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Full Length Research Paper

Stable *Agrobacterium*-mediated transformation of the halophytic *Leymus chinensis* (Trin.)

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In this study, an efficient procedure for stable *Agrobacterium*-mediated transformation of *Leymus chinensis* (Trin.) was established. *Agrobacterium tumefaciens* strain EHA105, harboring a binary vector pCAMBIA2300, was used for transformation, along with a sweet potato 2-cysteine peroxiredoxin (*2-Cys Prx*) gene under the control of the stress-inducible sweet potato anionic peroxidase 2 (*SWPA2*) promoter and the neomycin phosphotransferase (*nptII*) gene under the control of the cauliflower mosaic virus (CaMV) 35 S promoter. We found that a one-month-old callus derived from mature seeds could be efficiently transformed. Seven-day preculture followed by inoculation with the addition of 100 μmolL^{-1} acetosyringone (AS) and then a 3 day co-cultivation were performed before selection. Selection of transgenic shoots was done in the presence of 150 mgL^{-1} kanamycin (KM). An optical density at a wavelength of 600 nm (OD_{600}) of approximately 0.4 for *A. tumefaciens* infection solution and 20 min of infection time gave the highest transformation efficiency. Polymerase chain reaction (PCR) analysis of KM-resistant plants and newly regenerated rhizomes revealed stable transformation of the *2-Cys Prx* gene and the *nptII* gene, with the highest transformation frequency of 4.93%. RT-RCR analysis was conducted using salt stressed transgenic plants, and the results suggested that *2-Cys Prx* had low transcription levels under non-stressed conditions, and increased transcription after 6 h of 200 mM NaCl stress. This gene continued to demonstrate high levels of transcription until 6 h after withdrawal of stress, with a slow recovery. The method reported herein provides a direct opportunity for improvement of the quality traits of *L. chinensis* via genetic transformation.

Key words: *Leymus chinensis*, *Agrobacterium*-mediated transformation, *2-Cys* peroxiredoxin, gene transformation.

INTRODUCTION

The halophyte *Leymus chinensis* (Trin.), a perennial rhizome grass classified in the family Gramineae, is widely distributed throughout northern China, Mongolia and Siberia (Huang et al., 2004). Because of its strong rhizomes and intrinsic tolerance of high alkaline-sodic soil conditions (Chen, 1988), *L. chinensis* is used as a soil-binding plant to protect soil from desertification. Due to its high vegetative productivity, protein content, and good palatability for cattle, this species is also used as an important forage crop to meet grazing needs (Shu et al., 2005). However, climate changes, overgrazing and reclamation of grassland have resulted in severe deterioration of the grassland ecosystem, which significantly affects human life and is responsible for ecological problems. Thus, engineering a cultivated species of this grass with improved traits that include strong stress tolerance is urgently demanded.

Peroxiredoxins (Prxs) are a group of ubiquitous peroxidase enzymes in which redox-active cysteine residues

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participate in the reduction of hydrogen peroxide (Chae et al., 1994). Prxs were originally divided into two categories, *1-Cys* and 2-*Cys Prxs*, based on the number of cysteine residues (Chae et al., 1994). Due to different protein structures and catalysis mechanisms, *2-Cys Prxs* were further divided into typical and atypical *2-Cys Prxs*. Of these, the typical *2-Cys Prxs* are the largest class of Prxs and are identified by the conservation of their two redox-active cysteines, the peroxidatic cysteine (generally near residue 50) and the resolving cysteine (near residue 170, Hofmann et al., 2002). Typical *2-Cys Prxs* that can be oxidized directly by hydrogen peroxide have been found to be protective against the toxic effects of hydrogen peroxide (Wen and Van Etten, 1997; Veal et al., 2004; Jang et al., 2004; Moon et al., 2005). Other genetic studies have revealed that typical *2-Cys Prxs* play important roles in protecting against deoxyribonucleic acid (DNA) damage, oxidative stress and cancer (Lee et al., 2003; Neumann et al., 2003).

In addition, the sweet potato *2-Cys Prx* has been transformed into tobacco plants and cultured cells (Kim et al., 2003), and accumulating evidences suggest that the foreign gene enhances efficiently the development of stress-tolerant transgenic plants, especially driven under the control of a strong promoter. *2-Cys Prx* also has been transformed into sweet potato by *Agrobacterium*-mediated transformation, and transgenic plants that over-express of *2-Cys Prx* have shown an enhanced tolerance to oxidative stress (Kim et al., 2009). The use of transgenic techniques has become a common and convenient method for improving the traits of plants (Hiei et al., 1997; Akutsu et al., 2004). For *L. chinensis*, two reports of successful transformation and plant regeneration have been published. Shu et al. (2005) transformed the phosphinothricin acetyltransferase (PAT) gene using microprojectile bombardment transformation and successfully obtained transgenic plants. Later, Wang et al. (2009) transformed the wheat late embryogenesis abundant (LEA) gene using *Agrobacterium*-mediated transformation and obtained transgenic plants with high drought stress tolerance. For some other graminaceous plants such as rice (Dong et al., 1996; Hiei et al., 1997), wheat (Cheng et al., 1997), and barley (Tingay et al., 1997), *Agrobacterium*-mediated transformation is well understood.

However, studies on factors that affect *Agrobacterium*-mediated transformation in graminaceous plants are relatively few, especially in *L. chinensis*. Thus, a reliable protocol for efficient transformation and plant regeneration in graminaceous plants needs to be developed and improved. The requisite for efficient expression of foreign genes in plants is a powerful expression system with an appropriate promoter (Ryu et al., 2009). In this study, a strong oxidative stress-inducible *SWPA2* promoter that controls precisely the expression of target defense genes was used. Several evidences suggest the use of *SWPA2* promoter to conditionally induce the expression of antioxidant genes makes it possible to develop transgenic plants with an increased tolerance to multiple stresses (Lee et al., 2007; Lim et al., 2007; Tang et al., 2007). Here, we report a simple protocol for stable *Agrobacterium*-mediated transformation of a Type 1 embryogenic callus from mature seeds of China wild-type *L. chinensis* plants, LcWT07. We tested explant age, *A. tumefaciens* infection solution concentration and infection time and successfully obtained KM-resistant plants with a positive transgene insertion. The results of this investigation provide rapid and direct opportunities for improvement of the quality traits of *L. chinensis* via genetic transformation.

MATERIALS AND METHODS

Mature seeds of China wild-type *L. chinensis* plants, LcWT07 were collected at natural grassland in Siping, Jilin province, China, and were stored at 4°C. All seeds were de-husked and surface-sterilized with 70% ethanol for 1 min and then with 5% sodium hypochlorite for 20 min. The sterilized seeds were rinsed 5 times with sterile water and were then placed on callus induction medium.

Bacterial strains and plasmids

The *A. tumefaciens* strain EHA105 used in this study, harbors the plasmid pCAMBIA2300, which contains the *2-Cys Prx* and neomycin phosphotransferase (*nptII*) genes under the control of the stress-inducible sweet potato anionic peroxidase 2 (*SWPA2*) promoter (accession number: AF4532791, Kim et al., 2003) and the cauliflower mosaic virus (CaMV) 35S promoter, respectively (Figure 1).

Agrobacterium-mediated transformation

The *A. tumefaciens* strain EHA105 with the binary vector pCAMBIA2300 was grown in YEP liquid medium (20 gL^{-1} bacto-peptone, 10 gL^{-1} yeast extract) supplemented with 150 mg l^{-1} kanamycin (KM). The culture was grown at 28°C for 24 h with continuous shaking at 225 rpm until an optical density at a wavelength of 600 nm (OD_{600}) of 0.4 to 1.0 was reached. The solution was centrifuged at 5000 rpm for 10 min. The pellet was resuspended in the same volume of MMA medium containing Murashige and Skoog (MS) basal salt (Murashige and Skoog, 1962) and MES 10 mol l^{-1} pH 5.6 with the addition of 100 µmol l^{-1} acetosyringone (AS) and 20 g l^{-1} sucrose. A Type 1 embryogenic callus was induced through the efficient tissue culture and plant regeneration system described in our previous study (Sun and Hong, 2010), and the one-month-old Type 1 embryogenic callus was prepared for transformation. With a preculture on callus induction medium containing MS basic salt, 2.0 mgL^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 5.0 mgL^{-1} L-glutamic acid for 7 days, the embryogenic callus was immersed in an *A. tumefaciens* EHA105/pCAMBIA2300 suspension for various infection times (60, 30, 25, 20, 15 and 10 min). After the incubation the infected callus was drained on filter paper for removal of excess bacteria and co-cultivated on callus induction medium with the

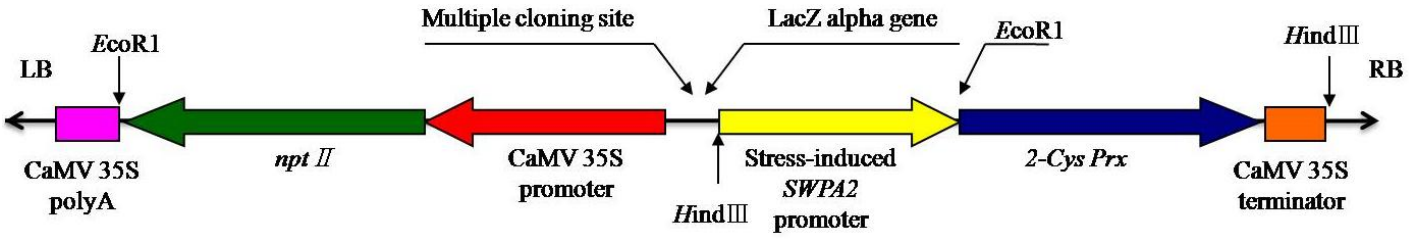

Figure 1. Structure of T-DNA regions in plant expression vector pCAMBIA2300-Prx. The sweet potato *2-Cys Prx* gene was inserted between the stress-inducible *SWPA2* promoter and the CaMV 35S terminator. The *nptII* gene was inserted between the CaMV 35S promoter and the CaMV 35S terminator. RB. Right border; LB. Left border; *nptII*. Neomycin phosphotransferasegene; *2-Cys Prx*. Typical 2-Cysteine Proxiredoxins.

addition of 100 µmol l^{-1} AS for an additional 3 days at 28°C in the dark. After co-cultivation, transgenic calli were washed 5 times with sterile water containing 250 mgL^{-1} cefotaxime (CE) and drained on filter paper for removal of excess water. The infected callus was then transferred onto callus induction medium with selection using 250 mg l^{-1} CE and 150 mg l^{-1} KM for 20 days of culture at 28°C in the dark.

The regeneration of KM-resistant shoots was carried out on plant regeneration medium with MS basic salt, 2.0 mgL^{-1} kinetin, 0.2 mgL^{-1} α-naphthalene acetic acid (NAA), and 2.0 gL^{-1} casamino acid with selection using 250 mgL^{-1} CE and 50 mgL^{-1} KM. The cultures were incubated under low light conditions (25 µE m^{-2} s^{-1}) with a 16/8 h (light/dark) photoperiod for the first week, and then maintained under high light conditions (70 µE m^{-2} s^{-1}) with the same photoperiod. Rooting of regenerated KM-resistant shoots was carried out on rooting medium with half-strength MS basic salt and selection using 50 mg l^{-1} KM, at 28°C under high light conditions with a 16/8 h (light/dark) photoperiod. Well-rooted KM-resistant plants were removed from the culture medium, rinsed with sterile water to remove the media, and then transplanted into pots with a mixture of sterilized soil and vermiculite (3:1) under greenhouse conditions (25°C, 60% humidity). All solid culture media were supplemented with 30.0 gL^{-1} sucrose and 4.0 gL^{-1} gelrite for solidification, with the pH adjusted to 5.8 prior to the addition of gelrite. After a one-month culture in the aforementioned greenhouse conditions, transformants with multiple regenerated rhizomes were split into many independent plants from the different rhizomes, to test the stability of the gene transformation. These independent plants from the transgenic plants were grown in pots with a mixture of sterilized soil and vermiculite (3:1) under the same conditions.

Salt stress treatment

One-month-old wild-type and transgenic plants cultured in the greenhouse conditions described were subjected to 200 mM NaCl until the final pH value of the outflow became stable. Sampling was carried out 6 h after the initiation of treatment. For the withdrawal of NaCl stress, stressed plants were immediately washed with Hoagland nutrient solution (Hoagland and Amon, 1950) until the final pH value of the outflow became stable. Sampling was conducted after 6 and 12 h of withdrawal from NaCl stress. Each experiment was performed three times.

DNA extraction and gene insertion assay

Genomic DNAs were extracted from fresh leaf tissues of wild-type and transgenic plants using the sodium dodecyl sulfate (SDS) method (Dellaporta et al., 1983). Polymerase chain reaction (PCR) analysis for the selection gene (*nptII*) was carried out following the method above: 0.5 µmol l^{-1} of each primer, 10 ng DNA template, 200 µmolL^{-1} each of dATP, dCTP, dGTP and dTTP mix, 1.0 units of Taq DNA polymerase (Promega, USA) and the corresponding buffer in a total volume of 20 µl. The PCR reaction was carried out as follows: 95°C for 5 min followed by 35 cycles of incubation at 95°Cfor 10 sec, 50°Cfor 30 sec, and 72°C for 30 sec, with final extension at 72°C for 7 min. The primers for amplification of *nptII* gene were designed according to the synthetic plasmid pK18 kanamycin resistance gene (accession number: M17626), named as npt2-f1, 5′-GAGGCTATTCGGCTATGACTG-3′ and npt2-r1, 5′-ATCGGGAGCGGCGATACCGTA-3′. To detect the presence of the transgene (*2-Cys Prx*, accession number: AF4532791), a gene-specific primer set was designed with the following sequences: prx-f1, 5'-TCTAGAATGGCGTCTGTTGCT-3' and prx-r1, 5'-GAGCTCCTAAATAGCTGAGAA-3', using the same PCR program used above. PCR reactions were performed using the ASTEC PC808 PCR detection system (ASTEC, PC808, Janpan). PCR products were analyzed by 1% agarose gel electrophoresis. Photos were taken and analyzed using the MultiDoc-It Digital imaging system (UVP, Cambridge, UK).

RNA extraction and RT-PCR

Total ribonucleic acids (RNAs) were isolated from fresh leaf tissues of various treated wild-type and transgenic plants using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The NanoPhotometer (IMPLEN, UK) was used to determine RNA concentration and quality. The quality of RNA was also assessed by agarose gel electrophoresis. The complementary deoxyribonucleic acid (cDNA) sequence of *β-actin* gene in the wild-type Chinese *L. chinensis* plants was cloned by RT-PCR and deposited in the NCBI database with the accession number HM623326. As a control, *Lcactin* was amplified with the following primers: β-actin-f1: 5'-TGGACTCTGGTGATGGTGTC-3', and β-actin-r1: 5'-CCTCCAATCCAAACACTGTA-3'. The *2-Cys Prx* gene (accession number: AF4532791) was amplified using the following primers: prx-f1, 5'-TCTAGAATGGCGTCTGTTGCT-3' and prx-r1, 5'-GAGCTCCTAAATAGCTGAGAA-3'. Each PCR reaction contained 0.5 µmolL^{-1} of each primer and 2 µg RNA template in a total volume of 20 µl using the Maxime RT PreMix Kit (Oligo dT Primer, iNtRON BIOTECHNOLOGY, Korea), according to the manufacturer's instructions. PCR amplification was performed with an initial denaturation at 95°C for 5 min followed by 35 cycles of incubation at 95°C for 10 sec, 50°C for 30 s, and 72°C for 30 s, with

Table 1. Growth rate of non-transgenic callus on callus induction medium as the culture period increased.

Culture period		0 (day)	1 (day)	3 (days)	5 (days)	7 (days)	10 (days)	15 (days)
Fresh weight of callus (mg)	1[a]	329.85	242.25	249.52	274.47	332.67	358.67	387.37
	2	300.95	302.15	308.20	332.85	404.42	444.62	480.46
	3	284.15	248.72	290.98	314.26	370.82	411.62	452.78
Daily growth rate (%)[b]	1	0.00	1.00	3.00	1.00	21.21	7.82	8.00
	2	0.00	0.40	2.00	8.00	21.50	10.00	8.00
	3	0.00	0.20	2.20	8.00	18.00	11.00	10.00
Growth rate mean (±SE, %)[c]		0.00 ± 0.00	0.53 ± 0.42[c]	2.40 ± 0.53[bc]	8.67 ± 1.15[bc]	20.24 ± 1.94[a]	9.61 ± 1.63[bc]	8.67 ± 1.15[bc]

[a]The numbers mean three independent experiments. [b]daily grow rate (%) means the increased amounts of fresh weight daily, calculated by the ratio of the increased callus fresh weight over certain culture period to the callus fresh weight before the culture period. Take the growth rate after culture period 3 days as example, growth rate = (fresh weight of callus at 3 days - fresh weight of callus at 1 day)/(fresh weight of callus at 1 day × culture period 2 days). [c]growth rate mean (%) is the average of the growth rates of three independent experiments. Means followed by the same letter in the same series are not significantly different with $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test.

a final extension at 72°C for 7 min. PCR reactions were performed using the ASTEC PC808 PCR detection system (ASTEC, PC808, Japan). PCR products were analyzed by 1% agarose gel electrophoresis. Photos were taken and analyzed using the MultiDoc-It Digital imaging system (UVP, Cambridge, UK).

Statistical analysis

Three identical, independent experiments were performed for each experiment. In each replicate, 85 to 100 mature seeds, or calli were used. The data presented are the average of three experiments. Statistical analysis was performed by two-way ANOVA using Duncan's multiple-range test with $P < 0.05$ (Duncan, 1955).

RESULTS

Preculture period of the callus

Undergoing a preculture before *A. tumefaciens* infection was equivalent to subculturing on callus induction medium in terms of transformation efficiency, as both improved the growth status of explants and allowed for maintenance of an optimal callus type. We first tested the relationship between the growth rate of the callus and the culture period on the callus induction medium. Our findings showed that the daily growth rates of the callus were very low at the beginning of culture, and then growth began to increase after 3 days of culture (Table 1). After a 7 days culture, the calli demonstrated a significant increase in daily growth rates, with maximal growth observed after 15 days of experimental culture period in this study. Later, the daily growth rates began to decline and remained at levels comparable to those of the fifth day. With a high growth rate, these calli were considered to possess active transgene integration and to be good potential targets for transformation. Thus, the duration of preculture was set at 7 days to enhance the transformation efficiency in the present experiment.

A. tumefaciens concentration and infection time

To test the effects of *A. tumefaciens* concentration as evaluated by optical density 600 (OD_{600}) and infection time, two groups of experiments were performed using various infection times, with *A. tumefaciens* of both high OD_{600} 0.8 to 1.0 and low OD_{600} 0.3 to 0.4. Under high OD_{600} infection solution, the survival probability after the first selection in callus induction medium was significantly influenced by the infection time (60, 30, 25, 20, 15 and 10 min, Table 2). The survival probability increased as the infection time decreased, and the calli exposed to a 60 min infection had the lowest survival probability, significantly lower than that after 20 min or after less than 20 min. Next, the growth rates of transgenic calli after various infection periods were compared in parallel in 3-fold increments. In these experiments, only 2 transgenic plants were obtained after the 20 min infection time. In addition, a relatively high frequency of albino transformation was obtained from the 60 min infection time. Using the low OD_{600} infection solution, shorter infection times (20, 15 and 10 min) were used for optimization of the transformation condition (Table 3). Relatively high survival probabilities after the first selection were obtained at all infection times, as compared to the equivalent infection times using high bacterial concentrations. The same results were found for the growth rates of the callus. The highest transformation frequency was obtained with 15 min of infection time, and

Table 2. Effect of infection time on callus growth rate and transformation frequency according to various infection times (60, 30, 25, 20, 15 and 10 min) using 0.8 to1.0 OD_{600} of *Agrobacterium* infection solution.

Infection time (min)	Survival probability after the first selection (%)[a]	Growth rate of the still lived callus after the first selection (100%)[b]	Transformation frequency (%) (number of transgenic plants)[c]	Albino transformation frequency (%) (number of albino transgenic plants)[d]
60	9.80 ± 1.2[c]	2.85 ± 0.6[a]	0.00 (0)	14.04 (4)
30	16.49 ± 0.8[bc]	2.97 ± 0.8[a]	0.00 (0)	2.10 (1)
25	26.92 ± 2.4[abc]	2.81 ± 0.5[a]	0.00 (0)	0.00 (0)
20	34.09 ± 1.8[ab]	3.02 ± 1.0[a]	2.21 (2)	0.00 (0)
15	37.89 ± 1.3[a]	2.98 ± 0.7[a]	0.00 (0)	0.93 (1)
10	32.65 ± 1.5[ab]	3.12 ± 0.8[a]	0.00 (0)	0.00 (0)

[a] Survival probability after the first selection is calculated by the ratio of the number of still lived callus after the first selection to all number of calli incubated on callus induction medium with selection. [b]growth rate of the still lived callus after the first selection is calculated by the increase frequency of the fresh weight when cultured on plant regeneration with selection. [c]transformation frequency is calculated by the ratio of the number of calli which regenerated KM-resistant shoots to all number of calli which were cultured on plant regeneration medium with selection. [d]albino transformation frequency is calculated by the ratio of the number of calli which regeneration albino plants to all number of calli which were cultured on plant regeneration medium with selection. Values are the mean of three repeated experiments. Means followed by the same letter in the same series are not significantly different with $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test.

Table 3. Effect of infection time on callus growth rate and transformation frequency according to various infection times (20, 15 and 10 min) using 0.3 to 0.4 OD_{600} of *Agrobacterium* infection solution.

Infection time (min)	Survival probability after the first selection (%)[a]	Growth rate of the still lived callus after the first selection (100%)[b]	Transformation frequency (%) (number of transgenic plants)[c]	Albino transformation frequency (%) (number of albino transgenic plants)[d]
20	51.67 ± 1.61[a]	3.15 ± 0.6[a]	1.02 (2)	0.00 (0)
15	51.58 ± 1.92[a]	3.52 ± 1.0[a]	4.93 (17)	0.00 (0)
10	53.33 ± 1.33[a]	3.20 ± 1.2[a]	0.78 (2)	0.00 (0)

[a] Survival probability after the first selection is calculated by the ratio of the number of still lived callus after the first selection to all number of calli incubated on callus induction medium with selection. [b]growth rate of the still lived callus after the first selection is calculated by the increase frequency of the fresh weight when cultured on plant regeneration with selection. [c]transformation frequency is calculated by the ratio of the number of calli which regenerated KM-resistant shoots to all number of calli which were cultured on plant regeneration medium with selection. [d]albino transformation frequency is calculated by the ratio of the number of calli which regeneration albino plants to all number of calli which were cultured on plant regeneration medium with selection. Values are the mean of three repeated experiments. Means followed by the same letter in the same series are not significantly different with $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test.

this system successfully produced 17 independent transgenic plant lines. In addition, 2 independent transgenic plant lines were obtained from 20 and 10 min of infection time, respectively. No albino transgenic plants were regenerated under any of the transformation conditions.

Regeneration and propagation of transgenic plants

KM-resistant shoots were regenerated following the process shown in Figure 2, taken the most efficient infection system as example. Type 1 embryogenic callus was infected with OD_{600} 0.3 to 0.4 of *A. tumefaciens* solution for 20 min, co-cultured with *A. tumefaciens* in the dark for 3 days and then transferred onto selection culture medium (Figure 2A). At the early stage of plant regeneration, some calli showed purple spots or shoots, and a few of them had green spots or shoots (Figures 2B and C). As they grew, purple shoots began to turn green (Figure 2D). Some embryogenic calli directly regenerated green shoots (Figure 2E). Most transgenic shoots were formed within 2 months after being transferred onto plant regeneration medium with selection. Until the regenerated shoots reached 3 cm in length, rooting was induced by transferring the shoots onto rooting medium containing 50 mgL^{-1} KM (Figures 2F and G). Nearly 100% of the transgenic shoots produced roots well after 10 days. Well-rooted kanamycin-resistant plants were transferred into pots containing a mixture of sterilized soil and vermiculite (3:1) under greenhouse conditions (Figures 2H and I). As a perennial multiple rhizome grass

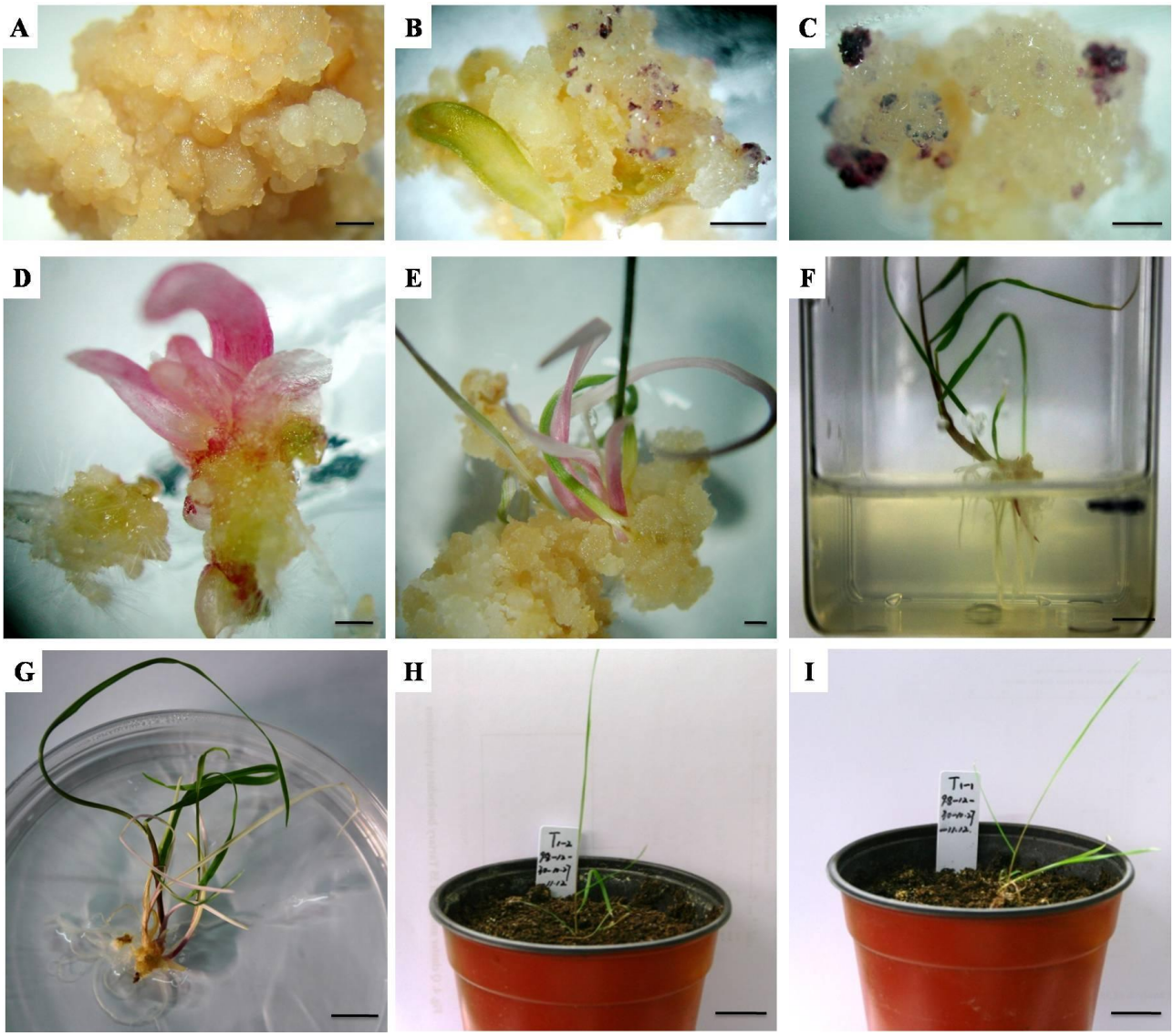

Figure 2. Transgenic plant regeneration mediated with *A. tumerfaciens* in *L. chinensis*. A. KM-resistant callus induction on selection culture medium; B, C. The early stage of KM-resistant callus with purple spots or shoots on plant regeneration medium with selection; D, E. The late stage of KM-resistant callus with shoots on plant regeneration medium with selection; F, G. Rooting of the KM-resistant shoots in high medium pot and Petridish; H, I. Transgenic plants grown in pots under greenhouse conditions. *Scale bar*: 1 mm (A, B, C, D, E); 10 mm (F, G); 20 mm (H, I).

with abundant horizontally creeping rhizomes (Figure 3A) and high vegetative propagation (Figure 3B), multiple rhizomes regenerated from regenerated shoots even on rooting medium (Figure 3C). After one month of culture in the greenhouse, more multiple rhizomes regenerated (Figure 3D). To allow for propagation, transformants were split into many independent plants from separate rhizomes and were grown in a mixture of sterilized soil and vermiculite (3:1) under greenhouse conditions (Figure 3E).

Molecular analysis of the transgenic plants

Fresh leaf tissues from independent KM-resistant clones were used for PCR analysis using primers designed to specifically amplify the *nptII* gene and an internal 800-bp fragment of the *2-Cys Prx* gene. Three transgenic plants were analyzed as model plants in this work, and the results showed clear bands for the selection gene (*nptII*) and the gene of interests (*2-Cys Prx*) (Figure 4). To test the stability of the gene transformation, fresh leaf tissues

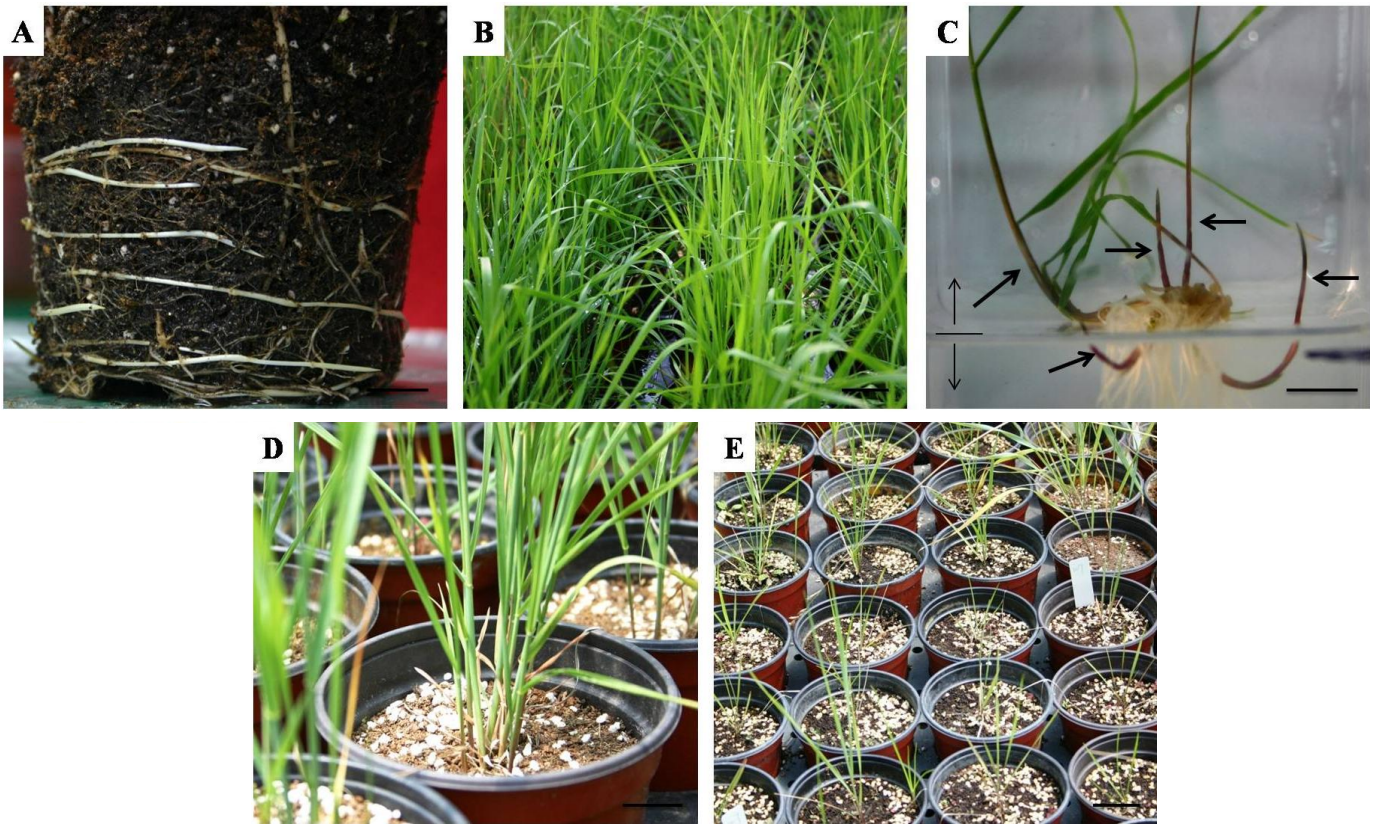

Figure 3. Natural characteristics of *L. chinensis* and the propagation of transgenic plants. A. Abundant horizontally creeping rhizomes; B. High vegetative propagation; C. The transgenic plant grown on rooting medium with MS basic salt and 50 mg l^{-1} KM in high tissue culture pot. The line and two arrows denote the interface of culture medium. Other five arrows shown in this picture denote independent rhizomes which regenerate from the shoot apical meristem (SAM) of the rhizomes. The biggest plantlet is the main one from a separate rhizome; D. The transgenic plants grown in pots under a greenhouse condition for one month, regenerated multiple rhizomes; E. The transgenic plants were divided into many independent plants based on separate rhizomes, and the independent plants were grown in pots under the greenhouse conditions. *Scale bar*: 20 mm (A, C); 10 mm (D); 60 mm (B, E).

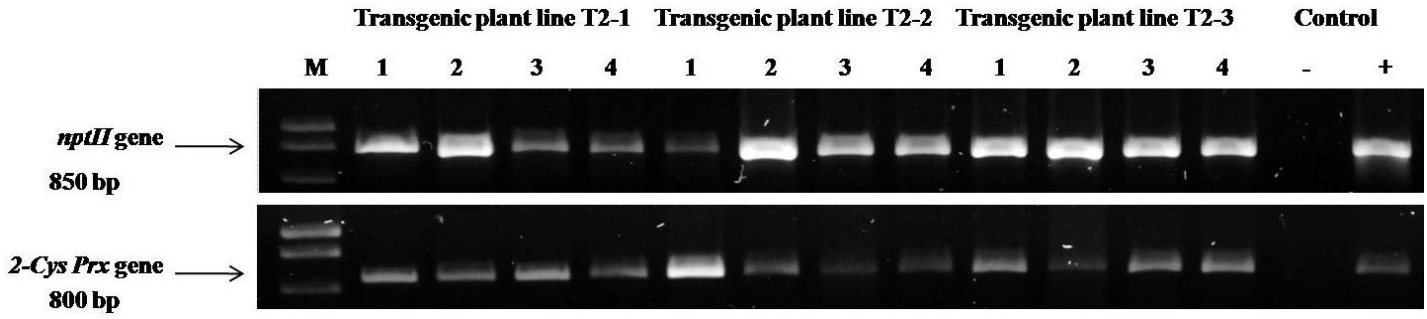

Figure 4. Molecular analysis of transgenic plants (T2-1, T2-2 and T2-3 line). PCR analysis of the selection gene, *nptII* gene and the gene of interests, *2-Cys Prx* gene. M. DNA ladder (1 Kb Plus DNA Ladder, Invitrogen, U.S.A.). + and -. A positive control from a plasmid containing T-DNA and a negative control from non-transgenic lines, respectively.

from independent plants were also used for PCR analysis using the above method. Clear bands of the correct sizes for the selection gene and the gene of interests were also amplified from all independent plants (Figure 4).

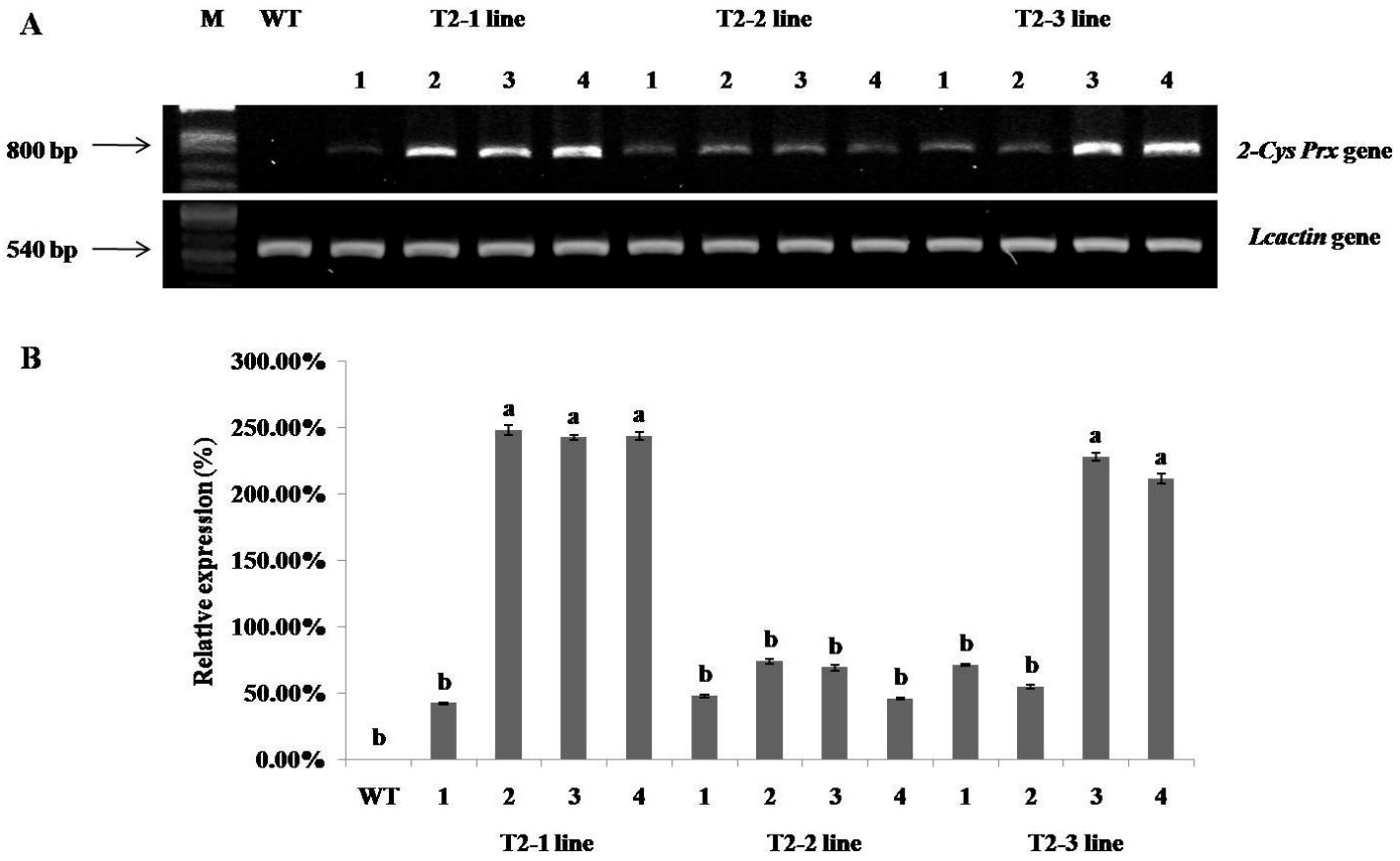

Figure 5. RT-PCR analysis (A) and relative gene expression (%, B) of *2-Cys Prx* in transgenic plants (T2-1, T2-2 and T2-3 line) under 6 h salt stress (200 mM NaCl) or the withdrawal of salt stress. Number 1 to 4 means non-stressed conditions, 6 h salt stress treatment with 200 mM NaCl, 6 h withdrawal of salt stress, and 12 h withdrawal of salt stress, respectively. The *Lcactin* gene is shown as control. Values are the mean of three repeated experiments. Means followed by the same letter in the same series are not significantly different with $P < 0.05$ according to two-way ANOVA using Duncan's multiple-range test.

2-Cys Prx gene expression in transgenic plants

To determine the transcription profile of the foreign gene and the expression responses to salt stress, we performed real time-polymerase chain reaction (RT-PCR) using RNAs extracted from fresh leaf tissues of various treated transgenic plants. The results revealed that *2-Cys Prx* could be expressed not only under non-stressed conditions but also under 6 h 200 mM NaCl stress (Figure 5). Under non-stressed conditions, only 42.36 to 71.13% of *2-Cys Prx* transcription was found in three selected transgenic lines, compared with the transcription levels of a control gene (*Lcactin*, Figures 5A and B). Salt stress treatment with 200 mM NaCl for 6 h could activate *2-Cys Prx* transcription in the T2-1 line but not the T2-2 or the T2-3 line. The responses of *2-Cys Prx* transcription to stress withdrawal were also line-specific: for the T2-1 line, the withdrawal of salt stress for even 12 h could not decrease the enhanced gene expression, whereas, due to the lack of transcriptional changes in the salt stressed T2-2 line, the withdrawal of salt stress did not cause any changes. Interestingly, no increase in gene expression was detected in the T2-3 line after 6 h of salt stress, but a significant increase occurred with stress withdrawal.

DISCUSSION

Here, we report a study detailing routine *Agrobacterium*-mediated transformation in the halophytic *L. chinensis*, China wild-type LcWT07. Until now, only one successful *Agrobacterium*-mediated transformation, with the *TaLea*$_3$ gene, has been reported in the Nongmu No. 1 variety (Wang et al., 2009). Thus, the further understanding of the critical factors that affect transformation is ungently needed. We followed the convincing demonstrations of *Agrobacterium*-mediated transformation in other plant species, such as rice (Hiei et al., 1994; 1997), barley

(Tingay et al., 1997), wheat (Cheng et al., 1997) and tall fescue (Bettany et al., 2003) and developed a reproducible protocol for transformation in this grass. This protocol was subsequently used to produce over 30 independent transgenic plants. Initially transformation in the callus was essential to establish the best tissue culture system to supply callus sources. A Type 1 embryogenic callus derived from the mature seeds of LcWT07, which has a high growth rate and a high plant regeneration potential, was used as the source of transformation.

Previous reports have shown that the incorporation of foreign DNA into a host genome, especially into the monocotyledonous plants, is related to cell division (An, 1985; Kudirka et al., 1986; Binns and Thomashow, 1988). Based on this knowledge, a preculture before infection with *Agrobacterium* solution is required, and the timing of the preculture period becomes an important factor in the success of the process. Our studies showed that the daily growth rate of calli increased slightly during the first week of culture, reached a maximum at 7 days after culture, and maintained that growth rate value until the next subculture. The slowly staggering growth rate during the beginning of 7 days suggested that explants callus was at the adaption process to surrounding nutrient conditions. The gradually degressive growth rate after 7 days culture was caused by the surrounding nutrient exhausting. Thus, the preculture period was regulated to 7 days after culture in this work, at which point the plant was determined to have the highest daily growth rate and cell division. Monocotyledonous plants, in particular cereal plants, were originally outside the host range of *A. tumefaciens*, but evidence has showed that some plant species may be susceptible to infection by *A. tumefaciens* (De Cleene and De Ley, 1976). Thus, the wounding for the entry of bacteria, the application of AS, and the integration of foreign DNA made the infected callus weaker, which resulted in the requirement for an appropriate infection time and an optimal infection solution concentration. Our results showed that less than or equal to 20 min of infection time with an OD_{600} value of 0.3 to 0.4 enhanced the survival probability after the first selection and optimized the transformation frequency.

In our experiments, the use of KM as a selective agent was effective for the selection of transformed calli and transformed shoots, although Sharma and Anjaiah (2000) have reported that selection on KM cannot eliminate the non-transformed cells efficiently. The T-DNA integration event occurred in all KM-resistant plants and in the subsequent vegetative propagation. As demonstrated by PCR analysis, the selection *nptII* gene and the *2-Cys Prx* gene remained stable in its clonal progeny plants. This result indicated that this transformation system, which could successfully produce transformants, was stable in terms of the transgene integration and vertical transmission. However, the leaves of newly regenerated shoots were usually purple and eventually turned green. This progression is the same as that of the coleoptile of new shoots regenerated from the rhizomes of a mature plant, which are also purple. The aim of this work was to establish a protocol for the routine transformation of the China wild-type *L. chinensis* plants, LcWT07. Our results show that it is possible to achieve this goal using a one-month-old Type 1 embryogenic callus. This study provides an efficient method to introduce genes of interests. In addition, the development of an *Agrobacterium*-mediated transformation system paves way for the introduction of stress-related genes into this grass to improve its traits. Detailed characterizations of the transgene expression and the physiological traits of transgenic plants currently needs further study.

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Abbreviations: ***2-Cys Prx*****,** 2-Cysteine peroxiredoxin; ***nptII*****,** neomycin phosphotransferase; **MS,** Murashige and Skoog; **AS,** acetosyringone; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **CE:** cefotaxime; **KM,** kanamycin; **NAA,** naphthalene acetic acid; **PAT,** phosphinothricin acetyltransferase; **LEA,** late embryogenesis abundant; **CaMV,** cauliflower mosaic virus; **OD_{600},** optical density at a wavelength of 600 nm; **SDS,** sodium dodecyl sulfate; **PCR,** polymerase chain reaction; **SAM,** shoot apical meristem.

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