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METHODOLOGY

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Optimized rice transformation protocol for transformation of the blast susceptible Indica rice accession CO39

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Abstract

Background: Many rice transformation protocols have been reported, but optimization is still required to ensure efficient transformation of specific rice accessions. The modified rice transformation protocol presented here builds upon the original protocol: 'An improved protocol for efficient transformation and regeneration of diverse Indica rice cultivars' volume 7, Article number: 49 (2011), Plant Methods.

Results: Following the aforementioned transformation protocol, calli browning occurred and no *Agrobacterium*-mediated transformation could be achieved, but this could be remedied by increasing the concentration of L-Proline. Improved callus health lead to successful transformation and proliferation of calli on selection media, but a low frequency of plantlet regeneration occurred when calli were transferred to regeneration media. The efficiency of plantlet regeneration was greatly improved by removing antibiotics from regeneration media, with the presence of escapes selected against during subsequent transfer of plantlets to antibiotic containing rooting media. Transformation of CO39 callus was found to be possible 8 days after callus induction resulting in a time saving of 10 days compared to the original protocol.

Conclusions: This optimized transformation protocol allows for the generation and survival of healthy CO39 calli, efficient transformation of calli using *Agrobacterium,* and produces a high frequency of regenerated transgenic plants. These protocol modifications will be useful for optimizing the transformation and regeneration of other recalcitrant Indica rice cultivars, particularly those sensitive to antibiotics during plantlet regeneration.

Keywords: Rice, Transformation, CO39, Indica, Mature seed-derived calli, *Agrobacterium*, Rice-blast susceptible

Background

Agrobacterium-mediated transformation of rice callus is favored for its ability to efficiently generate a large number of independent transformation events which can then be isolated on selective media and regenerated into transgenic plants. With the ability to induce callus from mature seed, rice transformation can be performed without the need for continuous propagation of plants. Numerous rice transformation protocols propose some variation of callus induction from mature seed (Sahoo et al. 2011; Tran and Sanan-Mishra 2015; Toki et al. 2006; Priya et al. 2018; Saika and Toki 2010). However, many protocols, particularly those focused on recalcitrant Indica rice accessions, achieve relatively low levels of plantlet regeneration from callus. In one study, between 16 (IR64) and 40% (IET-4786) of Indica derived hygromycin resistant callus regenerated into plantlets (Shri et al. 2013). For the Indica accession Kasalath, regeneration efficiencies of up to 66.9% have been reported (Saika and Toki 2010). Exceptional regeneration frequencies of 90% have been reported, but this efficiency drops once

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antibiotic selection is introduced to between 40 and 60% depending on the rice accessions and *Agrobacterium* strain tested (Sahoo et al. 2011).

Stable transformation of rice is an essential method for the rapid functional validation of rice genes, including those associated with disease resistance. The Indica rice accession CO39, which is often used as a susceptible control for rice blast infection assays (Telebanco-Yanoria et al. 2011), is a suitable genetic background for validating candidate rice-blast resistance genes. So far, reported transformation of CO39 has been limited to biolistic transformation of immature embryos, which produced four stable CO39 transformants (Narayanan et al. 2004). *Agrobacterium*-mediated transformation has the potential to improve the throughput and efficiency of CO39 transformation. To establish efficient *Agrobacterium*-mediated transformation of CO39, the Indica rice transformation protocol reported by Sahoo et al. (2011) was followed. However, when attempting to implement the protocol a number of issues arose (highlighted below), which needed to be optimized before efficient transformation and plantlet regeneration could be achieved. Here we report a transformation protocol which facilitates the successful transformation of the Indica rice variety CO39. Key optimizations include: an increased concentration of L-Proline to prevent callus browning, earlier transformation of embryonic callus representing a time saving of ten days, and removal of antibiotics from shoot regeneration media to greatly increase regeneration efficiency and overall callus health. Furthermore, we report a specific and suitable gelling strength of agarose essential to apply osmotic stress which is conducive to shoot formation, which was not reported in the original protocol. The alterations reported here are not only essential for the efficient transformation of CO39, but also provide additional tools for improving the transformation and regeneration efficiencies of other Indica rice accessions.

Results

Improving callus health to facilitate *Agrobacterium*-mediated transformation

Callus induction from CO39 mature seed was performed using the described MCI media, however MS basal salts and vitamins (Caisson laboratories, Catalog no. MSP09) were substituted for a combination of M & S Salt Base Powder (CAROLINA. 19–5703) and Gamborg's Vitamin Solution 1000x (SIGMA, G1019-50 ml) as this media was readily available and constitutes the same essential components. High rates of embryonic callus induction were observed (~90%; Additional file 1: Fig. S1), similar to those previously reported (Sahoo et al. 2011). Following callus induction callus browning began to occur, though calli did continue to grow slowly. Cutting calli into thirds after 14 days as originally reported further increased callus browning. After *Agrobacterium*-mediated transformation of subdivided calli, no calli survived the selection process. Poor callus health may explain the lack of transformation and survival on selective media. In order to reduce callus browning the concentration of L-Proline was increased from 0.6 to 2.8 g/l, as high concentrations of L-Proline may provide protection against plant stress responses and reduce callus browning (Toki et al. 2006; Hare and Cress 1997; Nanjo et al. 1999).

An additional batch of calli was prepared using the updated MCI media containing increased concentrations of L-Proline. Since callus browning previously intensified after cutting calli into thirds, this step was omitted and calli were instead directly subjected to *Agrobacterium*-mediated transformation 14 days after callus induction. To minimize damage to calli, transformation was performed while calli were still attached to the seed and coleoptile as has been proposed previously (Toki et al. 2006). Embryonic calli were separated from their seed and coleoptile prior to transfer to selection media (MSM). By leaving calli attached to seed and coleoptile, transformation can be performed shortly after callus induction. Previous examples in the Japonica rice cultivar Nipponbare have shown that 98.2% of callus could be successfully transformed after just 5 days of seed being plated on callus induction media (Toki et al. 2006). Since implementing a similar step in the modified Indica transformation protocol it has been possible to successfully transform CO39 calli attached to seed 8 days after plating sterile mature seed on the updated MCI media. Transforming calli 8 days after induction represents a time saving of 10 days compared to the original protocol.

Improved preparation of cultures for *Agrobacterium*-mediated transformation

During cultivation of either EHA105 or LBA4404 *Agrobacterium* strains in YEP media, slow growth and clumping of cells occurred despite using the reported media components and *Agrobacterium* strains. In order to simplify the preparation of *Agrobacterium* cultures, growth on solid *Agrobacterium* (AB) media rather than growth in liquid YEP media was performed. 4 days prior to calli infection with *Agrobacterium*, glycerol stocks of *Agrobacterium* cultures were streaked on Luria–Bertani (LB) agar containing appropriate antibiotics. After 48 h, single colonies were selected and transferred to solid AB media plates along with 100 µl of LB media to facilitate spreading across entire plate using glass beads. Alternatively, single colonies can be streaked into a small 'lawn' on solid AB media. After 48 h, a 0.5–1 cm^2 section of *Agrobacterium* was transferred from AB media plates to a 50 ml falcon tube containing 20 ml of MS resuspension media before mixing well by shaking,

pipetting or vortexing. Bacterial suspension was adjusted to an $O.D_{600}$ of ~0.1 by adding additional resuspension media or bacteria culture from plates. Bacterial suspension was left to shake at 28 °C for ~2 h. In the meantime, calli were prepared for inoculation by transferring pieces to one 200 ml sterile jar per construct/bacterial suspension. From this stage, *Agrobacterium* infection of calli followed the original transformation protocol except that Co-cultivation was carried out for 3 days at 25–26 °C instead of 2 days at 27–28 °C.

Selection of hygromycin resistant calli and elimination of Agrobacterium

In addition to the inclusion of a higher concentration of L-Proline in MSM selection media, the use of antibiotics to select against *Agrobacterium* were altered to further mitigate callus stress during selection. The reported protocol calls for cefotaxime to prevent growth of *Agrobacterium* during selection for transformed calli. However, it has been shown that while cefotaxime and carbenicilin are able to eliminate *Agrobacterium* at 250 mg/l, both cause severe necrosis to callus tissue derived from the Indica accession IR 64; whereas Timentin [ticarcillin disodium and clavulanate potassium (15:1)] was suitable for eliminating *Agrobacterium* without adversely influencing calli growth (Priya et al. 2018). Therefore, Timentin was used as a substitute for cefotaxime to eliminate *Agrobacterium* during the selection of hygromycin resistance calli.

Shoot regeneration from calli

To confirm that it was possible to regenerate plantlets from CO39 calli using the described protocol, shoot regeneration of untransformed calli cultured to a similar age of calli having been through *Agrobacterium*-mediated transformation and selection, was performed. Initially, it was difficult to find an agarose which solidified media, and promoted shoot regeneration as the type and brand are not reported by Sahoo and colleagues. Eventually regeneration rates for untransformed calli in the absence of antibiotic selection comparable to those reported in the original protocol were achieved (>90%) using SeaKem® LE Agarose. Similar regeneration rates have since been observed when using a different brand of agarose that also had a Gel strength of: (1%) > 1200 g/cm$^{2.}$

Efficient shoot regeneration from transformed calli is enhanced by removal of antibiotics from shoot regeneration media

With modifications to callus induction, co-cultivation, selection and regeneration media, a complete transformation procedure using CO39 mature seed was performed. The *Agrobacterium* strain EHA105 was transformed with binary vectors containing eight candidate rice blast resistance genes (Table 1). Seven of the constructs contained 2.5–3 kb of 'promotor' sequence, the full exon and intron sequence, and 1.5 kb of genomic sequence following the stop codon for each candidate gene. Genomic sequence was inserted into the pIPKb001 binary vector by In-fusion cloning (Takara Bio). pIPKb001 contains a *Hygromycin phosphotransferase* plant selection marker expressed under the maize ZmUbi1 promoter (Himmelbach et al. 2007). An additional construct used for transformation contained the gene *LOC_Os09g17870* in the binary vector pIPKb002 which contains two ZmUbi1 promotors for the expression of candidate genes and *Hyg* selection marker.

Following the first round of selection for transformed CO39 calli, 29 (*LOC_Os12g18729*) and 59 (*LOC_Os08g10440*) putative independent transformation events from 104 and 97 pieces of embryonic calli respectively, survived and proliferated on selection media. However, after transfer to the previously validated regeneration media from which high levels of shoot regeneration were obtained for un-transformed calli, transformed calli continued to grow but did not readily form shoots. From this round of transformation only two independent transformation events produced plantlets per construct (Table 1). During the same round of transformation the *LOC_Os08g10440* and *LOC_Os12g18729* constructs transformed into the *Agrobacterium* strain LBA4404 were used to transform 89 and 85 pieces of embryonic calli respectively. Poor regeneration meant only a single regenerated plantlet could be recovered per construct (Additional file 1: Table S1). Since marginally better results were obtained for *Agrobacterium* strain EHA105, all subsequent rounds of transformation used only this strain.

It has been reported for the Indica genotype IR64, that antibiotics can inhibit plantlet regeneration from transformed calli. For example, the presence of hygromycin in regeneration media reduced regeneration efficiency from between 6.94 and 12.44% to between 1.45 and 10.99% depending on the plant growth regulator (PGR) composition of regeneration media (BAP, Kinetin, TDZ or Zeatin), and when both hygromycin and cefotaxime were used, regeneration efficiency dropped to 0% regardless of the PGR used (Tran and Sanan-Mishra 2015).

To improve the regeneration efficiency of transformed calli, subsequent rounds of transformation were performed using regeneration media that did not contain antibiotics. Removal of antibiotics from regeneration media improved regeneration efficiency from 3.39% and 6.90%, to 17.81% and 32.43% for constructs containing the candidate genes *LOC_Os08g10440* and *LOC_Os12g18729,* respectively (Table 1, Round 1 vs 3). In addition to improving regeneration efficiency, excluding

Table 1 Transformation and regeneration statistics for eight constructs using *Agrobacterium* strain EHA105 to transform CO39 rice calli derived from mature seeds, utilizing a modified transformation protocol; including additional L-Proline (round 1–5), removal of antibiotics from regeneration media (rounds 2–5) and transformation of calli 8 days after induction instead of 18 days (4–5)

Transformation round	Candidate gene	Construct size LB-RB (kb)	Number of *Agrobacterium* infected calli	Independent proliferating calli lineages after selection	Putative transformation efficiency (%)	Regenerated plantlets (counting maximum 1 per event/ lineage)	Putative regeneration efficiency (%)	Escapes killed by antibiotic selection on rooting media	Escapes (surviving until genotyping)	Independent transformation events after genotyping	Overall efficiency (%)
1	LOC_Os08g10440	11.4	97	59	60.82474	2	3.389831	0		2	2.06
	LOC_Os12g18729	13.6	104	29	27.88462	2	6.896552	0		2	1.92
2	LOC_Os12g22839	6.6	180	46	25.55556	9	19.56522	4		5	2.78
	LOC_Os12g22880	8.3	180	47	26.11111	12	25.53191	3	1	8	4.44
3	LOC_Os08g10440	11.4	201	73	36.31841	13	17.80822	2		11	5.47
	LOC_Os12g18729	13.6	238	74	31.09244	24	32.43243	6	1	17	7.14
4	LOC_Os09g12350	14.5	340	64	18.82353	24	37.5	11	1	12	3.53
	LOC_Os09g12380	9	380	85	22.36842	35	41.17647	22		13	3.42
5	LOC_Os09g17810	10.3	100	23	23	9	39.13043	2		7	7.00
	LOC_Os09g17870	9.2	100	53	53	16	30.18868	4	1	11	11.00

For transformation rounds 2–5, antibiotics were excluded during plantlet regeneration. 'Lineage' refers to calli subsequently derived from a single independent transformation event. Putative transformation efficiency is calculated as the % of independent transformation events (Healthy, proliferating calli lineages surviving hygromycin selection) derived from the total number of *Agrobacterium* infected calli. Regeneration efficiency is calculated as the % of Calli lineages which survived selection that developed fully regenerated plantlets. Overall efficiency is calculated as the % of *Agrobacterium* infected calli which gave rise to a calli lineage with fully regenerated plantlets

antibiotics from regeneration media (MSRMa-II) also improved overall health of the calli. When comparing calli 14 days after transfer to MSRMa-II plus or minus antibiotics, calli were visibly healthier and readily produced shoots when transferred to MSRMa-II excluding antibiotics (Fig. 1A, C). CO39 calli transferred to MSRMa-II containing antibiotics experienced browning, grew slowly, produced very few green spots and very few shoots (Fig. 1A). Owing to the improved health of calli transferred to MSRMa-II excluding antibiotics, most putative transgenic events formed shoots (Table 1) with multiple shoots forming for each transformation event (Fig. 1C). In contrast, of the calli transferred to MSRMa-II containing antibiotics, only two putative transgenic events per construct tested produced any plantlets (Table 1). Furthermore, only a single plant was able to be recovered from these transformation events and this only occurred after 6 weeks of cultivation on regeneration media with transfers to fresh media performed every two weeks. In transformation rounds 2–5, where antibiotics were excluded from regeneration media, the number of calli lineages transferred to regeneration media which formed shoots was between 17.8 and 41.2%, compared to between 3.4 and 6.9% when antibiotics were included as in transformation round 1 (Table 1).

The results presented here indicate that removing antibiotics from regeneration media greatly improves callus health and the rate of shoot regeneration for transformed calli derived from the rice cultivar CO39. Importantly, antibiotics can still be included in selection media (MSM) allowing for the isolation of transformed calli before transfer to regeneration media.

Inclusion of antibiotics in rooting media does not prevent root formation and prevents non-transgenic escapes

Following the formation of shoots greater than 1–2 cm in length (Fig. 1D), shoots were transferred to rooting media which contained 30 mg/L of Hygromycin as suggested in the original protocol. Despite the removal of antibiotics during the regeneration stage, relatively few plantlets failed to survive when transferred to the hygromycin containing rooting media indicating that growth of calli on selection media prior to plantlet regeneration eliminates most non-transgenic calli (Fig. 1E). Transformation round 4 was an exception with 45.83% (11/24) and 62.86% (22/35) of lineages producing shoots failing to survive when transferred to rooting media (Table 1). Including antibiotics in rooting media was not detrimental to root formation and is a necessary step to ensure that non-transgenic escapes are selected against.

Validation of transgenic lines by qPCR, PCR and segregation analysis

Rice plantlets that were recovered from tissue culture were screened by qPCR to confirm the presence and copy number of the *hygromycin phosphotransferase* gene using genomic DNA as a template. The *Sucrose Phosphate Synthase* gene was used as a single copy endogenous reference gene (Ding et al. 2004). qPCR analysis showed that the majority of the 90 (88 derived from EHA105 (Table 1), 2 derived from LBA4404) independent transformation events for the 8 candidate genes were predicted to contain multiple copy insertions (≥ 2 copies), 32 had a single-copy insertion and 3 plants were non-transgenic escapes (Additional file 1: Table S1). To rule out the possibility of a truncation of the transgene occurring during transformation, the rice plantlets were screened with primers specific for the flanking regions of each candidate gene by PCR. This step uncovered a total of 7 truncated transgene copies across all the transgenic lines which did not retain part or all of their candidate gene, but did retain the *hygromycin phosphotransferase* gene (Additional file 1: Table S1).

Seeds from 15 single-copy, 11 double-copy and 3 multiple-copy (>2 copies) T0 plants were sown out and the segregation of the transgene was verified with a leaf-tip assay to test for the presence of the hygromycin resistance gene (Fig. 2) (Wang and Waterhouse 1997). The results of the segregation analysis were subjected to a chi-square goodness-of-fit test, to assess if the observed segregation ratio differed from the expected. Only 3 of the 15 single-copy transgenic lines did not show the expected segregation ratio. In contrast 7 of the 11 double-copy transgenic lines had segregation ratios corresponding to a single-copy insertion line (Additional file 1: Table S1), suggesting that the qPCR analysis overestimated the copy number in T0 parent plants (Fig. 2).

Discussion

The method reported here builds on the Indica rice transformation protocol proposed by Sahoo and colleagues to allow for the successful transformation of the rice blast susceptible Indica accession CO39. The modifications presented here were essential to enable callus survival, transformation and plantlet regeneration for calli derived from CO39. Plantlet regeneration from callus is a major bottleneck in rice transformation, particularly for Indica rice accessions. The transformation protocol reported by Sahoo and colleagues reports a high percentage of plantlet regeneration from embryonic calli which can hold true for the Indica accession CO39, but only when antibiotics are excluded from regeneration media as we propose. Importantly, antibiotic selection is used

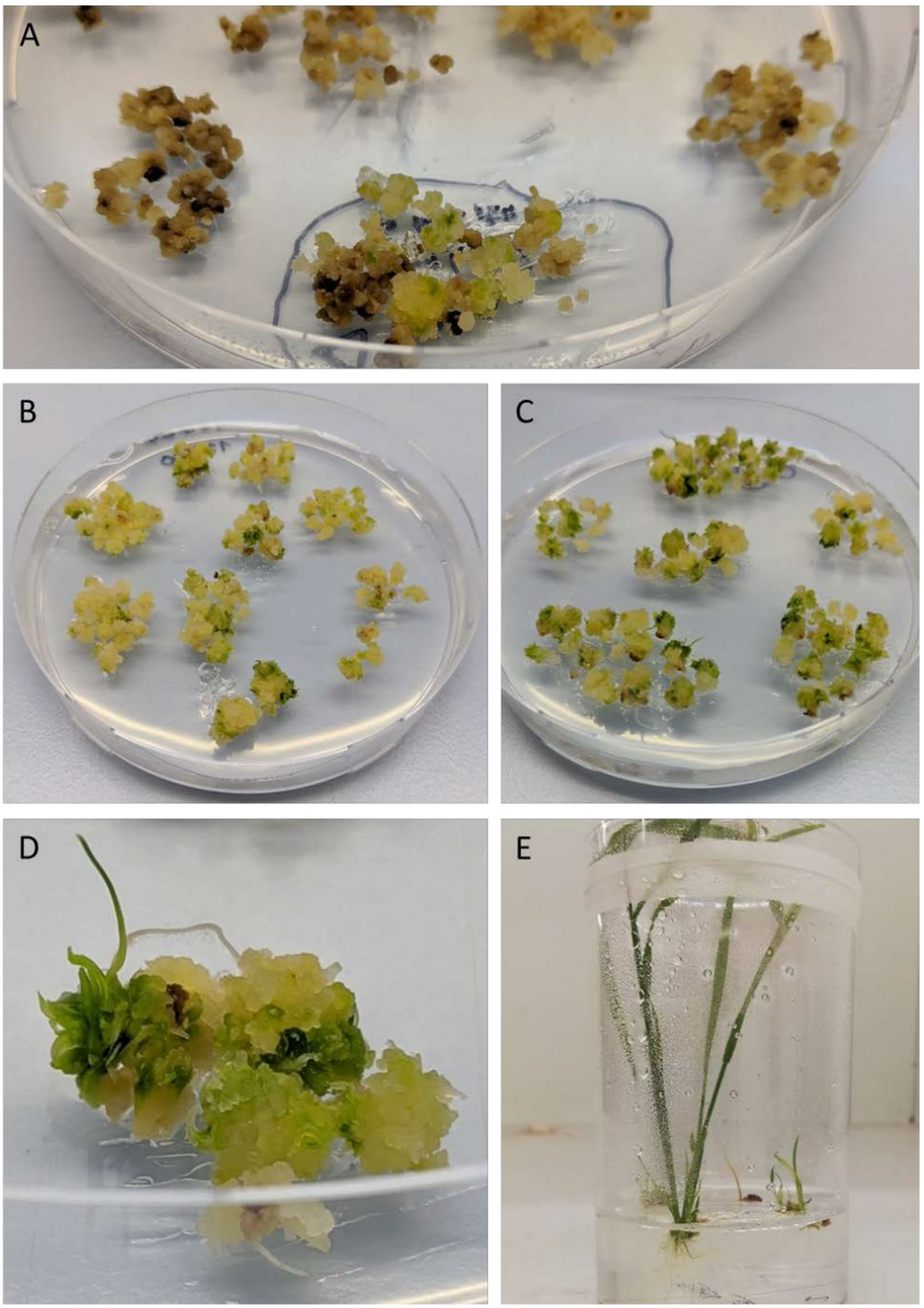

Fig. 1 Regeneration of CO39 calli is inhibited by presence of antibiotics. **A** Calli after 14 days on regeneration media MSRMa-II containing antibiotics. Calli browning and poor shoot regeneration can be observed. **B** Callus after 9 days on MSRMa-II media excluding antibiotics. **C** Callus after 14 days on MSRMa-II media excluding antibiotics. **D** Close up image of regenerating callus 14 days after transfer to MSRMa-II excluding antibiotics. **E** Image of regenerated shoots of different ages after transfer to rooting media, MROM containing 30 mg/l hygromycin. Shoots were transferred to rooting media after forming 1–2 cm shoots on regeneration media excluding antibiotics. A shoot from a putative transformation event at the back of the rooting media is a non-transgenic escape and is killed by transfer to hygromycin containing rooting media. For scale, petri dishes are 90 mm wide and jam jar in **E** is 50 mm wide

at all other stages following transformation (selection on MSM, and rooting on MROM) to limit the number of non-transgenic escapes. Transgene presence, and hygromycin resistance in subsequent generations of transgenic lines follows expected segregation ratios, indicating that any chimeric plants are selected against during antibiotic selection on rooting media.

In the case of CO39, the presence of antibiotics during plantlet regeneration on MSRMa media drastically reduces callus health and plantlet regeneration from putative transformed calli. Similarly, the presence of hygromycin during IR64 callus shooting causes reductions in regeneration frequency, or completely blocks regeneration when cefotaxime is also included (Tran and Sanan-Mishra 2015). Callus growth rates are also known to be reduced by the presence of hygromycin (Li et al. 1993). The removal of antibiotics from regeneration media is sufficient to improve CO39 callus health and increase shoot regeneration frequency, similar to reports for the Indica rice accession IR64 (Tran and Sanan-Mishra 2015). Excluding antibiotics during shoot regeneration allows for plantlets to be recovered from almost all transformation events, and multiple plantlets to be recovered from each transformation event. Although recovering multiple plantlets from a single transformation event is not essential when screening for independent transgenic events, this may be useful for recovering multiple CRISPR/Cas-9 containing plants where chimeric edits and inefficient homology directed repair edits can subsequently be detected.

Agrobacterium-mediated transformation of rice calli derived from mature seed is favored over biolistic transformation protocols for the efficient and stable transformation of rice (Li et al. 1993). Both *Agrobacterium*-mediated transformation and biolistic transformation are associated with genomic sequence abnormalities, though sequence disruptions caused by biolistic transformation can be severe (Liu et al. 2019). Still, the ability for biolistics to introduce proteins and genetic material, such as Cas9 ribonucleoproteins and repair templates for precise gene editing, and allele swaps (Liang et al. 2017; Ali et al. 2020), makes biolistics a favorable technique for genome editing applications. Importantly, the modifications presented here which result in improved calli health and plantlet regeneration rates are broadly applicable to biolistic approaches in rice.

Timentin has been shown to improve shoot regeneration rates in tomato, potentially through the release of auxin-like compounds (Costa et al. 2000). Although Timentin was not included in our modified regeneration media, we cannot discount the possibility that replacing cefotaxime with Timentin during selection of transformed calli helps to promote shoot formation when calli are subsequently transferred to regeneration media. Similarly, additional L-Proline included in selection media may have a positive influence on shoot formation after transfer. Proline has been reported to improve rice callus regeneration when included in regeneration media (Khaleda and Al-Forkan 2006).

We employed qPCR to rapidly identify positive transformants and to estimate transgene copy number. While qPCR was effective at identifying single copy transgenic lines for downstream analysis, it often overestimated the transgene copy number. A qPCR based approach which controls for variation in PCR efficiency could be employed in future (Huang et al. 2013). Regardless, qPCR represents a cheaper and more straightforward option for rapidly screening transgenics for single copy insertions compared to either southern blot analysis or digital droplet PCR (Yuan et al. 2007). Furthermore, combining qPCR with the hygromycin leaf-tip assay in T1 plants was sufficient for the isolation of single copy transgenic lines, and the identification of non-transgenic siblings for downstream phenotypic comparisons.

Conclusions

Building on the original protocol, the modifications highlighted here are essential for the transformation of CO39 and may help facilitate the transformation of other recalcitrant Indica rice accessions by improving callus health and plantlet regeneration rates.

Methods

A protocol flow chart (Fig. 3) and media list can be found after the written method. Unless stated otherwise, incubation steps are performed in the dark and plates are sealed with parafilm.

Generation of rice calli

Mature rice seeds of the Indica cultivar CO39, provided by the International Rice Research Institute (IRRI), were used to generate calli. The seeds were hulled, and then sterilized as follows: they were washed for 1 min with 70% Ethanol, rinsed with sterile distilled water and then washed again with 6–8% Sodium hypochlorite containing 2 µl 50% Tween per 1 ml for 5 min, followed by a rinse with sterile distilled water. Subsequently, the seeds were soaked for 20 min in 6–8% Sodium hypochlorite, rinsed 5 times with sterile distilled water and left to dry in a sterile petri dish.

Sterile petri dishes were prepared by half filling with molten MSM media. Approximately 12 seeds were distributed per plate and incubated at 28 °C for 8–14 days (Additional file 1: Fig. S1). 20 plates are sufficient to generate ~ 200 pieces of calli.

Agrobacterium-mediated transformation of rice calli and selection

Glycerol stocks of transformed *Agrobacterium* were streaked on LB agar containing Rifampicin (25 mg/l) and Spectinomycin (100 mg/l) and incubated at 28 °C for two days. Colonies were then plated on AB agar using glass beads and 100 µl of sterile LB media to facilitate even spreading. The plates were incubated for 48 h at 28 °C.

A 1–2 cm^2 patch of *Agrobacterium* from solid AB media was added to 30 ml of MS resuspension media (Sahoo et al. 2011). The OD_{600} of bacterial suspension was measured using a BioPhotometer® D30 (Eppendorf, Hamburg, Germany) and adjusted to OD_{600} 0.1–0.15. Bacterial suspensions were incubated for 2–3 h at 28 °C with gentle shaking (100–120 rpm).

The generated rice calli were transferred to sterile jam jars, or 50 ml falcon tubes and enough *Agrobacterium* suspension was added to cover calli. Calli were incubated with gentle rocking for 20 min at room temperature (22 °C). After incubation, the calli were left to dry between 4 layers of sterile filter paper. Subsequently, the coleoptile and seed were removed from the calli and calli were plated on MCCM media. The calli were then incubated for 3 days at 25–26 °C.

Calli were transferred to a sterile falcon tube and rinsed between 5 and 10 times with sterile distilled water until the water was clear. Then calli were rinsed 2 times with 25 ml of 250 mg/l Timetin in sterile distilled water. The calli were left to dry between 4 layers of sterilized filter paper and then replated onto selection plates containing MSM media. Plates were incubated for 12 days at 28 °C.

Any healthy white, or creamish white calli were replated onto fresh MSM media and incubated first for 10 days, before a final transfer of calli and incubation for 7 days at 28 °C. During transfer, clusters of proliferating calli originating from a single section of transformed calli were maintained in groups separate from other transformation events (Fig. 1A–D).

Plantlet regeneration from rice calli

Healthy calli groups were transferred to MSRMa-I media and incubated for 7 days at 28 °C. From this point on, all plates were sealed with micropore tape. Depending on the size of calli groups, between 4 and 10 groups of calli were maintained per petri dish (Fig. 1B, C).

Calli groups were then transferred to MSRMa-II media and incubated at 27 °C, 16 h light/8 h dark photoperiod and 71.8 $mMol/m^2/s$ light intensity, until they formed shoots of >1 cm. Calli that had not formed shoots after two weeks were transferred to fresh MSRMa-II media. Shoots generally began forming after one week, but could continue to appear for up to four weeks.

Calli with shoots were transferred into sterile 'jam jars' (Fig. 1E) containing MROM media. The plants were incubated at 27 °C, 16 h light/8 h dark photoperiod and 71.8 $mMol/m^2/s$ light intensity. As soon as the plants had formed a root system with multiple lateral roots over 3 cm in length, they were planted into damp soil (70% peat, 30% perlite mix) and grown in a greenhouse at 28 °C and 70–80% relative humidity. The plants were fertilized with 1 g/l 0.1% Sequestrene vital (Maag, Dielsdorf, Switzerland) and 2 ml/l 0.2% Wuxal+ Profi (Maag) after a week of establishing in soil.

Screening for the presence and copy number of hygromycin resistance gene in transgenic plants

The copy number of the t-DNA was assessed using a single-copy reference gene, the Sucrose Phosphate Synthase (SPS) gene (Ding et al. 2004). Detection of the hygromycin resistance gene and copy number assessment of the t-DNA was performed by real-time quantitative PCR (qPCR) on a real-time PCR thermal cycler CFX96TM (Bio-Rad Laboratories, Hertfordshire, UK) using compatible 96-wells plates. Each DNA sample from transgenic plants was amplified in a total reaction volume of 10 µl. This included 5 µl of 2 × KAPA SYBR® fast qPCR Mix, 3.5 µl sterile distilled water and 5 µM of the forward and reverse primers for either the hygromycin resistance gene (Collier et al. 2017) or the SPS gene. Plates were spun down briefly and then analyzed using the following thermal cycling profile: 3 min at 95 °C, followed by 39 cycles of 20 s at 95 °C, 20 s at 56 °C and 10 s at 72 °C. Acquisition was performed after each cycle. The results were analyzed with the CFX Maestro Software (Bio-Rad).

Screening for intact candidate transgene in transgenic plants

Each DNA sample from transgenic plants was screened for the presence of the candidate transgene by PCR in a 0.2 mL thin wall PCR tube Multiply®-µStrip (Sarstedt, Nümbrecht, Germany). Each 20 µL reaction contained 4 µL 5 × Green GoTaq® G2 reaction buffer (Promega, Fitchburg, US), 0.4 µL 10 mM dNTPs, 0.6 µL DMSO, 0.2 µL GoTaq® G2 polymerase (Promega), 11.8 µL deionized water and 0.5 µM of each primer for the respective candidate gene and flanking vector sequence. The PCR was performed using the following cycling profile: 3 min at 95 °C, followed by 33 cycles of 20 s at 95 °C, 30 s at the corresponding annealing temperature and 72 °C for 30 s with a final extension time of 10 min at 72 °C. The PCR products were visualised by agarose

gel electrophoresis using 1% agarose, 1 × Sodium-Borate (SB) buffer gel.

Leaf-tip assay to test for the presence of hygromycin resistance in segregating t1 plants

Leaf pieces of 2 cm where cut from seedlings and stuck into a sterile distilled water agar media (2.2 g/l bacto agar, and 30 mg/l hygromycin B added after cooling) and incubated at 22 °C, 16 h light/8 h dark photoperiod and 31.3 mMol/m^2/s light intensity. The results of the assay were scored after 5–7 days (Fig. 2).

Protocol modifications* as indicated in Fig. 3 are detailed below:

1. *Agrobacterium* stock streaked on LB agar;
2. Secondary culture by streaking lawn of *Agrobacterium* on AB agar;
3. Suspension of *Agrobacterium* in MS liquid media directly from secondary culture plate to O.D600 = 0.1 − 0.15. *Agrobacterium* suspension then incubated at 28 °C for around 2 h. O.D 600 should increase slightly during this time up to 0.15–2 from 0.1 to 0.15;
4. Embryonic calli including seed and shoot used directly for infection with *Agrobacterium* after 8–14 days on MCI media without sub-culturing small pieces, or additional 4-day incubation;
5. Co-cultivation of *Agrobacterium* infected calli performed at 25–26 °C for 3 days instead of at 27–28 °C for 2 days;
6. Washing of calli performed using 250 mg/l of Timentin;
7. Additional 2 days spent on third selection media;
8. Antibiotics excluded from regeneration media; and

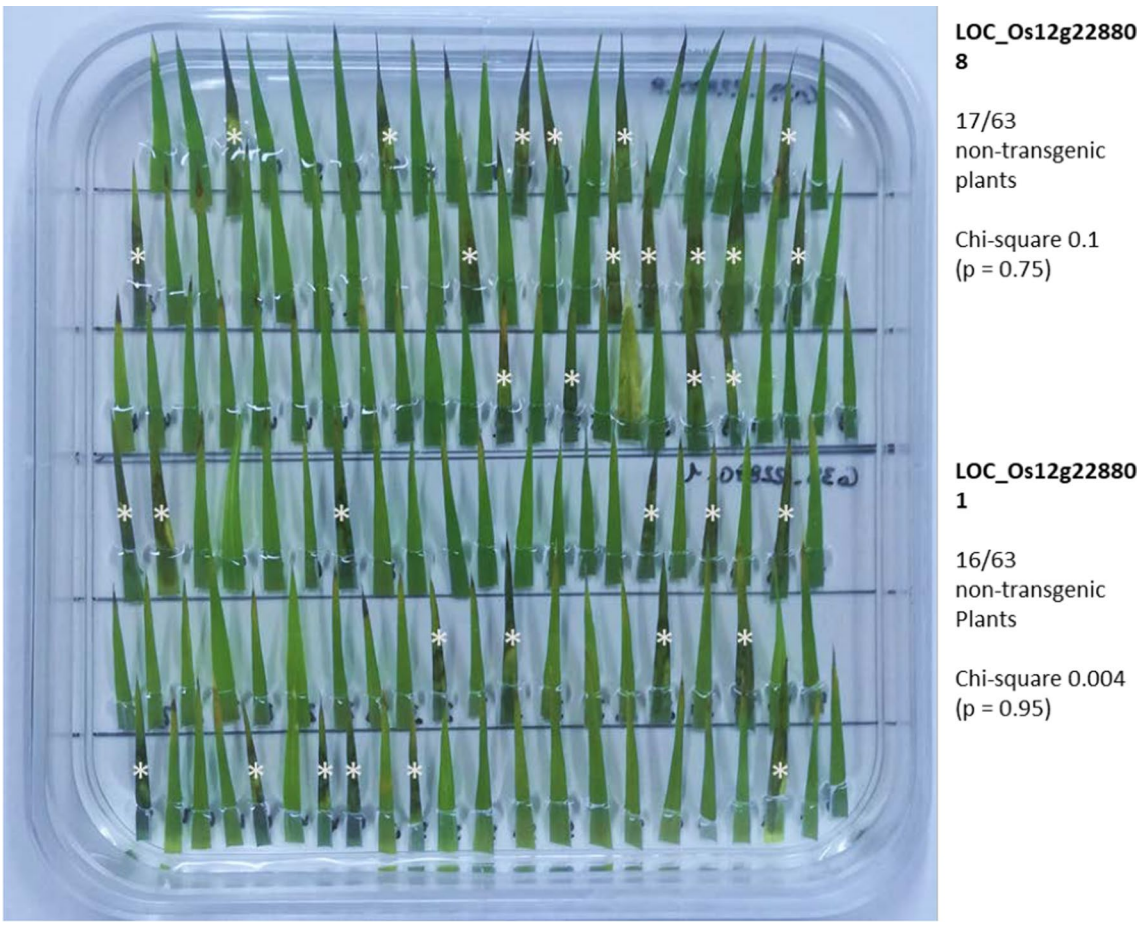

Fig. 2 Example of hygromycin leaf-tip assay for segregating T1 population. 17/63 and 15/63 T1 plants from the segregating T1 of LOC_Os12g22880_1 and LOC_Os12g22880_8 transformation events, respectively were likely homozygous non-transgenic individuals based on necrosis of leaf tissue* indicative of hygromycin susceptibility. The results of the chi-square test show that the observed segregation ratio does not differ significantly ($p > 0.05$) from the expected segregation ratio (1/4) of a single-copy, or single locus transformation event. Color saturation and color temperature have been adjusted to improve visibility of leaves. Original image is presented in Additional file 1: Fig. S2

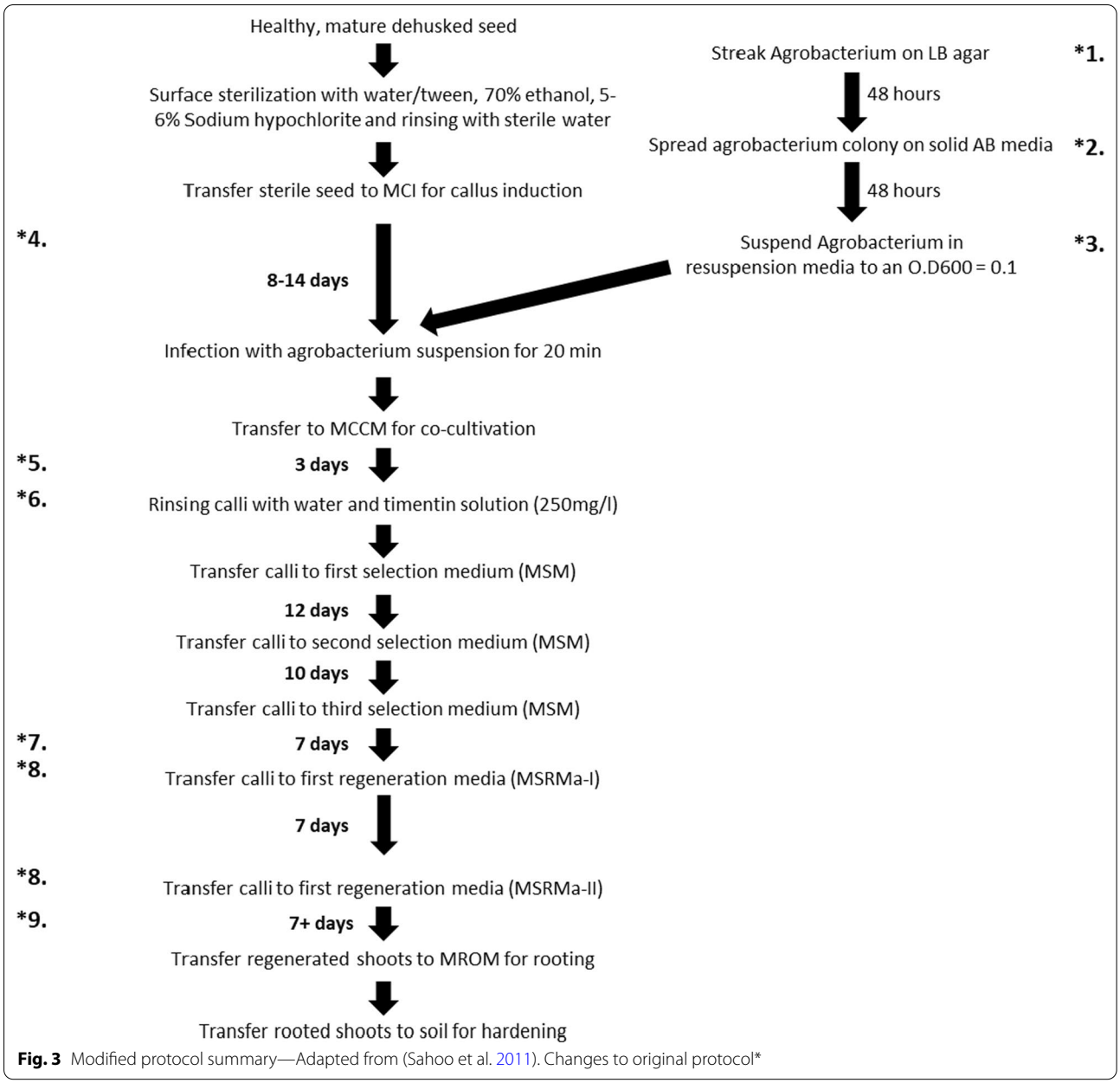

Fig. 3 Modified protocol summary—Adapted from (Sahoo et al. 2011). Changes to original protocol*

9. Transfer of regenerated shoots typically possible between 1 and 3 weeks. After 1.5–2 weeks, calli were transferred to fresh MSRMa-II media.

Summary of media alterations for CO39 transformation

M & S Salt Base and Gamborg's Vitamin solution were used as a direct substitute for MS basal salts and vitamins.

BAP excluded from MCI, MCCM and MSM media. Inclusion of BAP at 0.25 mg/l may promote embryonic callus induction rate of CO39, but was found to inhibit Callus growth rate (Additional file 1: Fig. S1). Successful transformation has been performed with or without BAP. Depending on the cultivar used, BAP may not be necessary for callus induction and propagation.

Casamino acids were used as a direct substitute for Casein hydrolysate. Both Casein hydrolysate and Casamino acids have been used successfully.

Increased concentration of L-Proline in MCI and MSM media (as described in main text)*.

AB agar used for culturing *Agrobacterium* (as described in main text)*.

SeaKem® LE Agarose used for regeneration media (as described in main text).

Antibiotics excluded from regeneration media.

*Media alterations adapted from (Toki et al. 2006).

Media modifications and ingredient list

Items which are underlined are substitutions or alterations to the original protocol.

*Indicates additional media component for modified protocol.

MCI

4.3 g/l M & S Salt Base Powder (CAROLINA. 19-5703), 1 ml/l Gamborg's Vitamin Solution 1000x (SIGMA, G1019-50 ml), 30 g/l maltose, 0.3 g/l casamino acids, 2.8 g/l l-proline, 3 mg/l 2, 4-D, pH 5.8 and 3 g/l phytagel or gelzan.

AB agar*

1000 mg/l NH_4Cl, 296 mg/l $MgSO_4{\cdot}7H_2O$, 1150 mg/l $NaH_2PO_4{\cdot}H_2O$, 3000 mg/l K_2HPO_4, 150 mg/l KCl and 10 ml/l of Stock containing 1000 mg/l $CaCl_2$ + 250 mg/l $FeSO_4$ (10 mg/l $CaCl_2$ and 2.5 mg/l $FeSO_4$ final concentration in AB media). pH 7.2, 5000 mg/l Glucose and 15 g Bacto agar added before autoclaving.

MS resuspension media

4.3 g/l M & S Salt Base Powder (CAROLINA. 19-5703), 1 ml/l Gamborg's Vitamin Solution 1000x (SIGMA, G1019-50 ml), 68 g/l sucrose, 36 g/l glucose, 3 g/l KCl, 4 g/l $MgCl_2$, 0.5 g/l casamino acids, pH 5.2 and 150 µM acetosyringone (Freshly prepared at a concentration of 100 mM by dissolving 0.3924 g in 12 ml 95% Ethanol and then adding 8 ml of miliQ water. Solution is filter sterilised before use).

MCCM

4.3 g/l M & S Salt Base Powder (CAROLINA. 19-5703), 1 ml/l Gamborg's Vitamin Solution 1000x (SIGMA, G1019-50 ml), 0.3 g/l casamino acids, 0.6 g/l L-proline, 10 g/l glucose, 3 mg/l 2, 4-D, pH 5.2, 3 g/l phytagel or gelzan and 150 µM acetosyringone added after autoclaving.

MSM

4.3 g/l M & S Salt Base Powder (CAROLINA. 19–5703), 1 ml/l Gamborg's Vitamin Solution 1000x (SIGMA, G1019-50 ml), 30 g/l maltose, 0.3 g/l casamino acids, 2.8 g/l l-proline, 3.0 mg/l 2, 4-D, pH 5.8 and 3 g/l phytagel or gelzan. 250 mg/l Timentin [Ticarcillin disodium and clavulanate potassium (15:1), (Bioworld, 420100123)] for first selection, 150 mg/l thereafter, and 50 mg/l hygromycin B added after autoclaving.

MSRMa-I

4.3 g/l M & S Salt Base Powder (CAROLINA. 19-5703), 1 ml/l Gamborg's Vitamin Solution 1000x (SIGMA, G1019-50 ml), 30 g/l maltose, 2 mg/l kinetin, 0.2 mg/l NAA, pH 5.8, 10 g/l SeaKem® LE Agarose (LONZA, 50004).

MSRMa-II

4.3 g/l M & S Salt Base Powder (CAROLINA. 19-5703), 1 ml/l Gamborg's Vitamin Solution 1000x (SIGMA, G1019-50 ml), 30 g/l maltose, 2 mg/l kinetin, 0.2 mg/l NAA, pH 5.8, 8 g/l SeaKem® LE Agarose (LONZA, 50004).

MROM

2.15 g/l M & S Salt Base Powder (CAROLINA. 19-5703), 0.5 ml/l Gamborg's Vitamin Solution 1000x (SIGMA, G1019-50 ml), 30 g/l sucrose, pH 5.8, 3 g/l phytagel or gelzan. 30 mg/l hygromycin B added after autoclaving. 150 mg/l Timentin may also be added after autoclaving, but *Agrobacterium* contamination is typically removed after selection on MSM.

Abbreviations

BAP: 6-Benzylaminopurine; SDN: Site directed nuclease; NAA: 1-Naphthaleneacetic acid; TDZ: Thidiazuron.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43170-022-00100-y.

Additional file 1: Figure S1. Callus induction from mature CO39 seed on MCI media +/- BAP after fourteen days. **A** Frequency of embryonic callus induction is greatest in the presence of BAP, but callus growth rate is slowed when compared to **B** callus induced in the absence of BAP. Scale bars are 5 cm. **Figure S2.** Unaltered hygromycin leaf tip assay image from Figure 2. **Table S1.** T0 screening results (copy number <1.5 = green, 1.5-2.5 = orange, >2.5 = red). For the purpose of the Chi-square test, T0 plants predicted to contain multiple copies (>1.5) are treated as two copy lines.

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Author contributions

JRG designed and performed experiments, and contributed to the writing of the manuscript. ANG designed and performed experiments, and contributed to the writing of the manuscript. Both authors read and approved the final manuscript.

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Availability of data and materials

All relevant data is contained in the manuscript.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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