

AGROBACTERIUM-MEDIATED GENE TRANSFORMATION OF POLLEN SPECIFIC GENE STK1 IN MAIZE (*ZEA MAYS*)

JAVEED, A.^{1#} – WU, S.^{3#} – HUANG, S.¹ – AHMED, M.² – SIKANDAR, A.² – IQBAL, M. F.¹ – DOGAR, U. F.⁴ – ZHANG, C.¹ – CHEN, M.¹ – LIU, C.¹ – HUSSAIN, K.⁴ – FENG, L.^{1*}

¹*College of Bioscience and Biotechnology, Shenyang Agricultural University, Shenyang, Liaoning, China*

²*College of Plant protection, Shenyang Agricultural University, Shenyang, Liaoning, China*

³*College of Science Institute, Shenyang Agricultural University Shenyang, Liaoning, China*

⁴*Department of Botany, University of Gujrat, Hafiz Hayat Campus, Gujrat, Pakistan*

[#]*These authors have equal contribution to this paper.*

^{*}*Corresponding author*

e-mail: fenglinsn@126.com; phone: +86-248-848-7086; fax: +86-248-848-7416

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Abstract. Maize is a major food, feed and fodder crop, grown extensively all over the world. Genetic engineering has a unique role in crop improvement. Serine-Threonine-Kinase1 (*ZmSTK1*) is a gene-based enzyme, which due to its biochemical action in maize pollen grains, lowers the survival capability and affects pollination. Out of different methods of genes transfer into the genome of the target cell, Agrobacterium-mediated gene transformation is an easy and convenient method in which required gene is insert into the Agrobacterium, which after multiplication carry foreign genes into the host cell's genome. In this research, a simple and easy *Agrobacterium tumefaciens*-mediated method used to establish the genetic transformation system of *zmstk1* into maize germinating embryo as receptor system. Transgenic plants obtained successfully through this method and the complicated culture process not needed. The expression of *zmstk1* in the tissue was determined by observing the histochemical localization of *zmstk1* in order to elucidate the expression of *zmstk1* in mature tassel or pollen.

Keywords: *agrobacterium tumefaciens, gene editing, gene mutation, pollen viability, transgenic maize*

Introduction

Maize is a major crop found all over the world and used extensively for food, feed and fodder. Because of its consumption at large scale, some high yielding varieties have to be selected to meet the requirement. Naturally, dominant and vigorous varieties also play an important role in production of healthy and disease free food but for high production of maize, some controlled experiments on cellular and molecular level are required. Public and government affiliated institutions of agriculture research are investigating reasons related to adjustment of maize plant for biotic and environmental stresses. Transfer of genes of cold regions maize varieties to increase production and to improve quality, breeders focus on the improvement of new varieties by using these techniques. One of the major hurdles in functional use of this technique to temperate varieties of maize is the selection of germplasm responsible for its renaissance in tissue culture and inoculation by Agrobacterium (Souza et al., 2017). There are many ways to enhance the ability of an organism to produce the required yield of product such as natural hybridization, cellular metabolism enhancement, quantitative trait loci, molecular and genetic recombination and gene transformation into the cell. For different

purposes, different methods and ways are used. One of the best and advanced research in genetic and molecular experiments is to transfer a specific target gene into the vegetative cells of plants. For this purpose, some experiments held in Shenyang Agricultural University Shenyang, Liaoning, China.

Agrobacterium-mediated transformation is a fast and more accurate method for gene transfer with high frequency and time saving approaches (Que et al., 2014). This method is more extensively adapted now days by both civil and private institutions, however, capability of transformation varies highly from lab to lab for the same plant genotype. Here, advanced Agrobacterium-mediated transformation technique in maize by using a simple form of binary vectors exemplified. This method uses undeveloped embryos as fundamental explants and the bar gene as a selectable marker together with bialaphos as a selective agent in maize (Shrawat and Good, 2011), rice (Hei and Komari, 2008) and in switchgrass (Xi et al., 2009). Development based on genetic transfer varies, in which gene can be inserted into plants more effectively which depend on the species or variety, or the genetic marker being used (Binka et al., 2012). In recent times, a CRISPR/Cas9 toolkit used for multiplex genomic editing in different host plants based on the pGreen and in pCambia backbone (Xing et al., 2014). Genetic changes in plants which effect pollen development can be easily observed by the unusual isolation of markers linked to it (Vasal et al., 2006) and in plants where the genetic changes are due to transposons that present a phenotype are by the abnormal isolation of responsible transposon itself (Lalanne et al., 2004; Boavida et al., 2009).

Serine threonine kinase1 (STK1) and serine threonine kinase2 (STK2) are closely related to paralogous genes of maize predicted to encode serine/threonine protein kinases. Those pollens, which have mutations of *ZmSTK1* or *ZmSTK2*, can compete poorly with wild pollens, indicating to an abnormality in germination or growth of pollen tube. Both genes show their expression only in pollens but not in most of other parts of plants. In medium of germination, *ZmSTK1* and *ZmSTK2* fluorescent fusion proteins are located in the cell membrane of the somatic cell of plant. RNA-sequencing experiments indicated 534 genes, which expressed differentially in *zmstk1* mutant pollens as compared to wild type. Gene ontology molecular functional analysis revealed many differently expressed genes with supposed ribosome initiation and elongation functions, signifying that *zmstk1* may show effect on function of ribosomes. Of these two mutant forms, *zmstk1* may show a more imperative role in pollen development as compared to *zmstk2*, as mutations of *zmstk2* have a smaller transmission effect in pollen development. However, *zmstk2* acts as a booster of *zmstk1* expression because the combination of double mutation shows that pollens are too weak to survive until fertilization as compared to wild and single mutants. Therefore, it is analyzed that the *ZmSTK* paralogs play an important role in pollen development (Huang et al., 2017).

New requirements need to develop some techniques for low copy transfer of genes into the target cell by *A. tumefaciens* (Sivamani et al., 2015). In these experiments, *ZmSTK1* gene transferred into the cells of maize embryo during its initial growth time. For transformation of gene there are different ways used in the bio-scientific field, out of these methods Agrobacterium-mediated method of transformation was found as an easy and best way to transform the target gene into the maize embryo cells (Yadava et al., 2017). In this experiment, *Agrobacterium tumefaciens* used as a vector for multiplication to carry the gene *ZmSTK1* into the target embryo cells and insert the gene into the genome of maize cells.

Materials and methods

Plant material

Wild-type McC and mutant *zmstk1* provided by Dr. Dooner, Rutgers University, USA. The *pMD18-T-pSTK1* recombinant plasmid and the *pCAMBIA1301* plasmid provided by the laboratory from the College of Bioscience and Biotechnology in Shenyang Agricultural University, China.

Instruments and equipment

Test thermostat incubator, PCR machine, refrigerated centrifuge, electrophoresis tank, gel electrophoresis, autoclave, water bath, gel imager, horizontal electrophoresis tank, DC power supply, shaker, 50 ml centrifuge tube, tweezers, scalpel, and 2.0 ml centrifuge tubes.

Major reagents

To make 1% Rifampicin, 0.1 g rifampicin dissolved in 10 ml methanol, sterilized after filtration and stored at -20 °C. To make 5% kanamycin, 1.0 g kanamycin dissolved in 20 ml distilled water, filtered, sterilized and preserved at -20 °C. To make 50 g/l ampicillin, 2 g ampicillin dissolved in 40 ml distilled water, sterilized after filtration and stored at -20 °C. For Acetosyringone solution, dimethyl sulfoxide dissolved in acetosyringone, 200 mmol/l solution was prepared, diluted with distilled water to make 100 mmol/l, filtered and stored at -20 °C. In addition, for 5% sodium hypochlorite solution, 50 ml of pure sodium hypochlorite dissolved in sufficient amount of distilled water, raised the volume to 1 L and stored at room temperature. 70% ethanol was prepared by adding 70 ml of pure ethanol, with the sufficient amount of distilled water and raised the volume 0.1 L. To make YEB medium, 10 g tryptone, yeast 10 g extract and 0.5 g MgSO₄ .7H₂O mixed with distilled water to raise the volume 1000 ml and stored at pH 7.2. LB medium prepared by dissolving 10 g tryptone, 5 g yeast extract, 10 g NaCl in distilled water, raised the volume to 1 L, and stored at pH 7.0. For sodium phosphate buffer (100 mmol/l, pH 7.0), Solution A (6.55 g NaH₂PO₄ .2H₂O dissolved in 50 ml of distilled water) and Solution B (1.83 g Na₂HPO₄ .12H₂O dissolved in 50 ml of distilled water) mixed and volume raised to 200 ml at pH 7.0. X-Gluc prepared using (5-bromo-4chloro-3 indolyl)glucuronide and mother liquor (0.5 mg/ml). 0.025 g of X-Gluc dissolved in 1 ml of N-N-dimethyl amide (DMF) and stored at -20 °C. For X-Gluc base solution (10 mmol/l), 0.1 g of K₃Fe (CN)₆ potassium ferricyanide (1 mmol/l) and 0.13 g of K₄Fe (CN)₆ were mixed in 200 ml of sodium phosphate buffer, followed by the addition of 300 ul 0.1% Triton X-100, 1.12 g Na₂EDTA Potassium ferricyanide (1 mmol/l) and completely dissolved to make 250 ml.

Construction of maize ZmSTK1 expression vector

First competent cells of *E. coli* were prepared by making LB medium, which used to flatten to activate Escherichia coli DH5a single colonies, stored overnight at 37 °C. Then pre-activated single colony was selected, inoculated in 5 ml LB liquid medium and culture was shacked for 12 h at 37 °C. After that 500 µl bacteria inoculated to absorb in 50 ml LB liquid medium, shacked it at 37 °C and bacterial suspension placed in the already cooled 1.5 ml centrifuge tube and ice bathed for 15 min. The cooled suspension centrifuged for 1 min at 4 °C at 8000 rpm. Supernatant discarded after

removing the net residue of the culture medium and preserved in ice bath. Then suspended bacteria in a pre-cooled CaCl₂ solution (0.1 mol/l) at 4 °C and centrifuged for 1 min at 8000 rpm. Then 0.1 mol/l suspension of bacteria centrifuged for 1 min at 8000 rpm at 4 °C and supernatant removed by using 0.1 mol/l CaCl₂ solution. Supernatant discarded, net residual culture medium removed and 2 ml pre-cooled CaCl₂ solution (0.1 mol/l) added for bacterial suspension. After that, 80% of the volume of glycerol added, mixed, and stored at -80 °C in refrigerator for further use.

Double digestion reaction

The recombinant plasmids *pMD18-T-pSTK1* and *pCAMBIA1301* plasmids digested with restriction endonuclease *HindIII* and *NcoI*. Table 1 shows the reaction system.

Table 1. Composition of system used for digestion

System composition	Consumption
<i>pMD18-T-pSTK1</i>	15 uL
<i>HindIII</i>	1 uL
<i>NcoI</i>	1 uL
10*buffer (Enzyme digestion buffer)	2 uL
Double distilled H ₂ O	1 uL
Total	20 uL

Recycled digested product

The enzyme digested with the kit and the digested product of *pMD18-T-pSTK1* digested about 1000 bp and named *HindIII-pSTK1-NcoI* and *pCAMBIA1301*. The large fragment digested product named *HindIII-pCAMBIA1301-NcoI*.

Binding with the expression vector

HindIII-pSTK1-NcoI ligated with the vector *HindIII-pCAMBIA1301-NcoI*, and the binding system incubated at 14 °C for 14 h under the conditions of Table 2.

Table 2. Components of enzymatic systems

System composition	Consumption
T4 DNA Ligase	2.0 uL
T4 Ligase buffer solution	2.0 uL
<i>HindIII</i> - <i>pCAMBIA1301-NcoI</i>	4.0 uL
<i>HindIII</i> - <i>pSTK1-NcoI</i>	12 uL
Total	20 uL

Conversion

Thawed *E. coli* competent cells to complete melting drew 100 µl into a new sterile centrifuge tube and immediately placed in ice. Then 5-µl ligase enzyme added, mixed and placed in ice for 30 min followed by putting it in a hot water bath at 42 °C for 90 s and quickly placed in ice for 5 min. Then 1-1 LB liquid medium added, mixed and shacked at 37 °C for 1.5 h and centrifuged for 1 min at 8000 rpm, discarded the centrifugal supernatant and added 100 µl LB liquid medium. The bacterial liquid

coated on LB solid medium containing 50 g/l of ampicillin and the surface placed up to the bacterial solution, completely absorbed by the culture medium and cultured at 37 °C for 16 to 24 h. Then a single colony picked and used PCR kit to mention the plasmid. After a double enzyme digestion, electrophoresis detection of recombinant plasmid used and digestion products tested in the positive plasmid named *pCAMBIA1301-pSTK1-GUS* saved in refrigerator at -80 °C.

Preparation of agrobacterium competent cells

Agrobacterium tumefaciens EHA105 cultured on YEB solid medium containing 1% rifampicin and cultured at 28 °C for 48 h. Activated single colony selected, inoculated with 1 ml liquid medium containing 1% rifampicin YEB and shaken at 28 °C overnight. 250 µl of the bacterial solution was taken and incubated in 25 ml of YEB liquid medium containing 1% rifampicin and shaken at 28 °C until OD600 = 0.5. Next, took the bacterial suspension, ice bathed for 15 min and centrifuged in a high speed centrifugal machine for 10 min at 5000 rpm and supernatant was discarded. 5 ml 0.15 mol/l NaCl suspension of bacteria was prepared, ice bathed for 15 min and centrifuged at 5000 rpm for 10 min. Then 2 ml 0.1 mol/l pre-cooled CaCl₂ solution was prepared with cell suspension, 80% glycerol added and refrigerated at -80 °C.

Transformation of agrobacterium competent cells

Thawing agrobacterium competent cells

Ten µl of *pCAMBIA1301-pSTK1-GUS* added into 100 µl competent cells, mixed and ice bathed for 30 min. Next, placed in liquid nitrogen for 5 min and immediately transferred it to a 37 °C water bath for 5 min. Then 1 ml of YEB added, shook for 5 h at 28 °C, centrifuged at 220 rpm and supernatant removed. 200-µl YEB liquid medium of bacterial suspension added centrifuged at 5000 rpm for 2 min and supernatant removed. YEB solid medium containing 1% rifampicin and 5% kanamycin added and cultured at 28 °C for 48 h. Finally, a single colony picked and PCR used to identify the positive results.

Genetic transformation of maize germination embryos

Strain activation

After preparation, YEB solid medium (1-l YEB medium + 7 g agar powder) placed in a high-pressure sterilization chamber of a steam sterilizer. Petri dishes, gun, gun head, a sealing film, an inoculation ring and other tools were also placed in ultraviolet radiation chamber for sterilization and then in autoclave chamber. Bacterial culture taken out of the refrigerator for 30 min for defrosting. When the medium temperature was moderate, 1% rifampicin and 5% kanamycin added in the ultraclean working medium and poured on a flat plate. When the medium solidified, the inoculation ring burned to red with alcohol lamps, and the front of the rod fixed about 7 cm. The outer ring heated for 10 s, and inoculated ring completely cooled. The positive colonies stained with rings, and the culture medium divided into three zones. After the completion of the inoculation, the inoculation ring placed on the alcohol lamp for burning and cooled. After inoculation on the edge of the line, the area crossed again to make two areas. At the end, the third area also inoculated in the same method as it inoculated into other areas. The inoculation completed after burning the inoculation

ring. After cooling, the metal ring removed and the petri dishes closed with sealing film. Next, placed the petri dish in the incubator in a dark chamber at 28 °C for 48 h.

Preparation of bacteria

A single colony of *Agrobacterium tumefaciens* EHA105 containing *pCAMBIA1301-pSTK1* recombinant expression plasmid transferred on the plate with a sterilized gun tip. Seeded in YEB liquid medium containing 1% rifampicin (7.5 µl) and 5% kanamycin (1.5 µl) in a centrifuge tube (size: 2.0 ml) of 1.5 ml, shaken overnight on a 200 rpm speed in a constant temperature shaker at 28 °C and found positive results as shown in *Tables 3 and 4*. Bacterial solution transferred into the 50 ml centrifuge tube, 25 ml of YEB liquid medium, 1% rifampicin 125 µl and 5% kanamycin, 25 µl of mixture added and culture shaken well at a constant temperature. The determined OD value was in the range of 0.4-1.2. The material saved at 4 °C for further use.

Table 3. Primers for *GUS*

Name of primer	Primer sequence
Upstream primers: GUS—F	5'-GCAACTGGACAAGGCACT-3'
Downstream primers: GUS—P	5'-GAGCGTCGCAGAACATTACA-3'

Table 4. Reaction components of bacterial culture medium for PCR

System composition	Consumption
PCR Master Mix	5.0 uL
GUS—F	0.5 uL
GUS—P	0.5 uL
bacterial fluid	4.0 uL
Total	10.0 uL

Seed sterilization

Healthy seeds selected with no damage, soaked into 70% ethanol for sterilization for 30 s; seeds transferred to 5% sodium hypochlorite for disinfection for 45 min and rinsed the seeds 5 to 7 times with sterile water. Finally, seeds packed into a sterilized jar (200 tablets), sterilized water added and shaken with the help of a shaker at 90 rpm and soaked overnight. The next day, tubes centrifuged at 4000 rpm for 10 min, supernatant removed and precipitate transferred to the sterile jar. Fresh 1% rifampicin 125 µl, 5% kanamycin 20 ml, YEB liquid medium 25 µl, 15% 5 µl of acetosyringone and agar added as transformation and growth medium. Tip of the sterilized blade used to scrape the embryo growth point and placed in the jar. Labeled flasks placed in a thermostatic shaker and incubated at 28 °C and 90 rpm for 12 h. After those 12 h, the seeds transferred into the nutrient rich soil containing vermiculite and perlite and cultured for T° plant stage. Then set different gradients of OD value to 0.4, 0.6, 0.8, 1.0 and 1.2 and acetyl acetone (AS) with concentration gradient of 50 mmol/l, 100 mmol/l, 150 mmol/l, 200 mmol/l and the conversion rate of maize plants was calculated.

DNA extraction and identification of plant genetic transformation

When the plant reached the three-leaf stage, 1-2 cm of blade was cut and put into the centrifuge tube containing 200 µl of NaOH 0.25 mol/l and the tube was centrifuged for 30 s at 100 °C constant temperature. 200 ul of 0.25 mol/l HCl and 100 ul of 0.5 mol/l of Tris-HCl (pH 8.0, NP-40 0.25%) added to the centrifuge tube. The tube again incubated in boiling water for 2 min. About 2 mm² of the leaves was picked up after few steps treatment of acid and alkali and this sample used as a template for PCR amplification. The PCR amplification reaction conditions shown in *Table 5* and the reaction system components shown in *Table 6*.

Table 5. PCR amplification reaction conditions

Temperature	Duration
94 °C (Pre-denaturation)	2 min
94 °C (Denaturation)	90 s 35 cycles
57 °C (Annealing)	1 min
72 °C	2 min
72 °C	5 min
4 °C	Saved permanently

Table 6. PCR reaction system components

System components	Consumption
GUS-F	2.5 µL
GUS-R	2.5 µL
PCR Master Mix	7.3 µL
Blade	≤ 2 mm ²
Double distilled H ₂ O	37.7 µL
Total	50.0 µL

Gel electrophoresis machine used to separate different sized DNA fragments. For the preparation of 10* TBE buffer, 108 g Tris, 7.44 g Na₂EDTA .2H₂O and 55 g of boric acid was taken in a beaker and 800 ml distilled water was added to the beaker stirred to fully dissolved, volume was raised to 1 L with addition of distilled water and stored at room temperature. 0.5* TBE buffer was prepared by mixing 25 ml 10* TBE and 475 ml distilled water and stored at room temperature. 5 mg/ml ethidium bromide(EB)aqueous solution was prepared and agarose gel plates were prepared by mixing 0.2 g agarose and 20 ml 0.5* TBE in a conical flask by flushing liquid in it. After mixing, the flask placed in the microwave for heating for 1 min to dissolve fully and then cooled to 60 °C to add EB drop by drop. After shaking the sample, the combs were fixed, the solution was poured into the sealed gel tank (thickness 3-5 mm) and air bubbles between the comb teeth discharged. Combs taken out after cooling and glue solidification, then gel plate placed into a horizontal electrophoresis tank containing 0.5* TBE buffer until the plane thickness of submerged glue became 2 mm. Further, ten to fifteen µl marker sample added in marked gel hole and input of 180 voltage, 100 mA current to the gel electrophoresis tank provided and samples were run in the gel. Power turned off; gel bands removed from the electrophoresis tank and placed in the gel imager to check the imaging results. To perform GUS

staining of transgenic plants the configured X-Gluc mother liquor and X-Gluc base solution completely mixed to a volume of 300 ml. T° genetically modified transgenic plants and wild-type plants immature and mature roots, young leaves, mature leaves, immature anthers and mature anthers placed in fixed solution of 90% acetone for 15 min. Rinsed with buffer three times, put into the GUS staining solution, vacuumed and placed in the incubator at 37 °C in the dark overnight. Next day, samples rinsed three times with 70% alcohol for decolorization, observed under the microscope, pictures taken and differences analyzed.

Results

The construction of expression vector

The digestion of recombinant plasmid *pMD18-T-pSTK1* and plasmid *pCAMBIA1301* done by *Hind III* and *NcoI* restriction enzymes. The digested *Hind III-pSTK1-NcoI* promoter and *Hind III pCAMBIA1301-NcoI* connected with *CaMV35S* promoter instead of *pCAMBIA1301* in the vector. The recombinant plasmid expressing *pCAMBIA1301-pSTK1* transferred into *Escherichia coli* competent cells. After successful transformation, plasmids extracted and transferred into the susceptible state of *Agrobacterium tumefaciens*. The screening of samples performed and selected colonies used for PCR. The results are shown in *Figure 1*, Lane 3, Lane 4 and Lane 5 in 1000 bp band.

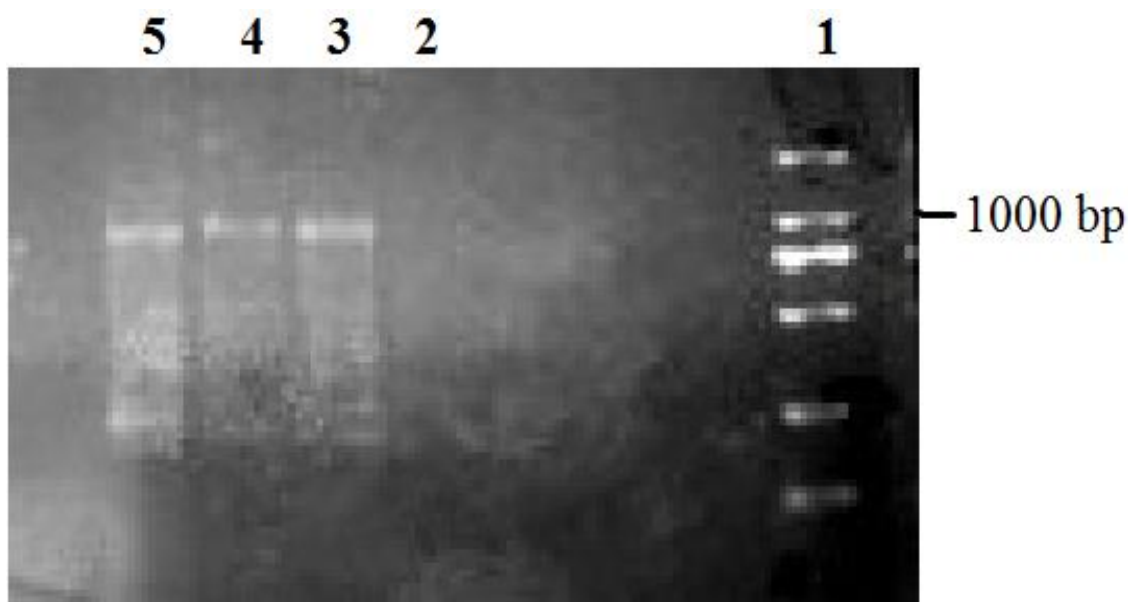


Figure 1. PCR detection of *pCAMBIA1301-pSTK1-GUS*. (Note: Lane 1 is 2000 marker; Lane 2 is control carrier *pCAMBIA1301*; Lane 3-5 are the product to be tested)

Identification of active colonies

Active colonies identified by their activities in PCR amplification and detection through agarose gel electrophoresis. The detection results are shown in *Figure 2*, Lane 2 and Lane 3 in about 750 bp.

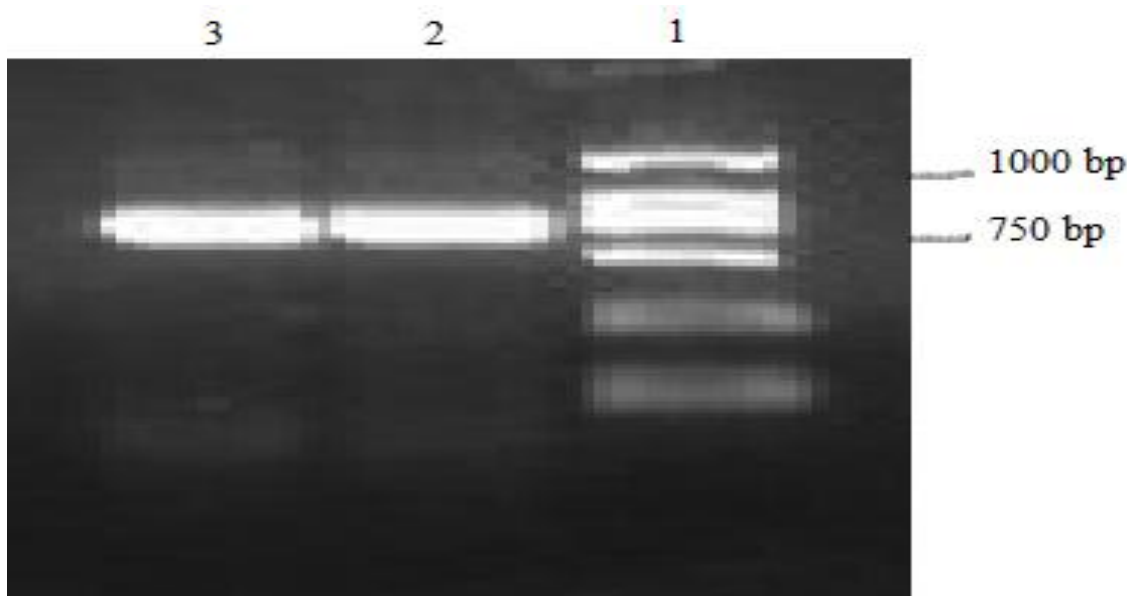


Figure 2. Detection of bacterial liquid PCR. (Note: Lane 1 is 2000 marker; Lane 2, 3 are colonies to be tested)

Optimization of transformation conditions

Acetosyringone AS can induce the activation of virulent gene in *Agrobacterium tumefaciens*, and promote the integration of foreign genes. The addition of AS can improve the conversion rate. The maize seeds were treated with different concentrations (OD value were 0.4, 0.6, 0.8, 1 and 1.2) of the bacteria by adding 0.1 mmol/l acetosyringone (AS) and cultivated at constant temperature of 28 °C. The results showed that different concentrations of bacteria had little effect on seed germination (around 20%) but affected the seed conversion rate. When the concentration of bacteria was up to 0.8 OD and acetosyringone concentration was 0.1 mol/l, the transformation rate was maximum.

Regeneration of germinating embryo by genetic transformation

The inoculated corn seeds cultured in nutrient rich soil containing vermiculite and perlite and 150 grains were sown in each pot (15 × 10) as shown in *Figure 3*.

Total 4000 seeds (200 per plot) incubated into germinating embryos and 807 seeds germinated after Incubation. Only three plants successfully transformed as shown in *Figure 4*. The *pstk-1* transgenic plants of T⁰ generation obtained by Agrobacterium-mediated method. The results showed that when OD value was at 0.8 and concentration of AS was 100 mmol/l, the transformation rate was the best. The germination rate was 20.2%, and the conversion rate was 0.075%.

Transgenic plant testing

After DNA extraction, PCR amplification and detection from the transgenic plants results showed that there were bands in the samples and detected at around 750 bp. The PCR results showed that three seeds out of 4000 seeds successfully transformed (*Fig. 5*).



Figure 3. Screening of positive plants



Figure 4. Transgenic regeneration of positive plants

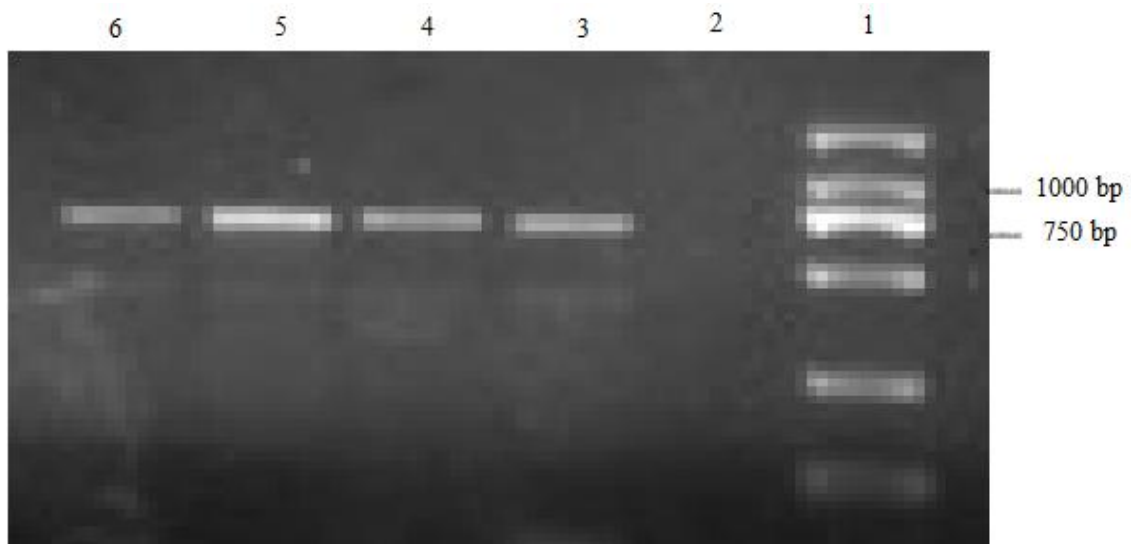


Figure 5. PCR detection of transgenic plants. (Note: Lane 1 is 2000 marker; Lane 2 is non-transgenic plants; Lane 3 is bacterial liquid; Lanes 4-6 are the transgenic plants to be tested)

GUS staining of regenerated plants

In GUS staining of non-transgenic plants and *PSTK1-GUS* plants parts such as immature roots, immature leaves and immature anthers, mature root, mature leaves and mature anthers and mature pollen performed. The results showed that all parts of the non-transgenic plants showed no GUS signal. *PSTK1-GUS* transgenic plant immature roots, mature roots, immature leaves, mature leaves and immature anthers also showed no GUS signal but the mature anthers and mature pollens showed GUS signal in the form of dark spots as shown in *Figure 6*.

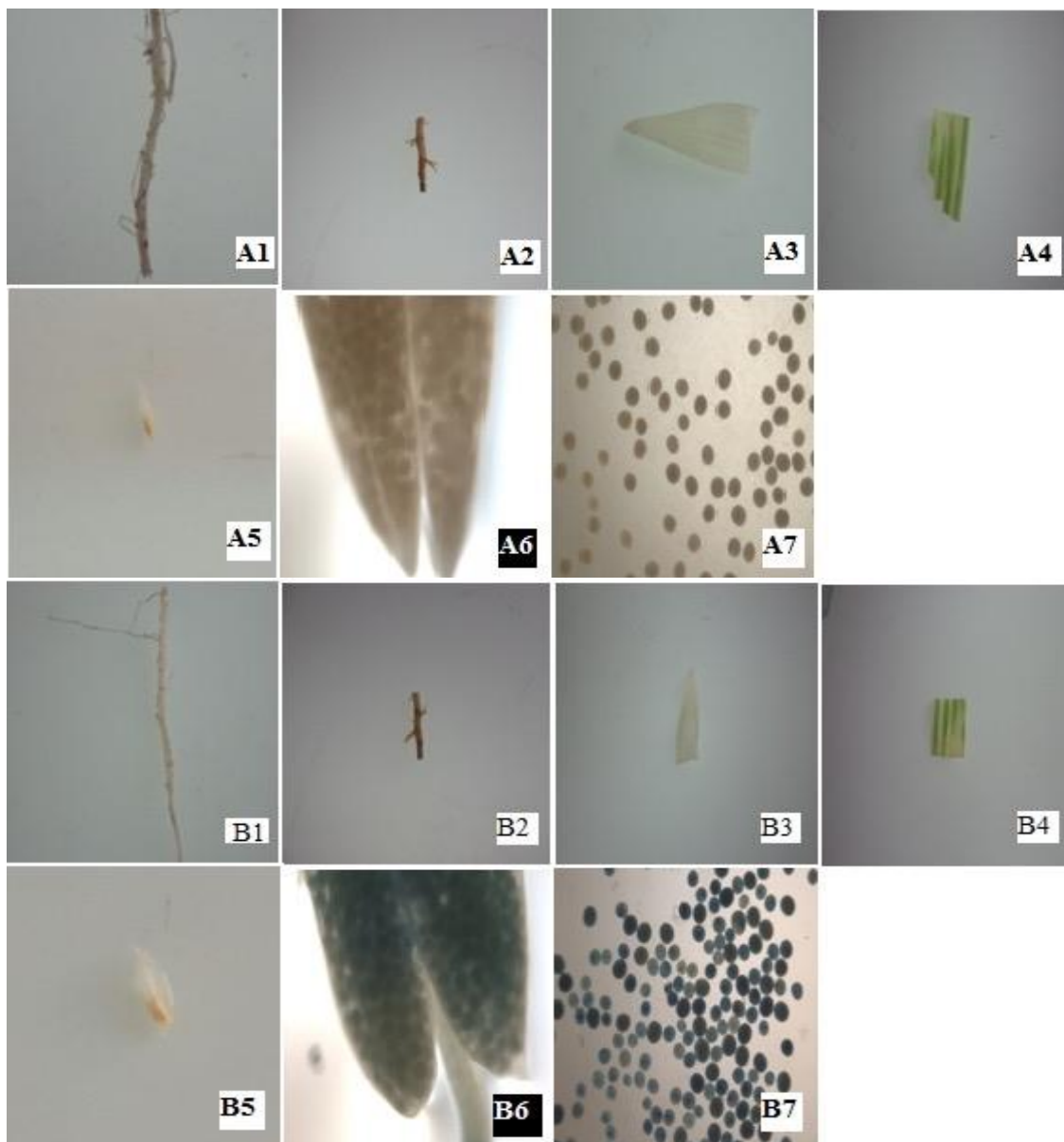


Figure 6. *GUS* staining for the non-transgenic and *PSTK1-GUS* plant parts. (Note: A1-A7 are non-transgenic plants; B1-B7 are *PSTK1-GUS* plants; A1 and B1 are radicles; A2 and B2 are mature roots; A3 and B3 are immature leaves; A4 and B4 are mature leaves; A5 and B5 are immature anther; A6 and B6 are mature anther; A7 and B7 are mature pollen)

Discussion

Genetic transformation is an effective way to increase crop yield and improve quality. With the progress of technology, the method of transgenic maize is continuously improved. In maize, there is a variety of transformation methods, such as electroporation, pollen tube pathway, gene gun, polyethylene glycol, and Agrobacterium-mediated transformation. The receptor of electroporation and polyethylene glycol (PEG) is through protoplast, which is very strict to the preparation and operation of receptor, so it cannot be widely used. The complete pollen tube transformation process is done by natural pollination, without tedious preparation work, breeding required time is short and used directly. Sometimes growing season and flowering stage can affect it. It is also affected by environmental conditions such as temperature, wind, light, rain and more. The bombardment carried out by using the tiny metal particles containing DNA to target the receptor, and DNA injected into the recipient cells by a bilayer lipid membrane. Without host restriction, biolistic method transforms into a variety of plants and improves the plant transformation rate. Moreover, receptor-mediated no-gene type restrictions can be applied to different species and different varieties. It can be applied to different organelles with high degree of controllability. However, because the bombardment is random and the conversion rate is relatively low, so multiple copies of the gene are inserted in genome during rearrangement. Prone to a variety of ways, the homologous sequence can be RNA-RNA, DNA-DNA, or DNA-RNA with each other, causing transcription or posttranscriptional level gene silencing. According to various methods, Agrobacterium-mediated transformation has the advantages of relatively stable inheritance, simple operation and high conversion rate. This transformation experiment using *Agrobacterium tumefaciens*-mediated transformation of maize germinating embryo was time saving, strong repeatability and no need of the corn growing season constraints. It also does not depend on tissue culture can also be done without the process of tissue culture transformation. The receptor of this study, and the concentration conditions of bacteria and AS optimized was also corn-germinating embryo.

After acid and leaf treatment, PCR amplification used to screen the positive plants, which was simple and effective. This method does not need to extract the detected leaf DNA according to traditional procedure, shortened the testing time, only needs leaf area of not more than 2 mm. At the same time, in this method, plant leaf injury is relatively low and the cost is decreased. Therefore, this method has the advantages of less time consumption and high detection efficiency.

GUS detection method is fast, simple and stable. The GUS gene is beta-D-glucuronidase (Gus) gene, X-Gluc can be hydrolyzed into blue material and staining of tissues showed blue spots with GUS activity (visible), so gene expression of GUS detected in specific tissues at exogenous gene site. This is not reported in other gene transfer method. In this study, the *PSTK1-GUS* gene of T⁰ transgenic plants only had GUS signal in mature anthers and mature pollens, which showed that *stk1* promoter only had transcriptional activity in mature pollen. However, for T⁰ transgenic plants, it is not possible to determine whether the inserted fragments are integrated successfully into the genomic DNA or not and further experiments are needed to verify them.

Conclusion

The results suggested that the transformation rate to obtain transgenic plants was very low. The GUS staining of non-transgenic and transgenic *PSTK1-GUS* plants showed no GUS signal. However, mature anthers and pollens showed GUS signal in the form of dark spots, which indicated *ZmSTK1* only influences during pollen development and germination. Agrobacterium-mediated gene transformation method is advance and easy for genetic researchers to solve the mysteries behind gene mutation and function at a specific stage.

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Ethical approval. This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interests. All authors declare that there is no conflict of interests.

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