LaCl₃ treatment improves *Agrobacterium*-mediated immature embryo genetic transformation frequency of maize

Shengnan Liu¹ · Yunlu Shi¹ · Fang Liu¹ · Yan Guo¹ · Minhui Lu¹

Received: 14 December 2021 / Accepted: 18 March 2022 / Published online: 4 April 2022
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2022

Abstract

**Key message** We report an optimized transformation system that uses a LaCl₃ pretreatment (a Ca²⁺ channel blocker) for enhancing *Agrobacterium*-mediated infection of immature embryos and improving the genetic transformation frequency of maize.

Abstract *Agrobacterium*-mediated genetic transformation of immature embryos is important for gene-function studies and molecular breeding of maize. However, the relatively low genetic transformation frequency remains a bottleneck for applicability of this method, especially on commercial scale. We report that pretreatment of immature embryos with LaCl₃ (a Ca²⁺ channel blocker) improves the infection frequency of *Agrobacterium tumefaciens*, increases the proportion of positive callus, yields more positive regenerated plantlets, and increases the transformation frequency from 8.40 to 17.60% for maize. This optimization is a novel method for improving the frequency of plant genetic transformations mediated by *Agrobacterium tumefaciens*.

Keywords Maize · *Agrobacterium tumefaciens* · Immature embryo · LaCl₃ · Transformation frequency

Introduction

Maize is a monocotyledon food crop, as well as a feed and energy crop. It is the most widely grown and productive crop in the world. Approximately, one-third of the world’s population depends on corn as a staple food. Maize is a C₄ plant, meaning that it is a model plant for photosynthesis studies (Mookkan, et al. 2017). Owing to the time and labor consumption, and the existence of interspecific reproductive barriers, which prevent the introduction of target traits into recipient plants, the utilization of excellent germplasm resources is limited to a certain extent by conventional breeding (Ahmar, et al. 2020). However, transgenic technology has significantly promoted the process of obtaining various resistance candidate genes and new varieties based on the gene-function studies. Still, the efficient, short-cycle, and stable genetic transformation system of maize remains a hindrance.

Transgenic technology is a powerful method for cultivating high-yield and high-quality crops resistant to biological and abiotic stress. During the development of the maize transgenic technology, scientists have invented many transformation methods, such as electroporation (Fromm, et al. 1986), particle bombardment (Klein, et al. 1988), polyethylene glycol (PEG) treatment of protoplasts (Golovkin, et al. 1993), silicon carbide fibers (Kaeppler et al. 1994), and *Agrobacterium*-mediated transformation (Ishida et al. 1996). Among these transgenic transformation methods, *Agrobacterium*-mediated transformation not only has a clear mechanism, simple operation, low cost, but it also exhibits stable inheritance of exogenous genes and low-copy number (Liu et al. 2017). Owing to its many advantages, *Agrobacterium*-mediated transformation is the most widely used genetic transformation method, especially in commercial production (Chen et al. 1998; Hiei et al. 1997).

In 1987, Grimsley et al. first used *Agrobacterium* for infecting maize, and demonstrated that this method could be used for transforming maize (Grimsley et al. 1987). Subsequently, in 1996, Ishida et al. established a relatively stable agrobacterium-mediated genetic transformation system.
with immature maize embryos as explants for the first time (Ishida et al. 1996). There have been many studies on the Agrobacterium-mediated optimization of the immature maize embryo genetic transformation system. Many factors, such as the vector, the explant genotype, the explant pretreatment condition, Agrobacterium strains, the Agrobacterium solution concentration, the infection duration, the co-culture duration, the infection medium, and the co-culture medium, affect the genetic transformation frequency (Sheikholeslam and Weeks 1987; Cho et al. 2014; Frame, et al. 2006; Hiei et al. 2006; Sivanandhan et al. 2015; Vega et al. 2008). Recently, a ternary vector system carrying extra copies of Vir genes has been shown to increase the transformation frequency of maize (Anand et al. 2018; Zhang et al. 2019). The application of morphogenic regulator genes such as BABY BOOM (BBM) and WUSCHEL (WUS) was a significant breakthrough in the genetic transformation of maize, significantly improving the transformation frequency and enabling to overcome the dependence on genotypes and explants to a certain extent (Salvo et al. 2014; Lowe et al. 2016, 2018; Mookkan et al. 2017). However, its application in commercial-scale production still has some problems. Although Agrobacterium-mediated genetic transformation frequency of maize immature embryos has significantly improved owing to continuous system optimization, and the method has been widely used in the commercialization of maize breeding, the low transformation frequency remains a bottleneck that precludes practical application of maize gene-function studies and molecular breeding.

It is well known that Agrobacterium tumefaciens, a naturally occurring Gram-negative bacterium, contains a tumor-inducing (Ti) plasmid, which contains transfer deoxyribonucleic acid (T-DNA) that can be integrated into recipient plant genomes after being horizontally transferred into plant cells. Hence, Ti plasmid molecules are modified to transform target genes into the plant genome, enabling the transformation of the target genes in the recipient species; Agrobacterium tumefaciens has been termed “the smallest genetic transformation engineer in nature” (Yuan and Williams 2012). In addition, evidence suggests that Agrobacterium tumefaciens triggers the activation of multiple mitogen-activated protein kinases (MAPKs), a defense mechanism that is rapidly triggered by the host perception of pathogen-associated molecular patterns (PAMPs) (Djamei, et al. 2007). However, A. tumefaciens can also induce the formation of plant crown galls (Escobar et al. 2003); thus, it is an exogenous pathogen in sessile plants (Cho and Winans 2005). When exogenous pathogenic microorganisms infect plant receptors, the innate immune response of the receptors is triggered to defend against pathogens and maintain growth (Gómez-Gómez 2004). During the plant-pathogen interaction, a sequence of signal transduction events occurs in plants, including an increase in the Ca2+ concentration, accumulation of reactive oxygen species (ROS), and activation of signaling cascades mediated by mitogen-activated protein kinases (MAPKs) and Ca2+-dependent protein kinases (Lamb and Dixon 1997; Boller and Felix 2009). Toyota et al. investigated the mechanisms of the long-distance transmission of Ca2+-dependent defense signals in plants; They applied LaCl3, a Ca2+ channel blocker, to the leaves of Arabidopsis thaliana, blocking the transmission of danger signals from caterpillars to nearby and distal parts of the plants (Toyota, et al. 2018). Inspired by this, here we propose that the Agrobacterium-mediated transformation frequency can be improved by dampening the innate immune response of plants to Agrobacterium tumefaciens.

In this study, we developed an efficient optimization system that used a Ca2+ channel blocker for pretreating immature maize embryos before the Agrobacterium infection. Optimization of the receptor pretreatment conditions revealed that immature maize embryos pretreated with 10 mM LaCl3 yielded twice as many positive regeneration plantlets as those in the control group, and stability was further validated using testing vectors.

## Materials and methods

### Plant materials

Immature embryos of transformed receptors were from maize inbred lines ND101 and ND88 created by the Center for Crop Functional Genomics and Molecular Breeding of China Agricultural University (Liu et al. 2021; Zhang et al. 2019). Maize plants were grown in a greenhouse under a 16/8 h light/dark cycle at 20–25 °C. Immature embryos were collected from fresh ears 9–12 days after pollination for genetic transformation.

### Agrobacterium strains and binary vectors

The Agrobacterium tumefaciens strain EHA105 was used for maize transformation. The binary vector contained the Ds-Red gene as the reporter driven by the ubiquitin-1 promoter, and bar as the herbicide resistance selection marker gene. The test vectors contained a single target and double targets, provided by the Center for Crop Functional Genomics and Molecular Breeding of China Agricultural University. The vectors’ structure and related construction methods were described by Xing et al. (2014). Gene models were obtained from the Gramene database (https://ensembl.gramene.org/Zeae_mays/Info/Index). Single-guide ribonucleic acids (SgRNAs) were designed using the CRISPOR database (http://crispor.tefor.net/).
**Agrobacterium-mediated transformation and immature embryo pretreatment**

Maize transformation followed published protocols with minor modifications (Sidorov and Duncan 2009). The 9–12 DAP (day after pollination) ears of maize inbred lines ND101 or ND88 were sterilized in 70% ethanol solution for 1 min after removing the bracts. Intact immature embryos with a length of 1.5–2.0 mm were isolated, and placed in a 2-mL centrifuge tube containing infection solution (2.16 g/L MS basal salt mixture, 10 ml/L 100× MS vitamins, 68.5 g/L sucrose, 36 g/L glucose, 0.115 g/L L-proline, pH 5.2). Following the collection of immature embryos, the infection medium in the centrifuge tubes was removed and a fresh infection medium containing LaCl₃ was quickly added to them. Then, the centrifugal tubes were placed in a 45 °C water bath for a 5-min-long pretreatment. After the LaCl₃ pretreatment, the supernatant was removed from the centrifuge tubes to the maximal possible extent, and a fresh Agrobacterium solution with OD₆₀₀ in the 0.6–0.8 range was added to the tubes. Under dark conditions, the centrifuge tubes containing immature embryos and the Agrobacterium solution were incubated at 22 °C for 30 min. After that, the Agrobacterium fluids were removed to the maximal possible extent. To facilitate subsequent observations and quantitative analysis of the RFP expression fluorescence, after the immature embryos were transferred into the co-culture medium (2.16 g/L MS basal salt mixture, 10 ml/L 100× MS vitamins, 3 mg/L 2,4-D, 10 g/L glucose, 20 g/L sucrose, 0.115 g/L L-proline, 200 μM acetosyringone, 3.4 mg/L silver nitrate, pH 5.2), sterile filter paper was used for gently absorbing the fluid around the embryos, to reduce background noise during photography. After cultivation for 2 days under dark conditions at 22 °C, the embryos were transferred to screening medium (4.33 g/L MS basal salt mixture, 10 ml/L 100× MS vitamins, 30 g/L sucrose, 1.38 g/L L-proline, 0.5 g/L casamino acids, 3.0 g/L phytogel, 0.5 mg/L 2,4-D, 2.2 mg/L picloram, and 3.4 mg/L silver nitrate, 250 mg/L carbenicillin and 5 mg/L bialaphos sodium salt, pH 5.8) for 14 days cultivation under dark conditions at 28 °C to induce calluses. Then, the calluses were transferred to pre-regeneration medium (4.33 g/L MS basal salt mixture, 1 ml/L 1,000× Fromm vitamins stock, 3 mg/L 6-benzyladenine, 3.0 g/L phytogel, 30 g/L sucrose, 1.36 g/L L-proline, 0.05 g/L casamino acids, 250 mg/L carbenicillin and 5 mg/L bialaphos sodium salt, pH 5.8) for 14 days cultivation under dark conditions at 28 °C to induce calluses. After all primary regeneration plantlets (T₀) were obtained, the transformation frequency was calculated as follows: the transformation frequency (%) = number of infected embryos × 100.

**Analysis of the transient RFP expression and statistical analysis of the RFP fluorescence intensity**

The red fluorescence distribution of immature embryos and resistant calluses was observed using a multifunctional zoom microscope (Nikon AZ100), for wavelengths in the 510–560 nm range. The fluorescence signal values were computed using a custom script by ourselves. Statistical significance was assessed using Student’s t-test.

**Statistical analysis of the infection frequency, rate of callus, rate of positive callus, regeneration rate, and transformation frequency**

After 2 days of co-culturing, the infection frequency was calculated as follows: the infection frequency (%) = number of embryos with RFP transient fluorescence expression/number of infected embryos × 100.

After 10 days of culturing using the selection medium, the rate of RFP-positive callus was calculated as follows: the rate of RFP-positive callus (%) = number of calluses with RFP transient fluorescence expression/number of total calluses × 100.

After 14 days of culturing using the selection medium, the rate of callus was calculated as follows: the rate of callus (%) = number of calluses/number of embryos in the co-culture medium × 100.

After 20 days of the first differentiation culture, the regeneration rate was calculated as follows: the regeneration rate (%) = number of calluses with shoots/number of total callus clumps × 100.

After all primary regeneration plantlets (T₀) were obtained, the transformation frequency was calculated as follows: the transformation frequency (%) = number of bar-positive T₀ events/number of infected embryos × 100.

After all bar-positive plantlets (T₀) were obtained, the single copy frequency was calculated as follows: the single copy frequency (%) = number of single copy T₀ plantlets/number of infected embryos × 100, and the low-copy frequency was calculated as follows: the low-copy frequency (%) = number of one/two-copy T₀ plantlets/number of infected embryos × 100.

All the above statistical significance analyses were assessed using Student’s t-test.
Molecular identification and copy number analysis of transgenic plantlets

Genomic DNA of transgenic plantlets was extracted using magnetic beads. Wild-type lines and putative transformations were confirmed by polymerase chain reaction (PCR) analysis with selection marker gene primers (forward primer: ATGAGCCCAGAACGC; reverse primer: TCAAAATCTCGGTGACCGG). Copy number analysis of transgenic plantlets was performed by the duplexed TaqMan assays (Liu et al. 2021). Bar gene was detected by TaqMan quantitative PCR and hmg was the reference gene. The bar gene primer and probe sequences are: bar-taq-F: ACAAGC ACGTGCAACTTCC, bar-taq-R: GAGGTCGTCGCTCCA CTC, bar-taq-probe: FAM-TACCGACGAGAACC -BQ1 (FAM is the fluorescent group at the 5' end, BQ1 is the quenching group at the 3' end). The hmg gene primer and probe sequences are: hmg-taq-F2: TGAGACTAGAAAT CTCGTCGCTGA, hmg-taq-R2: TACATAGGAGCCCT GTCC, hmg-taq-probe: VIC-CAATCCACACAAACG CAGCCGT-BQ1 (VIC is the fluorescent group at the 5' end, BQ1 is the quenching group at the 3' end). The copy numbers of bar of T0 transgenic plants were calculated by the Pfaffl formula (Pfaffl 2001) which took into account the amplification efficiency of the transgenic bar and reference gene hmg.

Results

Pretreatment of immature embryos with LaCl3 improves the infection frequency of Agrobacterium tumefaciens

To test the hypothesis above, we simultaneously pretreated immature embryos of ND101 with infection media containing different concentrations of LaCl3 at 45 °C for 5 min before the Agrobacterium infection; the infection medium without LaCl3 was used as the control. Twenty-five immature embryos were used for each treatment and kept in the infection medium for less more than 60 min. After the pretreatment with 10 mM LaCl3, immature embryos were infected with Agrobacterium tumefaciens EHA105 harboring the binary vector with the RFP reporter gene (Fig. S1). Then, they were transferred to a co-culture medium for co-cultivation.

To detect the infection frequency, fluorescence microscopy was used for investigating the transient expression of RFP after co-cultivation for 2 days, and the infection effect was assessed by the statistical analysis of the images’ fluorescence intensity. The results suggested that the fluorescence intensity after the pretreatment with 10 mM LaCl3 was significantly higher than that for the control group and those for the other tested concentrations (Fig. 1A and B); in addition, the infection frequency for the pretreatment with 10 mM LaCl3 was the highest (Fig. 1C). After that, we observed the callus induction after 14 days under the selection condition, and calculated the rate of callus; observation of pre-differentiated callus clumps cultured for 12 days were also followed (Fig. 1A and D). The results of these analyses indicated that the pretreatment with 10 mM LaCl3 yielded the best performance for the pre-differentiation state, had no effect on the callus formation, and significantly improved the infection frequency mediated by Agrobacterium.

Pretreatment of immature embryos with LaCl3 improves the rate of positive callus

Our experimental results confirmed that the pretreatment of immature embryos with 10 mM LaCl3 improves the infection frequency of Agrobacterium, but whether the recipient cells integrating the exogenous genes from the T-DNA of Agrobacterium undergo dedifferentiation and re-differentiation for forming embryogenic callus is a hinge that affects the transformation frequency.

To evaluate whether the transformed cells could form embryogenic callus through dedifferentiation, after 10 days of callus induction in the selection medium under dark conditions, we observed the transit expression of RFP in calluses using fluorescence microscopy, and counted the proportion of RFP-positive callus. We observed that a number of red-fluorescent adventitious buds appeared in embryogenic calluses following the pretreatment with 10 mM LaCl3, compared with the control group (Fig. 2A), and the proportion of RFP-positive callus was consistent with this (Fig. 2B). The results suggested that the pretreatment of immature embryos with LaCl3 increased the rate of RFP-positive callus, and did not negatively affect the formation of embryogenic callus.

Pretreatment of immature embryos with LaCl3 improves the transformation frequency

To investigate the effect of the pretreatment with 10 mM LaCl3 on the transformation frequency of immature embryos via Agrobacterium tumefaciens, we tracked the re-differentiation process of all calluses. After 14 days of callus induction in the selection medium under dark conditions, all calluses, including non-embryonic calluses, were transferred to the pre-differentiation medium for resistance screening under low light conditions for 12 days, and then were transferred to the regeneration medium for resistance selection under light conditions for 20–30 days. We compared the differentiated shoots developed in the experimental and control groups after culturing for 20 days in the regeneration medium, and it was obvious that the experimental
group pretreated with 10 mM LaCl₃ fared better than the control group (Fig. 3A). Furthermore, we estimated the regeneration rate as the proportion of tissues differentiated with elongated shoots relative to the overall number of calluses. The results showed that after the LaCl₃ treatment, the regeneration rate increased from 13.20 to 27.20%, which was

Fig. 1 Pretreatment of immature embryos with 10 mM LaCl₃ improves the infection frequency of Agrobacterium tumefaciens. Immature embryos were treated with 10 μM, 100 μM, 1 mM, 10 mM, and 100 mM of LaCl₃, respectively, for 5 min before the Agrobacterium infection. A. Transient expression of RFP in immature embryos after 2 days of co-cultivation; calluses induced in the selection medium after 14 days, and callus clumps cultured after 12 days of pre-differentiation cultivation. The red squares represent calluses in the randomly selected magnified area below. B. RFP fluorescence statistics for the transient expression. C. Statistical analysis of the infection frequency. D. Statistical analysis of the callus’ rate. Error bars are mean ± SEM. Statistical differences were analyzed using Student’s t-test, n = 3

Fig. 2 Pretreatment of immature embryos with 10 mM LaCl₃ improves the rate of positive callus. A. RFP-positive calluses cultured for 10 days in the selection medium, observed using a fluorescence microscope under the RFP field (left) and bright field (right). The white asterisks indicate red-fluorescent adventitious buds that appeared in calluses. B. Statistical analysis of the rate of RFP-positive callus. Error bars are mean ± SEM. Statistical differences were analyzed using the Student’s t-test, n = 10
more than twofold than the control group’s rate (Fig. 3B).
Subsequently, we identified bar-positive T₀ plantlets using
PCR, and calculated the transformation frequency. The
results revealed that the transformation frequency increased
from 8.40 to 17.60% after the LaCl₃ pretreatment (Fig. 3C
and D). The low-copy frequency also increased from 8.00
(20/250) to 15.20% (38/250). Moreover, the number of bar-
positive T₀ plantlets transplanted into the nutrition bowl of
the LaCl₃ pretreatment was twice that of the control group,
consistent with the above conclusion (Fig. 3E). Our results
indicated that pretreatment of immature embryos with LaCl₃
improved the Agrobacterium-mediated transformation fre-
quency in maize.

We also used EDTA and EGTA (two Ca²⁺ chelators,
although they also chelate other divalent metal cations,
such as Mg²⁺ and Mn²⁺) to pretreat immature embryos of
ND101. Preliminary results indicated that the transformation
frequency increased from 7.47 (5 bar-positive T₀ plantlets/32
immature embryos of ND101) to 27.78% by EDTA treat-
ment (10 bar-positive T₀ plantlets/36 immature embryos of
ND101).
ND101) and 31.43% by EGTA (11 bar-positive T₀ plantlets/35 immature embryos of ND101), respectively (Fig. S2A). In addition, we pretreated immature embryos of ND88 in the same manner, yielding a recalcitrant maize inbred line. The infection frequency nearly doubled from 46.88% (32 immature embryos of ND88) to 100% (EDTA: 29 immature embryos of ND88, EGTA: 32 immature embryos of ND88), and fluorescence microscopy-based analysis also suggested that the fluorescence quantity of the RFP transient expression in immature embryos after 2 days of co-culturing was significantly higher than that for the control group (Fig. S2B and C). These results suggest that the repression of calcium increasing triggered by Agrobacterium tumefaciens infection enhances the maize embryo transformation frequency.

To address whether the LaCl₃ pretreatment of immature embryos affected the morphology and fertility of regenerated plants, we followed the growth, development, and fructification of T₀ generation plants. As expected, no significant difference was observed in terms of the plant growth and development between the LaCl₃ pretreatment group and the control group (Fig. S3).

Based on the above, we established an optimized system for improving the frequency of the Agrobacterium-mediated genetic transformation by pretreating immature maize embryos with LaCl₃ (Fig. 4).

**Testing of the effectiveness and stability of the optimized genetic transformation system in maize**

To test the validity of the optimized protocol, we selected four adenosine triphosphate (ATP)-binding cassette
transporter genes (Zm00001d036986, Zm00001d047534, Zm00001d018522, and Zm00001d046662) and constructed six vectors, namely CAUC1828, CAUC1829, CAUC1831, CAUC1832, CAUC1834, and CAUC1871 (Fig. S4). After that, we introduced five of the vectors into the maize inbred line ND101, using the optimized protocol, while the remaining vector was transformed using the non-optimized protocol as the control. Then, we estimated the regeneration rates and the transformation frequencies for the different transformation protocols. The results suggested that the regeneration rate increased from 11.09 to 20.52–25.21% after the optimization, while the average regeneration rate was 23.03%. Consistently, the transformation frequency increased from 7.17 to 11.98–12.95% after the optimization, while the average transformation frequency was 13.25%. The regeneration rate and the transformation frequency both doubled compared with the corresponding control values (Table 1). Copy number is an important selection index in both gene-function research and production breeding. Therefore, we further analyzed the low-copy frequency. The results of our data analyses showed that the low-copy frequency in the control group was 4.35%, while it ranged from 7.18 to 12.18% for five of the vectors in the LaCl3 treatment group (Table 1). The average low-copy frequency for the LaCl3 treatment group was 8.84%, which was more than double that for the control group. This result showed that the protocol optimized with LaCl3 increased the transformation frequency without affecting the copy number of T0 plants. Thus, the results showed that the regeneration rate, the transformation frequency, and the low copy frequency were all doubled compared with the control group values.

In conclusion, we used LaCl3 to inhibit the Ca2+-dependent signal transduction triggered by Agrobacterium-infected immature maize embryos, effectively improving the frequency of Agrobacterium-mediated infection and increasing the transformation frequency.

**Discussion**

Based on the hypothesis that partial inhibition of Ca2+ transduction triggered by the Agrobacterium infection explants improves the infection frequency and thus possibly improves the transformation frequency, we successfully established a transformation system for pretreatment of immature embryos with LaCl3. This method can improve the infection frequency of Agrobacterium, the regeneration rate, and the transformation frequency. We introduced six vectors for system verification, which revealed that the proposed protocol indeed effectively improved the regeneration rate, the transformation frequency. Hence, in the present study, inhibition of the Ca2+ signal transduction triggered by the Agrobacterium infection in explants improved the transformation frequency.

Ca2+ is a universal second messenger that plays an important role in signal transduction in many physiological processes, including stress and immune responses in plants and animals (Ma et al. 2019). For an ever-increasing number of environmental stresses, pathogen attacks, drought stress, cold/heat stress, oxidative stress, and salt stress, it has been found that temporally and spatially defined rapid changes in the cytoplasmic Ca2+ concentration differ in the Ca2+ elevation duration, intensity, amplitude, frequency, and other aspects. Moreover, Toyota et al. showed that caterpillar feeding or wounding with scissors induced rapid [Ca2+]cys increased that propagated to distal parts. However, when plants were treated with LaCl3, systemic [Ca2+]cys was blocked. In addition, the relative expression levels of wound-induced defense marker genes, such as JAZ5, JAZ7, ZAT12, OPR3, and RBOHD, significantly decreased (Toyota et al. 2018). The downregulated expression of defense genes also weakened the plants’ defense against the invasion of exogenous pathogens, which made it easier for pathogens to infect plants. Based on this theory, LaCl3 was used to inhibit the increase in [Ca2+]cys and block this infection signal, allowing

| Vector ID | Number of embryos | Number of regenerated calluses | Regeneration rate % | Number of bar PCT+ | Transformation frequency % | Number of single copy events | Single copy frequency % | Number of low copy events | Low copy frequency % |
|-----------|-------------------|-------------------------------|--------------------|-------------------|---------------------------|----------------------------|------------------------|------------------------|----------------------|
| CAUC1828  | 224               | 52                            | 23.21              | 29                | 12.95                     | 13                        | 5.80                   | 20                     | 8.93                 |
| CAUC1829  | 238               | 60                            | 25.21              | 39                | 16.39                     | 20                        | 8.40                   | 29                     | 12.18                |
| CAUC1831  | 167               | 40                            | 23.95              | 20                | 11.98                     | 1                         | 0.60                   | 14                     | 8.38                 |
| CAUC1832  | 240               | 53                            | 22.08              | 29                | 12.08                     | 12                        | 5.00                   | 18                     | 7.50                 |
| CAUC1834  | 195               | 40                            | 20.51              | 24                | 12.31                     | 10                        | 5.13                   | 14                     | 7.18                 |
| CAUC1871  | 460               | 51                            | 11.09              | 33                | 7.17                      | 10                        | 2.17                   | 20                     | 4.35                 |

CAUC1828, CAUC1829, CAUC1831, CAUC1832, and CAUC1834 were treated with 10 mM of LaCl3, CAUC1871 was used as the control group. The low-copy events include one-copy and two-copy conversion events.
immature embryos to take the edge off their defense against Agrobacterium. Therefore, Agrobacterium-containing target genes or editing systems can more efficiently transfer Ti plasmids into plant receptor cells, and increase the integration opportunity of target genes and editing systems on the receptor genome. Finally, the genetic transformation frequency of immature maize embryos mediated by Agrobacterium is improved.

In the LaCl₃ concentration test experiment, we found that a high concentration of LaCl₃ affected the formation of embryonic callus and reduced the rate of callus (Fig. 1A and D). This result implied that the optimal concentration of LaCl₃ was key to this optimization, especially in other maize genotypes. We also inquired whether pretreatment of other Ca²⁺ inhibitors, such as EDTA and EGTA, could improve the frequency of the Agrobacterium infection, and the similar results as LaCl₃ treatment were obtained. These results further suggested that inhibition of the Ca²⁺ signal transduction pathways in explants could improve the infection frequency and transformation frequency.

In summary, the proposed optimized protocol provides a novel approach to improving the genetic transformation frequency of maize. In future studies, by further optimization, we expect to be able to overcome the genotype-dependent obstacles of the Agrobacterium-mediated genetic transformation during operation. This is expected to open new vistas for further basic research on the crop gene function and molecular breeding.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00299-022-02867-w.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant U1706201).

Author contribution statement MHL and YG conceived and designed the study. SNL and YLS conducted the experiments. FL conducted copy number analysis. SNL analyzed data. SNL, MHL, and YG wrote the manuscript. All the authors read and approved the manuscript.

Declarations

Conflict of interest There are no conflicts of interest to declare relevant to the contents of this article.

References

Ahmar S, Gill RA, Jung K, Faheem A, Qasim MU, Mubeen M, Zhou W (2020) Conventional and molecular techniques from simple breeding to speed breeding in crop plants: recent advances and future outlook. Int J Mol Sci 21:2590

Anand A, Bass SH, Wu E, Wang N, McBride KE, Annaluru N, Miller M, Hua M, Jones TJ (2018) An improved ternary vector system for Agrobacterium-mediated rapid maize transformation. Plant Mol Biol 97:187–200

Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60:379–406

Chen L, Zhang S, Beachy RN, Fauquet CM (1998) A protocol for consistent, large-scale production of fertile transgenic rice plants. Plant Cell Rep 18:25–31

Cho H, Winans SC (2005) Vira and Virg activate the Ti plasmid REPABC operon, elevating plasmid copy number in response to wound-released chemical signals. Proc Nat Acad Sci USA 102:14843–14848

Cho M, Wu E, Kwan J, Yu M, Banh J, Linn W, Anand A, Li Z, TeRonde S, Register JC, Jones TJ, Zhao Z (2014) Agrobacterium-mediated high-frequency transformation of an elite commercial maize (Zea mays L.) inbred line. Plant Cell Rep 33:1767–1777

Djamei A, Pitzschke A, Nakagami H, Rajh I, Hirt H (2007) Trojan horse strategy in Agrobacterium transformation: Abusing MAPK defense signaling. Science 318:453–456

Frame BR, McMurray JM, Fonger TM, Main ML, Taylor KW, Torney FJ, Paz MM, Wang K (2006) Improved Agrobacterium-mediated transformation of three maize inbred lines using MS salts. Plant Cell Rep 25:1024–1034

Fromm ME, Taylor LP, Walbot V (1986) Stable transformation of maize after gene transfer by electroporation. Nature (London) 319:791–793

Golovkin MV, Abraham M, Morocz S, Bottka S, Feher A, Dredits D (1993) Production of transgenic maize plants by direct DNA uptake into embryogenic protoplasts. Plant Sci 90:41–52

Gómez-Gómez L (2004) Plant perception systems for pathogen recognition and defence. Mol Immunol 41:1055–1062

Grimsley N, Hohn T, Davies J, Hohn B (1987) Agrobacterium-mediated delivery of infectious maize streak virus into maize plants. Nature 325:177–179

Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by Agrobacterium tumefaciens. Plant Mol Biol 35:205–218

Hiei Y, Ishida Y, Kasaoka K, Komari T (2006) Improved frequency of transformation in rice and maize by treatment of immature embryos with centrifugation and heat prior to infection with Agrobacterium tumefaciens. Plant Cell Tissue Organ Cult 87:233–243

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens. Nat Biotechnol 14:745–750

Kaeppler HF, Somers DA (1994) DNA delivery into maize cell cultures using silicon carbide fibers. The maize handbook. Springer, New York, pp 610–613

Klein TM, Fromm M, Weisinger A, Tomes D, Schaaf S, Sletten M, Sanford JC (1988) Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. Proc Natl Acad Sci USA 85:4305–4309

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251–275

Liu F, Cheng J, Liu X, Wang X (2021) High-throughput and accurate determination of transgene copy number and zygosity in transgenic maize: from DNA extraction to data analysis. Int J Mol Sci 22:12487

Liu Y, Zhang Z, Fu J, Wang G, Wang J, Liu Y (2017) Transcriptome analysis of maize immature embryos reveals the roles of cysteine in improving Agrobacterium infection efficiency. Front Plant Sci. https://doi.org/10.3389/fpls.2017.01778

Lowe K, Wu E, Wang N, Hoerster G, Hastings C, Cho M, Sclonoge C, Lenderts B, Chamberlin M, Cussatt J, Wang L, Ryan L, Khan T, Chow-Yiu J, Hua W, Yu M, Banh J, Bao Z, Brink K, Igo E, Rudrappa B, Shameser PM, Bruce W, Newman L, Shen B, Zheng P, Bidney D, Falco C, Register J, Zhao Z, Xu D, Jones T, Gordon-Kamm W (2016) Morphogenic regulators Baby boom
and Wuschel2 improve monocot transformation. Plant Cell 28:1998–2015
Lowe K, La Rota M, Hoerster G, Hastings C, Wang N, Chamberlin M, Wu E, Jones T, Gordon-Kamm W (2018) Rapid genotype “independent” Zea mays L. (maize) transformation via direct somatic embryogenesis. In Vitro Cell Dev - PL 54:240–252
Ma L, Ye J, Yang Y, Lin H, Yue L, Luo J, Long Y, Fu H, Liu X, Zhang Y, Wang Y, Chen L, Kudla J, Wang Y, Han S, Song C, Guo Y (2019) The SOS2-SCaBP8 complex generates and fine-tunes an AtANN4-dependent calcium signature under salt stress. Dev Cell 48:697–709
Moookkan M, Nelson-Vasilchik K, Hague J, Zhang ZJ, Kausch AP (2017) Selectable marker independent transformation of recalcitrant maize inbred B73 and sorghum P898012 mediated by morphogenic regulators Baby boom and Wuschel2. Plant Cell Rep 36:1477–1491
Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:2002–2007
Salvo SAGD, Hirsch CN, Buell CR, Kaeppler SM, Kaeppler HF, Zhang X (2014) Whole transcriptome profiling of maize during early somatic embryogenesis reveals altered expression of stress factors and embryogenesis-related genes. PLOS One 9:e111407
Sheikholeslam SN, Weeks DP (1987) Acetosyringone promotes high-efficiency transformation of Arabidopsis thaliana explants by Agrobacterium tumefaciens. Plant Mol Biol 8:291–298
Sidorov V, Duncan D (2009) Agrobacterium-mediated transformation: immature embryos versus callus. In: Scott MP (ed) Methods in Molecular Biology, p 47
Sivanandhan G, Kapil Dev G, Theboral J, Selvaraj N, Ganapathi A, Manickavasagam M (2015) Sonication, vacuum infiltration and thiol compounds enhance the Agrobacterium-mediated transformation frequency of Withania somnifera (L.) Dunal. PLOS One 10:e124693
Toyota M, Spencer D, Sawai-Toyota S, Jiaqi W, Zhang T, Koo AJ, Howe GA, Gilroy S (2018) Glutamate triggers long-distance, calcium-based plant defense signaling. Science 361:1112–1115
Vega JM, Yu W, Kennon AR, Chen X, Zhang ZJ (2008) Improvement of Agrobacterium-mediated transformation in Hi-II maize (Zea mays) using standard binary vectors. Plant Cell Rep 27:297–305
Xing H, Dong L, Wang Z, Zhang H, Han C, Liu B, Wang X, Chen Q (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol 14:327
Yuan Z, Williams M (2012) A really useful pathogen, Agrobacterium tumefaciens. Plant Cell 24:112–1012
Zhang Q, Zhang Y, Lu M, Chai Y, Jiang Y, Zhou Y, Wang X, Chen Q (2019) A novel ternary vector system united with morphogenic genes enhances CRISPR/Cas delivery in maize. Plant Physiol 181:1441–1448

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.