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Full Length Research Paper

Effective *Agrobacterium*–mediated transformation of pineapple with *CYP1A1* by kanamycin selection technique

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Initial calli was induced from leaf base of aseptic shoots. These calli were co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harboring a plant expression vector pUHA1 containing a human cytochrome P4501A1 (CYP1A1) gene for 3 days. Then, the infected calli were transferred to differentiation medium. Adventitious buds were generated after about 10 day's incubation. The generated shoots were cultured on a medium containing 30 to 50 mg L^{-1} Km for screening. The selected Km-resistant shoots were subsequently transferred to rooting medium for rooting. We derived 95 Km-resistant plants from four infection groups in total. The transformation frequency was 0.12 to 2.69%. Further analysis by polymerase chain reaction (PCR) and Southern blotting confirmed that the positive frequency of Km-resistant plants was 53.58%. We also confirmed that these protocols below were essential for *A. tumefaciens*-mediated genetic transformation of pineapple calli: taking agar as medium gelling agent, adding AS to co-culture medium, increasing selection times and gradually increasing the concentration of Km in the selection medium.

Key words: *Ananas comosus*, CYP1A1 gene, genetic transformation, *Agrobacterium tumefaciens.*

INTRODUCTION

Pineapple (*Ananas comosus*) is the number one agricultural commodity in certain parts of the world (Gangopadhyay et al., 2009), and the third most important fruit crop in the tropics and subtropics, after banana and citrus (Rohrbach et al., 2002). Pineapple is self-incompatible and its breeding is a lengthy process (Botella et al., 2000) owing to long generation time (about 4 years), and these traits lagged the improvement of physiological and horticultural traits. Although, extensive efforts have been made to produce improved varieties via hybridization of this self-incompatible monocot species, only a few new hybrids have been released worldwide (Wang et al., 2009). Due to high level of genomic heterozygosity and apparent genome instability, conventional breeding to develop improved varieties has been proved to be difficult (Kato et al., 2004). Genetic transformation is an important and effective means to modify single horticultural trait without altering their phenotype (Yabor et al., 2006). In contrast to conventional breeding programs, the direct introduction of foreign or novel DNA into potentially regenerative cells has been proved to be a very effective and useful tool for introducing new desirable traits into crops (Ko et al., 2006). Transgenes have been introduced to control many agronomic and postharvest problems, such as precocious flowering (Trusov and Botella, 2006), chilling-induced internal fruit browning (Ko et al., 2006). Although there have been some reports on regeneration of pineapple, little effort has been directed toward the development of pineapple regeneration methods that are suitable for the genetic transformation of pineapple (Firoozabady et al., 2006). Using microprojectile bombardment technique, herbicide-tolerant transgenic plants were obtained (Sripaoraya et al., 2001).

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Table 1. Comparison of medium used for calli induction, multiplication and differentiation of pineapple.

Medium	Concentration (mg L^{-1})		Calli induction efficiency (%)	Calli multiplication coefficient	Calli differentiation coefficient
	6-BA	NAA			
1	1	2	52	1.6	0.3
2	1	3	63	2.5	0.7
3	1	4	65	1.2	0.5
4	2	2	78	1.8	3.1
5	2	3	91	2.0	2.8
6	2	4	84	1.6	1.7
7	3	2	86	2.7	5.2
8	3	3	89	2.2	4.3
9	3	4	85	1.8	3.6
10	5	2	92	1.7	6.1
11	5	3	94	2.3	5.4
12	5	4	88	2.6	4.7

Calli induction efficiency (%) = (number of leaf base generated callus / number of leaf base) ×100%. Calli multiplication coefficient= weight of calli after cultured on multiplication medium for 28 days / weight of calli before cultured on the multiplication medium. Calli differentiation coefficient = number of shoots generated from the calli / number of calli.

The efficiency of *Agrobacterium* mediated transformation assisted by micropropagation in temporary immersion bioreactor reached 6.6% (Espinosa et al., 2002). A blackheart involved gene was introduced in pineapple by the same technique; however, the transformation efficiency was less than 1.5% (Ko et al., 2006). Using encapsulation-based antibiotic selection, the transformation efficiency is 20.58% (Gangopadhyay et al., 2009), and it is still low for the application of genetic transformation of pineapple. Furthermore, there was no report about the trans-formation technology of pineapple cultivar “Shenwan”. We have developed efficient regeneration methods of pineapple, “Shenwan”, parameters affecting the regene-ration of “Shenwan” have been optimized (He et al., 2008), and it can be used in the transformation system.

Cytochrome P450 (CYP) gene is a vital environmental gene which plays an important role in the oxidative metabolism of endogenous and exogenous lipophilic compounds in higher plants (Nelson et al., 1996). CYP1A1 is a member of the P450 gene family isolated from human. CYP1A1 showed high drug-metabolizing activity and relatively broad substrate specificity (Inui et al., 2000). So the plant with CYP1A1 can be used to monitor the Persistent Organic Pollutants (POPs) in environment (Inui et al., 2000) and degrade the POPs in the plant.

Ectopic expression analysis of CYPs in potato, tobacco and rice confirmed that the transformed plants can degrade most of the 57 POPs tested, and significantly reduce the pesticide residue in the plants as well as exhibit cross-tolerance to herbicides with different chemical structures and different modes of herbicide action (Inui et al., 1998, 1999, 2001). Transforming the CYP1A1 into pineapple can give light into understanding of POPs metabolism and increase the ability of degrading the residue of POPs in the plant. Furthermore, the transformation of CYP1A1 into pineapple would not bring risk to human health, because this gene was isolated from human.

In this study, a stable *A. tumefaciens* mediated transformation protocol for pineapple has been established using Km as screening agent. Transgenic plants were confirmed by PCR and Southern blotting. Km was suitable for the screening of positive transgenic plantlets. The positive efficiency of Km-resistant plants was 53.58%. We also found that a range of factors, such as preculture regimes, manipulation of inoculation, cocultivation conditions and components of culture medium played significant role in the transformation of pineapple.

MATERIALS AND METHODS

Development of tissue culture systems

The generation protocol of *Ananas comosus*. cv. Shenwan was according to the methods of the present group (He et al., 2008). The composition of the culture medium used to optimize the medium for the induction; multiplication and differentiation of calli are listed in Table 1. The optimal mediums were chosen for the transformation. The leaf base regions of aseptic shoots of pineapple were cultured on CI medium (Murashige and Skoog, 1962), medium (MS), 2.0 mg L^{-1} 6-Benzylaminopurine (6-BA), 3mg L-1 Naphtaleneacetic acid (NAA), 30 g L^{-1} sucrose, 8 g L^{-1}agar (pH 5.8) for calli induction. The induced surface loose calli were subcultured on CM medium (MS medium, 3.0 mg L^{-1} 6-BA, 2.0 mg L^{-1} NAA, 30 g L^{-1} sucrose, 8 g L^{-1}agar, pH 5.8) for multiplication for about a month. Then, these undifferentiated calli can be maintained and multiplied on CM medium, and then it can be used as starting material for subsequent *Agrobacterium* infection. The calli were transferred to fresh CM medium every 28 days to generate adventitious shoots. The shoots were then rooted on RM medium (MS medium, 1.0 mg L^{-1} indolebutyric acid (IBA), 30 g L^{-1} sucrose, 8 g L^{-1}agar, pH 5.8).

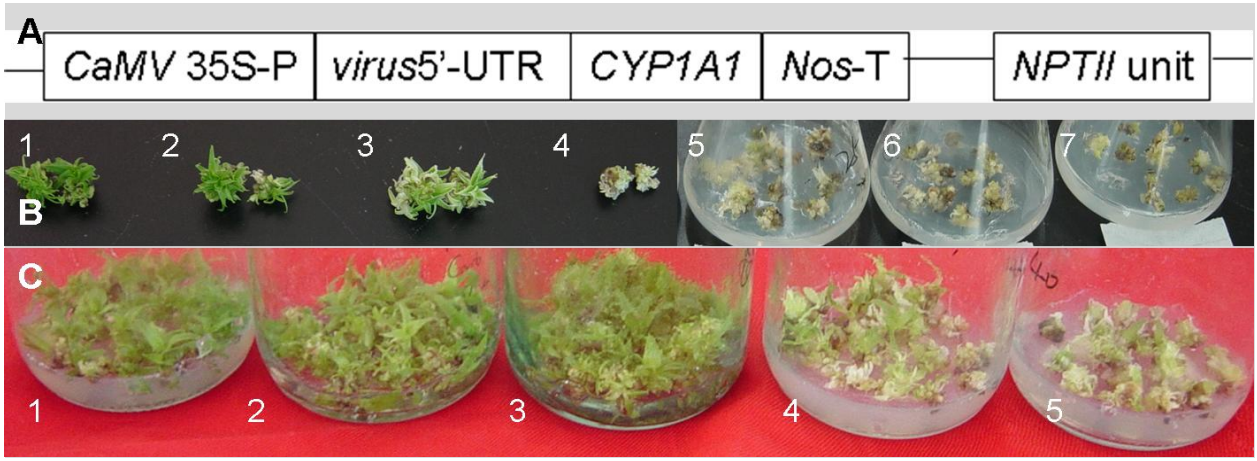

Figure 1. A Scheme of T-DNA of pUHA1 vector The T-DNA contains CYP1A1 gene flanked by the cauliflower mosaic virus 35S promoter at the 5′end and by the transcriptional terminator at the 3′end, and contains nptII gene at the 5′end (B) Shoots germinated on CM medium containing different concentration of Km; 1: free of Km (CK); 2: 5 mg L^{-1} Km; 3: 10 mg L^{-1} Km 4: 15 mg L^{-1} Km; 5: 25 mg L^{-1} Km; 6: 50 mg L^{-1} Km; 7: 100 mg L^{-1} Km. (C) Shoots germinated on CM medium with different gelling agent; 1: coagulated by agar and free of Km; 2: coagulated by carrageenan and supplemented 20 mg L^{-1} Km; 3: coagulated by carrageenan and supplemented 40 mg L^{-1} Km; 4: coagulated by agar and supplemented 20 mg L^{-1} Km; 5: coagulated by agar and supplemented 20 mg L^{-1} Km.

Unless otherwise stated, cultures were incubated in a culture room at 25 ± 2°C with a photoperiod of 14 h light (100 umol m^{-2} s^{-1}) and 75% relative humidity.

Bacterial strains and plasmid

A. tumefaciens (strain LBA4404) harboring the binary vector pUHA1-CYP1A1 (Research Center for Environmental Genomics, Kobe University) was used for transformation. The T-DNA of pUHA1 (Figure 1 A) contained the CYP1A1 and the NPTII gene, which confers kanamycin resistance in the plant cells. All these genes are driven by CaMV35S promoter.

A 50 ml bacterial culture initiated from a single *Agrobacterium* colony containing recombinant plasmid (pUHA1- CYP1A1) was cultured overnight (28°C, 200 rpm) in YEP medium (yeast extract 10, peptone 10 and sodium chloride 5 g L^{-1}, pH 7.5) containing 75 mg L^{-1} Km to obtain bacteria solution (OD_{600} 1.0). The bacteria were harvested by centrifugation at 4000 rpm for 5 min and resuspended in liquid MS-BM medium (MS medium, 30 g L^{-1} sucrose, pH 5.8) containing 75 mg L^{-1} Km. The resuspended bacteria solution were shaken at 150 rpm for 2 h to OD_{600} 1.0.

Determine of Km concentration for transgenic plants selection

Pineapple calli were cultured on the CM medium containing 0-100 mg L^{-1} Km to find the lethal concentration of Km for pineapple and to determine the suitable concentration of Km for resistant plant screening (Table 3). At the same time, we coagulated the medium with 8 g L^{-1} agar (Sangon) or 8 g L^{-1} carrageenan (Sangon) to analyze the effect of gelling agent on Km screening (Table 3). Four replicates were used for each treatment, and each replicates contains 20 pieces of calli.

Pineapple transformation

A summary of the protocol is presented in Table 2. The surface loose calli were cut into pieces of 0.5 cm (about 0.2 to 0.3 g) and subsequently pre-cultured on CM medium for 2 days. Then submerged in the bacterial solution for 5 min, and then blotted dry on sterile filter paper. Then these calli were co-cultivated on CM medium and 100 µmol L^{-1} acetosyringone (AS) in dark for 3 days. The same calli which were cultured on CM medium without AS was used as the control to analyze the effect of AS on transformation efficiency. Then these callies were transferred to CM medium containing 20 mg L^{-1} Km and 300 mg L^{-1} Carb for screening. The infected explants were regularly subcultured in fresh CM medium at 28-day intervals to maintain the selection pressure. The germinated green shoots were cut from the mother calli and then cultured on the SC medium (MS medium, 2.0 mg L^{-1} NAA, 30 g L^{-1} sucrose, 8 g L^{-1}agar, pH 5.8) plus 30 mg L^{-1} Km and 300 mg L^{-1} Carb, and SC medium plus 50 mg L^{-1} Km and 200 mg L^{-1} Carb for further screening. The transformation was performed 4 times.

After 3 times of Km selection, the obtained green shoots were rooted on RM medium plus 30 mg L^{-1} Km. When the well rooted plantlets were approximately 3 cm in height, the lid of the culture bottle was opened and the culture bottle was put in culture room for about 3 days. Then the culture bottles were moved out of the culture room to culture for another 3 days. The plants were subsequently transferred into pots in a high humidity greenhouse.

PCR analysis

Genomic DNA was isolated from 0.2 g young leaves of the putative transformed and control plants by cetyl trimethylammonium bromide (CTAB) following the method of Murray (1980). A fragment (332bp) of the CYP1A1 was amplified using primers CYP F (5′-GCCAAGCTTTATAACAATGC-3′) and CYP R (5′-AAGGACATGCTCTGACCATT-3′). Primer pair (ChvA-F: TCCATCAGCAACGTGTCGGTGCT and ChvA-R: GTGGAAAGGCGGTGAGCGATGAT) designed from the Chv region of *Agrobacterium* was used to detect the bacterial contamination. PCR condition were as follows: initial denaturation at 94°C for 5 min, subsequent denaturation at 94°C for 40 s, annealing at 55°C for 60 s, extension at 72°C for 1 min, 38 cycles, the final extension

Table 2. Procedure of calli induction and transformation.

Material	Medium	Time (day)
Leaf base	CI	28
↓		
Calli	CM	28
↓		
Calli (pre-culture)	CM	2
↓		
Calli + Agrobacterium	CM + 100 µmol l-1 AS	3
↓		
Calli	CM + 20 mg L^{-1} Km + 300 mg L^{-1} Carb	28
↓	SC + 30 mg L^{-1} Km, + 300 mg L^{-1} Carb	
Shoots	SC + 50 mg L^{-1} Km, + 200 mg L^{-1} Carb	28
↓	RM + 30 mg L^{-1} Km	
Shoots		28
↓		
Shoots		28

at 72°C for 10 min. The PCR mixture was composed of 0.7 units of Taq Polymerase, 1 × Taq Polymerase buffer (with $MgCl_2$), 500 µM dNTPs, 0.5 µM primers. PCR products were separated on a 1.2% agarose gel, and detected by staining with ethidium bromide.

Southern hybridization analysis

For Southern hybridization analysis, total DNA was isolated using the same method as PCR analysis. DNA of PCR-positive transgenic, non-transformed control shoots and pUHA1- CYP1A1 plasmid as positive control was used to be sample to amplify CYP1A1 fragment. The amplified production were separated on 0.8% agarose gels overnight, and the fragements were transferred onto nylon membranes (Roche) using standard protocols. Southern hybridization probes (specific fragment of CYP1A1) were labeled with DIG using the DIG DNA Labeling and Detection Kit (Roche), following the manufacturers protocol. Hybridization and autoradiography were carried out according to the instruction of the manufacturers (Roche).

Statistical analysis

All data scored were evaluated by analysis of variance using SPSS software package version 12.0 (SPSS Inc.). The least significant difference 5% probability between the means was evaluated by SNK test.

RESULTS

Preparation of calli for transformation

A good *in vitro* regeneration system is essential for the development of an effective genetic engineering system (Yasmeen, 2009). The optimize results are listed in Table 1. We chose the optimal culture medium for the transformation (Table 2). We did not choose No. 10 and 11 culture mediums for calli induction and differentiation, because the higher the concentration of hormone used, the higher mutation rate may exist. Furthermore, there was no apparent difference between the results of it with the optimal medium we chose. Application of the specific media and experimental conditions can obtain lots of high-quality calli from excised meristematic leaf base regions. These calli were capable of producing losts of shoot bud-like structures. Then these calli were subsequently used for transformation.

Determine of Km concentration for screening

In order to determine the appropriate concentration of Km for effective screening of transformed shoots, calli were cultured on agar solidified CM medium containing different concentrations of Km. The results show that pineapple was sensitive to Km. When the medium contained 10 mg L^{-1} Km, the white shoots frequency was only 28.6%, while Km concentration were more than 15 mg L^{-1}, all the shoots regenerated appeared to be bleached (Table 3, Figure 1 B). The inhibition effect of Km on calli regeneration ability aggravated while the concentration of Km increased. When the concentration of Km reached 50 mg L^{-1}, there were almost no shoots regenerated (Figure 1 B). In order to guarantee high differentiation frequency and low false positive frequency, we chose 20 mg L^{-1} Km for primary screening. The germinated shoots were sub cultured on CM medium containing 30 and 50 mg L^{-1} Km sequently for further screening.

We also found that the gelling agent (agar and carrageenan) had great influence on the Km-resistance of the shoots. When carrageenan was used as gelling agent, even if Km concentration reached 50 mg L^{-1}, the regenerated shoots were all green and grew normally (Table 3, Figure 1 C). So carrageenan could not be used for Km screening in pineapple transformation.

Table 3. Influence of Km on adventitious shoots differentiation of pineapple calli.

Gelling agent	Km concentration (mg/L)	Calli (piece)	Differentiation coefficient	White shoots frequency (%)
agar	0	80	4.8[a]	0[d]
agar	5	80	4.5[a]	5.7[c]
agar	10	80	3.8[a]	28.6[b]
agar	15	80	3.2[ab]	100[a]
agar	25	80	2.6[b]	100[a]
agar	50	80	0.15[c]	100[a]
agar	100	80	0.08[c]	100[a]
Carrageenan	50	80	4.0[a]	0[d]
Carrageenan	100	80	3.5[ab]	20.3[b]

*The mean values in the same list labeled with the same letter are not significantly different by SNK test (P ≤ 0.05).

Table 4. Transformants selection of different groups.

Group	Calli (piece)	First selection			Second selection		Third selection	
		Total number of adventitious shoots	Green shoots	Selection efficiency (%)	Green shoots/ cumulate selection efficiency (%)	Selection efficiency (%)	Green shoots/ cumulate selection efficiency (%)	Selection efficiency (%)
1	450	1485	180	12.12[ab]	67 / 4.51[a]	37.22[a]	40 / 2.69[a]	59.70[a]
2	500	1602	12	0.75[c]	4 / 0.24[c]	25.00[b]	2 / 0.12[c]	50.00[a]
3	640	2176	161	7.40[b]	36 / 1.65[b]	22.36[b]	19 / 0.87[b]	52.77[a]
4	500	1694	295	17.41[a]	68 / 4.01[a]	23.05[b]	36 / 2.13[a]	52.94[a]

*Selection efficiency = (number of green shoots/ number of adventitious shoots in each selection) ×100%, cumulate selection efficiency = (number of green shoots/ number of adventitious shoots in first selection) ×100%. The mean values in the same list labeled with the same letter are not significantly different by SNK test.

Selection of transformed plantlets

From a total of 2090 *Agrobacterium*-inoculated calli in four infection groups, we obtained 97 Km-resistant plantlets after 3 times of Km screening. There were apparent differences in transformation efficiency between the four inoculations. The final transformation efficiency (cumulative selection efficiency) was 0.12 to 2.69%, which showed comparatively large randomicity (Table 4). This transformation efficiency was close to the efficiency got by microprojectile bombardment (Ko et al., 2009). After first Km screening, adventitious shoots differentiation frequency was above 3, the shoots were 1 to 5 mm high with 2 to 5 leaves. Km-resistant shoots frequency (green shoots frequency) were 0.75 to 17.41% (Table 4, Figure 2). The green shoots frequency of comparative higher shoots (long than 4 mm) was higher than that of the comparative lower shoots (below 2 mm) (Table 4). It maybe because the blastema of comparative higher shoots has already been formed before *Agrobacterium* inoculation, so it had high Km-resistance, but it was not the real transformed shoots. In order to eliminate the influence of inoculate materials, we took three measures during the second and third screening culture. Firstly, we excised all the leaves of the green shoots after first screening; only transferred the stem tip for regeneration. Secondly, incubated the shoots on culture medium without 6-BA to prevent the proceeding differentiation of adventitious buds. Thirdly, we used high selection pressure of 30 mg L^{-1} Km

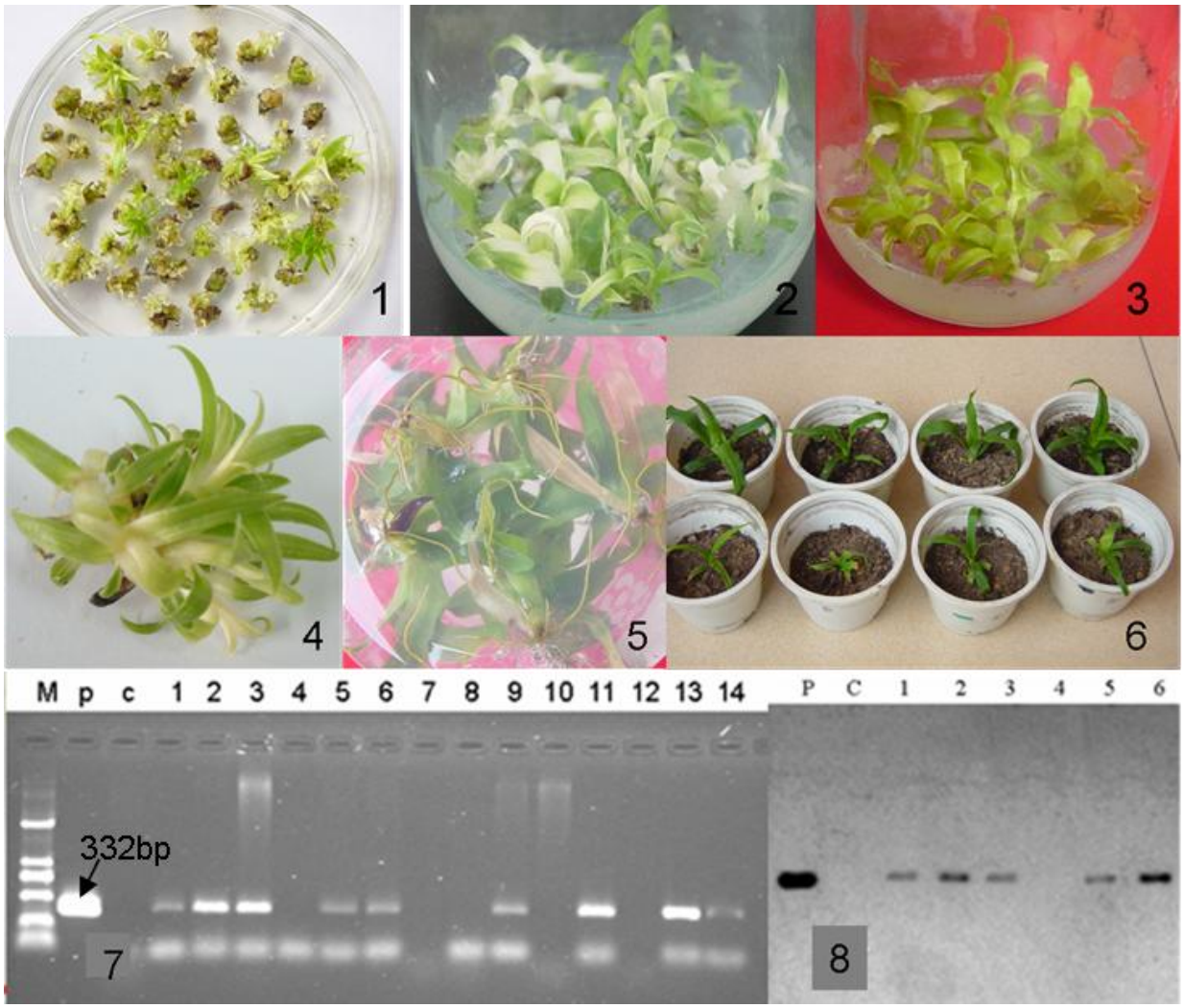

Figure 2. 1: Shoots after Km selection one time; 2: Shoots after Km selection two times; 3: Shoots after Km selection three times; 4: Non-resistant white shoots; 5: Rooted shoots; 6: Transformants transplanted to soil for a month; 7: PCR detection of Km-resistant plants; (M Marker p plasmid pUHA1- *CYP1A1*, c control, lanes 1-14 putative transformants); 8: Southern hybridization analysis of PCR-positive plants (p plasmid pUHA1- CYP1A1, c control, lanes 1-6 PCR-positive plants).

for screening. In this way, after the second screening culture, some of the initial green shoots bleached out gradually (Figure 2). The selection efficiency of the third screening was more than 50%. After three times of Km screening, almost all the shoots were green (Figure 2), and then there was no remarkable difference in selection efficiency between the four inoculations. Using CM medium without AS for co-culture, the cumulate selection efficiency after 3 times of selection was only about 0.01%.

After three times of Km screening, the Km-resistant shoots were subsequently subjected to rooting and hardening. When the resistant plantlets had more than eight leaves and five roots, being higher than 3cm, it was transplanted in soil with survival frequency above 80%. The transgenic plants could grow normally under conventional culture conditions, but there were notable differences between the growth vigour of them. Most of the transformants grew well; the leaves were green and 5 to 8 cm long. However, some transformants grew slowly; the leaves were yellowish and short (Figure 2).

Molecular detection of Km-resistant plants

14 Km-resistant plantlets were randomly chosen for PCR detection. Nine of the fourteen Km-resistant plants generated target band size of 332bp with the CYP1A1 primers (Figure 2, No. 1, 2, 3, 5, 6, 9, 11, 13 and 14). No band was detected in the untransformed control. The presence of CYP1A1 gene in putative transgenic plantlets DNA confirmed the validity of the transgenic plantlets. PCR positive ratio reached 64.29%. Primer pairs of

Agrobacterium did not generate any band from the DNA of putative transgenic plantlets. It confirmed that there was not bacterial contamination. Six of the PCR positive plants were randomly selected for Southern hybridization to verify the integration of the transgenes into the genomic DNA. Positive hybridization band was detected in five of the seven transgenic lines tested and the positive control (Figure 2). No signal was detected in the non-transformed control (Figure 2). This result demonstrated that the T-DNA region of the transformed vector was inserted in the genome of the five lines.

DISSCUSSION

Based on the regeneration system we established, a high efficient *Agrobacterium*-mediated transformation protocol of pineapple was developed. The composition and concentration of hormones in the culture medium for *in vitro* regeneration of pineapple have been optimized. Using the regeneration system, calli could be induced efficiently from leaf base region. And the calli can persistently give rise to adventitious bud-like structures through organogenesis. The mean number of adventitious shoots regenerated from initial calli explants was about 3. The callies were good starting material for pineapple transformation. The regenerated shoots were easy to be rooted, and after a procedure of hardening, the well rooted plantlets could survive and grow well. The efficient regeneration system was an important base for the genetic transformation. Compared to the recent work of Firoozabady (2006) and Espinosa (2002), our transformation protocol was more efficient and faster. It only took us about 4 months to get the transgenic plants from inoculation, while it took Firoozabady (2006) more than 7 months. This effective regeneration system provided a technique base for the effective transformation of pineapple.

Confirmed by PCR-analysis and Southern blotting analysis, the positive efficiency of Km-resistant plants was 53.58%. This high efficiency might be attributed to the co-cultivation medium, culture conditions, bacterium strains and agronomic and physiological characteristics of the genotypes. Beyond that following protocols applied in this study improved the efficiency of the technique. Firstly, the use of agar as gelling agent improved the sensibility of the shoots to Km. On agar solidified culture, the shoots were sensitive to Km. When the medium contained 10 mg L^{-1} Km, the white shoots frequency was only 28.6%, while Km concentration increased to more than 15 mg L^{-1}, all the shoots regenerated appeared to be bleached. When use carrageenan as gelling agent, even supplemented 50 mg L^{-1} Km in the medium, almost all the shoots germiznated were green. And there were only 20% shoots whitened even added 100 mg L^{-1} Km. These results suggest that the gelling agent has important effect on the sensitivity of the shoots to Km. Secondly, Km resistance was used to be plant selection marker. The NPT-II gene was a staple selection marker in plant genetic transformation. However, it was generally agreed that NPT-II was not suitable for the transformation of legumina and monocotyledon plants (Wang and Fang, 2003). Some study considered that NPT-II was a suitable selection mark gene for pineapple, but Km was not an appropriate selection antibiotic (Graham et al., 2000; Firoozabady et al., 2006). They often used hygromycin as selection antibiotic (Gangopadhyay et al., 2009; Wang et al., 2009). Our study proved that when agar was used as gelling agent, pineapple was sensitive to Km and the Km resistance was a suitable selection marker for transgenic plantlets. When cultured on medium containing 15 mg L^{-1} Km, all the adventitious buds generated were white. When the concentration of Km exceeded 50 mg L^{-1}, almost no adventitious buds generated. So we supplemented 20 to 30 mg L^{-1} Km in the medium for transgenic plants selection. Furthermore, it was easier to distinguish the non-resistant shoots from Km-resistant ones, and this increased the positive frequency of Km selection. Because young resistant shoots of pineapple were yellowish, and the non-transformed shoots on Km con-taining medium were white. However, the non-resistant shoots of other selection antibiotics were brown (Firoozabady et al., 2006), and it was more difficult to distinguish it from the yellowish resistant plants. Thirdly, we added acetosyringone in the co-culture medium to enhance the transformation efficiency. It was reported that acetosyringone was beneficial for the transformation of monocotyledon (Firoozabady et al., 2006). In our study, the adding of acetosyringone increased the transformation efficiency more than 10 times. Fourthly, In order to further eliminate the non-resistant shoots, we prolonged the selection period. The green shoots were selected on Km-containing medium for 3 times. There was apparent difference in green shoots frequency after first selection between the four inoculations, but after 3 times of Km selection, there was no apparent difference in green shoots frequency. It confirmed that after 3 times of Km selection, the false positive efficiency of Km selection reached the lowest level. Lastly, we increased selection pressure gradually by adding increased concentration of Km in the culture medium.

The Km resistance of the shoots was related to its size. When the shoots were bigger, the resistance was higher. So we increased the concentration of Km gradually along with the screening culture to eliminate the non-resistant shoots. The constant differentiation of adventitious buds from the infected calli after transferring to the differential medium would interfere with the Km-resistance screening seriously. So, we chose Km-resistant green shoots carefully and excised off the proximal calli to avoid the differentiation of adventitious buds, and this helped in increasing the selection efficiency.

During the proliferation of resistant shoots, we found that there was high ratio of non-resistant shoots in the regenerated shoots. This phenomenon showed that

transgenic plants obtained through organogenesis might be chimeric plants composing of transgenic and no-transgenic cells. It is well know that somatic embryogenesis exhibits a virtue of no chimeras, whereas organogenesis shows a merit of little variation (Shao et al., 2010). So we were going to prove the possibility of using embryonic cell as transformation receptor, obtaining transgenic plants through somatic embryogenesis.

We have observed that there were apparent differences in growth vigor between transgenic plants after transplanted a month. This may be attributed to the influence of the transformation procedure. It was reported that the transformed gene caused several side effects at the biochemical level during early stage of plant hardening (Lourdes et al., 2006). It is meaningful to carefully study the biochemical, physiological, agricultural and ecological characters of transformants in the future.

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