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EFFICIENT REGENERATION SYSTEM AND *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF *VETIVERIA ZIZANIOIDES* (L.) NASH

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Abstract

Vetiver (*Vetiveria zizanioides* (L.) Nash) has been widely used in South China for erosion control and ecological restoration, but it cannot be used in North China due to poor resistance to cold. To better improve the cold resistance of vetiver, a method was established to enhance cold resistance of vetiver. 2,4-dichlorophenoxyacitic acid (2,4-D) and 6-benzyladenine (6-BA) at a concentration of 1.0 and 0.5 mg l^{-1}, respectively were most successful in inducing embryonic calli with an induction frequency up to 96.7%. Cytology observation proved that embryonic calli originated from epidermal cells and parenchyma cells of vetiver, and formed typical embryonic structure of monocotyledon. The regeneration ability of embryonic calli could be maintained for over two years, and the regeneration frequency was over 80% regardless of subculture times. Plant expression vector p1301UN-*ots*A was constructed by inserting the *ots*A gene digested with *Sac* I/*Kpn* I into Multiple Colony Site (MCS) of binary vector p1301UN. The freeze-thaw method was used to mobilize the recombinant plasmid into DH5a. Both Restriction analysis and DNA sequence analysis confirmed that the construction of plant expression vector p1301UN-*ots*A was successful. Moreover, an efficient genetic transformation system of vetiver was determined as follows: embryonic calli were infected with *A. tumefaciens* EHA105/p1301 (OD_{600}=0.4-0.5) for 20 min., and then transferred to co-cultivation induction medium (CIM) in the dark at 25°C for 4 days; thereafter the infected calli were selected on screening induction medium (SIM) in the dark at 25 °C for 4 weeks. Using the optimized protocol, 18% of the infected calli were hygromycin B (Hyg B) resistant. Transient integration and expression were confirmed by GUS assay.

Introduction

Vetiver (*Vetiveria zizanioides* (L.) Nash), a perennial monocotyledon native to the tropics, has been widely used in South China for the purposes of soil erosion control and ecological restoration (Xia *et al.*, 1998; Xia, 2004). However, this plant can not thrive in low temperature, so it is difficult to be disseminated in North China in spite of the fact that the demand to vetiver in North China is becoming increasingly urgent for the above purposes. Therefore, it is necessary to improve the cold tolerance of vetiver through bio-techniques.

Genetic engineering opens a new avenue to improve cold tolerance of plants. Genetic transformation mediated by *Agrobacterium tumefaciens* was first reported in the beginning of 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 over 10 years ago (Hiei *et al.*, 1994). The key factors in this method are a 'super-binary' vector and the addition of acetosyringone (AS) to co-cultivation induction medium (CIM). Subsequently, *Agrobacterium*-mediated transformation of many plant species, such as barley (*Hordeum vulgare*) (Tingay *et al.*, 1997), maize (*Zea mays*) (Zhao *et al.*, 2001), and Italian ryegrass (*Lolium multiflorum*) (Bettany *et al.*, 2003) were reported.

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Efficient regeneration system is the foundation of genetic transformation. Embryonic calli are an effective approach to enhance regeneration frequency, such as sugarcane (*Saccharum* spp.) (Ahloowalia & Maretzki, 1983), bermudagrass (*Cynodon dactylon*) (Li & Qu, 2004), rice (*Oryza sativa*) (Noor *et al*., 2005), and alfalfa (*Medicago sativa*) (Szucs *et al.*, 2006). However, up to now, very few related studies on vetiver have been reported except for *In vitro* culture studies of vetiver (Mucciarelli *et al.*, 1993; Ma *et al.*, 2000; Ruth *et al.*, 2000).

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

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

Materials and Methods

Plant materials and media: Vetiver materials collected from the nursery of South China Botanical Garden, Guangzhou, China was used in this study. After vetiver had grown in the nursery for one year, its stalks with nodes containing axillary buds were cut using a sharp sterile stainless steel blade. Axillary buds were sterilized with 70% ethanol for 2 min., and then rinsed 3 times with distilled water and sterilized with 20% Sodium hypochlorite ($NaClO_3$) solution for 10 min., and with 0.1% Mercuric chloride ($HgCl_2$) for 20 min. MS basal medium (Murashige & Skoog, 1962) supplemented with different concentrations of growth regulators was used in this study (Table 1). The media contained 8.0% agar and their pH values were adjusted to 5.8; then they were autoclaved at 121^{o}C for 15 min.

Bacterial strains, plasmids and vectors: *Escherichiai coli* DH5a/ pWY (Wang *et al.*, 2000) containing 1.431 Kb *ots*A gene, provided by Beijing Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, and binary vector p1301UN (Fig. 1), derivatives of pCAMBIA1301 (CAMBIA, Canberra, Australia), were used in this transformation experiment. T-DNA of the binary vector includes Ubi-1 promoter, *hpt* selectable marker gene and *gus* reporter gene (Ohta *et al.*, 1990).

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.

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
Induction medium (IM)	MS supplemented with 2.0 mg l^{-1} 2.4-D and 0.5 mg l^{-1} KT. pH5.8
Co-cultivation induction medium (CIM)	IM supplemented with 200 µmol AS. pH 5.4
Screening induction medium (SIM)	IM supplemented with 50 mg l^{-1} Hyg B and 500 mg l^{-1} Cef. pH5.8
Differentiation medium (DM)	MS supplemented with 1.0 mg l^{-1} 6-BA. pH5.8
Screening differentiation medium (SDM)	DM supplemented with 25 mg l^{-1} Hyg B and 250 mg l^{-1} Cef. pH5.8
Rooting medium (RM)	Half strength MS supplemented with 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} PP_{333}. pH5.8
Screening rooting medium (SRM)	RM supplemented with 25 mg l^{-1} Hyg B and 250 mg l^{-1} Cef. pH5.8

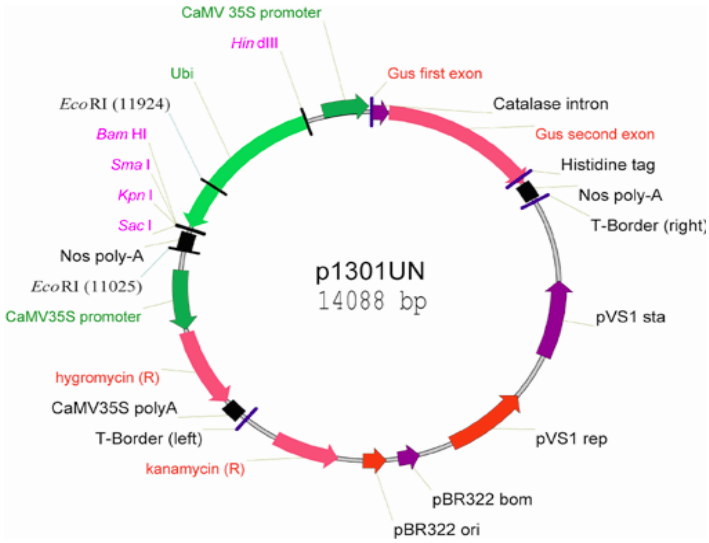

Fig. 1. Plasmid p1301UN.

Callus induction, subculture and differentiation: Sterilized axillary buds were cut longitudinally and then incubated on induction medium (IM) in dark at 25 ± 2°C for 2-4 weeks. Then induced calli were transferred into the same IM in dark at 25 ± 2°C for subculture. Six media containing different proportions of 2,4-D and 6-BA were used to induce embryonic calli. The general olefin slice method was used to observe the cyto-architecture of embryonic calli. Four weeks after incubation, light yellowish and compact embryogenic calli were transferred into differentiation medium (DM), illuminated with a light intensity of 1200 lux for 12 hr per day, and then transferred into the rooting medium (RM) for approximately 2 weeks. The incubation temperature was 25 ± 2°C. The plantlets were subsequently grown in a greenhouse and then were planted in nursery when they were 30-40 cm high.

Construction of plant expression vector p1301UN- otsA: According to the sequence of 5' and 3' terminus of *ots*A gene, specific primers were designed as follows: forward primer included a *Kpn* I recognition site at its 5' end, and reverse primer included a *Sac* I recognition site.

Forward primer: 5' - GGGCCGGTACCATGAGTCGTTTAGTCGTAGTATC - 3' (*Kpn* I)
Reverse primer: 5' - GCTACCTTTCCAAAGCTTGCGTAGGAGCTCGCCT - 3' (*Sac* I)
PCR amplification system was 50 µl, and reaction procedure was one cycle of 94°C for 5 min,30 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 2 min (extension); a final elongation at 72°C for 10 min., (one cycle). Then 1.431 Kb PCR products of *ots*A fragments were digested with *Sac* I/*Kpn* I, while binary vector p1301UN was also digested with *Sac* I/*Kpn* I, and then the target fragment was ligated into Multiple Colony Site (MCS) of binary vector p1301UN with T_4 DNA ligase to get the plant expression vector p1301UN-otsA. The freeze-thaw method was used to mobilize p1301UN-otsA into EHA105 (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. Then the bacteria were collected by centrifugation (12,000 g for 1 min) and re-suspended in AAM medium (Hiei *et al.*, 1994) supplemented with 200 µmol l^{-1} acetosyringone (AS) to make OD_{600} reach about 0.5 for co-cultivation with vetiver embryonic calli.

***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., and subsequently transferred to CIM in the dark at 25°C for 3-4 days. Thereafter, the calli were collected and rinsed with sterile distilled water and 500 mg l^{-1} cefotaxine (Cef) for several times, blot dried, and then incubated on SIM in the dark at 25°C to inhibit *Agrobacterium* growth. Four weeks later, the calli were subjected to two more rounds of selection. Calli growing vigorously under selection were then cultured on screening differentiation medium (SDM) for 4 weeks. The incubation temperature was kept at 25°C under a 12/12 hr (day/night) photoperiod (cool white fluorescent light). Regenerated shoots were transferred onto screening rooting medium (SRM) for rooting.

GUS histochemical assay: Assay for transient expression of *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°C and then observed under a microscope.

Results and Discussion

Induction conditions of embryonic calli: Embryonic calli did not initiate in the absence of 2,4-D even though the media contained different concentrations of 6-BA (Table 2). On the contrary, embryonic calli initiated when 2,4-D was present irrespective of the concentration of 6-BA. The best proportion of 2,4-D and 6-BA was with a combination of 1 and 0.5 mg/L respectively, with embryonic calli induction frequency up to 96.7%. From above analysis, 2,4-D played a key role in regeneration of vetiver. This result also has been demonstrated in many other species such as *Leymus chinensis* (Liu *et al.*, 2004) and *Oryza sativa* (Naqvi *et al*., 2005).

Formation and characters of embryonic calli: The axillary buds of vetiver began to swell two weeks after inoculation. The epidermal cells were active, and their cytoplasm became thick and divided quickly (Fig. 2A). Moreover, some deeply stained parenchyma cells around vascular bundles also divided quickly (Fig. 2B). This infers 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, couple cells, four cells, and multiple cells (Fig. 2C, 2D).

Every embryonic callus of vetiver was irregular, white granulose and compact (Fig. 2F), and had the typical embryo structure of monocotyledon such as scutellum, coleoptile and coleorhiza (Fig. 2E). Embryonic calli began to differentiate in the DM (Fig. 2G, 2H). Regeneration frequency of embryonic calli was 92.0% for 18 months, and 81.6% for 24 months, indicating that the regeneration ability of embryonic calli of vetiver was still very strong even two years later (Table 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 the full length of *ots*A gene in Plasmid pWY was 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 *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 construction of the plant expression vector p1301UN-*ots*A was successful (Fig. 3). T-DNA region of the recombinant plasmid p1301UN-*ots*A included Ubi-1 promoter, *hpt* gene, *ots*A gene and *gus* gene (Fig. 4). One of the 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 in monocotyledons (Christensen & Quail, 1996; Xu *et al.*, 2004).

Establishment of *Agrobacterium*-mediated transformation system of vetiver Screening press test to Hyg B: Hyg B, a strong cell growth inhibitor, has very high toxicity to many plants and, therefore, is usually 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 media for 4 weeks (Fig. 5). As a result, 50 mg l^{-1} Hyg B was a perfect selection concentration for screening transgenic calli (Fig. 5C), as 50 mg l^{-1} selection press could not only inhibit calli's growth effectively, but also avoid their quick death.

Concentration of *Agrobacterium tumefaciens*: *Agrobacterium tumefaciens* EHA105/p1301 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 OD_{600} value 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°C, 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 OD_{600} = 0.416 (about 4 hr), then the expression frequency declined as the concentration of EHA105 increased.

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

2, 4-D (mg l^{-1})	6-BA (mg l^{-1})	Number of explants	Number of embryonic calli	Induction frequency (%)
0	0.5	90	0	0
0	1.0	93	0	0
0.5	0	97	83	85.6
1.0	0.5	90	87	96.7
2.0	1.0	90	72	80.0
4.0	2.0	92	50	54.3

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

Subculture duration (months)	Number of embryogenic calli	Number of regeneration embryogenic calli	Regeneration frequency (%)
18	400	368	92.00
20	550	509	92.55
22	650	557	85.69
24	750	612	81.60

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

Inoculation time hr	OD_{600} value of *Agrobacterium*	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

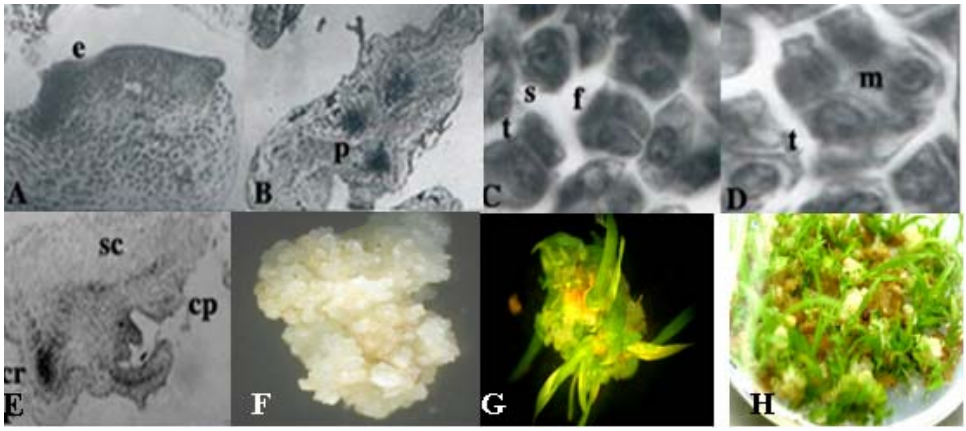

Fig. 2. Formation, characters and differentiation of embryonic calli.
A: Epidermal cells of the explant 2 weeks after inoculation (×100); B: Vascular bundle and parenchyma cells 2 weeks after inoculation (×100); C: Different stages of embryonic calli: single cell (s), two cells (t), and four cells (f) (×1000); D: Embryonic calli with multiple cells proembryo (m) (×1000); E: Embryonic calli including coleorhiza (cr), coleoptile (cp), and scutellum (sc) (×100); F: Visible embryonic calli; G and H: Differentiation of embryonic calli 2 weeks after inoculation

Table 5. The effect of co-cultivation temperature on frequency of GTE in vetiver.

Co-cultivated temperature (ºC)	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

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

Duration of co-cultivation (Days)	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

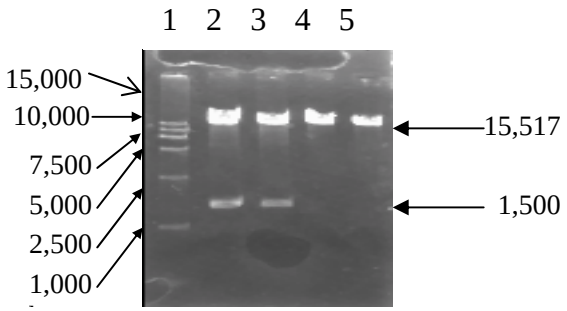

Fig. 3. Restriction analysis of recombinant plasmid p1301UN.
Line 1: Marker: DL15000; Line 2-3: p1301UN-otsA double digested with *Sac* I/*Kpn* I; Line 4-5: p1301UN-otsA digested with *Sac* I

Co-cultivation temperature: Low temperature at 22^{o}C improves *Agrobacterium*-mediated gene transferring to plant cells (Dillen *et al.*, 1997), and 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, embryonic calli of vetiver were co-cultivated at 18, 20, 22, 25, and 28^{o}C, respectively. As a result, the highest frequency of GTE was observed at 22-25^{o}C and 15.0-19.5% calli showed GUS activity in this temperature scope (Table 5). However, the frequency of GTE markedly decreased when temperature was increased to 28^{o}C, as contaminations produced by the rapid reproduction of *Agrobacterium* restrained the growth of calli and even killed them under the high temperature.

Duration of co-cultivation: The duration of co-cultivation to embryonic calli was observed on different days of 1, 2, 3, 4 and 5, respectively, at 25^{o}C. The frequency of GUS transient expression was only 2.7% after one-day co-cultivation, and was up to highest on day 4, and then decreased on day 5 due to abundant proliferation of bacteria (Table 6).

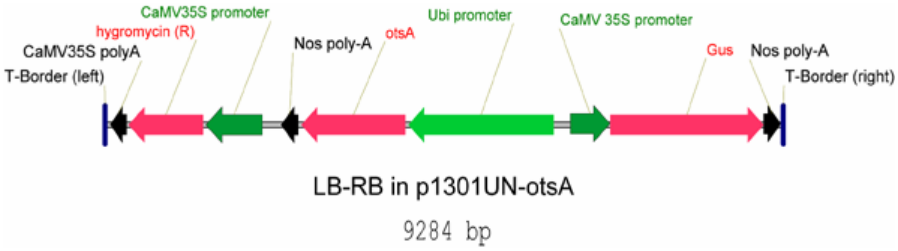

Fig. 4. Schematic structure of T-DNA region of p1301UN-*ots*A

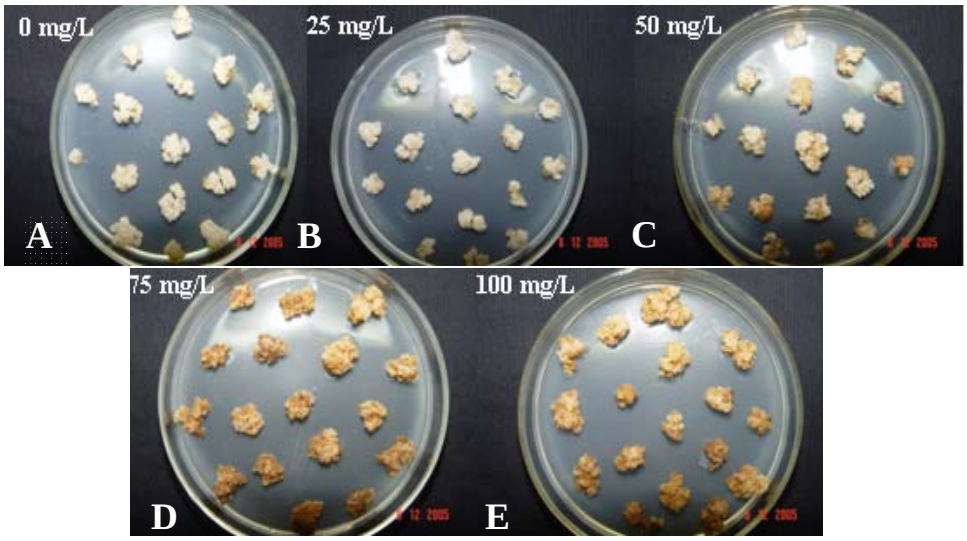

Fig. 5. The sensibility test to Hyg B
A and B: Embryonic calli grow well in 0 and 25 mg l^{-1} Hyg B; C: Embryonic calli stop growth in 50 mg l^{-1} Hyg B; D and E: Embryonic calli are killed in 75 and 100 mg l^{-1} Hyg B

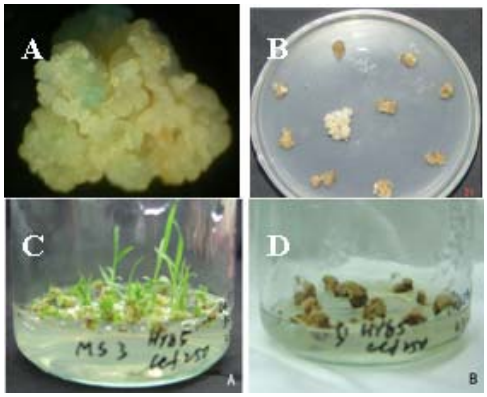

Fig. 6. Screening of resistant calli
A: GUS assay 4 days after co-cultivation; B: Hyg B resistant calli 4 weeks after cultivation on SIM; C: Hyg B resistant buds 4 weeks after cultivation on SDM; D: control.

Through measurement to the four above-mentioned parameters, an effective genetic transformation system of vetiver mediated by EHA105/ p1301 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 dark at 25°C for 4 days; thereafter the calli were incubated on SIM containing 50 mg l^{-1} Hyg B and 500 mg l^{-1} Cef in dark at 25°C to yield resistant calli.

***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°C for 4 weeks. During the period of selection with 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 taking place 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 Hyg B selection. However, the differentiation and growth of Hyg B resistant calli and buds were delayed for 10-15 days in comparison with the control, and some of them showed multiple phenotypic alterations, such as yellow wrinkling leaves, slow growth, short and thin shape. Similar growth retardation phenomena have 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).

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. 6B showed Hyg B-resistant calli after 4 weeks of cultivation on SIM. After 4 weeks of cultivation on SDM, the Hyg B-resistant calli sprouted buds (Fig. 6C), but no buds in the control (Fig. 6D).

Conclusion

To our knowledge, this is the first report related to genetic engineering of vetiver. In this study, a detailed observation on embryonic calli and regeneration of vetiver was conducted, and an effective and stable regeneration system for gene transformation was established. 2,4-D played an important role in the process of embryonic calli development. The calli development was from epidermal cells and parenchyma cells with typical embryonic structure of monocotyledons, including racidele, embryonic bud, hypocotyl, scutellum, coleoptile and coleorhiza. The regeneration ability of embryonic calli of vetiver was very strong, which could be maintained for 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 MCS of binary vector pCAMBIA1301UN. In addition, an efficient *Agrobacterium*-mediated transformation system of vetiver was also established and four 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. In sum, the obtained results have laid a good foundation for screening transgenic cold-tolerant cultivars of vetiver.

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