along with 0.5 mg/L of kinetin in MS medium showed highest callus induction efficiency (85.33%), whereas it was only 57.33% in the routinely used medium *i.e.* MS medium containing 1.0 mg/L of 2,4-D (Supplementary Table 1). In shoot tip explants, maximum callus induction (38.67%) was observed in MS medium containing 2.0 mg/L 2,4-D along with 2.0 mg/L kinetin and it was only 4% in 1.0 mg/L 2,4-D containing MS medium (Supplementary Table 2). These results showed that callus induction in *Allium cepa* cv. Bhima super is highly dependent upon the explants and the media composition as reported in other plant species. In this study, we report that the seedling radicle of 72-h-old germinated seedlings are better choice of explants for callus induction and further regeneration of onion. It also shows that between 2,4-D and picloram, the former is more effective for callus induction from seedling radicle explants in presence of kinetin rather than alone (Fig. 2).

The mean calli weight per treatment varied significantly according to explants as well as plant growth regulators. The calli mass from seedling radicle explants was higher by mean callus weight than shoot tip explants. Calli weight per treatment induced from seedling radicle ranged from 0.035 g in 2.0 mg/L picloram + 2.0 mg/L kinetin treatment to 8.715 g in 1.0 mg/L 2,4-D + 0.5 mg/L kinetin treatment after 8 weeks, whereas it was 4.571 g in MS medium containing 1.0 mg/L 2,4-D (Supplementary Table 1). With shoot tip explants, the mean calli weight per treatment ranged from 1.441 g in 2.0 mg/L 2,4-D+ 1.0 mg/L after 8 weeks, whereas it was 0.107 g in 1.0 mg/L 2,4-D treatment (Supplementary Table 2).

The physical appearance of the callus is an important indicator of its ability for shoot induction. Induced calli from the seedling radicle and shoot tip in different treatments exhibited variable morphology. The induced calli were white, yellowish white and yellow in color. Calli were either compact, friable or highly friable in nature (Supplementary Table 1 and 2). In the best treatment, embryogenic calli induced from seedling radicle on MS medium supplemented with 1.0 mg/L 2,4-D + 0.5 mg/L kinetin were yellow, friable with a nodular type (Fig 1h).

Further, we cultured 8-week-old calli (from the best treatment) on different SIMs and calculated the shoot regeneration efficiency as number of callus-producing green shoots (Fig 1i) from the total number of calli used in that treatment. The calli that turned green but failed to produce shoots (supplementary fig 1.) were not counted while calculating shoot regeneration efficiency. The shoot regeneration efficiency varied from 7.41% in MS medium containing 1.0 g/L kinetin to 73.15% in MS medium supplemented with 1.5 kinetin mg/L and 0.125 ABA mg/L (Table 1). MS medium containing 1.5 mg/L kinetin and 0.125 mg/L / 0.250 ABA mg/L ABA have generated 2 to 4 shoots per callus (data not shown). Altogether, these results showed that the calli obtained from seedling radicles of dark-grown seedlings of Bhima super have relatively higher regeneration efficiency via somatic embryogenesis in the presence of specific hormone combinations.

Optimization of selection pressure for transgenics production

The use of an efficient selection system is crucial for successful transformation. In the case of monocots, the well-established selection system, kanamycin, is not useful as the monocot cell and tissues are relatively insensitive to kanamycin (Wilmink and Dons 1993). In this study, we used hygromycin B and geneticin markers for selection. The selection pressure for these markers was calculated based on the percentage of browned/bleached calli after 4 weeks of incubation on the selection medium. Hygromycin B and geneticin at 25 mg/L showed 40% and 33.33% lethality of embryogenic calli, respectively; whereas at concentrations higher than 50 mg/L resulted in 100% lethality (supplementary fig. 2). In the control experiment, there was no loss of calli. Therefore, in further experiments, we used hygromycin B at 50 mg/L concentration.

Agrobacterium-mediated genetic transformation of *Allium cepa* cv. Bhima super

In our study, 1212 embryogenic calli derived from seedling radicle explants, were co-cultivated with *A. tumefaciens* harbouring pCAMBIA 1305.1 plasmid (Fig. 3a), in 6 independent batches. The different stages of *Agrobacterium*-mediated genetic transformation and development of putative T_0 transformants are shown in Fig. 3b-i. Out of 6 batches, calli from 3 batches were used for *GUS* staining (Experiment 1), while the other 3 batches were forwarded for shoot regeneration, rooting and hardening (Experiment 2).

In experiment 1, a total of 614 calli were infected with *Agrobacterium* in 3 batches with an average of 204.67 ± 4.01 calli per batch, of which 52.63% and 20.84% calli survived after the 1st and 2nd round of selection, respectively. In the control experiment (non-infected calli), 46.39% calli were survived after the 1st round of selection and no calli were survived after the 2nd round of selection (Table 3). Survived calli were subjected to histochemical *GUS* staining analysis. 1.15% of total co-cultivated calli retained the blue stain (Table 3), whereas blue stain failed to develop in the negative control callus (Fig. 4).

In experiment 2, a total of 598 calli were infected with *Agrobacterium* in 3 batches with an average of 199.33 ± 1.78 calli per batch, of which 54.32% and 18.90% calli survived after the 1st and 2nd round of selection, respectively. In the control experiment (non-infected calli), 43.57% calli survived after the 1st

round of selection and no calli survived after the 2nd round of selection (Table 4). Calli surviving after 2 rounds of selection were transferred to SIM for the regeneration of shoots. Of these, 3.85% calli survived on SIM (containing hygromycin B 30 mg/L) after incubation for 3 weeks and 1.62% calli showed shoot induction after 3 to 4 weeks of sub-culture and later sub-cultured again in SIM devoid of hygromycin. After 3-4 weeks of 2nd sub-culture, multiple shoots (2 to 4) regenerated from each one of the 6 independent calli and were transferred to rooting media. Well-developed plantlets were transferred to pots for hardening and maintained till maturity under green house conditions.

Molecular and histochemical analyses of transgenics

One fresh and healthy T_0 plant was selected from each event, and tested by PCR and RT-PCR analysis. PCR studies resulted in amplification of 687 bp and 512 bp of internal sequences of *GusPlus* (Fig. 5a) and *hptII* (Fig. 5b) genes in all the 6 independent events whereas no amplification was found in wild type Bhima super plant. RT-PCR studies of *GusPlus* gene also showed similar results (Fig. 5c). Tender leaf bits of PCR positive plants showed blue staining, whereas blue stain failed to develop in the negative control plant when analyzed by *GUS* assay (Fig. 6).

Discussion

The development of an efficient regeneration and transformation protocol is a pre-requisite for introducing agronomically important traits for crop improvement. So far, there have been only two reports for standardization of *in vitro* regeneration (Khar et al. 2005; Ramakrishanan et al. 2013) and only one study on transgenic development (Naini et al. 2019) of Indian short-day onion.

Embryogenic callus induction in onion has been achieved by using auxins alone in the culture media such as 2,4-D and/or picloram (van der Valk et al. 1992; Saker 1997; Eady et al. 1998; Zheng et al. 1998; Khar et al. 2005; Ramakrishnan et al. 2013; Sivanesan et al. 2015). In our experiment, the callus induction efficiency was low when auxin alone was used either 2,4-D or picloram and it varied from 4 to 22.67% and 24 to 57.33% in shoot tip and seedling radicle explants, respectively. This clearly depicts the explant-specific response for callus induction study, as reported in other onion regeneration studies (van der Valk et al. 1992; Khar et al. 2005). Supplementation of kinetin to auxin-containing MS medium enhanced the callus induction efficiency in both the explants *i.e.* up to 38.67% in shoot tip and 85.33% in seedling radicle. Picloram (5.0 mg/L) and 2,4-D (1.0 mg/L) was reported best to induce embryogenic calli from immature embryos (Eady et al. 1998) and zygotic mature embryos (Zheng et al. 1998), respectively. In our experiment, among auxins; 2,4-D in combination with kinetin was significantly more effective in embryogenic callus induction as compared to picloram. Further, it was better than routinely used callus induction medium *i.e.* 1.0 mg/L 2,4-D in transformation experiments (Zheng et al, 1998). This effect of cytokinin on callus induction efficiency could be genotype-specific response. High callus induction efficiency in Indian onion genotypes has been reported in presence of auxin and cytokinin using shoot tip explants (Khar et al. 2005; Ramakrishnan et al. 2013). Similarly, addition of cytokinin (kinetin or BAP) along with 2,4-D-containing callus induction medium enhanced the induction of somatic embryogenic

calli of onion (Saker 1997). The callus induction efficiency was positively correlated with calli weight per treatment in both the explants (Fig. 2) indicating callus induction is more critical than the callus proliferation.

Two cytokinins (BAP and kinetin) have been extensively studied for shoot regeneration in onion either alone or in combination with auxins, vitamins and ABA (van der Valk et al. 1992; Saker 1997; Eady et al. 1998; Zheng et al. 1998; Khar et al. 2005; Ramakrishnan et al. 2013). The microshoot regeneration potential of onion explants varied with cytokinin concentration and was higher in kinetin containing culture medium (Kamstaityte and Stanys 2004). In our study, the regeneration potential of the embryogenic calli induced from seedling radicle (on MS medium containing 1.0 mg/L 2,4-D + 0.5 mg/L kinetin) was significantly affected by variable concentration of cytokinin alone and varied between 7.41 to 31.2%. The addition of low concentration of ABA i.e. up to 250 mg/L further enhanced the shoot regeneration with highest (73.15%) in 1.5 mg/L kinetin + 0.125 mg/L ABA treatment. This is due to high shoot regeneration efficiency of embryogenic calli induced on 2,4-D-containing media (van der Valk et al. 1992; Luthar and Bohanec 1999; Sivanesan et al. 2015) and ability of low concentration ABA for increasing the number of shoot-forming embryogenic onion calli in MS medium containing kinetin (van der Valk et al. 1992). Khar et al. (2005) also reported shoot regeneration in kinetin-containing MS medium from the calli induced from root tip, shoot tip and whole seed explants of onion.

Hygromycin B and geneticin have been reported as selective agents for the selection of transformed onion explants (Eady et al. 2000, 2003; Zheng et al. 2001, 2005; Kamata et al. 2011, Naini et al. 2019). In our study, hygromycin B of concentration 50 mg/L and 30 mg/L was sufficient for selection during calli proliferation and shoot regeneration steps, respectively. Hygromycin B at 50 mg/L concentration was found to be optimal for the selection of transformed onion embryogenic calli (Zheng et al. 2001, 2005; Kamata et al. 2011; Naini et al. 2019). These results were consistent with our histochemical analysis that showed 1.15% GUS-positive calli among total infected calli. In our study, use of Hygromycin B at 30 mg/L concentration during shoot regeneration phase was found effective and did not affect regeneration. However, Naini et al. (2019) used 3 rounds of 50 mg/L hygromycin B during callus proliferation stage, but shoot induction was obtained from media devoid of hygromycin B. Thus use of selective agent during shoot induction improved the selection process and reduced time spent in the callus proliferation phase. In the study to evaluate geneticin as selective agent, 50 mg/L geneticin was found to be useful for selectively discriminating transformed calli from non-transformed ones, but survived calli failed to regenerate into shoots when transferred to SIM containing no geneticin and 30 mg/L geneticin (data not shown).

Several reports on onion transformation by *Agrobacterium*-mediated or particle bombardment showed 0.2% to 25% transformation efficiency (Eady et al. 2000, 2003; Zheng et al. 2001, 2005; Aswath et al. 2006; Kamata et al. 2011; Naini et al. 2019). In our study, transformation efficiency of seedling radicle induced calli was around 1 percent which was calculated based on the number of shoot producing calli from the total number of initially infected calli. Only shoot producing calli were counted as many of the selected calli turned green or proliferated but failed to regenerate into shoots. Around 2-4 shoots are

produced from each regenerative calli (data not shown), and we counted shoot-producing calli rather than number of shoots to avoid inflation of transformation efficiency. In this perspective, there are 3 reports of *Agrobacterium*-mediated transformation based on seedling radicle induced calli (Aswath et al. 2006; Kamata et al. 2011; Naini et al. 2019). Aswath et al. (2006) have reported a high 25% average transformation efficiency, based on the average of survived calli in each selection stages and not based on initial infected calli. Another report with higher efficiency of 6.4% by Kamata et al. (2011) actually counts total number of plants regenerated from only two calli out of 1751 co-cultivated calli. Similarly, a comparable report on transformation of Indian variety by Naini et al. (2019) showed 2.5% transformation efficiency based on number of survived calli after 3 rounds of selection from the initially infected calli and not based on shoot producing calli. Hence, the seemingly low transformation efficiency in our experiment is mainly due to the method of calculation of transformation efficiency, and is actually higher than or comparable with previous reports based on seedling radicle explant.

In conclusion, the results of the present study showed that the regeneration efficiency of onion is dependent on explants type and plant growth regulators. Supplementation of kinetin in the callus induction media with auxins has improved the callus induction efficiency in both the explants chosen in the study. Among explants, seedling radicle was better than shoot tip explant for callus induction. Shoot regeneration of the calli induced from seedling radicle was optimal in the presence of kinetin with minimal concentration of ABA. Thus, a stable transformation protocol for onion was established with 1% transformation efficiency. Overall, we report a regeneration system for the Indian onion cultivar that is suitable for *Agrobacterium*-mediated transformation and would be useful for researchers interested in introducing any trait-of-interest into onion.

Declarations

Funding

Indian Council of Agricultural Research- ICAR National Fellow project (Project code: HORTDOGRSOL201700800088)

Conflicts of interest

The work is presented in the manuscript with the consent of all authors. The authors declare that they have no conflict of interest.

Availability of data and material:

All data are included in the manuscript.

Code availability

Not applicable

Authors' contribution

MTK and AS conceived and designed the experiments. MTK conducted all the experiments. MTK, SS and AS drafted the manuscript. AS, VS and SS carried out the data analysis, manuscript editing. AS supervised the entire work. All authors read and approved the final manuscript.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent to publication

All authors consent for publication

Acknowledgements

Financial support and fellowship to MTK provided by Indian Council of Agricultural Research (ICAR National Fellow project, Project code: HORTDOGRSOL201700800088) is gratefully acknowledged.

References

Anbu P, Arul L (2013) *Beta glucuronidase* activity in early stages of rice seedlings and callus: A comparison with *Escherichia coli beta glucuronidase* expressed in the transgenic rice. Int J Biotechnol Mol Biol Res 4:52–59. https://doi.org/10.5897/ijbmbr2013.0152

Aswath CR, Mo SY, Kim DH, Park SW (2006) *Agrobacterium* and biolistic transformation of onion using non-antibiotic selection marker phosphomannose isomerase. Plant Cell Rep 25:92–99. https://doi.org/10.1007/s00299-005-0022-4

Degenhardt J, Poppe A, Montag J, Szankowski I (2006) The use of the phosphomannose-isomerase/mannose selection system to recover transgenic apple plants. Plant Cell Rep 25:1149–1156. https://doi.org/10.1007/s00299-006-0179-5

Eady CC (1995) Towards the transformation of onions (*Allium cepa*). New Zeal J Crop Hortic Sci 23:239–250. https://doi.org/10.1080/01140671.1995.9513895

Eady CC, Davis S, Farrant J, Reader J, Kenel F (2003) *Agrobacterium tumefaciens*-mediated transformation and regeneration of herbicide resistant onion (*Allium cepa*) plants. Ann Appl Biol 142:213–217. https://doi.org/10.1111/j.1744-7348.2003.tb00243.x

Eady CC, Lister CE (1998) A comparison of four selective agents for use with *Allium cepa* L. immature embryos and immature embryo-derived cultures. Plant Cell Rep 18:117–121. https://doi.org/10.1007/s002990050542

Eady CC, Weld RJ, Lister CE (2000) *Agrobacterium tumefaciens*-mediated transformation and transgenic-plant regeneration of onion (Allium cepa L.). Plant Cell Rep 19:376–381. https://doi.org/10.1007/s002990050743

Hemerly AS, Ferreira P, de Almeida Engler J, Van Montagu M, Engler G, Inze D (1993) cdc2a expression in *Arabidopsis* is linked with competence for cell division. Plant Cell 5:1711–1723. https://doi.org/10.1105/tpc.5.12.1711

Holsters M, Silva B, Van Vliet F, Hernalsteens J-P, Genetello C, Van Montagu M, Schell J (1978) In vivo transfer of the Ti-plasmid of *Agrobacterium tumefaciens* to *Escherichia coli*. MGG Mol Gen Genet 163:335–338. https://doi.org/10.1007/BF00271963

Joersbo M, Donaldson L, Kreiberg J, Peterson SG, Brunstedt J, Okkels FT (1998) Analysis of mannose selection used for transformation of sugar beet. Mol Breed 4:111–117. https://doi.org/10.1023/A:1009633809610

Kamata Y, Masamura N, Miyazaki A, Nagata T (2011) A novel autofluorescence-based selection of calli amenable to *Agrobacterium*-mediated transformation in onion (*Allium Cepa* L.). Plant Biotechnol 28:361–371. https://doi.org/10.5511/plantbiotechnology.11.0513a

Kamstaityte D, Stanys V (2004) Micropropagation of onion (*Allium cepa* L.). Acta Universitatis Latviensis, Biology 676:173-176Karam NS, Jawad FM, Arikat NA, Shibl RA (2003) Growth and rosmarinic acid accumulation in callus, cell suspension, and root cultures of wild *Salvia fruticosa*. Plant Cell Tissue Organ Cult 73:117–121. https://doi.org/10.1023/A:1022806420209

Khar A, Bhutani RD, Yadav N, Chowdhury VK (2005) Effect of explant and genotype on callus culture and regeneration in onion (*Allium cepa* L.) Akdeniz üniversitesi ziraat fakültesi dergisi 18:397-404.

https://dergipark.org.tr/en/pub/akdenizfderg/issue/1579/19595

Luthar Z, Bohanec B (1999) Induction of direct somatic organogenesis in onion (*Allium cepa* L.) using a two-step flower or ovary culture. Plant Cell Rep 18:797–802. https://doi.org/10.1007/s002990050664

Mostafa HHA, Wang H, Song J, Li X (2020) Effects of genotypes and explants on garlic callus production and endogenous hormones. Sci Rep 10:1–11. https://doi.org/10.1038/s41598-020-61564-4

Naini R, Pavankumar P, Prabhakar S, Kancha RK, Rao KV, Reddy VD (2019) Evolvement of nutraceutical onion plants engineered for resveratrol biosynthetic pathway. Plant Cell Rep 38:1127–1137. https://doi.org/10.1007/s00299-019-02432-y

Ramakrishnan M, Ceasar SA, Duraipandiyan V, Daniel MA, Ignacimuthu S (2013) Efficacious somatic embryogenesis and fertile plant recovery from shoot apex explants of onion (*Allium cepa*. L.). Vitr Cell Dev Biol - Plant 49:285–293. https://doi.org/10.1007/s11627-013-9510-3

Saker MM (1997) In vitro regeneration of onion through repetitive somatic embryogenesis. Biol Plant 40:499–506. https://doi.org/10.1023/A:1001788700469

Sivanesan I, Kyoung KE, Kyoung KM, Young KE, Park SW (2015) Somatic embryogenesis and plant regeneration from zygotic embryo explants of onion. Hortic Bras 33:441–447. https://doi.org/10.1590/S0102-053620150000400006

Stoykova P, Stoeva-Popova P (2011) PMI (manA) as a nonantibiotic selectable marker gene in plant biotechnology. Plant Cell. Tissue Organ Cult. 105:141–148

van der Valk P, Scholten OE, Verstappen F, Jansen R, Dons J (1992) High frequency somatic embryogenesis and plant regeneration from zygotic embryo-derived callus cultures of three *Allium* species. Plant Cell Tissue Organ Cult 30:181–191. https://doi.org/10.1007/BF00040020

Wilmink A, Dons JJM (1993) Selective agents and marker genes for use in transformation of monocotyledonous plants. Plant Mol. Biol. Report. 11:165–185

Zheng S, Henken B, Sofiari E, Jacobsen E, Krens FA, Kik C (1998) Factors influencing induction, propagation and regeneration of mature zygotic embryo-derived callus from *Allium cepa*. Plant Cell Tissue Organ Cult 53:99–105. https://doi.org/10.1023/A:1006034623942

Zheng S, Henken B, Sofiari E, Keizer P, Jacobsen E, Kik C, Krens F (1999) Effect of cytokinins and lines on plant regeneration from long-term callus and suspension cultures of *Allium cepa* L. Euphytica 108:83–90. https://doi.org/10.1023/A:1003652211389

Zheng SJ, Henken B, De Maagd RA, Purwito A, Krens FA, Kik C (2005) Two different *Bacillus thuringiensis* toxin genes confer resistance to beet armyworm (*Spodoptera exigua* Hübner) in transgenic Bt-shallots (*Allium cepa* L.). Transgenic Res 14:261–272. https://doi.org/10.1007/s11248-005-0109-2

Zheng SJ, Khrustaleva L, Henken B, Sofiari E, Jacobsen E, Kik C, Krens FA (2001) *Agrobacterium tumefaciens*-mediated transformation of *Allium cepa* L.: The production of transgenic onions and shallots. Mol Breed 7:101–115. https://doi.org/10.1023/A:1011348229189

Tables

Table 1 Shoot regeneration efficiency: (Values are represented in the form of mean ± standard error of 3 replicates).

S. No.	Treatment	Average shoot regeneration efficiency (%)
1	1.0 Kin mg/L + 0.000 ABA mg/L	7.41 ± 3.70
2	1.0 Kin mg/L + 0.125 ABA mg/L	19.44 ± 4.24
3	1.0 Kin mg/L + 0.250 ABA mg/L	15.28 ± 3.50
4	1.0 Kin mg/L + 0.500 ABA mg/L	15.74 ± 4.63
5	1.0 Kin mg/L + 1.000 ABA mg/L	15.28 ± 3.50
6	1.5 Kin mg/L + 0.000 ABA mg/L	31.02 ± 4.56
7	**1.5 Kin mg/L + 0.125 ABA mg/L**	**73.15 ± 3.34**
8	1.5 Kin mg/L + 0.250 ABA mg/L	34.72 ± 6.56
9	1.5 Kin mg/L + 0.500 ABA mg/L	34.26 ± 5.63
10	1.5 Kin mg/L + 1.000 ABA mg/L	26.39 ± 6.94
11	2.0 Kin mg/L + 0.000 ABA mg/L	22.22 ± 0.00
12	2.0 Kin mg/L + 0.125 ABA mg/L	23.33 ± 6.42
13	2.0 Kin mg/L + 0.250 ABA mg/L	11.57 ± 6.43
14	2.0 Kin mg/L + 0.500 ABA mg/L	15.74 ± 4.63
15	2.0 Kin mg/L + 1.000 ABA mg/L	11.57 ± 6.43

Table 2 Primers used in the study

Primer Name	5’ to 3’ sequence	Amplicon size and region	PCR conditions
GusPlus-F	TACGGGAAAGGACTGGAAG	687 bp of internal sequence of *GusPlus*	1 cycle of 94 °C for 5 min; 35 cycles of 94 °C for 45 sec, 55 °C for 45 sec, 72 °C for 1 min and 1 cycle of 72 °C for 10 min
GusPlus-R	GTAGAGATACGTGTTCAGTGG		
*Hpt*_F	GACGTCTGTCGAGAAGTTTC	512 bp of internal sequence of *hptII*	1 cycle of 94 °C for 5 min; 35 cycles of 94 °C for 45 sec, 60 °C for 45 sec, 72 °C for 45 sec and 1 cycle of 72 °C for 10 min
*Hpt*_R	GCCTCCAGAAGAAGATGTTG		

Table 3 Histochemical GUS staining analysis of onion calli: (Values are represented in the form of ean ± standard error of 3 replicates).

	No. of calli			
	co-cultivated	selected after 1st selection	selected after 2nd selection	GUS positive
Co-cultivated calli	204.67 ± 4.01	107.67 ± 2.84	42.67 ± 1.52	2.33 ± 0.27
% of co-cultivated calli		**52.63 ± 1.29**	**20.84 ± 0.85**	**1.15 ± 0.16**
Control calli	18.67 ± 0.27	8.67 ± 0.27	0.00 ± 0.00	-
% of control calli		**46.39 ± 2.61**	**0.00 ± 0.00**	-

e 4 Transformation efficiency of onion cv. B. super: (Values are represented in the form of mean ± standard error of 3 replicates).

	No. of calli					Events generated
	co-cultivated	selected after 1st selection	selected after 2nd selection	selected after SIM I	selected after SIM II	
cultivated i	199.33 ± 1.78	108.33 ± 3.41	37.67 ± 1.19	7.67 ± 0.54	3.17 ± 0.68	2 ± 0.00
of co-tivated calli		54.32 ± 1.25	18.90 ± 0.65	3.85 ± 0.31	1.62 ± 0.33	1.00 ± 0.01
ıtrol calli	18.33 ± 0.27	8.00 ± 0.47	0.00 ± 0.00	-	-	-
f control li		43.57 ± 2.03	0.00 ± 0.00	-	-	-

Figures

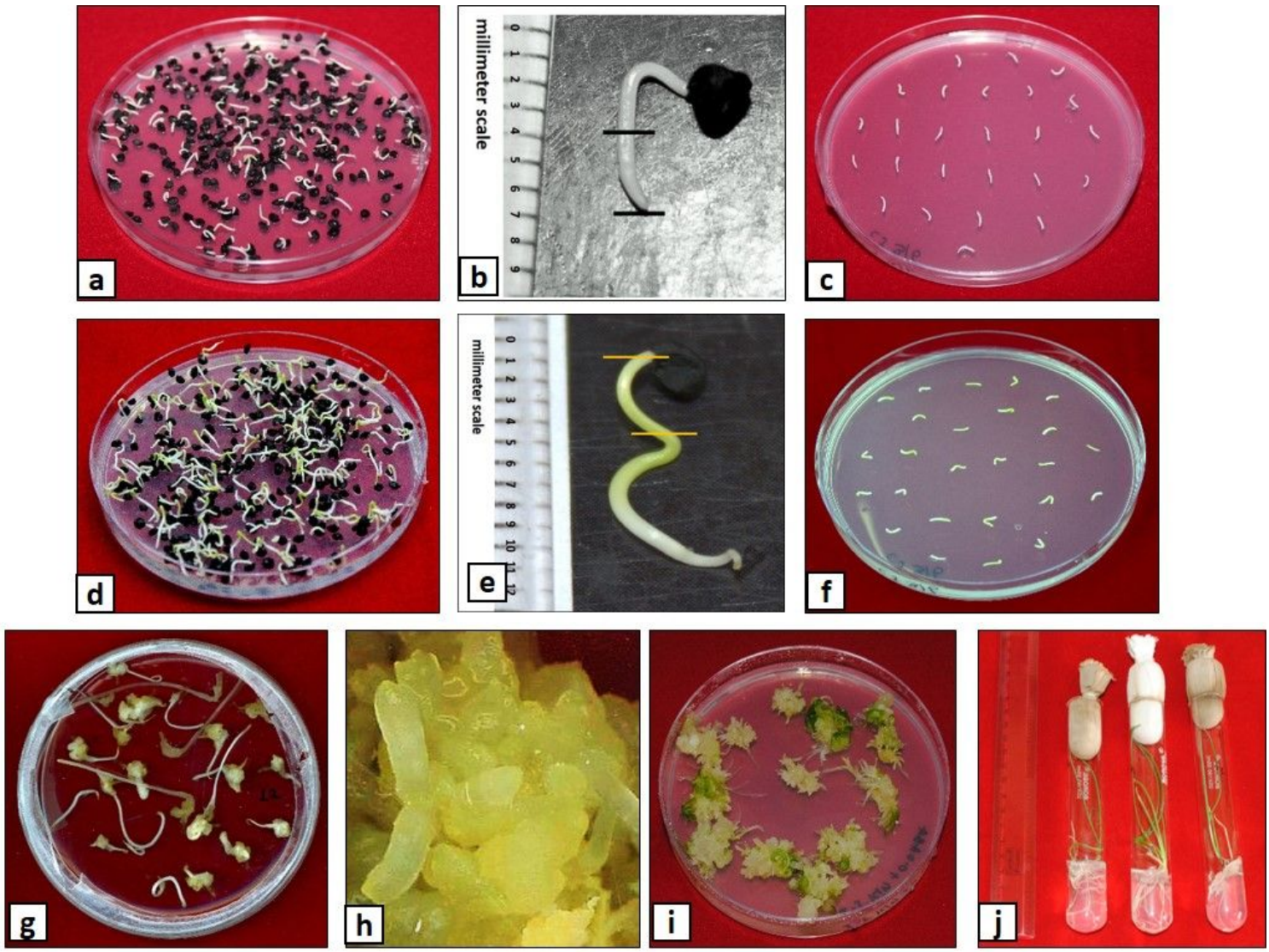

Figure 1

Somatic embryogenesis and regeneration of onion: a Germinated seeds on B5 medium after 72 h; b Hatched area represents seedling radicle explant; c Seedling radicle explants on CI medium; d Germinated seeds on B5 medium after 120 h; e Hatched area represents shoot tip explant; f Shoot tip explants on CI medium; g Induced calli after 6 weeks; h Nodular embryogenic callus after 8 weeks; i Embryogenic calli on shoot induction media after 6 weeks; j Rooting of onion plantlets.

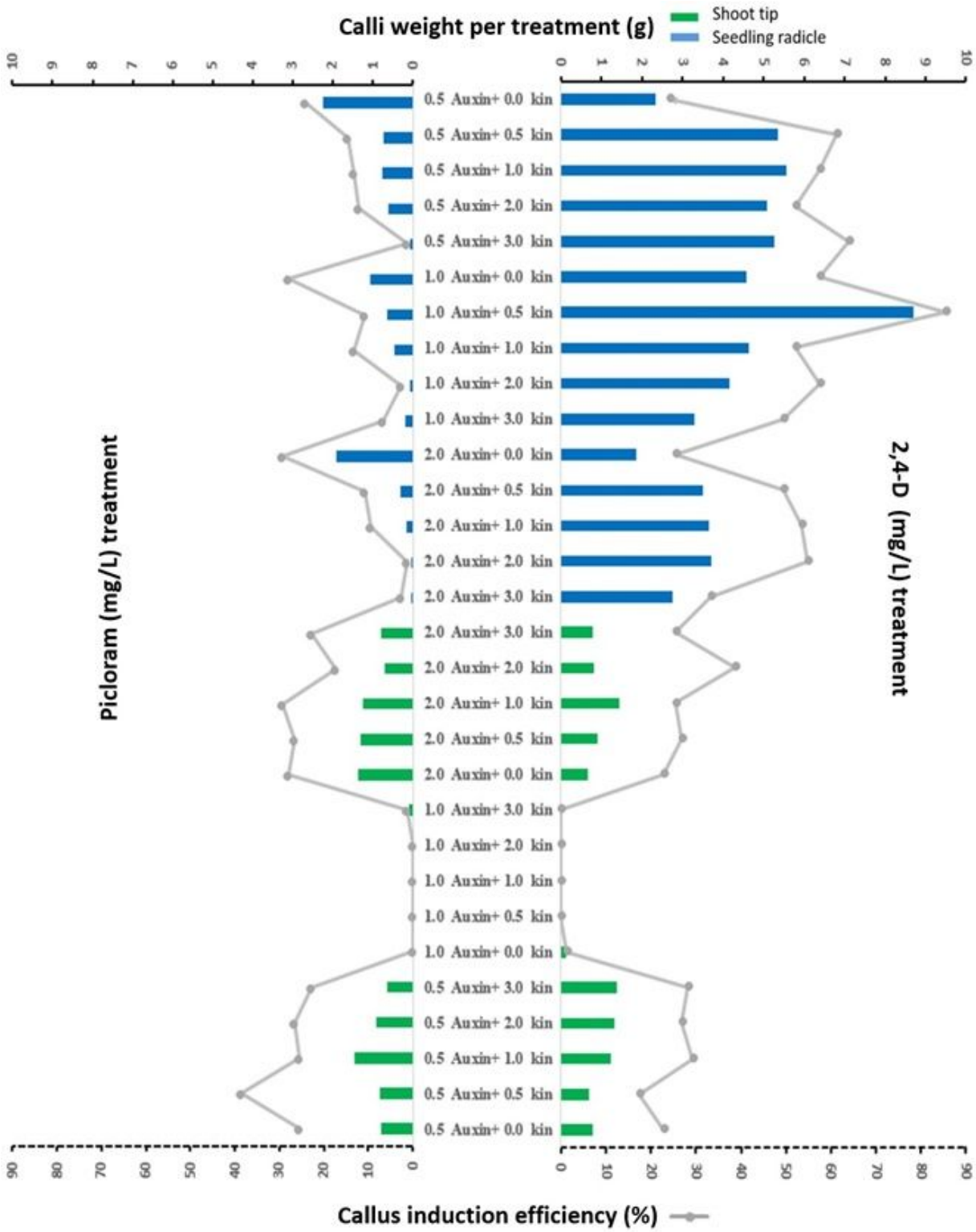

Figure 2

Response of explants and hormones in callus induction: Seedling radicle (72 h old) and shoot tip (120 h old) explants were cultured on MS medium containing 15 different hormone combinations. Calli weight per treatment (g) and callus induction efficiency were evaluated after 8 weeks of culture.

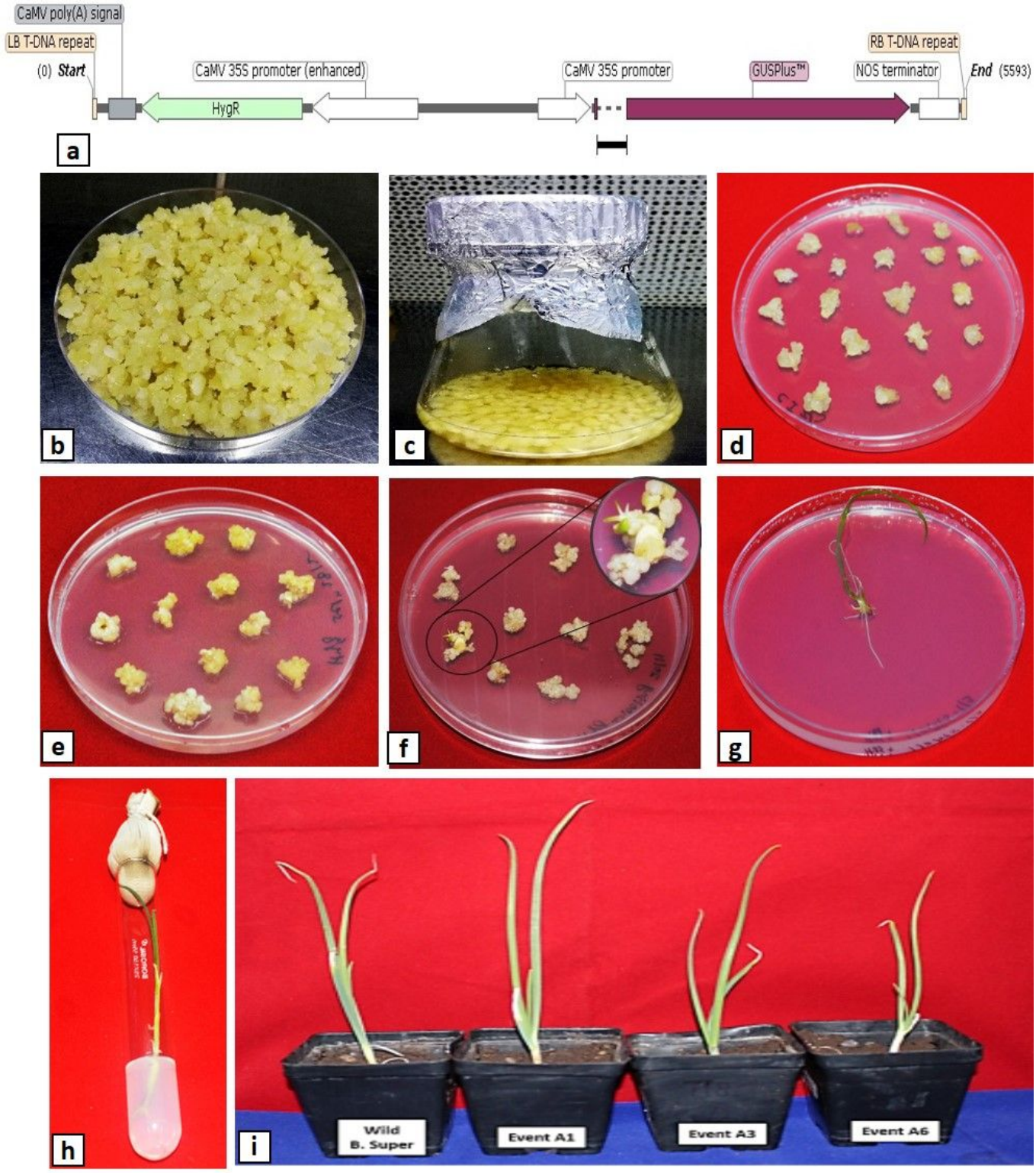

Figure 3

Different stages of Agrobacterium-mediated transformation of onion: a Binary vector pCAMBIA1305.1 harboring AcCenH3 GFP-tailswap construct; b Chopped 8 weeks old embryogenic calli; c Agrobacterium-mediated infection of embryogenic calli; d Calli on resting medium; e Calli on selection medium; f Regenerating shoot on shoot induction medium; g Regenerated shoot on rooting medium; h Plantlets sub-cultured on ½ MS basal media; i. Hardened T0 plants.

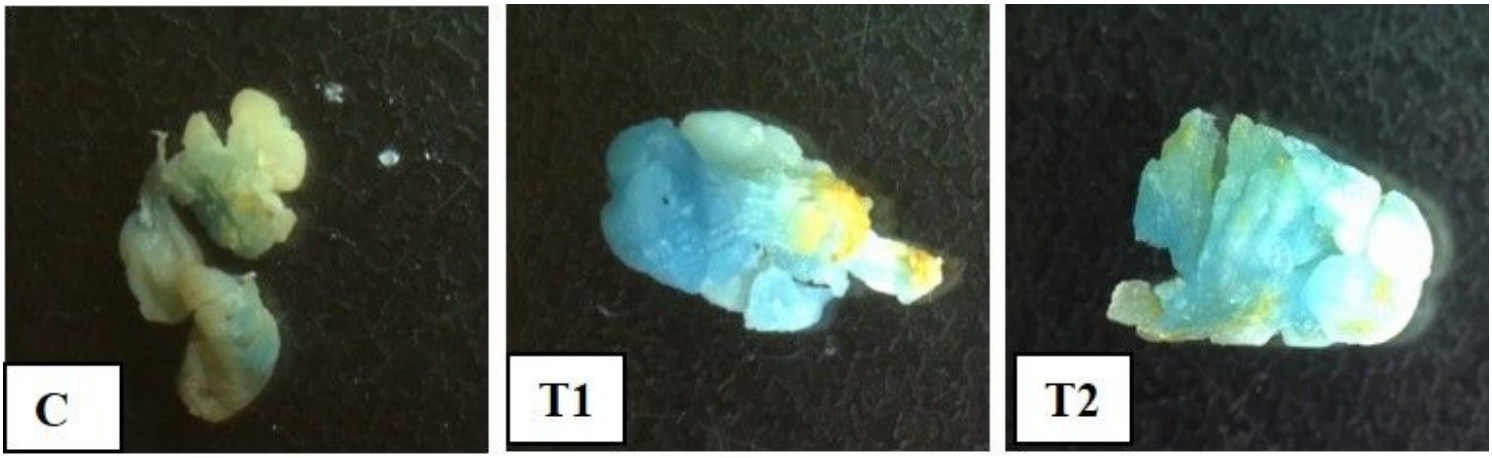

Figure 4

Histochemical GUS staining analysis of transgenic calli: Non-brown and yellowish mass of the non-infected (C) and Agrobacterium infected calli (T1 and T2) was selected from the calli after 2 rounds of selection on selection medium and stained with GUS solution and image was recorded using bright field stereo microscope.

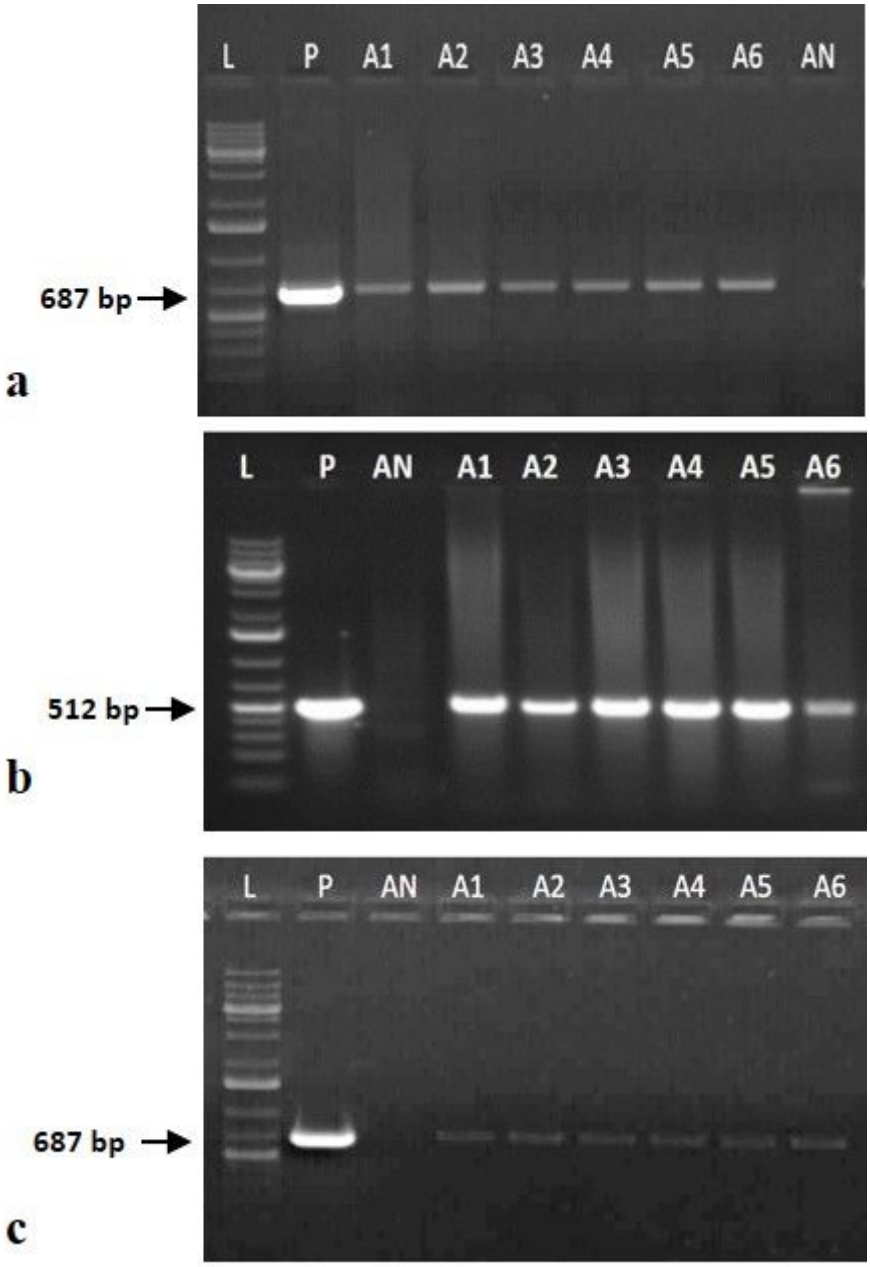

Figure 5

Molecular analysis of putative transgenic plants: a PCR analysis of putative transgenic plants to confirm the presence of GUSplus gene; b PCR analysis of putative transgenic plants to confirm the presence of hptII gene; c RT-PCR analysis to confirm the presence of mRNA of GUSplus gene, [L: 1 kb plus ladder, P: Pcambia1305.1 plasmid (Positive control), A1 to A6: Transgenic events, AN: Negative control (Wild type B. super)]

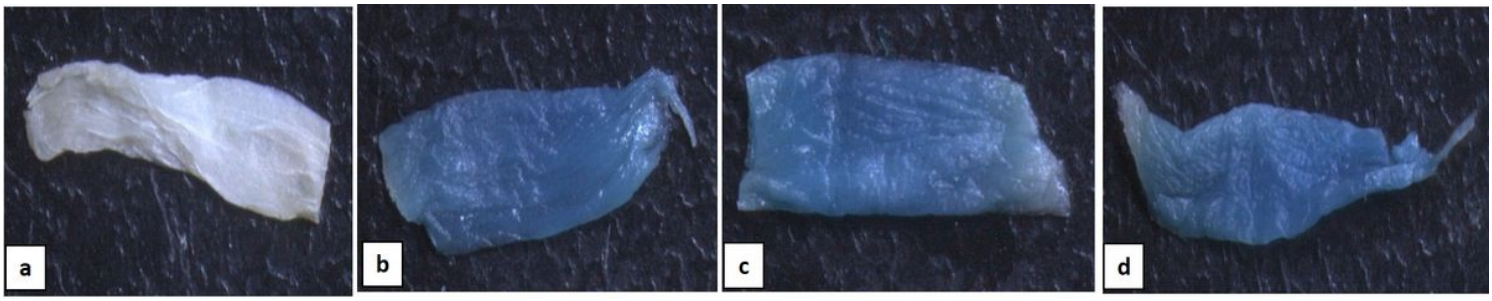

Figure 6

Histochemical GUS staining analysis of transgenic plants: Tender leaf bits of wild type B. super plants and (a) PCR positive transgenic plants stained (b, c and d) were stained with GUS solution after removing the chlorophyll and image was recorded using bright field stereo microscope.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supple.Fig.Tables.docx