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Full Length Research Paper

Genetic modification of maize inbred line for resistance against *Exserohilum turcicum* via immature embryo culture and incorporation of balsam pear class I Chitinase Gene

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Maize is one of the most important food crops worldwide and is subject to many diseases. Genetic engineering offers unique way to improve the current elite line via introduces single or multiple traits. In this report, we transformed an elite maize line, Jiao 51, with a balsam pear class I chitinase gene (*Mcchit 1*) driven by the maize ubiquitin-1 (ubi1) promoter. Conditions for callus induction from immature embryos were optimized using a Jiao 51 by culture on induction medium. The highest Type-I and Type-II callus induction efficiency of 91.33 and 89.10% were obtained on N6 media supplemented with 2.5 mg L^{-1} 2, 4-D. Both types of calli grew fast. Type-I callus could easily change to Type-II callus. *Agrobacterium*-mediated transformation was used to generate eight transgenic lines that expressed the chitinase transgene. Assessing the functionality of the *Mcchit1* protein in the transgenic plants was conducted using an excised leaf challenge assay. The transgenic maize expressing a balsam pear class I chitinase gene exhibited enhanced tolerance to *Exserohilum turcicum* in greenhouse conditions. After 5 days inoculation, the number of lesions was significantly fewer, and the size of lesions was significantly smaller compared to the controls.

Key words: Maize (*Zea mays.* L.), chitinase gene, transformants, fungal disease tolerance, Northern corn leaf blight, *Exserohilum turcicum*.

INTRODUCTION

Maize is one of the three most important food crops in the world, which is essential for global food security. However, maize yield is greatly impacted by fungal diseases. The most important fungal disease of maize is leaf blights, including Southern corn leaf blight, Northern corn leaf blight (NCLB), Gray leaf blight. NCLB was one of the most damaging corn leaf diseases, which was caused by the fungus *Exserohilum turcicum*. Severe corn leaf blights can weaken the corn plants and cause as much as a 50% reduction in yield. Thus control of this fungal disease is a worldwide major challenge to corn production. Growing concern about the environment and the high cost of chemicals has encouraged farmers and researchers to look for substitutes of chemical fungicides.

It was reported that "pathogen-related" (PR) protein gene could play an important role in defense response of plant against fungal infections (Van Loon, 1997). Chitinase (EC 3.2.1.14) is characterized as a subgroup of PR proteins. Since chitin is a main component of the fungi cell wall and the enzymatic activity of chitinase could specifically break bonds between the C1 and C4 of

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two consecutive N-acetylglucosamines of chitin, and consequently result in the cell wall degradation and death of pathogenic fungi, and then inhibit fungal infection (Roby et al., 1990). Plant chitinase is a member of chitinase and classified into seven classes (I-VII) based on their primary structures (Stirpe et al., 1992). Schlumbaum et al. (1986) have proved that plant chitinases were potent inhibitors of fungal growth. Many plants expressing plant chitinase genes in a number of agriculturally important crops have been shown to have enhanced fungal resistance fungal pathogens, such as transgenic rice (Kim et al., 2003), cacao (Maximova et al., 2006), wheat (Shin et al., 2008), Taro (He et al., 2008), and tobacco (Kern et al., 2009).

Balsam pear (*Momordica charantia* L.) is a vegetable crop and also a medical plant, highly resistant to various pathogens. A chitinase gene (*Mcchit1*) was cloned from balsam pear previously and characterized as class-I chitinase gene (Xiao et al., 2007). It was shown that overexpression of *Mcchit1* could enhance resistance to *Phytophthora nicotiance* in transgenic *Nicotiana benthamiana* and against *Verticillium* wilt in transgenic cottons (Xiao et al., 2007). Results reported above indicated that the *Mcchit1* gene was a useful gene in plant engineering against fungal diseases.

However, a basic prerequisite for the production of genetically modification plants is efficient regeneration system of normal and fertile plants from cells. The regeneration ability of maize is influenced by plentiful factors. The type of explants is considered one of the main factors. Green and Philips (1975) first reported that the plants were regenerated from immature embryos of maize. And then, the regeneration have been successfully achieved from a numbers of different organs, such as anthers (Ting et al., 1981), shoot apical meristems (Zhong et al., 1996), mature embryos (Huang et al., 2004), seedling shoot apex (Gould et al., 1991; Sidorov et al., 2006), leaf segments (Ahmadabadi et al., 2007), immature embryos (Ishida et al., 2007; Frame et al., 2002, 2006; Binott et al., 2008). Whereas, callus derived from immature embryo is more efficient and the most widely used for plant regeneration than callus from other explants. The genotype of the explants is regarded as another important factor, which influences the regeneration potential of explants. To further harness the benefits of genetic transformation in breeding program, it is necessary to develop protocols of regeneration and transformation with maize elite inbred lines. The third factor is culture conditions. Different mediums in combination with 2, 4-D have been used for the induction of embryogenic callus of *Zea mays* (Kamm et al., 1990; Binott et al., 2008; Bronsema et al., 2001; Rakshit et al., 2010). Concentration of 2, 4-D directly influences growth response depended on presence of 2, 4-D (Bronsema et al., 1997). Moreover, Jiao 51 is an important elite inbred line of maize in local. Most of high quality and high yield crossed hybrids are derived from this line. Therefore, the objectives of the present research were:

(i) To develop a highly-efficient protocol of maize elite inbred line and produce transgenic maize plants expressing a *Mcchit1* gene via *Agrobacterium*-mediated transformation, and
(ii) Demonstrate ability of increased disease resistance against *E. turcicum* in transgenic lines.

MATERIALS AND METHODS

Plant materials

Elite inbred line (Jiao 51) was stored in the laboratory (Guizhou Key Laboratory of Agro-Bioengineering, Guizhou University, Guiyang city, China). F1 immature zygotic embryos used for these experiments were from plants grown in the field. Immature ears are collected 11-13 days after self-pollination when immature embryos were about 1.0-2.0 mm in length and surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 8 min. The sterilized ears were rinsed five times with sterilized distilled water. Aseptic immature embryos dissected from ear are placed with scutellar side up and flat surface down on the callus induction medium (CIM) for Type-II callus induction.

Callus induction

Basal culture medium for callus induction was N6 medium (Chu et al., 1975), and supplemented with 1, 1.5, 2.5, 3.5 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D). Other reagents supplemented in N6 basal medium were described in Table 1. Different levels of 2, 4-D were evaluated to study the effect of this growth regulator on callus induction. Each treatment was repeated at least three times. Explants were incubated in the dark for 24h at 28°C. After 2 weeks, the number of explants producing callus was recorded. According to Bronsema et al. (1997), callus induced from maize immature embryos were Type-I and Type-II callus. Subculture of Type-II callus was conducted on callus induction medium (CIM) every 2 weeks to keep the embryogenesis ability. Effects of various concentration of 2, 4-D on callus induction were compared using SPSS 18.0. Mean comparisons were conducted using respective critical differences.

Construction of the plasmids

Agrobacterium tumefaciens strain EHA105 harboring the plasmid Pc Mcchit1 (Figure 1) containing the selectable bar gene (Rathore et al., 1993) encoding the phosphinothricin acetyltransferase (*PAT*) enzyme driven by the *35S* promoter of a cauliflower mosaic virus (*CMV*), β-glucuronidase marker gene (*GUS*) (Jefferson, 1989) driven by the maize ubiquitin (*Ubi-1*) promoter (Toki et al., 1992), as well as chitinase gene (*Mcchit1*, DQ407723) driven by the maize ubiquitin (*Ubi-1*) promoter. Agrobacterium containing the plasmid DNA was a gift from Pro. Pei Y (Biotechnology Center, Southwest University, China).

Plant transformation, selection and regeneration

Bacteria preparation, infection, co-cultivation, selection and plant regeneration procedures were according to Frame et al. (2002). To get efficient transformation, the callus was pre-cultured on CIM for 6-9 days, and agrobacterium suspension precultured in InfM+AS (2 h) was diluted to OD_{600} 0.3-0.4.Callus was inoculated with suspension for 10 min, blotted twice on sterile filter paper to remove

Table 1. Media formulations.

Media	Components (mg L^{-1})
CIM	N6 salts and vitamins (Chu et al., 1975), 0.7 g/L L-proline, 0.1 g/L Hydrolyzed casein, 0.1 g/L myoinositol, 20 g/L sucrose, 1.0 g/L Filter sterilized silver nitrate, 8.0 g/L agar, pH 5.8.
Inf M	N6 salts and vitamins, 2.0 mg/L 2,4-D, 0.7 g/L L-proline, 100 mg/L myoinositol,68.4 g/L sucrose, 36 g/L glucose, 1.0 mg/L Filter sterilized silver nitrate, pH 5.2. Add 100 μM acetosyringone before using.
CoM	N6 salts and vitamins, 2.0 mg/L 2,4-D, 0.7 g/LL-proline, 100 mg/L Hydrolyzed casein, 100 mg/L myoinositol, 20 g L^{-1} sucrose, 1.0 mg/L Filter sterilized silver nitrate, 300 mg/L Cysteine, 4.0 g/L Gelrite, pH 5.2.
RM	N6 salts and vitamins, 2.0 mg/L 2,4-D, 0.7 g/L L-proline, 100 mg/L Hydrolyzed casein, 100 mg/L myoinositol, 20 g/L sucrose, 1.0 mg/L Filter sterilized silver nitrate, 8.0 g/L agar, pH 5.2.
SMI	N6 salts and vitamins, 2.0 mg/L 2,4-D, 0.7 g/L L-proline, 100 mg/L Hydrolyzed casein, 100 mg/L myoinositol,20 g/L sucrose, 1.0 mg/L Filter sterilized silver nitrate, 1.5 mg/L bialaphos, 8.0 g/L agar, pH 5.8.
SMII	N6 salts and vitamins, 2.0 mg/L 2,4-D, 0.7 g/L L-proline, 100 mg/L Hydrolyzed casein, 100 mg/L myoinositol, 20 g/L sucrose, 1.0 mg/L Filter sterilized silver nitrate, 3.0 mg/L bialaphos, 8.0 g/L agar, pH 5.8.
PRM I	MS salts and vitamins (Murashige and Skoog, 1962), 700 mg/L L-proline, 100 mg/L Hydrolyzed casein, 100 mg/L myoinositol, 60 g/L sucrose, 3.0 g/L Gelrite, pH 5.8.
PRM II	MS salts and vitamins (Murashige and Skoog, 1962), 100 mg/L myoinositol,0.7 g/L L-proline, 100 mg/L Hydrolyzed casein, 30 g/L sucrose, 3.0 g/L Gelrite, pH 5.8.

a, Filter sterilized silver nitrate(10mg/L) is added prior to use. b, AS stocks were dissolved in DMSO to 200mM then diluted 1:1 with water and stored at -20ºC for use as needed.

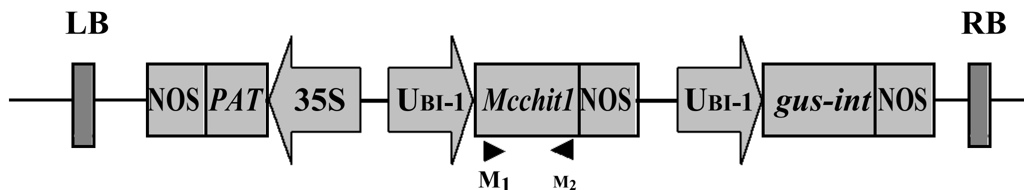

Figure 1. T-DNA regions of transformation vectors. *Ubi-1*, a maize-constitutive ubiquitin-1 promoter; *35S*, 35S CaMV gene promoter; *NOS*, 3'-untranslated sequence from the *Agrobacterium* NOS gene; *Mcchit1*, balsam pear chitinase gene; *PAT*, phospinothricin acetyltransferase gene; *gus-int*, β-glucuronidase gene containing an intron; LB, Left border ; RB, Right border.

excess liquid, and then transferred infected calli to the surface of co-cultivation medium (CoM), and incubated in the dark (23°C) for 3 days. After the co-cultivation stage, the calli were transferred onto resting medium (RM) containing antibiotics but no PPT and incubated at 28°C in dark for 10 days after which they were transferred onto selection medium (SM) containing 1.5 mg/L bialaphos to begin the first selection. Second selection was enhanced to 3 mg/L at week 4. Regeneration of callus (friable, stocked somatic embryos present) was accomplished by transferring resistant callus to plant regeneration medium (PRM) and incubating for 2-3 weeks at 28°C in the light. Once shoots reached 2-3 cm in height, plantlets were moved to MS rooting medium (RM) in 16:8 h light: dark cycle at 28°C. Regenerated plants were transferred to soil (KLASMANN Base Substrate 422, Germany), accomplished as described in Frame et al. (2002). Transgenic plants were grown to maturity in the greenhouse under fluorescent white light in 16:8 h light: dark cycle.

Histochemical analysis of GUS expression

Histochemical GUS assays (Jefferson et al., 1987) were used to assess stable expression of the *uidA* gene in transformed control and non-transformed plants in the T0 generations. Explants were incubated at 37°C for overnight in buffer containing 1 mM X-Gluc, 100 mM sodium phosphate buffer (pH 7.0), 10 mM Na_2EDTA, 0.5 mM potassium ferricyanide, and 0.1% (v/v) Triton X-100. Tissues were submerged in the substrate, vacuum infiltrated (25 inch Hg) for 5 min, and incubated at 37°C for overnight. Blue staining tissues were visualized by soaking tissues in 75% followed by 95% (v/v)

ethanol to remove chlorophyll.

Polymerase chain reaction (PCR) analysis

Total genomic DNA was extracted from approximately 20 mg of maize leaf tissues by the cetyltrimethylammonium bromide protocol (CTAB) (Saghai-Maroof et al., 1984). The 401-bp coding region of *Mcchit1* gene was amplified using the following primers: forward M_1:5'-ATCGGGAGACGTTGGCAG-3' and reverse M_2:5'-CTAGTGCTCTACCTGCTG-3'. The amplification reaction was carried out using a thermal cycler (MyCyler TM, BIO-RAD, U.S.A) under the following conditions: one cycle of 94°C for 5 min; 30 cycles of 94°C for 40 s (denaturation), 51°C for 40 s (annealing) 72°C for 40 s (extension); a final extension at 72°C for 5 min (one cycle). To ensure that reagents were not contaminated, DNA from non-transformed (control) plant was included in the experiments. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide (EB).

Southern blot analysis

Leaf genomic DNA was prepared from 2 to 3 g of fresh leaf tissue from putative transgenic maize plants using the CTAB method. Twenty micrograms of genomic DNA per sample was digested with the Kpn I restriction enzyme at 37°C overnight and separated on a 0.8% (w/v) agarose gel prior to transferring to positive charged nylon membrane (Bio-Rad, CA, USA). DNA was fixed to the membrane by UV cross-linking. Hybridization and washing conditions for southern blot analysis followed the Dig probe detection kit-I manufacturer's instructions (Innogent, Shenzhen, China). The *Mcchit1* gene from vector pCambia- Mcchit1-PAT was used to generate a Dig-labeled probe. The probe was prepared by the M1/M2 primer incorporating Dig-dUTP utilizing the Dig DNA Labeling kit-I (Innogent, Shenzhen, China).

RNA isolation and transcript analysis

To examine whether *Mcchit1* gene expressed in maize tissues, PCR-positive maize plants at the five-leaf stage were selected for RT-PCR analysis. Using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) to isolate total RNA from approximately 100 mg of fresh plant leaves ground to a fine powder with liquid N_2. Reverse transcription for cDNA synthesis with a one-step reverse transcription polymerase chain reaction (RT-PCR) kit (Qiagen, Valencia, CA). The reverse transcripts were used as templates for PCR. The M1/M2 primers for the *Mcchit1* gene and the PCR amplification reaction conditions were described above in the section on PCR analysis. As an internal control, primers for the coding region of the maize actin-1gene (GenBank: accession NO. J01238) were: forward 5'- tgtatgttgctatcgaggctgttc-3' and reverse 5'-tcattaggtggtcggtgaggtc-3'. The products were separated by electrophoresis on a 3.0% agarose gel.

Herbicide leaf painting assay

To determine the activity of PAT, which is the product of *PAT* gene, it was assayed indirectly by the resistance of transgenic maize to herbicide application. The herbicide BASTA (Crescent Chemical Islandia, NY) containing 200 mg L^{-1} glufosinate ammonium was used for leaf painting of the putatively transgenic plants. Healthy leaves of non-transformed control and transformed (T0) plants at the five-leaf stage were selected for leaf painting. Using a writing brush, 0.001% BASTA with 0.1% Tween X-100 was applied to paint the upper surface of the third or fourth leaf. Leaves were scored for herbicide damage ten days after application.

Screening for resistance against *Exserohilum turcicum*

To ensure virulence of isolate *E. turcicum* fungi from stock cultures were inoculated onto maize seedlings and re-isolated from lesions. The procedure of inoculation was as described by Juliana et al. (2005). *E. turcicum* obtained from infected maize tissues were used the leaves were cut from non-transformed control, To transformed to prepare inoculum for inoculations and disease evaluation. After plants were inoculated with *E. turcicum*, which were transferred to humid Petri dish. Parafilm sealed Petri dish was used to raise the relative humidity so as to enhance infection. In order to prevent dehydration of leaves, the cut of the leaves were kept in moisture condition with wet gauze. Detected infection leaves after 3 days and 5 days, respectively.

RESULTS

Callus formation on induction medium

Zygotic embryos were dissected from ear and placed with scutellum side up on the callus induction medium containing different levels of 2, 4- D to evaluate genotype culture ability. For each concentrations of 2, 4- D, a total of 150 immature embryos in 3 replicates were used as explants for experiments of callus induction. In general, during the first day of culture, the size of immature embryo obviously increased (Figure 2a). Callus initiation was firstly observed on basal sides of the scutellum after 4 days of culture. After 1 week of culture, calli were formed from both the swelling of the middle and the surface of scutellum (Figure 2b). The callus obtained on induction medium could be classified as Type-I and Type-II calli. Type-I callus was compact, and Type-II callus was soft friable, embryogenic with globular somatic embryos (Figure 2c). Then, partial Type- I callus could turn into Type-II within two week following culture.

Based on analysis of variance, concentrations of 2, 4-D were directly determined on the frequency of callus induction from 'Jiao51' inbred line, which also greatly influenced the quality and quantity of embryogenic callus (Table 2). The frequency of primary callus induction ranged from 44.67% to 91.33 % on induction medium. In addition, rates of callus induction were relatively low at 1.0 mg/L 2, 4-D, while the embryos readily germinated and the coleoptiles fast elongated with a straight shape. The size of Type-II callus was also small (0.53 cm). On the contrary, callus appeared at relatively high frequency at the high concentration of 2, 4-D, which also inhibits cell growth of the coleoptiles. The shape of coleoptile was rotated or corkscrew. The highest frequency (89.1%) of Type-II callus induction was observed on the medium supplemented with 2.5 mg/L 2, 4-D. The size of Type-II callus was 0.90 cm. On medium containing 2.5 mg/L 2, 4-D, response of callus initiation was highest and the quality of callus derived from this medium was better than the callus obtained upon incubation under mediums

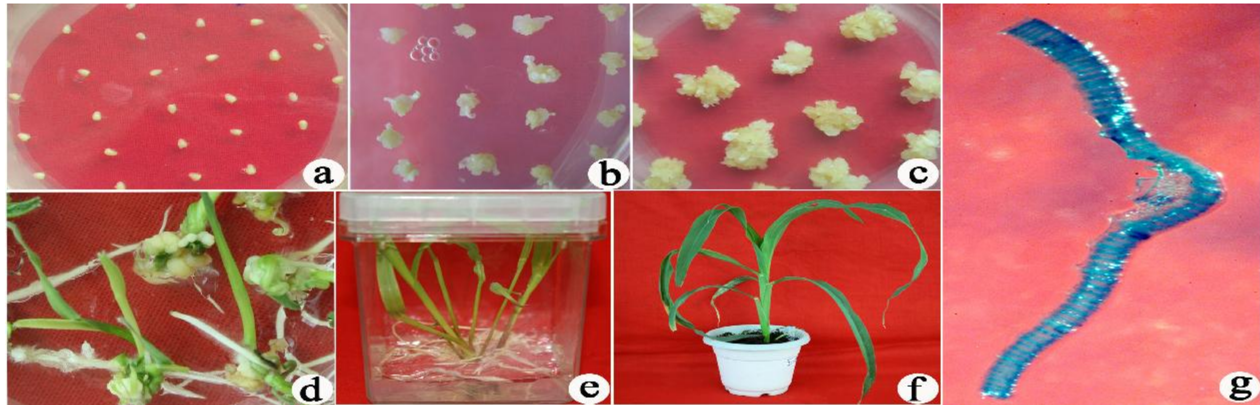

Figure 2. Callusing and regeneration of plantlets from immature embryos of Jiao 51 and histochemical analysis of GUS expression. a) Immature embryos dissected from 11-13 days ear after self-pollination; b) Callus initiation from immature embryos on N6 supplemented with 2.5 mg L-1 2, 4-D; c) Type-II embryogenic friable callus derived from Type-I callus; d) Differentiation of Type-II embryogenic callus; e) Plantlets with roots in rooting medium; f) The plant regenerated from immature embryos; g) GUS activity detected in leaf of a T0 (line-3).

containing other levels of the 2, 4-D concentrations. On the basis of the above, in terms of quality and quantity of organogenic callus, CIM (N6 supplemented with 2.5 mg/L 2, 4-D) was the most optimum medium. Type-II embryogenic friable callus were subcultured on proliferation medium (the same as CIM) to proliferate somatic embryos. Such callus remained embryogenic for ong time and could be used for transformation via *Agrobacterium*-mediated.

Generation of putative transgenic maize plant

Callus induced from immature embryos was infected with *Agrobacterium* containing the vector pCambia- *Mcchit1-PAT* (Figure 1), which contains chitinase gene, *PAT* gene and *GUS* gene. To get more efficient transformation, callus was transferred onto fresh CIM for 6-9 days proliferation, and then was infected with *A. tumefaciens*. Because the *GUS* gene contains an intron in this construct, blue staining was indicative of plant rather than *A. tumefaciens* expression of the transgene. So at the end of the co-culture period, histochemical GUS assays were carried out on callus to determine transformation efficiency after co-cultivation period. Result showed that callus was pre-cultured 6-9 days before infection on CIM, resulting in high rate of resistant callus (data not shown).

Transformed callus lines were selected based on their resistance to PPT. Resistant calli were placed on regeneration medium (RM) with 5% sucrose. The primary putative transformed shoots were formed after 2 weeks of culture (Figure 2d), and then placed onto PRM II to induce roots and elongate further (Figure 2e). When the plantlets were stronger enough, all were transplanted to soil (Figure 2f). Histochemical GUS assays was carried out on leaves from the surviving plant (Figure 2g). A total of 8 PPT-resistant transformants were obtained by the end of all of the experiments. All of transformants were grown to maturity in the greenhouse.grown to maturity in the greenhouse.

Transgenes integration and expression

PCR analysis was conducted using leaves from the nontransformed control and GUS positive maize plant. The expected 401 bp PCR product for the *Mcchit1* gene fragment was observed in total DNA extracted from 7 independent GUS-positive plantlets and in the plasmid pCambia- Mcchit1-PAT positive control, total DNA of the non-transformed control plant did not exhibit this 401 bp PCR product (Figure 3a). Also, expression analysis using RT-PCR suggested that the expected 401 bp *Mcchit1* gene transcripts was amplified in the reverse-transcription products of total RNA extracted from independent plantlets (Figure 3b).

Southern-blot analysis was carried out to assess stable integration of the *Mcchit1* transgenes in the T0 independent transgenic events. The results were presented in Figure 3c. All T0 plants except wild-type (Ctr-) plants showed one or two hybridization bands. Line 1, line 2, line 3, line 4 and line 7 had single cope of the *Mcchit1* gene. These results indicated that all gene cassettes were integrated into the genome of transgenic maize, and expressed successfully also.

In vitro screening for resistance against herbicide

Transgenic plants were resistant to PPT due to the presence of the *Pat* gene, but the wild-type was susceptible. The functional activity of PAT in all of the transformed plants was assessed by localized application of BASTA^{TM}.Leaf painting assay was conducted as

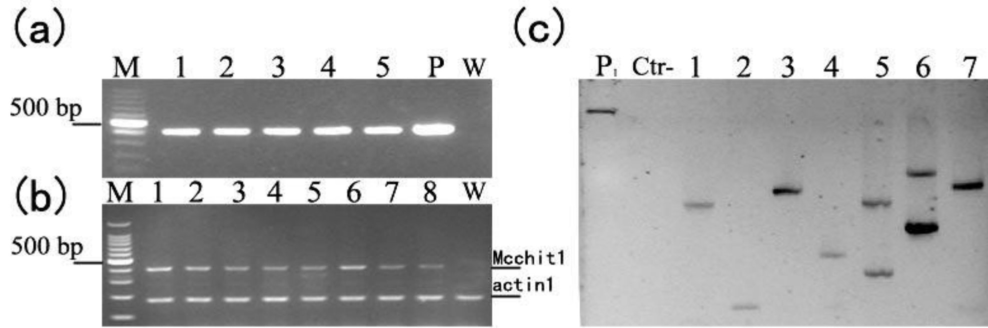

Figure 3. Molecular characterization of transgenes integration and expression in T0 transgenic maize plants. (a) PCR results that amplified the 400-bp fragments of the *Mcchit1* gene. M, DNA size marker (100 bp ladder); Lane1-5, independent line1-5; P, plasmid DNA; W, wild type. (b) RT-PCR analyses of *Mcchit1* transcript in leaves of transgenic plants. The transcript of the maize actin-1 gene was used as internal control. Lane1-8, T0 independent transgenic line1-8. (c) Southern-blot analysis of transgenic plants. Leaf tissue was sampled from 20 independent events in the T0 generation (1, 2, 3, 4, 5, 7 and 8); Total genomic DNA was digested with KpnI and hybridized with the *Mcchit1* probe. Ctr-, Negative control (genomic DNA from non-transformed plant); P_1, positive control (pCambia- Mcchit1-PAT plasmid DNA digested with Kpn I).

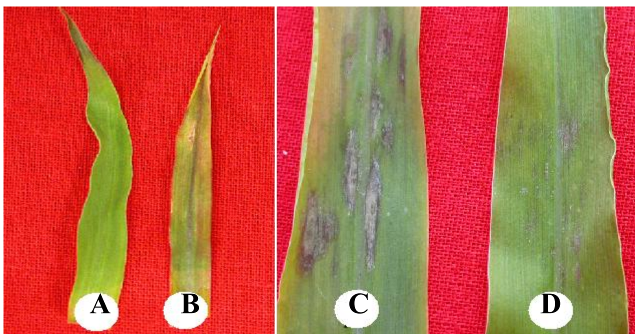

Figure 4. Analysis of resistance against herbicide and *E. turcicum* on leaves of transgenic maize. Leaves from transgenic plants (a) and non-transgenic plants (b) treated by 200 mg L^{-1} PPT; excised leaves of wild type (c) and transgenic lines (d) were inoculated with *E. turcicum* on day 5.

described in ‘Materials and Methods’. Within 6-9 days, leaves of wild-type seedling plants began to show injury symptoms and turned yellow, and apex of leaves produced necrotic lesions after BASTA^{TM} application. In comparison, transgenic seedlings continued to grow and remained green (Figures 4a and b).

Excised leaf challenge assay against *E. turcicum in vitro*

In order to evaluate the *in vivo* functionality of the Mcchit1 protein in the transgenic maize, an excised leaf challenge assay was developed. To ensure virulence of the *E. turcicum,* fungi strain used in this study were inoculated onto maize seedlings and isolated from lesions again. When excised leaves of transgenic lines and wild type were inoculated with the fungus strain, they exhibited different symptoms. Necrotic lesions of untransformed control plant leaves appeared on day 3 after inoculation with *E. turcicum.* In contrast, transgenic maize expressing the *Mcchit 1* gene was visible only on day 5 after inoculation. The number of lesions was significantly fewer, and the size of lesions was significantly smaller compared to the controls (Figures 4c and d).

DISCUSSION

Since the first report of regeneration from immature embryos of *Z. mays* published by Green and Phillips (1975), many explants from different genotypes of maize have been regenerated (Sujay et al., 2010; Ahmadabadi et al., 2007; Bronsema et al., 1997). Immature embryos have been shown to be most efficient in their regeneration procedure. It has been reported that 2, 4-D is an effective plant growth regulator (PGR) for callus induction from immature embryos of maize (Bronsema et al., 2001). Induction of callus from immature embryo was found to be dependent on the presence of 2, 4-D (Bronsema et al., 1997). Rakshit et al. (2010) reported that callus formation decreased as the level of 2, 4-D level increased and finally resulted in browning of calli at level 4.0 mg/L 2, 4-D. In present research, 89.1% of the Type-II callus induction was observed on mediums supplemented with 3.0 mg/L 2, 4-D. This may be due to mitotic activity in the cells of inbred line ‘Jiao 51’ that were sensitive to high level of 2, 4-D. According to Armstrong et al. (1985), maize embryogenic callus can be classified into two different types of embryogenic callus. Type-I callus is a kind of compact organized and slow-growing callus. Type-II callus is soft, friable and fast-growing one and characterized by its high regeneration capacity. Type-I calli were easily obtained from immature

Table 2. The frequency of callus induction on CIM medium supplemented with different 2, 4-D concentration.

Concentration of 2,4-D (mg/L)	No. of immature embryo	Frequency of type-I callus induction (%)	Frequency of type-II callus Induction (%)	The size of type-II callus (cm)
1.0	150	44.67 ±9.78 de	10.67 ±0.89 f	0.53 ±0.09 e
1.5	150	47.00 ±8.00 de	39.50 ±2.50 ef	0.65 ±0.05 d
2.0	150	62.67 ±5.78 bc	50.00 ±2.67 cd	0.80 ±0.03 b
2.5	150	91.33 ±6.22 a	89.10 ±4.89 a	0.90 ±0.67 ab
3.0	150	77.33 ±9.78 b	58.00 ±13.33 c	0.93 ±0.11 ab
3.5	150	58.33 ±2.22 d	50.00 ±2.67 cd	1.00 ±0.07 a

Means followed by the same letter are not significantly different according to SNK test at ($p < 0.05$).

embryos of inbred line ‘Jiao 51’ at low levels 2, 4-D, which could be changed to Type-II callus after two weeks subculture. Type-II callus obtained from immature embryos of inbred line ‘Jiao 51’ proliferated rapidly. When the embryogenic calli developed on CIM with 3.0 mg/L 2, 4-D were transferred to regeneration medium for plantlet formation, inbred line ‘Jiao 51’ yielded high frequency of shoot development without any PGR, but increasing concentration of sucrose up to 5%. PRM II medium without hormone led to higher rooting efficiency. Regenerated plants were successfully established into complete plants.

Chitinases are hydrolytic enzymes in response to fungal infection, which catalyzes the hydrolytic cleavage of the β-1, 4-glycoside bond present in the biopolymer of N-acetyl glucosamine (chitin) found in fungal cell walls (Sahai et al., 1993). *Mcchit1* gene used here belongs to the class I family and encodes the secretary endochintinase. Overexpression of *Mcchit1* gene increased resistance to fungal phytopathogens (*P. nicotiance* in *N. benthamiana* and *V. wilt* in cotton) (Xiao et al., 2007). Previous reports demonstrated that transgenic plants expressing chitinase gene of plant and fungal displayed disease reduction. Transgenic rice expressing a rice basic chitinase gene confers enhanced resistance to sheath blight (Kim et al., 2003). Cacao expressing a cacao class I chitinase gene enhances resistance against the pathogen *Colletotrichum gloeosporioides* (Maximova et al., 2006). Transgenic wheat expressing a barley class II chitinase gene has enhanced resistance against *Fusarium graminearum* (Shin et al., 2008). In present research, levels of chitinase expression in transgenic maize were likely sufficiently high enough to reduce fungal growth, resulting in reduced disease development. Excised leaf challenge assay exhibited that seven transgenic lines expressing *Mcchit1* gene had increased tolerance to the fungal pathogen *E. turcicum*.

In summary, regeneration system for maize inbred lines ‘Jiao 51’ was successfully developed using immature embryo explants. Thus, the established regeneration protocol could be used to produce novel transgenic maize plants by introduction of foreign genes conferring resistance to insect, fungal diseases, and other commercially important traits into inbred lines ‘Jiao51’. These transgenic inbred lines may be used for the construction of hybrid combinations for maize breeding.

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REFERENCES

Ahmadabadi M, Ruf S, Bock R (2007). A leaf-based regeneration and transformation system for maize (*Zea mays* L.). Transgenic Res., 16(4): 437-448.

Armstrong CL, Green CE (1985). Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta, 164: 207-214.

Binott JJ, Songa JM, Ininda J, Njagi EM, Machuka J (2008). Plant regeneration from immature embryos of Kenyan maize inbred lines and their respective single cross hybrids through somatic embryogenesis. Afri. J. Biotechnol., 7: 981-987.

Bronsema FBF, van Oostveen WJF, Lammeren AAM (2001). Influence of 2, 4-D, TIBA and 3, 5-D on the growth response of cultured maize embryos. Plant Cell, Tiss. Organ Cult., 65: 45-56.

Bronsema FBF, van Oostveen WJF, van Lammeren AAM (1997). Comparative analysis of callus formation and regeneration on cultured immature maize embryos of the inbred lines A188 and A632. Plant Cell, Tiss. Organ Cult., 50: 57-65.

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975). Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen source. Sci. Sin., 18: 659-668.

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(10): 1024-1034.

Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Pegg SE, Li B, Nettleton DS, Pei D, Wang K (2002). Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol., 129(1): 13-22.

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Jr, Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG (1990). Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell, 2(7): 603-618.

Green CE, Phillips RL (1975) Plant regeneration from tissue culture of maize. Crop Sci., 15: 417-421.

Huang XQ, Wei ZM (2004). High-frequency plant regeneration through callus initiation from mature embryos of maize (Zea Mays L.). Plant Cell Rep 22: 793-800.

Ishida Y, Hiei Y, Komari T (2007). Agrobacterium-mediated transformation of maize. Nat Protoc 2(7): 1614-1621.

Jean G, Michael D, Osamu H, Eugenio CU, Gregory P, Smith RH (1991). Transformation of *Zea mays* L. Using *Agrobacterium tumefaciens* and the shoot apex. Plant Physiol., 95: 426-434.

Jefferson RA (1989). The gus reporter gene system. Nature, 342(6251): 837-838.

Jefferson RA, Kavanagh TA, Bevan MW (1987). Gus fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., 6(13): 3901-3907.

Juliana BO, Marco AG, Isaias OG, Luis E (2005) New resistance gene in the Zea mays *Exserohilum turcicum* pathosystem. Genet Mol. Biol., 28: 435-439.

Kern MF, Maraschin SD, Vom Endt D, Schrank A, Vainstein MH, Pasquali G (2009) Expression of a chitinase gene from metarhizium anisopliae in tobacco plants confers resistance against rhizoctonia solani. Appl. Biochem. Biotechnol., 160(7): 1933-1946.

Kim JK, Jang IC, Wu R, Zuo WN, Boston RS, Lee YH, Ahn IP, Nahm BH (2003) Co-expression of a modified maize ribosome-inactivating protein and a rice basic chitinase gene in transgenic rice plants confers enhanced resistance to sheath blight. Transgenic Res., 12(4): 475-484.

Maximova SN, Marelli JP, Young A, Pishak S, Verica JA, Guiltinan MJ (2006). Over-expression of a cacao class I chitinase gene in *Theobroma Cacao* L. Enhances resistance against the pathogen, colletotrichum gloeosporioides. Planta, 224 (4): 740-749.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant, 15: 473-497.

Roby D, Broglie K, Cressman R, Biddle P, Chet IL, Broglie R (1990) Activation of a Bean Chitinase Promoter in Transgenic Tobacco Plants by Phytopathogenic Fungi. Plant Cell, 2(10): 999-1007.

Sahai AS, Manocha MS (1993). Chitinases of fungi and plants: Their involvement in morphogenesis and host-parasite interaction. FEMS Microbiol. Rev., 11: 317-338

Schlumbaum A, Mauch F, Vogeli UTB (1986). Plant chitinases are potent inhibitors of fungal growth. Nature, 324: 365-367.

Shin S, Mackintosh CA, Lewis J, Heinen SJ, Radmer L, Dill-Macky R, Baldridge GD, Zeyen RJ, Muehlbauer GJ (2008). Transgenic wheat expressing a barley class ii chitinase gene has enhanced resistance against fusarium graminearum. J. Exp. Bot., 59(9): 2371-2378.

Sidorov V, Gilbertson L, Addae P, Duncan D (2006). Agrobacterium-mediated transformation of seedling-derived maize callus. Plant Cell Rep., 25(4): 320-328.

Stirpe F, Barbieri L, Battelli MG, Soria M, Lappi DA (1992). Ribosome-inactivating proteins from plants: Present status and future prospects. Nat. Biotechnol., 10(4): 405-412.

Sujay R, Zerka R, Sekhar JC, Fatma T, Dass S (2010). Callus induction and whole plant regeneration in elite indian maize (*Zea mays* L.) inbreds Plant Cell, Tiss. Organ Cult., 100: 31-37.

Ting YC, Yu M, Zheng WZ (1981). Improved anther culture of maize (*Zea Mays*). Plant Sci. Lett., 23:139-145.

Toki S, Takamatsu S, Nojiri C, Ooba S, Anzai H, Iwata M, Christensen AH, Quail PH, Uchimiya H (1992) Expression of a maize ubiquitin gene promoter-bar chimeric gene in transgenic rice plants. Plant Physiol., 100(3): 1503-1507.

Van Loon LC (1997) Induced resistance in plants and the role of pathogenesis-related proteins. Eur. J. Plant Pathol., 103(9): 753-765.

Xiao YH, Li XB, Yang XY, Luo M, Hou L, Guo SH, Luo XY, Pei Y (2007) Cloning and characterization of a balsam pear class I chitinase gene (Mcchit1) and its ectopic expression enhances fungal resistance in transgenic plants. Biosci. Biotechnol. Biochem., 71(5): 1211-121.

Zhong H, Sun B, Warkentin D, Zhang S, Wu R, Wu T, Sticklen MB (1996). The competence of maize shoot meristems for integrative transformation and inherited expression of transgenes. Plant Physiol., 110(4): 1097-1107.