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ORIGINAL ARTICLE

Development of an efficient and reproducible in vitro regeneration and transformation protocol for tropical maize (*Zea mays* L.) using mature seed-derived nodal explants

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Abstract

Maize is an important crop for billions of people globally. The existing immature embryo-based regeneration protocol of maize has major limitations due to the non-availability of explants throughout the year, limited durability for culturing, and its laborious nature. Mature embryos, especially in tropical maize, are considered recalcitrant towards tissue culture. Therefore, standardization of a robust regeneration and transformation protocol in tropical maize using mature embryos or seeds as starting material is long envisaged. Considering this, in this study, 28 diverse tropical maize genotypes were evaluated for their embryogenic callus induction potential using two different explants (nodal explants and split embryo region) under two different callusing media. Out of 28 genotypes, better callus induction was achieved in four genotypes (BML 6, DHM 117, DMRH 1301, and DMRH 1308) from nodal explants. Further, in vitro regeneration was standardized using 22 different combinations of various auxins and cytokinins. Out of 28 genotypes, two recently commercialized and high-yielding cultivars (DMRH 1301 and DMRH 1308) demonstrated the best callusing and regeneration capability with an average regeneration percentage of 60.4% and 53.6%, respectively. Using the nodal explants-derived embryogenic calli, the genetic transformation was successfully carried out using the 'Biolistic' approach, and up to ~ 5% transformation efficiency was achieved. This efficient regeneration and transformation protocol can overcome the major limitations associated with the existing immature embryo-based protocol in tropical maize as mature seeds can be obtained easily in ample quantity round the year. Such a generalized and reproducible protocol has the potential to be a major tool for maize improvement using transgenic and genome-edited techniques.

Key message

The standardized protocol not only overcomes the major limitations associated with the existing and predominately used immature embryo-based protocol but it is easier, reproducible, and has either higher or comparable callusing, regeneration, and transformation efficiency.

Keywords Maize · Mature seed · Somatic embryogenesis · In vitro regeneration · Biolistic transformation

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Introduction

Maize (*Zea mays* L.) is one of the major cereal crops with wide adaptability under diverse agro-climatic conditions. It has multifarious uses as animal feed, fodder, food, and in starch and bioethanol industry, etc. It contributes ~ 45% to global grain production (FAO 2018). It is a prime driver of the global agricultural economy as well as the livelihoods of millions of farmers (Shah et al. 2016). Considering its high demand and ever-increasing global population, the global maize production as well as its nutritional quality, particularly in Asia, has to increase significantly in near future and that too with limited resources, shrinking arable land, and a changing climate.

Although conventional plant breeding has contributed remarkably towards maize improvement in the last few decades, it is time-consuming and also has a major limitation due to requirements of species compatibility and the dearth of germplasm diversity. Further, its actual yield potential has not been fully achieved due to various biotic and abiotic stresses. To overcome these limitations, it is of utmost importance to utilize genetic engineering (transgenic and genome editing) based breeding approaches for inserting novel traits (Agarwal et al. 2018; Kumar et al. 2018, 2020) and improving maize yield. However, for realizing the application of genetic engineering-based breeding strategies, robust and highly efficient regeneration and transformation protocols are imperative. So far different groups have tested different explants for maize callusing and regeneration globally, such as immature embryos (Duncan et al. 1985; Ishida et al. 1996; Bohorova et al. 1999; Aguado-Santacruz et al. 2007; Rakshit et al. 2010; Joshi et al. 2014; Malini et al. 2015), protoplast (Mórocz et al. 1990), tassel and ear meristems (Pareddy and Petolino, 1990), anthers (Barloy and Beckert 1993), shoot meristems (Sairam et al. 2003), nodal regions (Sidorov et al. 2006), leaf (Ahmadabadi et al. 2007), and mature embryos (Huang and Wei 2004; Tiwari et al. 2015), etc. Among these, till today, immature zygotic embryos are the most preferred and widely used explants for maize tissue culture and transformation experiments globally because of their very high regeneration potential (Ahmadabadi et al. 2007; Rakshit et al. 2010; Yadava et al. 2017). This is more so in tropical germplasm, which shows poor regeneration ability as compared to temperate maize (Yadava et al. 2017). However, immature embryo explants have a major drawback as these are not available year-round unless sophisticated glasshouse or phytotron facilities are available. Many times quality of immature embryos harvested from glass house-grown plants is not adequate for performing tissue culture and transformation experiments as compared to field-grown plants. Furthermore, 10–15 days post-anthesis is the appropriate age/stage of immature embryos for callus induction and regeneration (Abhishek et al. 2014) and these must be used immediately after extracting kernels from ears i.e. could not be stored, limiting their application in maize tissue culture and transformation endeavor. The excision and culturing of immature embryos are also laborious and time-consuming. Considering above mentioned limitations of predominately used immature embryo-based regeneration protocol, the establishment of a robust regeneration system using mature seeds as a starting material (i.e. season-independent explant source) is long envisaged in tropical maize breeding programme. Contrary, mature embryos (seed) from most tropical maize genotypes are considered recalcitrant for tissue culture and transformation as compared to immature embryos.

The present study was carried out to establish a robust and reproducible regeneration and transformation system in tropical maize using mature seeds as starting material. First efforts were made to identify the best genotype(s) producing embryogenic calli with higher frequency. Best media compositions were optimized for callus induction and regeneration. Furthermore, efforts were made to use the nodal explants-derived embryogenic calli for genetic transformation and independent transgenic lines were produced.

Materials and methods

Plant material and seed sterilization

Mature dry seeds of 28 different tropical/subtropical maize genotypes having a diverse genetic background, viz., 23 well-adapted elite inbred lines and 5 high-yielding hybrids were used in the present study (Table 1). The seeds were surface-sterilized with 1% bavistin for 20 min followed by washing with sterile distilled water for 3–4 times. Subsequently, the seeds were treated with 0.2% SDS for 10 min followed by washing with sterile distilled water for 3–4 times. Finally, these seeds were treated with 0.1% mercuric chloride ($HgCl_2$) for 5 min followed by washing with sterile distilled water for 3–4 times (Tiwari et al. 2015). After sterilization, seeds were dried on autoclaved tissue paper and inoculated on germination medium. The germination medium contained MS salts and vitamins (Himedia, Catalog No. PT021-100X1L; Murashige and Skoog 1962) supplemented with 40 g/l maltose, 0.1 g/l tryptone (casein enzyme hydrolysate), 1.95 g/l MES, 0.75 g/l magnesium chloride, gelled with 3 g/l gelrite (Duchefa Biochemie) and pH adjusted to 5.8 before autoclaving. Filter sterilized 0.5 g/l L-glutamine, 0.1 g/l L-ascorbic acids, 10 mg/l picloram (Sigma, Catalog No. PP-5575) and 3 mg/l BAP (Himedia) were added after autoclaving. Picloram and BAP help in the

Table 1 The response of 28 diverse maize genotypes for callus induction using callusing media-2 (CM2)

S. no.	Name of genotype	Pedigree/source population/parentage	Total no. of explants		Type of callus observed
			Embryo axis/split embryo (hypocotyl-epicotyl) region	Nodal explants/split node	
1.	DQL 1017-2	HQPM-1-1-4-6-2-B-#-BBB	460	360	Only watery callus[a]
2.	HKI 161	CML 161	554	327	Only watery callus[a]
3.	LM 13	JCY3-7-1-2-2-1-1 f	280	190	Only watery callusa
4.	CML 420	G16C19MH219-5-1-1-3-2-B-#-BBBB	258	126	Only watery callus[a]
5.	UMI 1210	Selection W2619-3	244	172	Only watery callus[a]
6.	IML 418-1	Waxy 418-1-2-1-2-B-#-BBB	282	141	Only watery callus[a]
7.	DMPRE 6-1	DMRPE	362	231	Watery callus with split embryo region and 21 embryogenic calli (~ 9%) from nodal explants
8.	HKI 42,050	BLS42050-1	362	181	Only watery callus[a]
9.	BML 5	NIZAMBADOP7B97K1-1-⊗B	194	97	Only watery callus[a]
10.	V 373	(JKMH-175-4 (O.P.) ⊗-16-7-12-1- ⊗b ## ⊗b#)	97	144	Only watery callus[a]
11.	IML 15-268	CML 269 X HKIPC 4B -268-1-2-2	84	94	Only watery callus[a]
12.	HKI 193-2	CML193	225	180	Only watery callus[a]
13.	CML 152	S8662Q-1-4-4-5-B-#	95	95	Only watery callus[a]
14.	IML 16-194	P72clxbrasil1117-2 x ESM11-194	95	90	Only watery callus[a]
15.	BML 10	[X_2Y Pool × Suwan 1 (T)]-B98k-1-2-1-1-2-3-⊗-2-⊗-1-⊗b-⊗b-⊗b	213	234	Only watery callus[a]
16.	HKI 323	Pool28	245	256	Only watery callus[a]
17.	BML 7	X2Y pool and CML 287	94	144	Only watery callus[a]
18.	VQL 2	[(CM 145 x CML 170) BC3P1 ⊗b⊗b⊗b-##]	100	465	Only watery callus[a]
19.	CML 409	P24STEC2-29-BBBB-#-7-BBBBBBB	100	178	Watery callus with split embryo region and 9 embryogenic calli (~ 5%) from nodal explants
20.	HKI 1128	SIRNTUXW-13-3-1-2-B-B	100	165	Only watery callus[a]
21.	BML 6	SRLL65	100	300	Watery callus with split embryo region and 104 embryogenic calli (~ 34%) from nodal explants
22.	IML 12-9	BML 6X DML 177-1-2-#-5-BB	170	105	Only watery callus[a]
23.	HKI 163	CML 163	100	150	Only watery callus[a]
24.	VH 33	V 372 × CM 212	83	100	Only watery callus[a]
25.	VH 25	V 341 × V 346	88	100	Only watery callus[a]

Table 1 (continued)

S. no.	Name of genotype	Pedigree/source population/parentage	Total no. of explants		Type of callus observed
			Embryo axis/split embryo (hypocotyl-epicotyl) region	Nodal explants/split node	
26.	DHM 117	BML 6 × BML 7	266	114	Watery callus with split embryo region and 39 embryogenic calli (~ 34%) from nodal explants
27.	DMRH 1301	BML 6 × IML 418-1	100	132	Watery callus with split embryo region and 106 embryogenic calli (~ 80%) from nodal explants
28.	DMRH 1308	BML 6 × HKI 163	100	110	Watery callus with split embryo region and and 83 embryogenic calli (~ 76%) from nodal explants
Total no. of explants used			5451	4981	

[a]In both the explants

development of conspicuous node (bulging). For germination, five to six sterile seeds per 400 ml capacity jam bottle were incubated at 27 °C ± 1 °C under 16/8 h light and dark period for 10–14 days.

Embryogenic callus induction and multiplication

The seedlings having well-developed and prominent nodes were selected and used further. The bulged nodes and hypocotyl-epicotyl axis (leftover region of embryo or tigellum) were excised from germinated seedlings under aseptic condition. Excised nodes (nodal region about 0.3 cm above and below nodes) and hypocotyl-epicotyl/embryo axis were split longitudinally into two halves. These two different explants i.e. split nodes (subsequently referred to as nodal explants) and split embryo regions were used for evaluating various maize genotypes for their callus induction potential. For finding the best callusing medium (CM), two different types of callusing media were tested i.e. callusing medium 1 (CM1) [CHU/N6 medium containing vitamins (Himedia, Catalog No. PT030-1L) supplemented with 2.76 g/l L-proline, 100 mg/l myo-inositol, 36.7 mg/l Na–Fe EDTA, 3% sucrose, 2 mg/l 2,4-D and 4 mg/l $AgNO_3$ (Merck), 3.0 g/l gelrite, pH 5.8] and callusing medium 2 (CM2) [MS salts and vitamins (Himedia), supplemented with 1.38 g/l L-proline, 0.5 g/l tryptone (casein enzyme hydrolysate), 3% sucrose, gelled with 3 g/l gelrite and pH adjusted to 5.8 before autoclaving. Filter sterilized 2.2 mg/l picloram, 0.5 mg/l 2,4-D (Himedia) and 3.4 mg/l $AgNO_3$ (Merck) were added to the medium after autoclaving]. Both CM1 and CM2 were plated on petri plates (90 × 15 mm, Himedia) for solidification. The explants were placed on the CM containing Petri plate by keeping the split/cut side of explants facing down i.e. touching CM. After inoculation, the plates were sealed with parafilm and incubated under the dark condition at 27 °C ± 1 °C for 4–5 weeks.

Only embryogenic primary calli (globular and non-mucilaginous) were selected for further sub-culturing while non-embryogenic (mucilaginous/watery/fragile/non-friable) were discarded (Fig. 1). For better proliferation, these embryogenic primary calli were sub-cultured every 15–20 days twice on the fresh CM2 of the same composition and incubated under dark at 27 °C ± 1 °C. Subsequently, these embryogenic calli were used for either in vitro regeneration or genetic transformation experiments.

Plant regeneration and rooting

Based on callusing response four genotypes, namely, BML 6, DMRH 1301, DMRH 1308, and DHM 117 were carried forward for further regeneration studies. After two subculture, the nodal explants-derived embryogenic calli (friable) were transferred to Petri plates (100 × 20 mm, Himedia) having regeneration media [RM: MS salts and vitamins (Himedia) supplemented with 1.0 g/l tryptone (casein enzyme hydrolysate), 30.0 g/l sucrose, 3.0 g/l gelrite, pH 5.8] containing several growth regulators in various combinations. In total, 22 different regeneration media (RM1 to RM22) having RM along with various

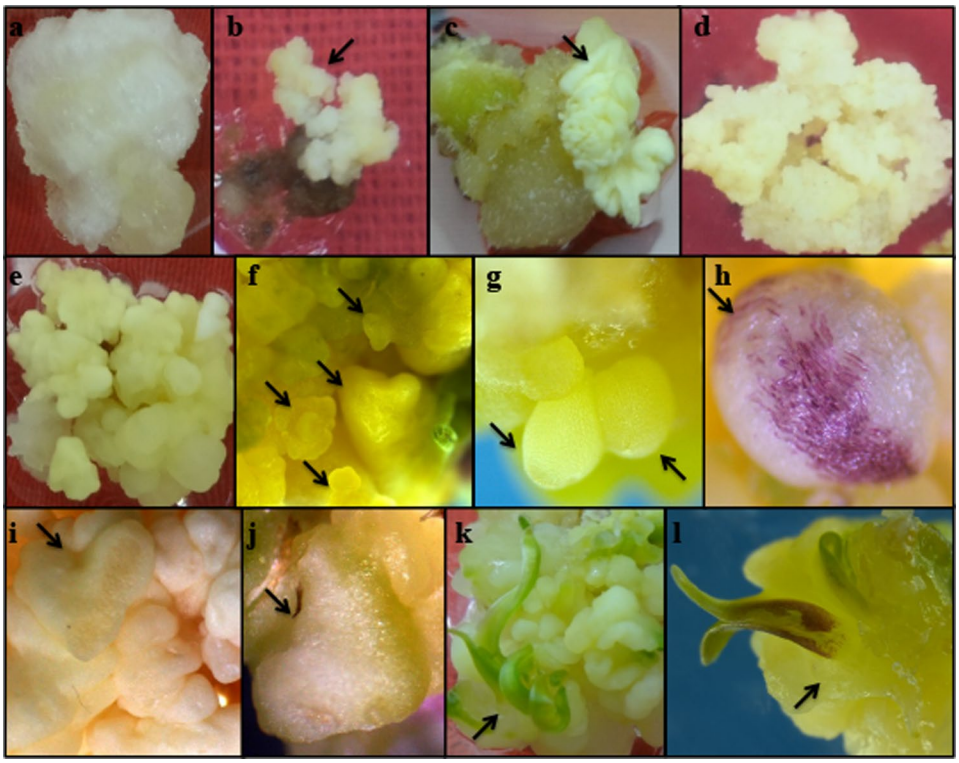

Fig. 1 Morphological characteristic of non-embryogenic and embryogenic calli of maize and different developmental stages of somatic embryos. **a** Non-embryogenic callus which is watery, mucilaginous, non-friable, and non-regenerable; **b–c** Primary embryogenic callus induced from nodal-explants; **d** Type-I embryogenic callus which is slow-growing, and less regenerative; **e** Type II embryogenic callus which is friable, fast-growing, loose, and more regenerative; **f** Globular, heart shape, and torpedo stage embryos; **g, h** Globular stage embryos; **i, j** Heart shape stage embryos; **k, l** Torpedo stage with shoot initiation

concentrations and combinations of auxins (IAA and NAA) and cytokinins (BAP and Kinetin) were tested for optimizing in vitro regeneration (Table 2). At least 20 calli per genotype per RM were used in triplicates. These embryogenic calli were incubated under 16 h photoperiod (16 h light and 8 h dark) at 27 °C ± 1 °C in the culture room. After 2–3 weeks, regenerated individual shoots (2–5 cm in length and also may have primary roots) were separated and transferred in jam bottles containing rooting medium (RM media without any hormone i.e. RM1) for root development under 16 h photoperiod at 27 °C ± 1 °C.

Hardening and acclimatization

After 10–15 days, the healthy plantlets possessing well-established roots were taken out carefully from media so that minimum damage to roots happens, followed by a gentle wash of roots with sterile water to remove entangled media particles from roots, and then transferred in autoclaved vermiculite and coco peat mixture (1:1) in small plastic glass/pots (300 ml) and watered with 40–50 ml autoclaved RO water. The plastic glass containing plantlets was covered with a transparent polythene bag having 4–6 small holes for hardening in the culture room. After one week, hardened plants were shifted in greenhouse conditions in the same plastic glass for 4–5 days and then transferred to 12″ × 10″ sized pots containing soil and grown up to maturity.

Biolistic transformation, and selection and regeneration of transformed calli

Based on callusing and regeneration response two best genotypes, DMRH 1301 and DMRH 1308 were subsequently used for transformation studies. The nodal explants-derived embryogenic calli (after two sub-culture cycles) were bombarded with pDM803 (plasmid size ~ 8.95 Kb; contains *bar* gene imparting Basta/phosphinothricin resistance for plant selection and the *gusA* reporter gene). In brief, intact plasmid DNA was precipitated and coated to gold microparticles (diameter 1.0 µm) after following Wang and Frame's (2009) procedure. The embryogenic calli were placed in the center of the Petri plate containing an osmotic medium [OSM; CHU/N6 medium containing vitamins (Himedia, catalog no. PT030-1L) supplemented with 0.69 g/l L-proline, 100 mg/l myo-inositol, 36.4 g/l sorbitol, 36.4 g/l mannitol, 3% sucrose, 36.7 mg/l Na-Fe EDTA, 3.0 g/l gelrite, pH 5.8] and incubated under dark conditions for 4 h at 27 °C ± 1 °C. These calli were bombarded twice with the plasmid DNA-coated gold particles using the following parameters: 6 cm target distance, 1100 psi rupture disk, under a vacuum of 25 mm of Hg, by particle delivery system (PDS-1000/He, Bio-Rad, USA) and were then maintained overnight (~ 16 h) at 27 °C ± 1 °C in the dark. The next day, the calli were shifted to selection medium (SM) [CM2

Table 2 Details of various hormone combinations used in RM for optimizing regeneration

Medium name	Plant growth regulators (PGRs) (mg/l)	Medium name	Plant growth regulators (mg/l)
RM1	RM without any PGRs	RM12	IAA (0.5) + BAP (2.0)
RM2	BAP (1.0)	RM13	IAA (0.5) + BAP (2.5)
RM3	BAP (2.5)	RM14	IAA (0.5) + BAP (3.0)
RM4	BAP (3.0)	RM15	IAA (0.25) + BAP (2.0) + Kinetin (1.0)
RM5	Kinetin (0.5)	RM16	IAA (0.5) + BAP (2.5) + Kinetin (1.25)
RM6	Kinetin (1.0)	RM17	IAA (0.5) + BAP (4.0) + Kinetin (2.0)
RM7	BAP (2.0) + Kinetin (1.0)	RM18	NAA (0.25) + BAP (0.75) + Kinetin (0.5)
RM8	IAA (0.5) + Kinetin (1.0)	RM19	NAA (0.375) + BAP (1.25) + Kinetin (0.625)
RM9	IAA (0.25) + BAP (1.0)	RM20	NAA (0.5) + BAP (1.5) + Kinetin (1.0)
RM10	IAA (0.5) + BAP (1.0)	RM21	NAA (0.5) + BAP (4.0) + Kinetin (2.0)
RM11	IAA (0.5) + BAP (1.5)	RM22	NAA (0.75) +BAP (2.5) + Kinetin (1.25)

IAA indole-3-acetic acid, *NAA* 1-naphthaleneacetic acid, *BAP* 6-benzylaminopurine

+ 2 mg/l DL-Phosphinothricin (PPT or Basta) (P0159.0250, Duchefa Biochemie)] to allow only transformed cells to grow further and incubated for 2–3 weeks in dark at 27 °C ± 1 °C. After the first selection, only creamish proliferating calli were transferred to fresh SM for two more rounds of selection of 2–3 weeks each while black or brown calli were discarded. After a total of three rounds of callus selection in dark, only healthy and proliferating calli were shifted to RM5S (the RM5 supplemented with 2 mg/l PPT as selection; Refer Table 2 for RM5 composition) for regeneration under 16/8 h photoperiod conditions. The regenerated shoots were transferred into RM1S (RM1 supplemented with 2 mg/l PPT) for rooting while hardening was done as per the procedure described above.

Molecular confirmation of putative transformants

Histochemical staining of β-glucuronidase (GUS) expression in transformed calli after 10 days of transformation was performed as described elsewhere (Jefferson 1987). The healthy putative transformants were analyzed for their transgenic status via Polymerase chain reaction (PCR) amplification, southern hybridization, northern blotting and histochemical staining of GUS expression. To confirm the presence of the transgene through PCR analysis, genomic DNA was extracted from leaf tissues of putative transformants and wild-type/untransformed plants using the CTAB extraction method (Saghai-Maroof et al. 1984). For PCR analysis, *gusA* gene-specific primers (forward: 5′ GATGTCACGCCGTATGTTATTGCC 3′ and reverse: 5′ TGGCAATACTCCACATCACCAC 3′) were used. The amplicons were analyzed on 1% agarose gel containing ethidium bromide and visualized under a gel documentation system.

To confirm transgene integration through Southern blotting, randomly six independent T_0 lines from DMRH 1301 and DMRH 1308 each were selected. Genomic DNA was isolated using the CTAB method from untransformed maize plant (wild type; WT) and putative transformants and 25 µg DNA subjected to restriction digestion with *Hind*III enzyme. The digested DNA was resolved on 0.8% agarose gel and subsequently blotted onto a positively charged nylon membrane (GE Healthcare Limited, UK) and cross-linked with UV light. The probe was designed against the GUS gene using PCR DIG Probe Synthesis kit (Roche Applied Science, Catalog No. 11636090910) as per the manufacturer's instructions. Further, the probe hybridization was detected by an anti-DIG antibody conjugated with alkaline phosphatase using CSPD (chemiluminescent substrate for alkaline phosphatase) using DIG luminescent detection kit (Roche Applied Science, Catalog No. 11363514910) as per the manufacturer's instructions.

Transgene expression was analyzed through northern blotting. The PCR-amplified fragment of *gusA* gene was labeled with α-^{32}P dCTP using a nick translation system (Invitrogen, Life Technologies) according to the manufacturer's instruction and used as a prob. The total RNA was isolated from WT and the same transformants (independent T_0 lines used for southern analysis) using TRIzol reagent (Invitrogen, Life Technologies, USA). About 15 µg RNA was fractionated in a 1.2% agarose–formaldehyde gel and transferred onto a nylon membrane (Amersham Hybond N+, GE, USA) by capillary flow method (Sambrook et al. 1989), and UV cross-linked. The nylon membrane was hybridized with a radiolabeled *gusA* probe using a standard protocol (Sambrook et al. 1989). The hybridized blot was developed by exposing them to a Phosphoimager screen for 12–15 h and scanned using the Typhoon Scanner (GE, USA). Further, the leaf and root tissues from

PCR, southern and northern positive plants were used for histochemical staining of reporter gene i.e. GUS activity.

Data analysis

The embryogenic callus induction frequency, regeneration frequency, and transformation efficiency were calculated as given below:

Embryogenic callus induction frequency (%)

= (Number of explants producing embryogenic (friable) calli ÷ Total number of explants plated) × 100%.

Regeneration frequency (%)

= (Number of healthy shoots regenerated ÷ total number of embryogenic calli incubated) × 100%.

Transformation efficiency (%)

= (Number of PCR positive plants exhibiting GUS activity ÷ total number of calli bombarded) × 100%.

Mean, standard error, and analysis of variance (ANOVA) were performed using SAS statistical software version 9.3 (https://sscnars.icar.gov.in/) to see the level of significance. Post-ANOVA comparisons were carried out by Tukey's HSD (Honest Significant Difference) test and differences between means were compared with HSD (at 1%) values obtained for the particular dataset.

Results and discussion

Evaluation of maize genotypes for callus induction using mature seeds as a starting material

Since mature seeds are mostly recalcitrant towards tissue culture, 28 diverse tropical maize genotypes were evaluated for good embryogenic callus induction potential (Table 1). All the selected inbreds are well-adapted to tropical/subtropical conditions and are either already used as a parent in released commercial cultivars or being used in the Indian maize breeding program. With limited success in inbred efforts were made to study tissue culture response of hybrids. The hybrids were selected based on their high-yielding ability and extent of cultivation (Kaul et al. 2012; Kumar et al. 2019). Initially, callus induction using mature seed-derived nodal explants and split embryos were tested on CM1. However, embryogenic callus induction was not observed in any genotype but only mucilaginous (watery and non-embryogenic) calli were formed. As mucilaginous calli are not suitable for regeneration due to very poor or no regeneration potential, other callusing media (CM2) were tested for evaluating all the maize genotypes. A total of 5451 and 4981 split embryos and nodal explants, respectively, from 28 genotypes were evaluated for callus induction on CM2 (Table 1). Out of 28 genotypes tested, embryogenic callus (friable, proliferative, and fast-growing) formation was achieved in three inbreds, viz., CML 409, DMRPE 6-1, BML 6, and three hybrids, viz., DHM 117, DMRH 1301, and DMRH 1308 with nodal explants (Table 1) while only mucilaginous (watery and non-embryogenic) calli formation was observed in the remaining genotypes (Fig. 1). Further, there was no embryogenic callus induction with split embryos explants in all the tested genotypes. Previously, in rice, a higher concentration of gelling agents has been shown to enhance embryogenic callus induction by imposing desiccation stress to calli (Kumar et al. 2005). Therefore, to further improve the calli induction in the genotypes producing watery non-embryogenic calli, higher concentrations of gelrite (3.5, 4 and 4.5 g/l) and porous Micropore tape (Millipore) for sealing Petri plate (instead of parafilm) were used. This was done to impose mild dehydration stress but without much success (data not shown). It has been shown by several groups that the capability of embryogenic callusing in maize is highly influenced by genotypes as only a few genotypes are amenable for tissue culture, the media compositions, and type of explants (Carvalho et al. 1997; El-itriby et al. 2003; Frame et al. 2011; Manivannan et al. 2010; Rakshit et al. 2010; Abhishek et al. 2014; Muppala et al. 2020). Our results are consistent with these previous reports and emphasize the importance of these three factors in embryogenic callus induction.

The callusing repeated five times in the best four genotypes having a higher callusing frequency, viz., BML 6, DHM 117, DMRH 1301, and DMRH 1308 (Supplementary Table 1). The average callus induction percentage of 87%, 82%, 32%, 30% was achieved in DMRH 1301, DMRH 1308, BML 6, and DHM 117, respectively, which were statistically significant at $P < 0.001$. The differences in callusing percentage among the genotypes while using mature seed-derived explants are in conformity with Huang and Wei (2004), Sidorov et al. (2006), and Tiwari et al. (2015). These four genotypes might have some genes or genomic

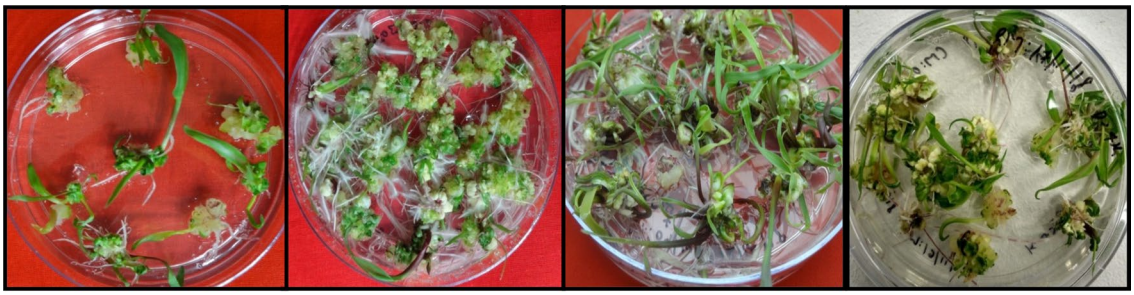

Fig. 2 Shoot regeneration from nodal explants-derived calli in four different genotypes (BML 6, DHM 117, DMRH 1301 and DMRH 1308) in RM5 media containing 0.5 mg/l kinetic hormone

regions responsible for their in vitro callusing and regeneration potential. Further, it may be noted that among the inbred, BML 6 recorded the best callusing percentage (32%,) and it is the female parent of three single cross hybrids, viz., DHM 117, DMRH 1301, and DMRH 1308, which also demonstrated much higher callusing percentage (30%, 82%, 87%, respectively). This indicated that good callusing in all these hybrids might be contributed by BML 6 as embryogenic callus induction was not observed in the other parents of all these three hybrids (Table 1). Efficient callusing in other hybrids, such as VH 25 and VH 33 could not be observed where BML 6 is not a parent. Independently, BML 6 did not give higher callusing over the hybrids where it is used as a parent. This may be because inter-allelic heterotic interactions give better callusing, which needs to be studied in the recombinant inbred lines (RILs)-derived from the best performing hybrids. Importantly, in this study, a higher callus induction percentage was obtained in DMRH 1301 and DMRH 1308 from mature seed-derived nodal explants, and it is comparable to the callusing frequency reported from frequently used explants i.e. immature embryos by different groups from various temperate, tropical, and subtropical genotypes (Bohorova et al. 1995; Aguado-Santacruzet al. 2007; Joshi et al. 2014; Malini et al. 2015).

Standardizing in vitro regeneration

A total of 22 different combinations of auxins and cytokinins hormones were assessed for testing the regeneration capability of embryogenic calli from four genotypes, viz., BML 6, DHM 117, DMRH 1301, and DMRH 1308 (Table 2). Distinct responses of calli with different combinations of hormones in regeneration media were observed. These include vigorous root formation with very less or no-shoot initials, faster callus multiplication without shoot regeneration, formation of leaf-like structure without proper shoot initial, formation of few shoot initials which lack further elongation, etc. (Supplementary Fig. 1 and Fig. 2). In 15 combinations (out of 22), most of the calli died finally without regenerating either into good quality or quantity of shoots (very low regeneration percentage) (Table 3). The proper shoot initial formation was found in the remaining combinations, which subsequently developed into the healthy shoot, but with a variable efficiency (Table 3). The effects of various combinations of growth regulators in RM and genotypes for regeneration were found highly significant ($P < 0.0001$) i.e. regeneration frequency was highly affected by the growth regulators in media as revealed by ANOVA. The importance of various growth regulators in achieving higher regeneration from embryogenic calli belonging to different genotypes has been shown in rice (Sahoo et al. 2011), wheat (Kumar et al. 2017), and maize (Huang and Wei 2004; Rakshit et al. 2010; Tiwari et al. 2015).

Based on Tukey's HSD (Honest significant difference), RM5 (regeneration media supplemented with 0.5 mg/l kinetin) was found as the best for regeneration. All the four genotypes exhibited the maximum regeneration frequency ranging from 32 to 60% in this media, which was significantly higher than all other RM (Table 3; Figs. 2 and 3; Supplementary Fig. 2). Further, ANOVA was performed separately for the dataset of regeneration in RM5, and the genotype effect was found significant ($P < 0.001$). The genotype-dependent variability in regeneration frequency in crops, including maize, has historically been a common observation (Bohorova et al. 1995; Aguado-Santacruzet al. 2007; Frame et al. 2011; Abhishek et al. 2014; Yadava et al. 2017). Furthermore, among all the four genotypes, two (DMRH 1301 and DMRH 1308) were found as the best based on HSD. Henceforth, in vitro regeneration was repeated five times in both these genotypes under RM5 and an average regeneration frequency of 60% and 53% was achieved in DMRH 1308 and DMRH 1301, respectively (Supplementary Table 2; Fig. 3). This confirms the reproducibility of our regeneration protocol. Previously, two studies have shown regeneration in different genotypes using mature seed-derived explants, viz., up to 32% frequency by Huang and Wei, 2004 and 34% by Tiwari et al. (2015). However, the regeneration frequency (up to 60%) obtained in the present

Table 3 The response of four maize genotypes (BML 6, DHM 117, DMRH 1301, and DMRH 1308) for in vitro regeneration in various RM media

Media	Regeneration percentage (%)				
	BML 6	DHM 117	DMRH 1301	DMRH 1308	Average of all four genotypes
RM1	22.76^{BCD}	11.10^{DEFG}	32.40^{BC}	22.75^{DE}	22.25^{C}
RM2	12.78^{EFG}	1.70^{G}	2.28^{G}	2.23^{GH}	4.75^{FGHI}
RM3	2.76^{HIJ}	2.31^{FG}	1.82^{G}	2.12^{GH}	2.25^{HI}
RM4	0.00^{J}	1.62^{G}	1.67^{G}	1.71^{GH}	1.25^{HI}
RM5	35.80^{A}	32.38^{A}	53.70^{A}	60.66^{A}	45.63^{A}
RM6	21.84^{CDE}	24.63^{ABC}	40.17^{B}	31.56^{C}	29.55^{B}
RM7	13.75^{DEF}	16.93^{BCDE}	31.49^{BC}	49.96^{B}	28.03^{B}
RM8	26.88^{ABC}	25.29^{AB}	29.38^{CD}	44.69^{B}	31.56^{B}
RM9	2.97^{HIJ}	14.64^{BCDE}	1.71^{G}	2.35^{GH}	5.42^{EFGH}
RM10	12.46^{FG}	22.87^{ABC}	0.00^{G}	1.95^{GH}	9.32^{DEF}
RM11	11.06^{FGH}	1.76^{G}	1.80^{G}	1.65^{GH}	4.00^{GHI}
RM12	14.63^{DEF}	2.48^{FG}	2.22^{G}	2.49^{GH}	5.46^{EFGH}
RM13	26.44^{BC}	6.83^{EFG}	12.01^{F}	10.00^{FG}	13.82^{D}
RM14	4.38^{GHIJ}	2.72^{FG}	2.86^{G}	1.70^{GH}	2.92^{HI}
RM15	1.31^{IJ}	0.00^{G}	22.49^{DE}	16.04^{EF}	9.96^{DE}
RM16	2.02^{HIJ}	0.54^{G}	14.55^{EF}	0.00^{H}	4.28^{GHI}
RM17	0.00^{J}	0.00^{G}	0.00^{G}	0.00^{H}	0.00^{I}
RM18	31.31^{AB}	13.57^{CDEF}	20.10^{EF}	44.49^{B}	27.37^{B}
RM19	15.37^{DEF}	21.36^{ABCD}	12.75^{F}	29.67^{CD}	19.79^{C}
RM20	2.47^{HIJ}	10.29^{DEFG}	1.13^{G}	2.13^{GH}	4.05^{GHI}
RM21	9.99^{FGHI}	19.82^{BCD}	1.73^{G}	2.25^{GH}	8.47^{EFG}
RM22	0.00^{J}	0.00^{G}	0.00^{G}	0.00^{H}	0.00^{I}

The individual column of variables having the same superscript letters is not significantly different according to Tukey's HSD test at 1% with P value < 0.0001

study is much higher (approximately double) than in these previous reports. One of the probable reasons for getting a much higher regeneration frequency in DMRH 1308 and DMRH 1301 than BML 6 is the hybrid vigor phenomenon which might be responsible for the higher capabilities of calli to survive stress faced during tissue culture. To the best of our knowledge, hybrid vigor for callusing and regeneration response has not been reported earlier. Previously, Al-Abed et al. (2006) reported callusing and regeneration in two hybrids (Hi-II and LH198 × LH227) and two inbreds (R23 and B73) using split-seed but no significant difference was found between the four genotypes.

The optimum root system with root hairs development was observed when well-developed individual healthy shoots were transferred in regeneration media without any hormone i.e. RM1. This is in agreement with Aguado-Santacruz et al. (2007) and Kumar et al. (2017). Subsequently, healthy regenerated plants were successfully established in the greenhouse following hardening and acclimatization (for details see "Materials and methods" section). However, phenotypic abnormalities, viz., tassel seed formation, dwarf phenotype without tassel, and the emergence of multiple ears were observed in 10–15% established regenerated plants, while remaining regenerated plants exhibited normal morphology and were able to produce normal cobs bearing normal kernels/seeds. The phenotypic abnormalities in the regenerated maize plants might be due to activations of transposable elements or alterations of heterochromatic knobs during the tissue culture process (Anami et al. 2010).

Genetic transformation of maize using nodal explants-derived calli

The subcultured embryogenic calli from both the high yielding and popular maize cultivars i.e. DMRH 1301 and DMRH 1308 were subjected to particle bombardment-mediated transformation (for details see "Materials and methods" section). The essential steps followed in the protocol have been summarized in the form of a flowchart (Fig. 4). The scrutiny of embryogenic (globular, friable, fast-growing, loose, nodular) calli from non-embryogenic (mucilaginous, fragile, slow-growing) ones was found to be a critical and essential step before in vitro regeneration and genetic transformation as this would affect the regeneration as well as transformation capability of the

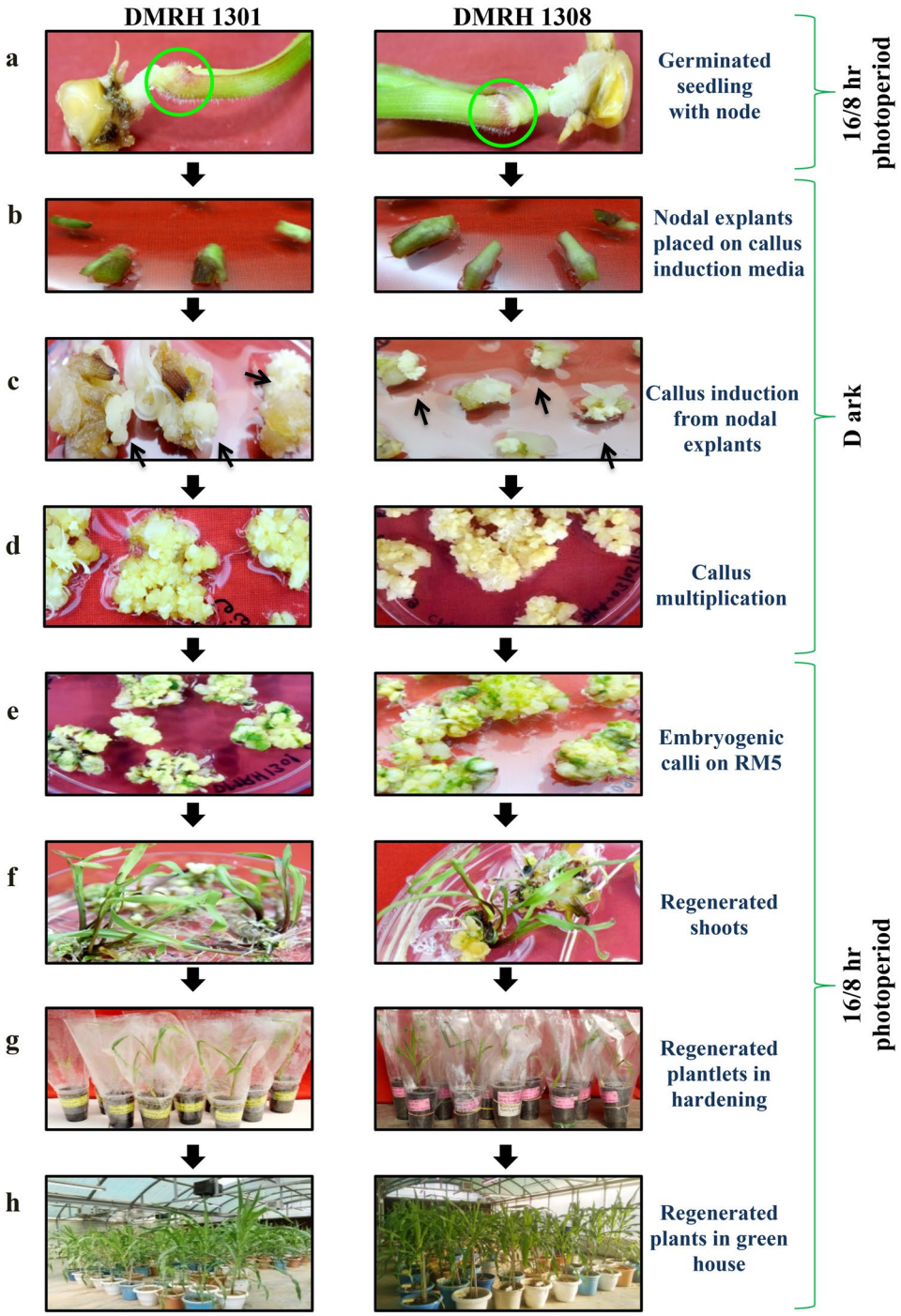

Fig. 3 Schematic representation of various steps for callusing and whole plant regeneration in tropical maize cultivars i.e. DMRH 1301 and DMRH 1308 from nodal explants. Steps b–d has to be performed under dark conditions

calli. This observation is in agreement with Manivannan et al. (2010), Sahoo et al. (2011), and Muppala et al. (2020). The histochemical staining of β-glucuronidase (GUS) activity exhibited the presence of blue spots in the calli even ten days after biolistic transformation and hence indicated the expression of GUS protein within transformed cells (Fig. 5). During the selection process under dark, some of the calli turned brownish and died while others remained creamish or yellowish and started proliferating. Subsequently, the proliferating and healthy microcalli regenerated shoots in RM5 supplemented with PPT selection (RM5S). Further, normal roots development was observed in the regenerated healthy shoots shifted to the rooting medium (RM1S) (Fig. 5).

PCR analysis of putative maize transformants using β-glucuronidase (*gusA*) specific primers confirmed the presence of the expected size band (~ 0.9 Kb) in many transformants as compared to untransformed maize plants (Fig. 5).

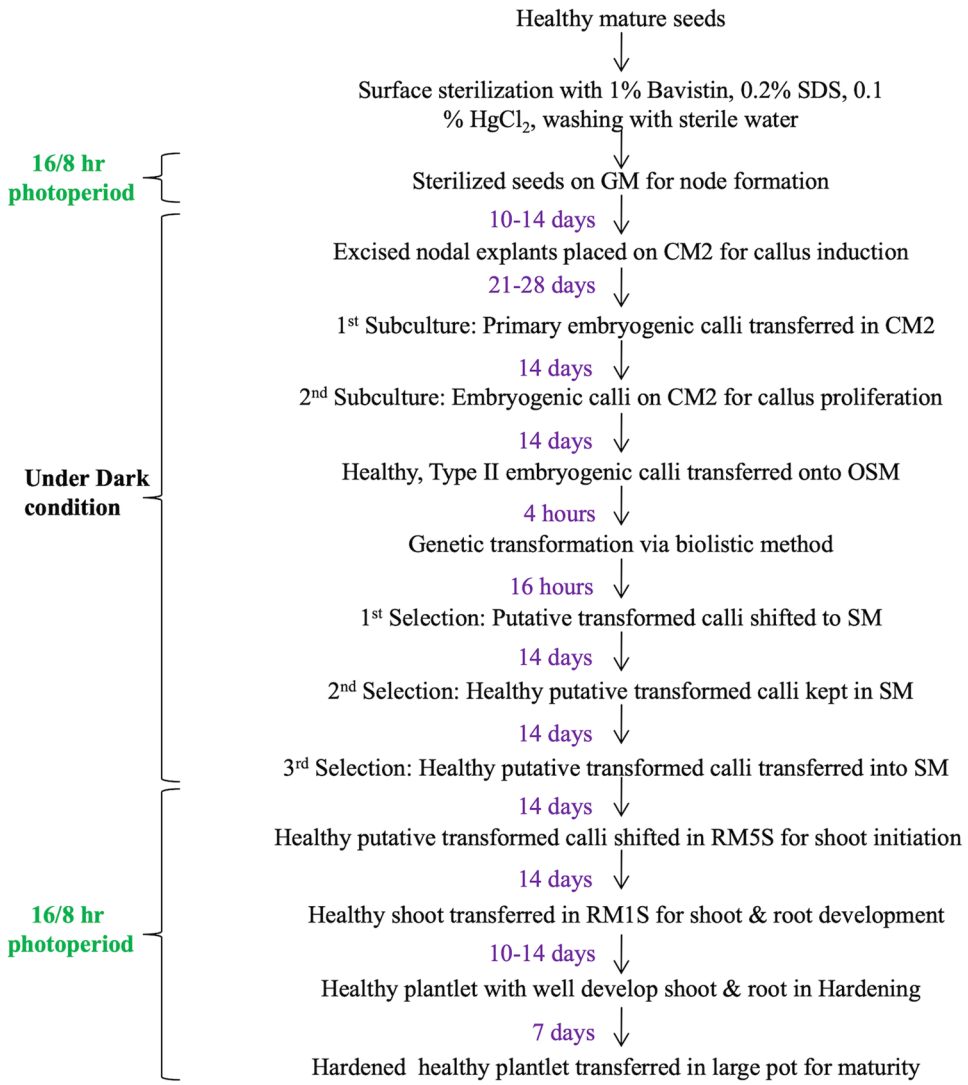

Fig. 4 Schematic workflow of transformation protocol in tropical maize using mature seeds as starting material. The flow chart showing the main steps and timeline followed in the protocol to transform nodal explants-derived embryogenic calli using the particle bombardment method. See "Materials and methods" section for details including the composition of different media

Southern analysis of randomly selected transformants confirmed the integration of *gusA* transgene into the genome of PCR positive maize plants (Supplementary Fig. 3). The number of hybridizing bands indicated single to multiple copy insertions at random locations in the transgenic plants. This analysis revealed that these plants are independent transgenic events. Similarly, northern blot analysis showed the presence of *gusA* transcripts of variable intensity in southern positive plants, confirming varied expression of the transgene in different transgenic lines (Supplementary Fig. 3). Further, histochemical analysis of transgenic plants (PCR, southern and northern positive) exhibited the presence of GUS activity in the leaf and root tissues while no GUS activity was observed in these tissues from the non-transformed wild-type plant (Fig. 5). Thus, the molecular analysis confirms the stable integration, and expression of transgene and hence, the transgenic nature of putative transformants. The genetic transformation in both the genotypes has been repeated three times and transformation efficiency of 4.25% and 5.61% was achieved in DMRH 1301 and DMRH 1308, respectively (Supplementary Table 3). Globally, immature embryos as initial material are the most preferred explants in maize and using the biolistic approach in these explants, transformation frequency ranges from 2 to 4% (Brettschneider et al. 1997), 1–2% (Bohorova et al. 1999), 0–11% (Frame et al. 2000), 0-7.3% (Wang et al. 2006), 0.9–2.31% (Petrillo et al. 2008), 1–2% (Assem et al. 2008), 1–5% (Shiva et al. 2009), 1.6% (Geetha et al. 2019) has been reported by various groups. Despite using mature seeds as the starting material which is generally considered recalcitrant for tissue culture, the transformation efficiency achieved in our study is comparable with these reports on immature embryos. Using seedling-derived maize callus, Sidorov et al. (2006) reported up to 42% callus induction and 2-11% transformation efficiency in temperate maize through *Agrobacterium*-mediated transformation. The transformation efficiency achieved in the present study is comparable to this study while the callusing frequency is significantly higher.

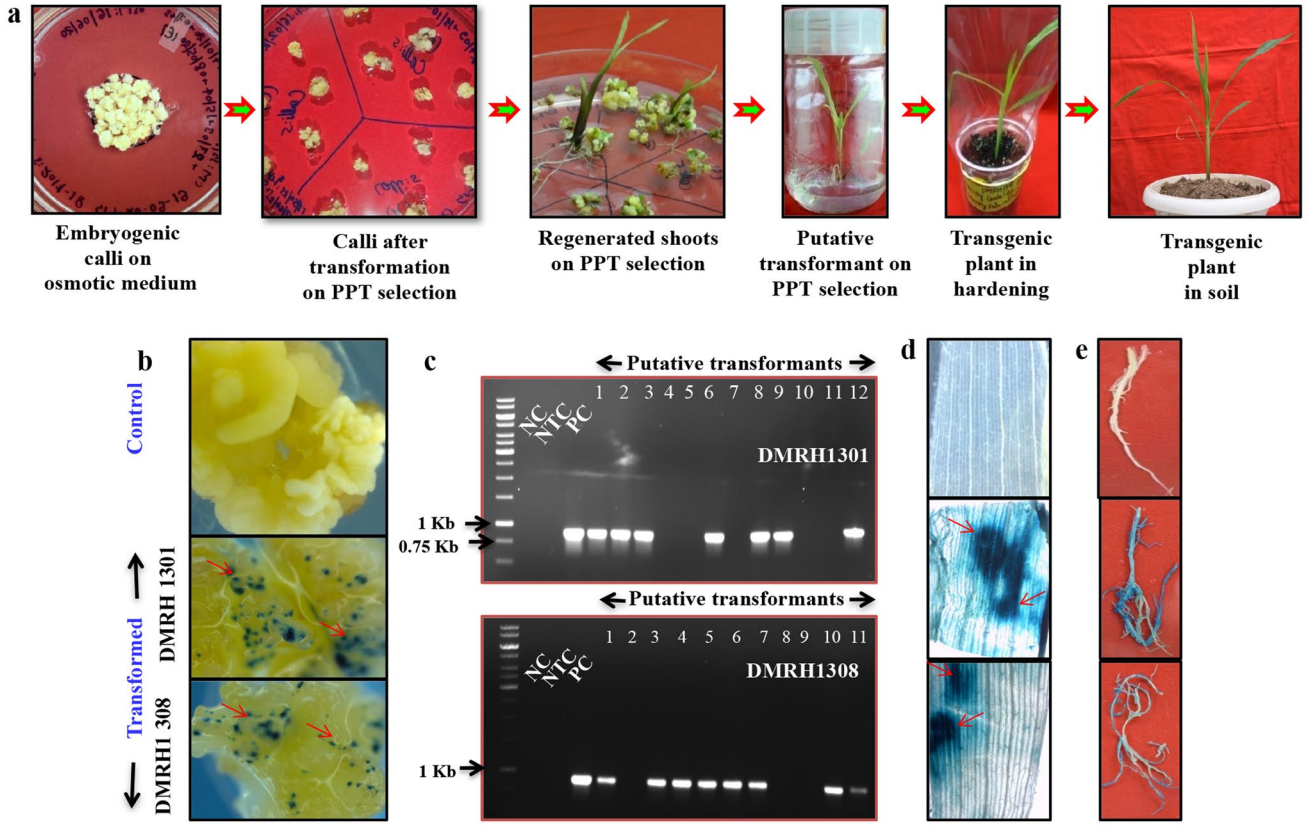

Fig. 5 Particle bombardment-mediated transformation of maize using mature seed derived-nodal explants based calli and confirmation of the transgenic status of the regenerated plants. **a** Different steps are followed during genetic transformation. Phosphinothricin (PPT) at 2 mg/l concentration was used for selection (three rounds of selection of calli in dark, followed by selection at shoot regeneration stage, and finally selection in rooting media). **b** GUS expression in calli 10 days after biolistic transformation. The upper panel represents untransformed calli, while the middle and lower panels correspond to transformed calli of DMRH 1301 and DMRH 1308, respectively. **c** PCR analysis using β-*gusA* specific primers to screen putative transgenic plants. Upper and lower gel images correspond to DMRH 1301 and DMRH 1308 putative transformants, respectively. GUS expression in leaves (**d**) and roots (**e**) from PCR, southern and northern positive plants. The upper panel represents leaf (**d**) and root (**e**) from wild-type plants, while the middle and lower panels correspond to these tissues from DMRH 1301 and DMRH 1308 T_0 transgenic plants, respectively

In the current study, maximum callusing and regeneration frequency in inbred lines could be obtained was 32% and 35%, respectively, in BML 6. Such frequency is not efficient enough to ensure a highly effective genetic transformation programme. Hence, hybrids were also explored and a very good regeneration and transformation system could be established. By integrating the Double Haploid technique with successful transgenic/genome-edited events from these hybrids (DMRH 1301 and DMRH 1308), fixed events may be obtained in the quickest time, rather than relying on inefficient transformation and regeneration protocol involving inbred lines. Later the fixed events may easily be deployed in the breeding programme. To the best of our knowledge, this is the first report in tropical maize on biolistic transformation using mature seed derived-nodal explants based calli. Taken together, our protocol has high regeneration (53–60%) and transformation efficiency (4–5%) and utilizes easily available explants. Therefore, it can be a good alternative to the existing immature embryos-based protocols in tropical maize. The present protocol would facilitate maize improvement, especially in tropical and subtropical maize, through transgenesis and genome-editing techniques.

Conclusions

The lack of a mature seed-based efficient transformation and regeneration system in tropical and sub-tropical maize is considered to be a major hurdle in developing transgenic and genome-edited lines with novel traits. To overcome this obstacle, we have standardized a highly efficient and repeatable regeneration and transformation protocol in high-yielding and popular maize cultivars, DMRH 1301

and DMRH 1308. The protocol has desirable advantages, viz., high embryogenic callus induction (up to 87%), regeneration (up to 60%), and transformation efficiency (up to 5%) which are either comparable or higher over the existing immature embryos based protocols. Furthermore, the usage of mature seeds as starting material imparts an extra advantage because of their unlimited availability round the year (season-independent) as compared to immature embryos that are not readily available but still globally used. In addition to this, mature seeds are easy to handle and kept viable for a long time. Despite using hybrids as a source of explants, fixed events could be generated quickly by employing the Double haploid technique. This easy, simple, and efficient protocol would facilitate the faster application of transgenic and genome-editing technologies for molecular breeding in maize. This will help in the augmentation of novel and economic traits which are intractable through conventional breeding.

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Author contributions SR and KK conceived the idea. AKJ, KK, AA, GG, CA, AT, and PS performed the experiments. AKJ, KK, and BK analyzed the data. PP carried out Southern and northern blot experiments. KK wrote the primary draft, which was further augmented, edited, and improved by SR and BK. BK and CGK provided the seeds of genotypes used in the study. All the authors read and approved this article for publication.

Data availability The datasets supporting the conclusions are included in the article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate Not applicable.

Ethical approval Not applicable.

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