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

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Received: 10 March 2021 / Accepted: 26 August 2021 / Published online: 1 September 2021
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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.

Key message
The regeneration protocol is standardized for Indian short-day onion cultivar and Agrobacterium-mediated transformation of onion is established, following in vitro co-cultivation of calli induced from seedling radicle explant.

Keywords Agrobacterium · Callus induction · Onion · Seedling radicle · Transformation

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.

In 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 and Lister 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 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), were 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, 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 onion transformation studies. 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. By contrast, 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 of Indian short-day onion cultivar, Bhima super. Hence there was a need to significantly improve the regeneration and transformation protocols.

The present study, therefore, was conducted with the following objectives: to select the best explant between shoot tip and seedling radicle; optimize the concentration of plant growth regulators to realise maximum callus induction efficiency; and use of the best performing explant to develop an efficient regeneration and stable Agrobacterium-mediated transformation protocol for Indian short-day onion cultivar, Bhima super.

Materials and methods

Plant material and growth conditions

Seeds of Allium cepa cv. Bhima super were used to get explants. Surface-sterilized seeds were germinated on B5 medium at 26 °C temperature 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, e) 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 explants in each replicate (Fig. 1c, f). The best medium for callus induction and growth is designated as CI medium (shown by bold letters in supplementary Table 1).

In shoot regeneration experiment, 8-week-old calli obtained from CI medium were transferred to 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) (Table 1). The culture was maintained in 16 h light/8 h dark photoperiod at 25 ± 2°C temperature. The experiment was carried out with 3 replicates of each treatment with 8 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 greenhouse 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 mm²) 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 25 ± 2 °C 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 embryogenic 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...
constantly for half an hour at 25 ± 2 °C temperature and blot and immersed in Agrobacterium infection medium, agitated and incubated at 25 ± 2 °C temperature for 3 days in dark (2,4-D, 100 µM acetosyringone and 7 g/L agar, pH 5.8).

Dried calli were transferred to CI medium (MS basal broth + 100 µM acetosyringone, pH 5.8) and incubated for 2 weeks at 25 ± 2 °C temperature. 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 µg/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/8 h light–dark cycle).

| 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 |

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

Agrobacterium-mediated genetic transformation of onion

Eight-week-old embryonic calli obtained from the seedling radicle explants were chopped into 3–4 mm² sized pieces and immersed in Agrobacterium infection medium, agitated constantly for half an hour at 25 ± 2 °C temperature and blotted 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 25 ± 2 °C 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 25 ± 2 °C 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 25 ± 2 °C 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.

For preliminary analysis of putatively transformed calli, 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-min-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 T0 and wild type plants were permeabilized in chilled 90% acetone (v/v), incubated at −20 °C for 2 h (Hemerly 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 T0 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 hptII genes using 1 × Dream Taq mastermix (Thermo Scientific) 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 T0 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 and Lister 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 explant 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 were 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 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).

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 s, 55 °C for 45 s, 72 °C for 1 min and 1 cycle of 72 °C for 10 min |
| GUSplus-R   | GTAGAGATACGTGTTCACTGG |                       |                |
| Hpt_F       | GACGTCTGTGAGAAGATTTTC | 512 bp of internal sequence of hptII | 1 cycle of 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s and 1 cycle of 72 °C for 10 min |
| Hpt_R       | GCCTCCAGAAGAGATGTTG |                       |                |
Fig. 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.
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 mg/L kinetin to 73.15% in MS medium supplemented with 1.5 mg/L kinetin and 0.125 mg/L ABA (Table 1). MS medium containing 1.5 mg/L kinetin and 0.125 mg/L or 0.250 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 the antibiotics hygromycin B and geneticin for selection. The selection pressure for these antibiotics 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 T₀ 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 survived after the 1st round of selection and no calli 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 30 mg/L hygromycin B) 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 T₀ plant was selected from each event, and tested by PCR and RT-PCR analysis. PCR studies
Fig. 3 Different stages of Agrobacterium-mediated transformation of onion: a Binary vector pCAMBIA1305.1; 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
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). Ten-day 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).

### Table 3 Histochemical GUS staining analysis of onion calli: (values are represented in the form of mean ± standard error of 3 replicates)

|                  | 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                  | 8.67 ± 0.27                   | –            |
| 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                   | –                             | –            |

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

|                  | Co-cultivated | Selected after 1st selection | Selected after 2nd selection | Selected after SIM I | Selected after SIM II | Events generated |
|------------------|---------------|-------------------------------|-------------------------------|-----------------------|-----------------------|-----------------|
| Co-cultivated calli | 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-cultivated calli | 54.32 ± 1.2   | 18.90 ± 0.65                  | 3.85 ± 0.31                   | 1.62 ± 0.33           | 1.00 ± 0.01           |                |
| Control calli     | 18.33 ± 0.27  | 8.00 ± 0.47                   | 0.00 ± 0.00                   | –                     | –                     |                |
| % of control calli | 43.57 ± 2.03  | 0.00 ± 0.00                   | –                             | –                     | –                     |                |

**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; Ramakrishnan 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 and Lister 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 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 explants, respectively.
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 and Lister 1998; Zheng et al. 1998, 1999; 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 at 50 mg/L and 30 mg/L concentrations were...
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 explant 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.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02161-9.

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

Authors’ contributions 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.

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

Data availability All data are included in the manuscript.

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/ijbmr2013.0152
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/manose 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*). NZ J Crop Hort Sci 23:239–250. https://doi.org/10.1080/01140671.1995.9513895

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

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

Hemerly AS, Ferreira P, de Almeida EJ, Van Montagu M, Engler G, Inze D (1993) cdca2 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 Hemerly AS, Ferreira P, de Almeida EJ, Van Montagu M, Engler G, Inze D (1993) cdca2 expression in *Arabidopsis* is linked with competence for cell division. Plant Cell 5:1711–1723. https://doi.org/10.1105/tpc.5.12.1711

Kamata Y, Masamura N, Miyazaki A, Nagata T (2011) A novel autofluorescence-based selection of calli amenable to *Agrobacterium tumefaciens*-mediated transformation in onion (*Allium Cepa* L.). Plant Biotechnol 28:361–371. https://doi.org/10.1051/plantbiotechnology.11.0513a

Kamstaityte D, Stanys V (2004) Micropropagation of onion (*Allium cepa* L.). Acta Universitatis Latviensis, Biology 676:173–176

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 universitesi ziraat fakultesi dergisi 18:397–404. https://dergipark.org.tr/en/pub/akdenizifderg/issue/1579/19595. Accessed 23 Mar 2021

Luther 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

Naini R, Pavankumar P, Prabhaker 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.). In *Vit* 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, 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/S0102-05362015000400006

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

Wilming M, Dons JIM (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 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

Zheng SJ, Krustaleva 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

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