$LaCl_3$ treatment improves the *Agrobacterium*-mediated immature embryo genetic transformation efficiency in maize

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Abstract

Agrobacterium-mediated genetic transformation of immature embryo plays an important auxiliary role in the study of gene function and molecular breeding in maize. However, the relatively low genetic transformation efficiency is still the bottleneck of the application of this method, especially in commercial scale production application. In this study, we found that pretreatment of immature embryos with $LaCl_3$, a Ca^{2+} channel blocker, could improve the infection efficiency of *Agrobacterium tumefaciens*, increase the proportion of resistant calluses, obtain more positive regenerated plantlets, and finally improve the transformation efficiency in maize. This optimization provides a new direction for improving the efficiency of plant genetic transformation mediated by *Agrobacterium tumefaciens*.

Keywords: Maize; *Agrobacterium tumefaciens*; Immature embryo; $LaCl_3$; Transformation efficiency

Introduction

Maize, a monocotyledon food crop, is also a feed crop and an energy crop. It is the world's most widely grown crop and one of the most productive food crops in the world. About one-third of the world's population depends on corn as a staple food. Maize is a C_4 plant, which is also a model plant for studying the photosynthesis (Mookkan, et al. 2017). Due to time and labor consuming and the existence of interspecific reproductive barrier, which prevents target traits from being introduced into recipient plants, so the utilization of excellent germplasm resources is limited to a certain extent by conventional breeding (Ahmar, et al. 2020). However, transgenic technology has greatly promoted the process of obtaining various resistance candidate genes and new varieties based on gene function research. Even so, the efficient, short cycle and stable genetic transformation system of maize is still a hindrance.

Transgenic technology is a powerful method to cultivate high yield, high quality, resistant to biological and abiotic stress varieties of crop. During the development of maize transgenic technology, scientists have invented many transformation methods, such as electroporation (Fromm, et al. 1986), particles 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 also has stable inheritance of exogenous genes and low copy number (Liu, et al. 2017). Due 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).

Since 1987, Grimsly et al. first used *Agrobacterium* to infect maize, and proved that this method could transform maize. Subsequently, Ishida et al. established relatively stable *Agrobacterium*-mediated genetic transformation system used maize immature embryos as the explant for the first time in 1996. There have been many studies on *Agrobacterium*-mediated optimization of immature maize embryo genetic transformation system. Among them, many factors such as the vector, the genotype of the explant, the pretreatment condition of the explants, *Agrobacterium* strains, the concentration of *Agrobacterium* solution, the infection duration, the co-culture duration, the component of infection medium and the co-culture medium, affect the genetic transformation efficiency (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, the ternary vector system carrying extra copies of *Vir* genes could increase 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*) is a great breakthrough in genetic transformation of maize, which greatly improves the transformation efficiency and overcomes the dependence on genotypes and explants to a certain extent (Salvo, et al. 2014; Lowe, et al. 2018; Lowe, et al. 2016; Mookkan, et al. 2017). However, its application in commercial scale production still has some problems. Although *Agrobacterium*-mediated genetic transformation efficiency of immature embryo in maize has been greatly improved through continuous system optimization, and this method has also been widely used in the commercialization of maize breeding, the low transformation efficiency is still an urgent bottleneck to overcome in the application of maize genes function study and molecular breeding.

It is well known that *Agrobacterium tumefaciens*, as a naturally occurring gram-negative bacterium, contains tumor induce (Ti) plasmid, which contains T-DNA that can be integrated into recipient plant genomes after being horizontally transferred into plant cells. Hence, Ti plasmid is modified to transform the target genes into the plant genome, so as to achieve the transformation of the target genes in the recipient species, therefore, known as “the smallest genetic transformation engineer in nature” (Yuan and Williams 2012). In addition, there is an evidence that *Agrobacterium tumefaciens* triggers the activation of multiple the activation of mitogen-actived protein kinases (MAPKs), which is one of defense mechanisms rapidly triggered by host perception of pathogen-associated molecular patterns (PAMPs) (Djamei, et al. 2007). However, *A. tumefaciens* can also induce the formation of plant crown galls (Matthew A Escobar et al., 2003), so it is an exogenous pathogen to sessile plants (Cho and Winans 2005). When the exogenous pathogenic microorganisms infect plant receptors, the innate immune response of the receptors will be triggered, so as to defend pathogen and maintain growth (GOMEZGOMEZ 2004). In the process of interaction between plant and pathogen, a series of signal transduction occurs in plant, including the increase of Ca^{2+} concentration, the accumulation of reactive oxygen species (ROS), and the activation of the signaling cascades mediated by mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (Lamb and Dixon 1997; Boller and Felix 2009). In turn, we propose a hypothesis as to whether improving Agrobacterium-mediated transformation efficiency can be achieved by dampening innate immune response of plants to Agrobacterium tumefaciens.

In this study, we developed an efficient optimize system that used the Ca^{2+} channel blocker to pretreat immature embryos of maize before *Agrobacterium* infection. The optimization of the pretreatment conditions of the receptor revealed that immature embryos of maize pretreated with 10 mM $LaCl_3$ obtained twice as much positive regeneration plantlets as those in the control group, and the stability

was further verified by CRISPR/Cas9 system.

Materials and Methods

Plant Materials

Maize inbred lines ND101 and ND88 were used in this study. The two maize inbred lines are provided by Center for Crop Functional Genomics and Molecular Breeding of China Agricultural University. Maize plants were grown in the greenhouse under a 16/8 h light/dark cycle at 20-25℃. The immature embryos were collected from the fresh ears 9-12 days after pollination for genetic transformation.

Agrobacterium Strains and binary vectors

Agrobacterium tumefaciens strain EHA105 was used for maize transformation. The binary vector contains *Ds-Red* gene as the reporter was drove by the ubiquitin-1 promoter, and the *bar* as the herbicide resistance screening marker gene. The editing vector pBUE411 contains two sgRNA targets, provided by Center for Crop Functional Genomics and Molecular Breeding of China Agricultural University. The related test methods were referred to Xing, et al. (2014).

Agrobacterium-Mediated Transformation and Immature Embryo Pretreatment

Maize transformation followed published protocols with minor modifications (Sidorov and Duncan 2009). After immature embryos were collected, the infection medium in the centrifuge tubes was removed and the fresh infection medium containing $LaCl_3$ was quickly added to it. Then, the centrifugal tubes were placed in a 45℃ water bath for pretreatment for 5 min. After the pretreatment, the supernatant was removed from the centrifuge tube immediately, and the liquid was cleaned as much as possible, and the freshly prepared *Agrobacterium* solution was added in the centrifuge tube, whose OD_{600} is between 0.6 and 0.8. Under dark condition, the centrifuge tube containing immature embryos and *Agrobacterium* solution was incubated at 22℃ for 30 min. After that, *Agrobacterium* fluids need to be removed as much as possible. In order to facilitate subsequent observation and analysis of *RFP* expression fluorescence value, after the immature embryos were transferred into the co-culture medium, sterile filter papers were used to gently absorb the fluid around the embryos to remove background noise during the photographing.

Analysis of Transient *RFP* Expression and Statistical Analysis of Intensity of RFP Fluorescence

The red fluorescence distribution of immature embryos and resistant calluses

were observed with a multifunctional zoom microscope (Nikon AZ100) at 510 nm -560 nm. The fluorescence signal value in the pictures was calculated by self-written script. The significance of the data was analyzed by t-test.

Statistical Analysis of the Infection Frequency, Rate of Calluses, Rate of Resistant Calluses, Regeneration Frequency and Transformation Efficiency

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

After 14 days of culture on screening medium, the rate of calluses was calculated as: the rate of callus (%) = number of calluses/number of embryos on co-culture medium × 100.

After 10 days of culture on screening medium, the rate of resistant callus was calculated as: the rate of resistant callus (%) = number of calluses with *RFP* transient fluorescence expression/number of total calluses × 100.

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

After all primary regeneration plantlets (T_0) were obtained, the transformation efficiency was calculated as: the transformation efficiency (%) = number of positive regeneration events / number of infected embryos × 100.

After all positive regeneration plantlets (T_0) were obtained, the editing efficiency was calculated as: the editing efficiency (%) = number of edited regeneration plantlets / number of positive regeneration plantlets × 100.

The significance of the data was analyzed by t-test.

Molecular identification and mutant analysis

Genomic DNA of T_0 seedlings was extracted by magnetic beads method. Wild type lines and putative transformations were confirmed by polymerase chain reaction (PCR) analysis with selection maker gene primers (forward primer: ATGAGCCCAGAACGACGC; reverse primer: TCAAATCTCGGTGACGGG). Mutation analysis of T_0 regenerated lines was performed using the first generation sequencing techniques according to Xing, et al. (2014).

Results

Pretreatment of immature embryos with $LaCl_3$ improved the infection efficiency of *Agrobacterium tumefaciens*.

To test the hypothesis mentioned above, we simultaneously pretreated the immature embryos of ND101 with infection medium containing different concentrations of $LaCl_3$, which is a calcium channel blocker, at 45℃ for 5 minutes. Meanwhile, the infection medium without $LaCl_3$ was used as the control. The intact immature embryos isolated from tassels which 9 to 12 days after pollination as explants, and used 25 immature embryos for each treatment and kept the embryos in the infection medium for no more than 60 minutes. After the pretreatment, the immature embryos were infected by *Agrobacterium tumefaciens* EHA105 harboring the binary vector with *RFP* reporter gene (fig. S1). Then they were transferred to co-culture medium for co-cultivation respectively.

To detect the infection efficiency, confocal microscopy was performed to investigate the transient expression of *RFP* after co-cultivation for 2 days, and the infection effect was reflected by the statistical analysis of fluorescence intensity in the images. The results suggested that the fluorescence intensity with 10 mM $LaCl_3$ pretreatment were significantly higher than the control group and those of other concentrations (Fig. 1 A and B); in addition, the infection efficiency of 10 mM $LaCl_3$ pretreatment was the highest (Fig. 1C). After that, we observed the callus induced after 14 days under the screening condition and calculated the rate of callus, and pre-differentiated callus clumps cultured for 12 days were also followed (Fig. 1A and D). The results indicated that pretreatment with 10 mM $LaCl_3$ had the best performance in the state of pre-differentiation, had no effect on callus formation and significantly improved the infection efficiency mediated by *Agrobacterium*.

Pretreatment of immature embryos with $LaCl_3$ improved the rate of resistant callus.

Our experimental results confirmed that the pretreatment of immature embryos with 10 mM $LaCl_3$ could improve the infection efficiency of *Agrobacterium*, but whether the recipient cells integrating the exogenous genes from T-DNA of *Agrobacterium* underwent dedifferentiation and redifferentiation to form embryogenic calluses is the hinge to affect the transformation efficiency.

In order to evaluate whether the transformed cells could form embryogenic callus though dedifferentiation, after 10 days of calluses induction on the screening medium under dark condition, we observed the transit expression of *RFP* in calluses by laser confocal microscopy and counted the proportion of resistant callus. We observed that the number of red-fluorescent adventitious buds appeared in embryogenic calluses with 10 mM $LaCl_3$ pretreatment more than of the control group (Fig. 2A), and the proportion of resistant callus was consistent with this (Fig. 2B). The results suggested that pretreatment with $LaCl_3$ of immature embryos improved the

ratio of resistant callus, furthermore did not have a negative effect on the formation of embryogenic calluses.

Pretreatment of immature embryos with $LaCl_3$ improved transformation efficiency.

To investigate the effect on the conversion efficiency of immature embryos with 10 mM $LaCl_3$ pretreatment via *Agrobacterium tumefaciens*, we tracked the redifferentiation process of all calluses. After 14 days of calluses induction on the screening medium under dark condition; all calluses, including non-embryonic calluses were transferred to the pre-differentiation medium for resistance screening under low light condition for 12 days, and then transferred to the regeneration medium for resistance screening under light condition for 20-30 days. We compared the differentiated shoots developed in the experimental and control groups after culturing for 20 days on regeneration medium, and it was obvious that the experimental group pretreated with 10 mM $LaCl_3$ was better than the control group (Fig. 3A). Furthermore, we counted the regeneration frequency that the proportion of tissues differentiated with elongated shoots are in the total calluses. The results showed that after $LaCl_3$ treatment, the regeneration frequency increased from 13.2% to 27.2%, more than twice as high as in the control group (Fig. 3B). Subsequently, we identified the *bar* positive T_0 plantlets by PCR and calculated the transformation efficiency. The results revealed that the transformation efficiency increased from 8.40% to 17.60% after $LaCl_3$ pretreatment. (Fig. 3C and D). Moreover, the number of positive T_0 plantlets transplanted into the nutrition bowl of $LaCl_3$ pretreatment was twice that of the control group, which was basically consistent with the above conclusion (Fig. 3E). Our results indicated that pretreatment with $LaCl_3$ of immature embryos improved *Agrobacterium*-mediated transformation efficiency in maize.

To address whether $LaCl_3$ pretreatment of immature embryos had effects on the morphology and fertility of regenerated plants, we followed the growth, development and fructification of T_0 generation plants. Within expectation, no significant difference was observed in the plant growth and development between $LaCl_3$ pretreatment group and the control group (fig. S2).

Base on the above, we established an optimized system to improve the efficiency of *Agrobacterium* mediated genetic transformation by pretreating immature embryo with $LaCl_3$ in maize (Fig. 4).

The optimized protocol was validated by CRISPR/Cas9 system

To verify the validity of the optimized protocol, we constructed 6 CRISPR/Cas9 vectors targeting four maize genes, including four single-editing-target vectors and

two double-editing-target vectors. After that, we transformed the five of the vectors into maize inbred line ND101 using the optimized protocol, while the other one transformed using the non-optimized protocol as the control, and estimated the regeneration frequency, transformation efficiency and editing efficiency of the different transformation protocol respectively. The results suggested that the regeneration frequency increased from 11.09% to 20.52%-25.21% after optimization, and the average regeneration frequency was 23.03%. Consistently, the transformation efficiency increased from 7.17% to 11.98%-12.95% after optimization, and the average transformation efficiency was 13.25%. The regeneration frequency and transformation efficiency were both doubled compared with control (Table 1). So, it turns out that the optimization protocol is indeed effective in improving the regeneration efficiency and transformation efficiency of genetic transformation mediated by *Agrobacterium*.

Finally, we analyzed the genes editing of the T_0 transgenic plants, and the results illustrated that all vectors had detected target gene editing plants. The editing efficiencies were 84.0% and 71.43%-92.86% (the average is 84.16%) of the transgenic plants obtained by the non-optimized system and optimized system respectively (Fig. S3A and B). Based on the above results, it is further confirmed that $LaCl_3$ pretreatment improves the genetic transformation efficiency of immature embryos mediated by *Agrobacterium* and has no effect on editing efficiency.

Discussion

Based on the hypothesis that partial inhibition of Ca^{2+} transduction triggered by *Agrobacterium* infection explants could improve the infection efficiency and thus possibly improve the transformation efficiency, we successfully established a transformation system for pretreatment of immature embryos with $LaCl_3$. This method can improve the infection efficiency of *Agrobacterium*, regeneration frequency and transformation efficiency. We transformed 6 CRISPR/Cas9 vectors for batch system verification, which revealed that the method protocol is indeed effective in improving the regeneration frequency, transformation efficiency, and also the delivery efficiency of editing vectors, and that had no effect on editing efficiency. Hence, in the present investigation, inhibition of Ca^{2+} signal transduction triggered by *Agrobacterium* infection in explants improves the efficiency transformation.

Ca^{2+}, as a universal second messenger, plays an important role in signal transduction in many physiological processes including stress and immune response in plants and animals (Ma, et al. 2019). For an ever increasing number of environmental stresses, pathogens attack, drought stress, cold/heat stress, oxidative stress and slat stress, it has been found that temporally and spatially defined rapid of

changes of Ca^{2+} concentration in the cytoplasm differs in elevation duration, intensity, amplitude, frequency and other aspects. Moreover, Masatsugu Toyota et al.'s study showed that caterpillar feeding or wounding with scissors could cause a rapid $[Ca^{2+}]_{cyt}$ increase and propagate to distal parts. However, when plants were treated with $LaCl_3$, systemic $[Ca^{2+}]_{cyt}$ was blocked. In addition, the relative expression levels of wound induced defense maker genes, such as *JAZ5*, *JAZ7*, *ZAT12*, *OPR3* and *RBOHD*, were significantly decreased (Toyota, et al. 2018). The down-regulated expression of defense genes also weakened the plant's defense against the invasion of exogenous pathogens, which made it easier for pathogens to infect plants. We are based on such a theory conjecture, $LaCl_3$ was used to inhibit the increase of $[Ca^{2+}]_{cyt}$ and block this infection signal, allowing immature embryos to take the edge off their defense against *Agrobacterium*. Therefore, *Agrobacterium* containing target genes or editing systems can more efficiently transfer Ti plasmid into plant receptor cells, and increase the integration opportunity of target genes or editing systems on the receptor genome. Finally, the genetic transformation efficiency of immature maize embryos mediated by *Agrobacterium* is improved.

In the $LaCl_3$ concentration test experiment, we found that high concentration of $LaCl_3$ could affect the formation of embryonic calluses and reduce the rate of callus (Fig1. A and D). This result implied that the optimum concentration of $LaCl_3$ was the key to this optimization, especially in other genotypes of maize. Otherwise, we were also curious about whether pretreatment of explants with other Ca^{2+} inhibitors could improve the efficiency of *Agrobacterium* infection. Thus, we used EDTA and EGTA (the two Ca^{2+} chelators) to pretreat immature embryos of ND101. Preliminary results indicated that the transformation efficiency increased from 7.47% to 27.78% and 31.43%, respectively (fig. S4A). Besides, we pretreated the immature embryos of ND88 in the same way, which is one recalcitrant maize inbred line. Infection efficiency was nearly doubled from 46.88% to 100%, and confocal results also showed that the fluorescence quantity of RFP transient expression in immature embryos after 2 days of co-culture was significantly higher than that in the control group (fig. S4B and C). The results further confirmed the feasibility that inhibition of Ca^{2+} signal transduction in explants could improve the infection efficiency and transformation efficiency.

In summary, this optimized protocol provides a new idea for improving the genetic transformation efficiency of maize. Meanwhile, perhaps in the near future, by further deepening the optimization system, we may overcome the genotype dependent obstacles of the *Agrobacterium*-mediated genetic transformation during the operation, and also provide more opportunities for further basic research on crop gene function and molecular breeding.

Author Contribution Statement

MHL and YG conceived and designed research. SNL and YLS conducted experiments. SNL analyzed data. SNL , MHL and YG wrote the manuscript. All authors read and approved the manuscript.

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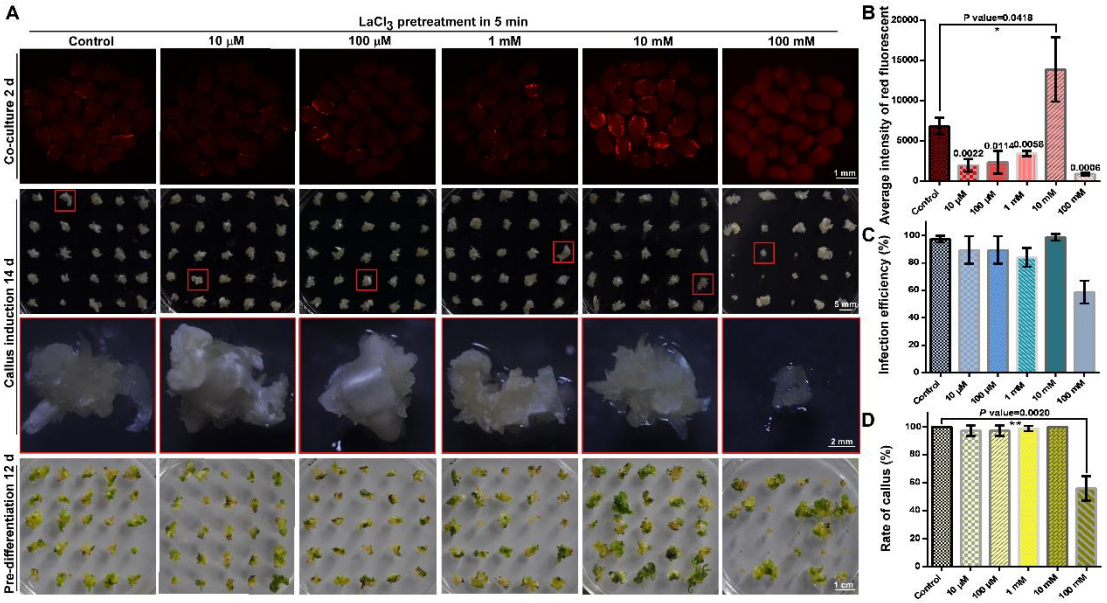


Figure 1. Pretreatment of immature embryos with 10 mM $LaCl_3$ improved the infection efficiency of *Agrobacterium tumefaciens*. Immature embryos were treated with 10 μM, 100 μM, 1 mM, 10 mM and 100 mM $LaCl_3$ for 5 min before *Agrobacterium* infection respectively. **A.** Transient expression of *RFP* in immature embryos after 2 days of co-cultivation; calluses induced on selection medium after 14 days and callus clumps cultured after 12 days pre-differentiation cultivation. **B.** Transient expression of *RPF* fluorescence statistics. **C.** Statistical analysis of infection efficiency. **D.** Statistical analysis of the rate of callus. In the above statistical analysis, error bars represent means ± SEMs. Statistical differences were analyzed by student's t-test, n=3.

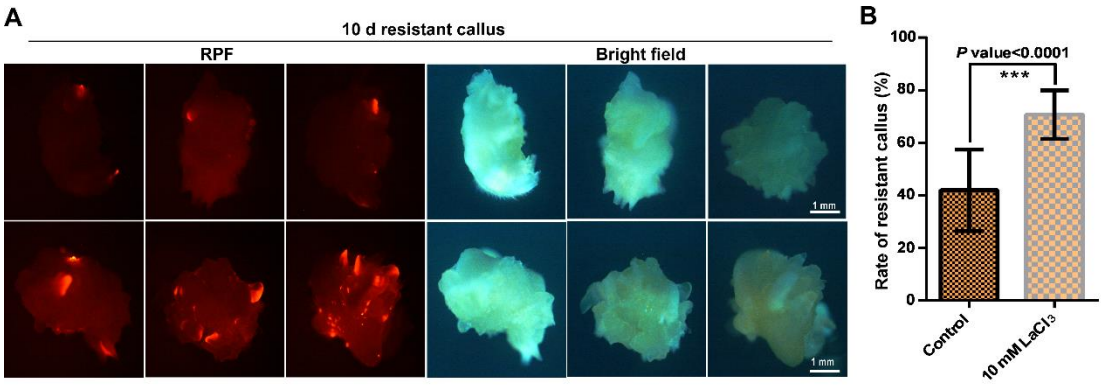


Figure 2. Pretreatment of immature embryos with 10 mM $LaCl_3$ improved the rate of resistant callus. A. The resistant calluses cultured for 10 days on selection medium observed by laser confocal microscopy under RFP field (left) and bright field (right). **B.** Statistical analysis of the rate of resistant callus. The error bars represent means ± SEMs in statistical analysis. Statistical differences were analyzed by student's t-test, n=10.

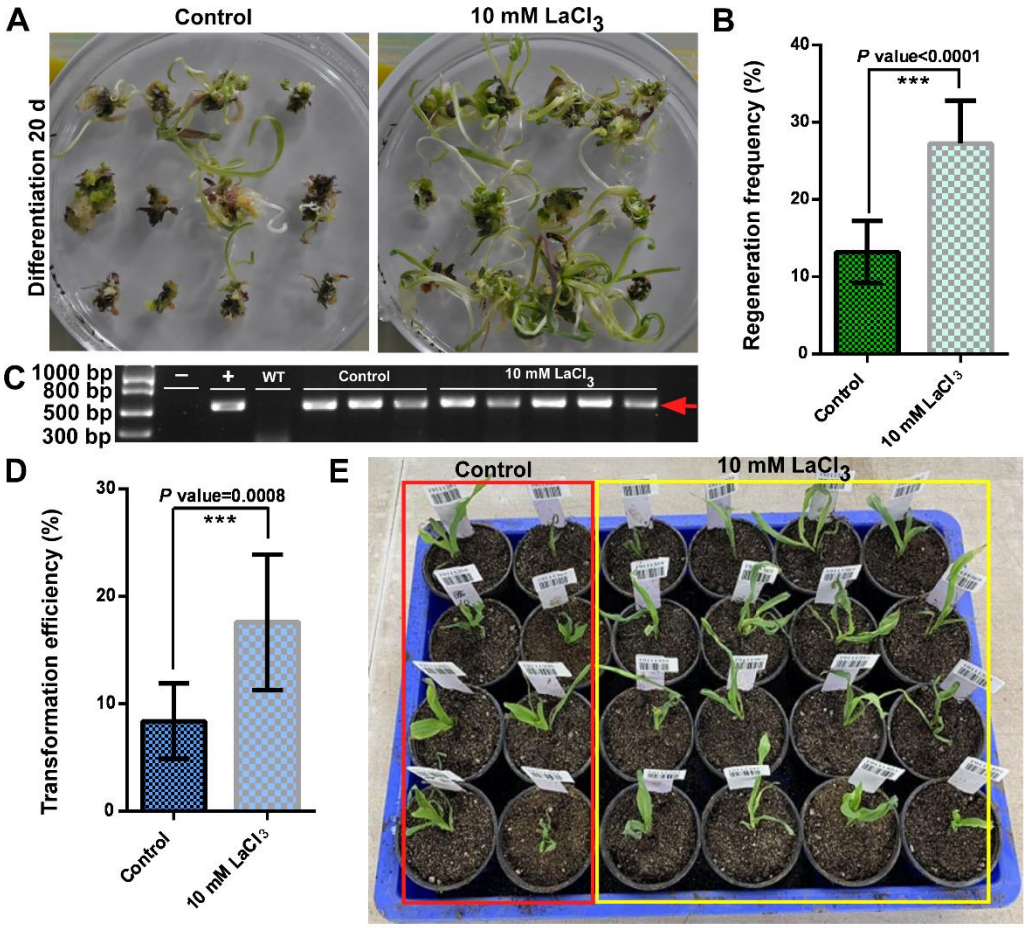


Figure 3. Pretreatment of immature embryos with 10 mM $LaCl_3$ improved the transformation efficiency. A. The morphology of callus cultured for 20 days on regeneration medium. **B.** Statistical analysis of the regeneration frequency. **C.** Analysis of PCR detection results of T_0 plantlets. **D.** Statistical analysis of the transformation efficiency. **E.** Bar positive T_0 plants were transplanted into vegetative soil. In the above statistical analysis, error bars represent means ± SEMs. Statistical differences were analyzed by student's t-test, n=10.

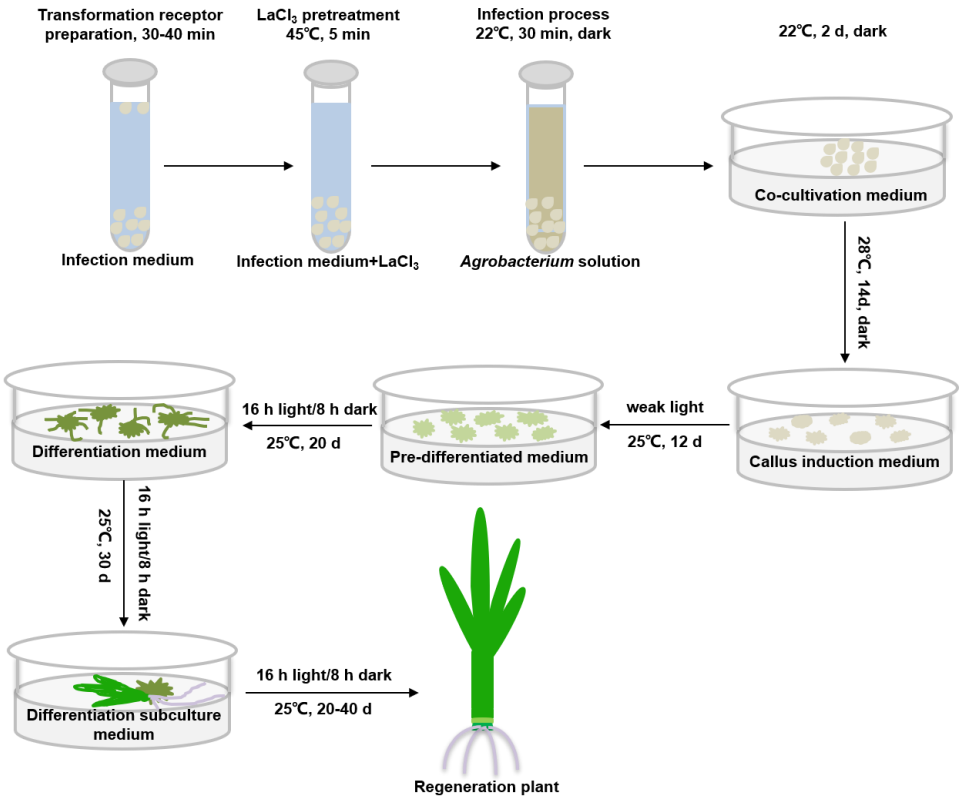
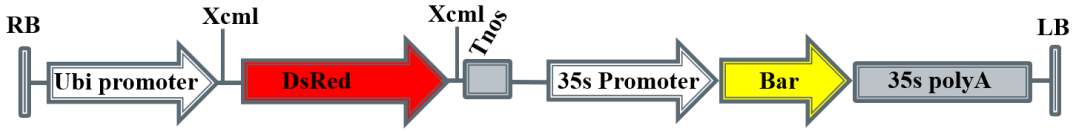


Figure 4. Schematic diagram of genetic transformation. Genetic transformation process mediated by *Agrobacterium* of immature embryos pretreated with $LaCl_3$ in maize.

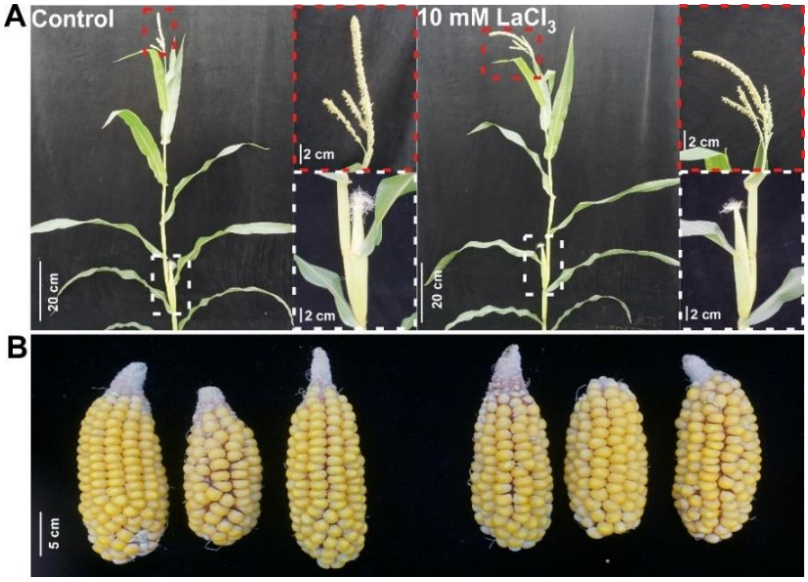
Vector ID	Targeted gene ID	sgRNA Seq.	The number of embryos	The number of regenerated lines	The regeneration efficiency	The number of positive evens	The transformation efficiency
CAUC1828	Zm00001d036986	AACGCAGGGGAGATGATCGTTGG	224	52	23.21%	29	12.95%
CAUC1829	Zm00001d047534	CCTGTGCTATTGTGCAGAACCCG	238	60	25.21%	39	16.39%
CAUC1831	Zm00001d018522	TTTGGGCAGAGCTCTGACAAGGG	167	40	23.95%	20	11.98%
CAUC1832	Zm00001d046662	AAGAGTGGTCGCAGCATACAGGG	240	53	22.08%	29	12.08%
CAUC1834	Zm00001d036986+Zm00001d047534	AACGCAGGGGAGATGATCGTTGG&CCTGTGCTATTGTGCAGAACCCG	195	40	20.51%	24	12.31%
CAUC1871	Zm00001d018522+Zm00001d046662	TTTGGGCAGAGCTCTGACAAGGG&AAGAGTGGTCGCAGCATACAGGG	460	51	11.09%	33	7.17%

Table 1. Genetic transformation statistical analyses of CRISPR/Cas9 system

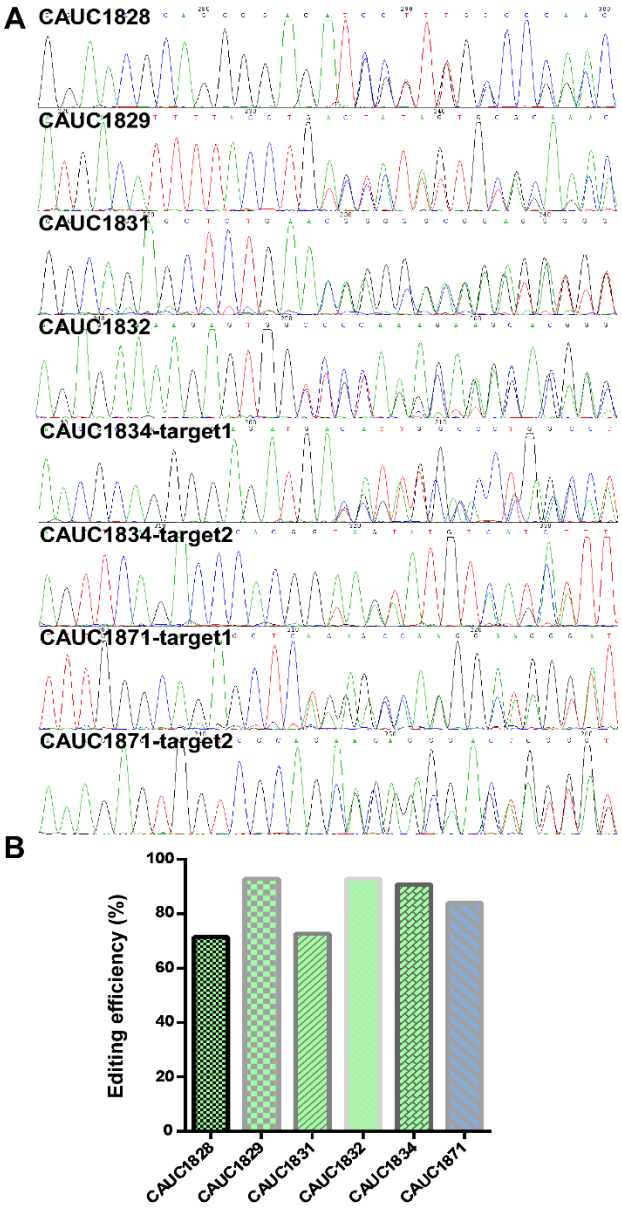
Supplemental data



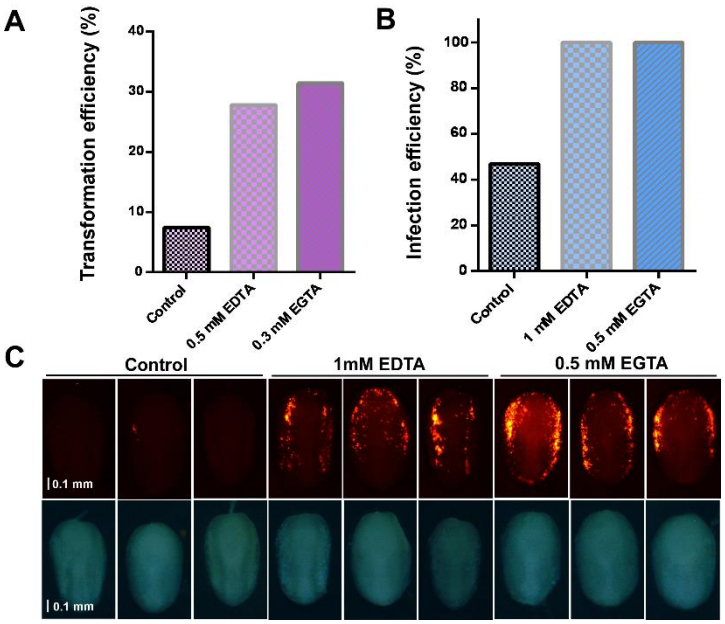
Supplemental figure 1. Diagram of the binary vector used in genetic transformation. *DsRed* is the reporter gene, and *Bar* is the herbicide selectable maker gene.



Supplemental figure 2. The morphology of T_0 generation plant of control group and $LaCl_3$ pretreatment group. A. The tassel and silking of T_0 generation plant. **B.** The mature ears of T_0 generation plant.



Supplemental figure 3. The target gene mutation analysis of the transgenic plants. **A.** Sequencing chromatograms from transformation plants. **B.** Statistical analysis of the editing efficiency.



Supplemental figure 4. The preliminary results of pretreatment of immature embryos with Ca^{2+} chelators EDTA and EGTA. A. Transformation efficiency of EDTA and EGTA treatments in ND101 immature embryos respectively at a time. **B.** Infection efficiency of EDTA and EGTA treatments in ND88 immature embryos respectively at a time. **C.** Transient expression of *RFP* in ND88 immature embryos pretreated with EDTA and EGTA after 2 days of co-cultivation respectively.

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