STUDY ON EFFICIENT REGENERATION SYSTEM AND AGROBACTERIUM-MEDIATED TRANSFORMATION OF VETIVERIA ZIZANIOIDES

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Abstract: Vetiver (*Vetiveria zizanioides*) has been widely used in South China for erosion control and ecological restoration. Vetiver cannot be applied to northern China due to its poor resistance to cold although there is a huge need and demand. To improve its cold resistance, a vetiver regeneration system was first established. Axillary buds and aseptic adventitious buds of vetiver were used as explants to investigate the factors that affected its somatic embryogenesis and plant regeneration. Explants were cultured on MS (reference MS in Abbreviations) medium supplemented with 2,4-D or 6-BA.

The results showed that 2,4-D was an important factor to induce somatic embryogenesis, for explants could be regenerated via somatic embryogenesis as long as the medium contained 2,4-D without or with 6-BA. Cytology observation, moreover, proved that embryonic calli originated from epidermal cells and parenchyma cells of vetiver, which had the typical embryonic structure of monocotyledon. Regeneration ability of embryonic calli could be maintained for almost two years, and regeneration frequency kept over 80% regardless of subculture times from 0 to 24. The whole process from callus induction to green plantlets transplanted to soil needed about 3-4 months.

Efficient regeneration system is the foundation of genetic transformation. *Agrobacterium*-mediated transformation system was also established. Plant expression vector p1301UN-*ots*A was constructed by inserting the *ots*A gene digested with *Sac* I/*Kpn* I into MCS of binary vector pCAMBIA1301UN. The freeze-thaw method was used to mobilize the recombinant plasmid into DH5a. Restriction analysis and DNA sequence analysis confirmed that the construction of plant expression vector p1301UN-*ots*A was successful. This recombinant plasmid contains an Ubi promoter, a *hpt* gene, an *ots*A gene, a km^r gene and a *gus* gene, so it is very useful and efficient for gene transformation of monocotyledon. Moreover, an efficient genetic transformation system of vetiver was described, embryonic calli were infected with *A. tumefaciens* EHA105/pCAMBIA1301 (OD_{600}=0.4-0.5) for 20 min and then transferred to CIM in the dark at 25_ for 3-4 d; infected calli were then selected on SIM in the dark at 25_ for 4 weeks. Using the optimized protocol, 18% of the infected calli were Hyg B resistant. Transient integration and expression were confirmed by GUS assay. However, Hyg B resistant calli and plantlets showed obvious growth retardation and phenotypic alterations compared to the control. On the whole, this paper initiated the study on gene engineering of vetiver, and established its regeneration system and *Agrobacterium*–mediated transformation system, which formed a good basis for screening cold tolerant cultivar of vetiver.

Key words: somatic embryogenesis, embryonic calli, *Agrobacterium*-mediated transformation, plant expression vector, resistant calli

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacitic acid; 6-BA – 6-benzyladenine; AS – acetosyringone; Cef – cefotaxine; CIM – co-cultivation induction medium; CM – co-cultivation medium; DM – differentiation medium; GTE – GUS transient expression; GUS – _-glucuronidase; *gus* – _-glucuronidase reporter gene; *hpt* – hygromycin phosphotransferase gene; Hyg B– hygromycin B; IBA – indole-3-butyric acid; IM – induction medium; Kan –

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Kanamycin; *km*^{r} – kanamycin selection gene; KT – Kinctin; MCS – Multiple Colony Site; NAA – naphthaleacetic acid; otsA – trehalose-6-phosphate synthase; PCR – Polymerase chain reaction; PP_{333} – poclobutrazol; Rif – Rifampicin; SDM – screening differentiation medium; SIM – screening induction medium; SRM – screening rooting medium

1 Introduction

Vetiver (*Vetiveria zizanioides*), a magical perennial monocotyledon in the tropical and subtropical regions, has been widely used in South China for the purpose of erosion control and ecological restoration. However, the plant is poorly resistant to low temperature that it is completely restricted for its dissemination and application in north China. In order to improve its cold tolerance, gene engineering opens new avenues to meet the specific demand.

Genetic transformation mediated by *Agrobacterium tumefaciens* was first reported in the 1980s (Block *et al.*, 1984). Since then *Agrobacterium*-mediated transformation has become the standard method to genetically modify dicotyledonous plants. Repeatable and efficient *Agrobacterium*-mediated transformation of monocotyledons was demonstrated first in rice a decade ago (Hiei *et al.*, 1994). The key factors in this method are a "super-binary" vector and the addition of AS to CIM. Subsequently, *Agrobacterium*- mediated transformation of many plant species, such as maize (Zhao *et al.*, 2002), barley (Tingay *et al.*, 1997) and Italian ryegrass (Bettany *et al.*, 2003) were reported.

Efficient regeneration system is the foundation of genetic transformation. Somatic embryogenesis is an effective approach to enhance regeneration frequency. There have been well documented on somatic embryogenesis in many plants, such as sugarcane (Ahloowalia and Maretzki, 1983), corn (Vasil *et al.*, 1984), bermudagrass (Li and Qu, 2004) and alfalfa (Szucs *et al.*, 2006), etc. However, very few related studies on vetiver, up to now, have been conducted except for calli induction and culture *In vitro* of vetiver (Mucciarelli *et al.*, 1993; Ruth *et al.*, 2000; Ma *et al.*, 2000).

Trehalose, a non-reducing disaccharide synthesized by *ots*A gene, is widely used as a protectant of enzymes and membranes in many microorganisms under adverse environment stress, such as drought, salt and cold, so it can enhance the resistant ability of plants (Crowe *et al.*, 1990; Strom and Kaasen, 1993; Drennan *et al.*, 1993). In recent years, transgenic plants such as tobacco, potato and sugarcane containing *ots*A gene have been developed through application of some new bio-techniques, including electroporation, microprojectile bombardment, *Agrobacterium*-mediated, and so on (Goddijn *et al.*, 1997; Yeo *et al.*, 2000; Wang *et al.*, 2003). Among these methods, *Agrobacterium*-mediated transformation is often preferred over other plant transformation systems because of the simplicity, low cost, high transformation efficiency and lower transgene copies integrated into the plant genome (Ishida *et al.*, 1996; Matzke *et al.*, 2001; Dong and Qu, 2005).

This present study aims to enhance cold-resistance of vetiver by transforming *ots*A gene into vetiver cells through establishing a regeneration system, a plant expression vector system and an *Agrobacterium*-mediated transformation system.

2 Materials and Methods

2.1 Plant materials and medium

All vetiver samples used in this study were collected from the South China Botanical Garden nursery. Vetiver axillary buds were sterilized with 70% ethanol for 2 min, and then rinsed 3 times with distilled water and sterilized with 20% $NaClO_3$ for 10 min and with 0.1% $HgCl_2$ for 20 min. MS basal medium (Murashige and Skoog, 1962) supplemented with different concentrations of growth regulators was used in the study (Table 1); the solid

medium contained 8.0% agar, pH was adjusted to 5.85 with 1 N NaOH or 0.1 N HCl, then autoclaved them at 121_ for 15 min.

Table 1 Medium composition of bacterial culture, tissue culture and transformation for vetiver

Medium	Composition
YEP	10 g l^{-1} Tryptone, 10 g l^{-1} Yeast Extract, 5 g l^{-1} NaCl and 1.5% agar. pH7.0
IM	MS supplemented with 2.0 mg l^{-1} 2.4-D and 0.5 mg l^{-1} KT. pH5.8
CIM	IM supplemented with 200 μmol AS. pH 5.4
SIM	IM supplemented with 50 mg l^{-1} Hyg B and 500 mg l^{-1} Cef. pH5.8
DM	MS supplemented with 1.0 mg l^{-1} 6-BA. pH5.8
SDM	DM supplemented with 25 mg l^{-1} Hyg B and 250 mg l^{-1} Cef. pH5.8
SRM	Half strength MS supplemented with 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} PP_{333}, 25 mg l^{-1} Hyg B and 250 mg l^{-1} Cef. pH5.8

2.2 Bacterial strains, plasmids and vectors

E. coli DH5a/ pWY (Wang *et al.*, 2000) contains a 1.431 Kb *ots*A gene, provided by Beijing Institute of Microbiology, the Chinese Academy of Sciences. *E. coli* DH5a/1301UN (Fig. 1) was provided by Beijing Agriculture University. The binary vector p1301UN, derivatives of pCAMBIA1301 (CAMBIA, Canberra, Australia), was used in this transformation experiment. The T-DNA of the binary vector includes Ubi-1 promoter, *hpt* selectable marker gene and *gus* reporter gene (Ohta *et al.*, 1990).

Fig. 1 Plasmid p1301UN

2.3 Enzymes and reagents

All restriction enzymes, T4 DNA ligase and DNA Purification Kit were purchased from TaKaRa Biotechnology Co. Ltd., Japan; Hyg B was purchased from Roche Diagnostics Corporation Indianapolis, USA; Rif, Kan, Cef and other reagents were purchased from Ding Guo Biotechnology Corporation, China. PCR primers were synthesized by Shanghai Shengon Biology Coporation, China, and DNA sequence analysis was identified by TaKaRa.

2.4 Callus induction, subculture and differentiation

Sterilized axillary buds were cut longitudinally and then incubated on IM supplemented with 30 g l^{-1} sucrose, 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} KT in the dark at 25±2_ for about 2-4 weeks. Then induced calli were transferred into IM in the dark at 25±2_ for subculture, once a month. Six mediums containing different proportions of 2,4-

D and 6-BA were used to induce embryonic calli from non-embryonic calli and aseptic adventitious buds. The general olefin slice method was used to observe the cytoarchitecture of embryonic calli. 4 weeks after incubation, light yellowish and compact embryogenic calli were transferred into DM containing 2.0 mg l^{-1} 6-BA and 2.0 mg l^{-1} NAA, and then illuminated with 1200 lx light for 12 h per day. The incubation temperature was approximately 25±2_. After the regeneration plants were formed from embryonic calli, they were transferred into the rooting medium containing 0.1 mg l^{-1} IBA for approximately 2 weeks. The plantlets were subsequently grown in a greenhouse at 25±2_ and then planted in the nursery when 30-40 cm high.

2.5 PCR amplification and construction of plant expression vector p1301UN-*ots*A

PCR was used to obtain *ots*A gene from the Plasmid pWY. According to the sequence of 5' and 3' terminus of *ots*A gene, specific primers were designed as follows: the forward primer included a recognition site for *Kpn* I at its 5' end, and the reverse primer included a recognition site for *Sac* I. The PCR amplification system was 50 μl, and the reaction procedure was one cycle of 94_ for 5 min; 30 cycles of 94_ for 30 s (denaturation), 55_ for 30 s (annealing), 72_ for 2 min (extension); a final elongation at 72_ for 10 min (one cycle).

Forward primer: 5' - GGGCCGGTACCATGAGTCGTTTAGTCGTAGTATC - 3' (*Kpn* I)
Reverse primer: 5' - GCTACCTTTCCAAAGCTTGCGTAGGAGCTCGCCT - 3' (*Sac* I)

To construct the plant expression vector p1301UN-*ots*A, 1.431 Kb PCR products of *ots*A fragments were digested with *Sac* I/*Kpn* I, and the binary vector p1301UN was also digested with *Sac* I/*Kpn* I, and then the target fragment was ligated into the MCS site of the binary vector p1301UN with T4 DNA ligase. The freeze-thaw method was used to mobilize p1301UN-otsA into EHA105 (An *et al.*, 1988; Hood *et al.*, 1993), The resulted EHA105/p1301UN-otsA was inoculated in YEP liquid medium at the presence of 50 mg l^{-1} Rif and 50 mg l^{-1} Km until OD_{600} reached about 1.0. The bacteria were collected by centrifugation (12,000 g for 1min) and re-suspended in AAM medium (Hiei *et al.*, 1994) supplemented with 200 μmol l^{-1} AS to make the OD_{600} reach about 0.5 for co-cultivation with vetiver embryonic calli.

2.6 *Agrobacterium*-mediated transformation and selection

Embryonic calli were sliced into small pieces (1-2 mm in diameter), and then immersed in AAM supplemented with 200 μmol l^{-1} AS suspension for 20 min, then transferred to CIM in the dark at 25_ for 3-4 d. The calli were then collected and rinsed with distilled water and 500 mg l^{-1} Cef for several times, blot dried, and then incubated on SIM in the dark at 25_ to inhibit *Agrobacterium* growth. 4 weeks later, the calli were subjected to two more rounds of selection. Calli growing vigorously under selection were then cultured on SDM for 4 weeks. The incubation temperature was kept at 25°C under a 12/12 h (day/night) photoperiod (cool white fluorescent light). Regenerated shoots were transferred onto SRM for rooting.

2.7 GUS histochemical assay

Assay for transient expression of the *gus* gene was performed by histochemical assays with X-gluc (Jefferson, 1987) as the substrate of the enzyme. Hyg B resistant calli were immersed in the GUS assay buffer overnight at 37_ and then observed under a microscope.

3 Results and Discussion

3.1 Induction conditions of embryonic calli

Embryonic calli did not form if no 2,4-D was present in the medium regardless of the 6-BA concentration (Table 2); on the contrary, embryonic calli did formed if 2,4-D existed irrespective of the concentration of 6-BA. The best proportion of 2,4-D:6-BA for aseptic adventitious buds was found to be 1.0:0.5, with the embryonic calli induction frequency of up to 77.9%; the best proportion 2,4-D:6-BA for non-embryonic calli, however, was 0.5:0, with the induction frequency up to 96.7%.

From above analysis, 2,4-D played a key role in regeneration of vetiver. Explants regenerated via somatic embryogenesis when the medium contained only 2,4-D without or minor concentration of 6-BA; explants, however, regenerated via organogenesis when the medium did not contain 2,4-D but only 6-BA.

Table 2 Effects of growth regulators on induction frequency of embryonic calli

2, 4-D (mg L^{-1})	6-BA (mg L^{-1})	Material	Number of materials	Number of E-calli	Induction frequency (%)
0	0.5	C	90	0	0
		B	72	0	0
0	1.0	C	93	0	0
		B	64	0	0
0.5	0	C	90	87	96.7
		B	70	0	0
1.0	0.5	C	97	83	85.6
		B	77	60	77.9
2.0	1.0	C	90	72	80.0
		B	78	30	38.5
4.0	2.0	C	92	50	54.3
		B	90	34	37.8

C=Non-embryonic callus; B=Aseptic adventitious bud; E-calli=embryonic calli.

3.2 Formation, characters and regeneration ability of embryonic calli

The axillary buds of vetiver began to expand about 10 d after inoculation. The epidermal cells were active, and their cytoplasm became thick and divided quickly (Fig. 2A-e). Moreover, some parenchyma cells stained deeply around vascular bundles also divided quickly (Fig. 2B-p). This indicated that the origin of calli came from both epidermal cells and parenchyma cells. In strong division areas, cells with typical embryonic structure could be found, including single cell (Fig. 2C-s), couple cells (Fig. 2C,D-t), four cells (Fig. 2C-f), and multiple cells (Fig. 2D-m).

Every embryonic callus of vetiver was irregular, white granulose, and compact (Fig. 2E), and had the typical embryo structure of monocotyledon, such as scutellum (Fig. 2F-cp), coleoptile (Fig. 2F-cr) and coleorhiza (Fig. 2F-sc), etc. Embryonic calli began to differentiate in the DM (Fig. 2G, H). Regeneration frequency of embryonic calli was 92.0% for 18 months, and 81.6% for 24 months; that is to say, regeneration ability of embryonic calli of vetiver was still very strong even two years later (Table 3).

Table 3 Effects of subculture duration on regeneration frequency of embryogenic calli

Subculture duration (months)	Number of E-calli	Number of regeneration E-calli	Regeneration frequency (%)

18	400	368	92.00
20	550	509	92.55
22	650	557	85.69
24	750	612	81.60

Data were collected every month in the last six months; E-calli = embryogenic calli

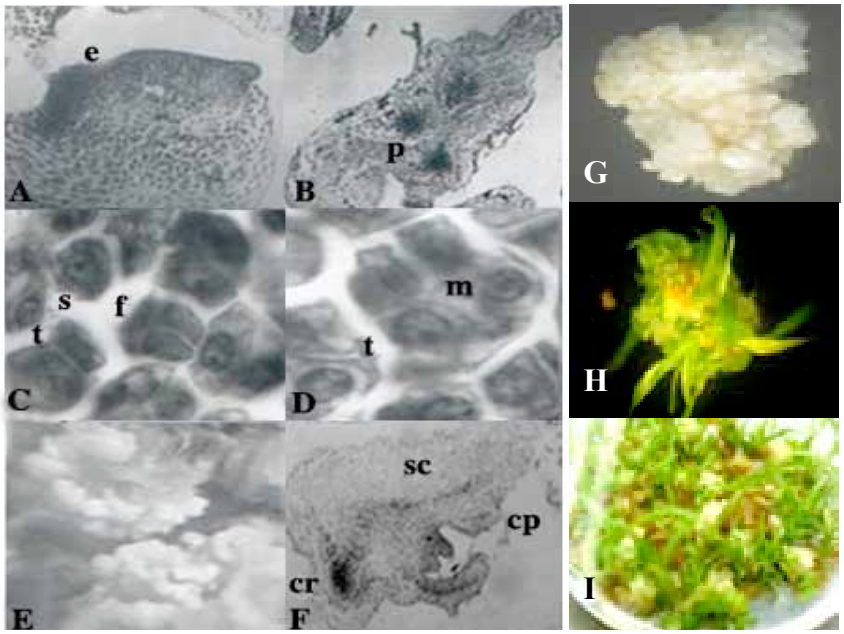
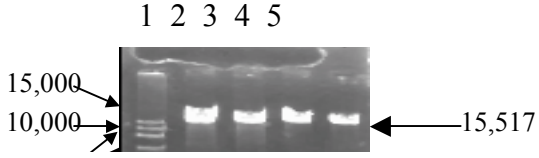


Fig. 2 Formation, characters and differentiation of embryonic calli

A: Epidermal cells of the explant 2 weeks after inoculation (_100); B: Vascular bundle and parenchyma cells 2 weeks after inoculation (_100); C: Different stages of embryonic calli: single cell (s), two cells (t), four cells (f) (_1000); D: Embryonic calli with multiple cells proembryo (m) (_1000); E and G: Visible embryonic calli (_30); F: Embryonic calli including coleorhiza (cr), coleoptile (cp), scutellum (sc) (_100); H and I: Differentiation of embryonic calli 2 weeks after inoculation

3.3 Constrcution of plant expression vector p1301UN-*ots*A

PCR amplification products were separated in a 1% agarose gel electrophoresis. Restriction analysis and DNA sequence analysis showed that full length of *ots*A gene in Plasmid pWY is 1.431 Kb, coding 477 amino acids. Binary victor p1301UN was digested with *Sac* I/*Kpn* I and then recovered using DNA Purification Kit. Plant expression vector p1301UN-*ots*A was constructed by inserting the *ots*A fragment digested with *Sac* I/*Kpn* I into the MCS of binary vector p1301UN. The freeze-thaw method was used to mobilize the recombinant plasmid into DH5a. Restriction analysis and DNA sequence analysis confirmed that the construction of the plant expression vector p1301UN-*ots*A was successful (Fig. 3). T-DNA region of the recombinant plasmid p1301UN-*ots*A includes Ubi-1 promoter, *hpt* gene, *ots*A gene and *gus* gene (Fig. 4). One of this vector's advantages is that *ots*A gene inhabiting the vector is driven by Ubi-1 promoter, which could reduce the copy number of foreign genes in transgenic maize plants, so it might be useful in avoidance of gene silencing and highly active in monocotyledons (Christensen and Quail, 1996; Xu *et al.*, 2004).



Lane 1: Marker: DL15000; Lane 2-3: p1301UN-otsA double digested with *Sac* I/*Kpn* I; Line 4-5: p1301UN-otsA digested with *Sac* I

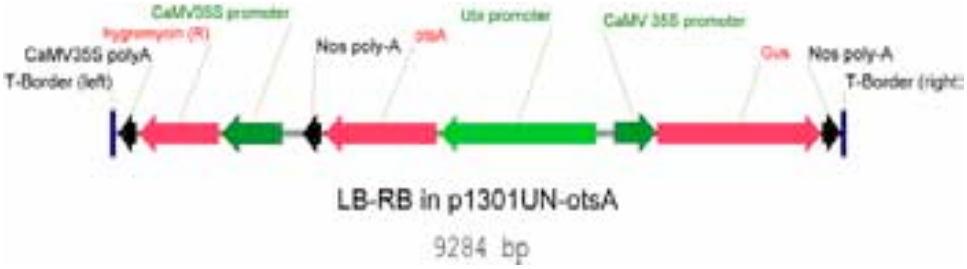


Fig. 4 Schematic structure of T-DNA region of p1301UN-*ots*A

3.4 Establishment of *Agrobacterium*-mediated transformation system of Vetiver

3.4.1 Screening press test to Hyg B

Hyg B, a strong cell growth inhibitor, has very high toxicity to many plants and, therefore, usually is used as a selection marker for genes. Different concentrations (0, 25, 50, 75 and 100 mg l^{-1}) of Hyg B were added into IM respectively, and then embryonic calli of vetiver were cultured on these mediums for 4 weeks (Fig. 5). The results showed 50 mg l^{-1} Hyg B was a perfect selection concentration for screening transgenic calli (Fig. 5C), as 50 mg l^{-1}selection pressure could not only inhibit calli's growth effectively, but also avoid their quick death.

(Fig.5)

3.4.2 Concentration of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens EHA105/pCAMBIA1301 was inoculated in YEP liquid medium in the presence of 50 mg l^{-1} Rif and 50 mg l^{-1} Km until OD_{600} reached about 1.0. The bacteria were collected by centrifugation (12,000 g, 1 min) and re-suspended in AAM supplemented with 200 μmol l^{-1} AS. The value of OD_{600} of AAM was observed once per hour, and then embryonic calli were inoculated in the AAM for about 20 min. After 3 days of co-cultivation on CIM in dark at 25_, the frequency of GUS transient expression (GTE) was evaluated by number of GUS-expressing calli over the number of calli infected (Table 4). The result showed that high expression frequency could be obtained when the OD_{600}=0.416 (about 4 h), then the expression frequency declined as the concentration of EHA105 increased.

Table 4 The effect of *Agrobacterium* concentration on frequency of GTE in vetiver

Inoculation time_h_	Concentration of A*grobacterium*	Number of calli treated	Number of GUS-expressing calli	Frequency of GTE (%)
1	0.050	31	0	0
2	0.115	37	2	5.4
3	0.224	41	5	12.2
4	0.416	40	8	20.0
5	0.560	38	5	13.1
6	0.737	37	3	8.1

3.4.3 Concentration of AS

Phenolics like AS, are well-known virulence inducers for *Agrobacterium*, which plays an important role in the transformation process especially for monocotyledon's (Dion *et al.*, 1995; Suzuki and Nakano, 2001). However, the concentration of AS added into the medium should be limited, otherwise calli would be killed due to *Agrobacterium*'s luxuriant growth. Different concentrations of AS were added into AAM and CIM; as a result, the frequency of GTE was highest when adding 200 μM AS into AAM and CIM (Table 5).

Table 5 The effect of AS concentration on frequency of GTE in vetiver

Conc. of AS (μM)	Number of calli treated	Number of GUS-expressing calli	Frequency of GTE (%)
0	40	0	0
100	36	3	8.3
200	38	7	18.4
300	41	6	14.6
400	40	4	10.0

3.4.4 Co-cultivation temperature

The lower temperature of 22_ improves *Agrobacterium-mediated* gene transferring to plant cells (Dillen *et al.*, 1997). Low temperature also promotes pilus assembly leading to an increase in the number of pili on the cell surface (Fullner *et al.*, 1996). To determine the influence of temperature during co-cultivation of vetiver, embryonic calli were co-cultivated at 18, 20, 22, 25, and 28_, respectively. The highest frequency of GTE was observed at 22-25_, in which 15.0-19.5% calli showed GUS activity (Table 6). However, the frequency of GTE markedly decreased when the temperature was increased to 28_, because contamination produced by the rapid reproduction of *Agrobacterium*, restrained calli growth or even killed them if temperature became high.

Table 6 The effect of co-cultivation temperature on frequency of GTE in vetiver

Co-cultivated temperature (_)	Number of calli treated	Number of GUS-expressing calli	Frequency of GTE (%)
18	37	2	5.4
20	38	3	7.8
22	40	6	15.0
25	41	8	19.5
28	37	3	8.1

3.4.5 Duration of co-cultivation

The duration of co-cultivation was observed by co-cultivated calli for different days of 1, 2, 3, 4 and 5 respectively. The frequency of GUS transient expression was only 2.7% after 1 day of co-cultivation, and was up to highest on day 3 or 4, and then decreased on day 5 due to abundant proliferation of bacteria (Table 7).

Table 7 The effect of co-cultivation time on frequency of GTE in vetiver

Days of co-cultivation	Number of calli treated	Number of GUS-expressing calli	Frequency of GTE (%)
1	37	1	2.7
2	38	2	5.3
3	40	5	12.5
4	41	6	14.7
5	37	3	8.1

Through the study on the five above-mentioned parameters, effective genetic transformation system of vetiver mediated by EHA105/ pCAMBIA1301 was established as follows: embryonic calli were immersed in AAM supplemented with 200 μmol l^{-1} AS until the OD_{600} was up to 0.4-0.5, then transferred to CIM containing 200 μmol AS in the dark at 25_ for 3-4 d, the calli were then incubated on SIM containing 50 mg l^{-1} Hyg B and 500 mg l^{-1} Cef in dark at 25_ to yield resistant calli.

3.5 *Agrobacterium*-mediated transformation

Approximately one to two-month-old, light yellowish and compact embryonic calli were used for transformation. A total of 450 calli were co-cultivated with EHA105/p1301UN-*ots*A. After 4-day co-cultivation, embryonic calli were inoculated on SIM in dark at 25_ for 4 weeks. During the selection period on Hyg B (50 mg l^{-1}), a majority of calli gradually turned brown or even died whereas some yellowish Hyg B resistant calli were observed 4 weeks after selection. These Hyg B resistant calli were subjected to a lower level selection (25 mg l^{-1} Hyg B) of the regeneration process. In the three transformation experiments, 18% calli showed resistance to the Hyg B selection. However, the differentiation and growth of Hyg B resistant calli and buds was commonly delayed for 10-15 d compared to those of the control, some of them showed multiple phenotypic alterations, such as yellow wrinkling leaves, slow growth, short and thin shape. Similar growth retardation phenomenon has also been found in transgenic tobacco, potato and sugarcane due to the over expression of *ots*A gene (Goddijn *et al.*, 1997; Yeo *et al.*, 2000; Wang *et al.*, 2003).

3.6 Analyses of Hyg B resistant calli

After 4-day co-cultivation, GUS expression was observed from resistant calli. Parts of calli had already become distinctly blue, indicating that T-DNA of vector

EHA105/1301UN-*ots*A had been delivered into the plant cells (Fig. 6A), although the GUS expression levels varied among them. Fig. 7B showed Hyg B resistant calli after 4 weeks of cultivation on SIM. Fig. 6C-a showed Hyg B resistant buds after 4 weeks of cultivation on SDM, but no buds in the control (Fig. 6C-b).

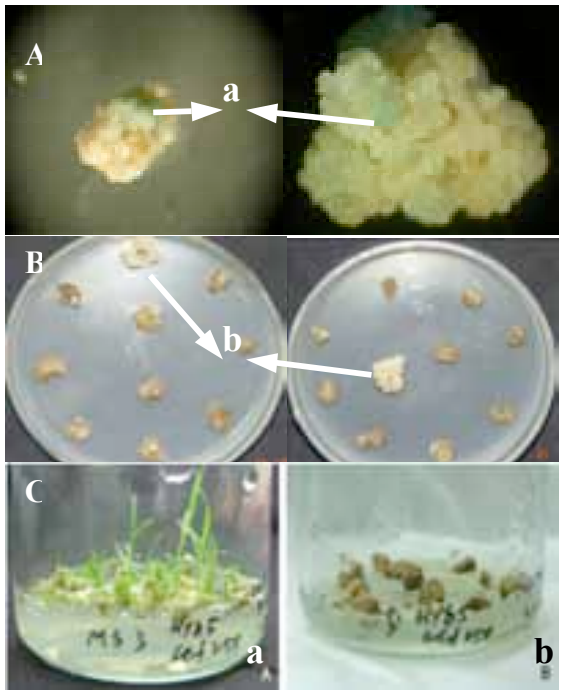


Fig. 6 Screening of resistant calli

A: GUS assay 3 d after co-cultivation (arrow a); B: Hyg B resistant calli (arrow b) 4 weeks after cultivation on SIM; C: (a) Hyg B resistant buds 4 weeks after cultivation on SDM; (b) control

4 Conclusion and Perspective

This is the first report to our knowledge related to gene engineering of vetiver. In this study, we gave a detailed observation on somatic embryogenesis and regeneration of vetiver, and established an effective and stable regeneration system for gene transformation. 2,4-D played an important role during the somatic embryogenesis. Explants could be regenerated via somatic embryogenesis when the medium contained only 2,4-D without or with 6-BA. Embryonic calli came from epidermal cells and parenchyma cells, which have the typical embryonic structure of monocotyledons, including racidele, embryonic bud, hypocotyl, scutellum, coleoptile and coleorhiza. Hence the regeneration ability of embryonic calli of vetiver was very strong, and even could be maintained over two years. The whole process from callus induction to green plantlets transplanting to soil took about 3-4 months.

In the present study, plant expression vector p1301UN-*ots*A was constructed by inserting *ots*A gene digested with *Sac* I/*Kpn* I into the MCS site of binary vector pCAMBIA1301UN. In addition, an efficient *Agrobacterium*-mediated transformation system of vetiver was also established and five transformation parameters were optimized. Gus assay confirmed that the target gene was integrated into the Hyg B-resistant calli and plantlets, although some of them showed obvious growth retardation and phenotypic alteration.

So far, the obtained results have laid a good foundation for screening out transgenic cold-tolerant cultivars of vetiver. At present we are doing molecular analysis and cold resistance analysis to the Hyg B resistant plantlets, and will conduct further observation

after they are transplanted to the outside field and to the northern regions of China with the objective of obtaining true transgenic cold-tolerant cultivars of vetiver.

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A Brief Introduction to the First Author

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