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Plant regeneration from petiole callus of *Amorphophallus albus* and analysis of somaclonal variation of regenerated plants by RAPD and ISSR markers

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ABSTRACT. A simple procedure has been outlined for plant regeneration of *Amorphophallus albus* Liu & Wei, a native medicinal plant in China, from petiole-derived callus. Calli were induced at a high frequency of 76.4±3.2% from petiole explants excised from two-month-old plants on Murashige and Skoog (MS) medium supplemented with 5.37 μM α-naphthaleneacetic acid (NAA) and 4.44 μM 6-benzyladenine (BA). Of the different types of callus induced, type III callus was selected for morphogenesis induction. Culture of the callus on MS medium containing proper NAA and BA or KT combinations resulted in formation of corm-like structure (CLS) that produced shoots and roots during further culture. The optimal morphogenetic response was observed on the media with a cytokinin/auxin ratio of about 4:1, which resulted in more than 70% CLS formation and 6~8 CLSs per callus. Complete plantlets with well-developed root systems were obtained from these CLSs by subculturing them on the original media from which they had been derived without a separate rooting culture. Transfer of the plantlets with roots to soil resulted in a more than 90% survival rate. Analysis of 20 regenerated plants by two molecular markers, randomly amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR), revealed somaclonal variation in the regenerated plants. The percentage of polymorphic bands in RAPD and ISSR analysis were respectively 20.8% and 39.0% for the 20 plants. Cluster analysis indicated that the genetic similarity values calculated on the basis of RAPD and ISSR data among the 21 plants (20 regenerated and one donor plant) were, respectively, 0.973 and 0.917, which allowed classification of the plants into distinct groups. A high-frequency somaclonal variation induced in *A. albus* tissue culture may help in the selection of useful variants that may be induced to improve this important corp.

Keywords: *Amorphophallus albus* Liu & Wei; Organogenesis; Molecular marker; Somaclonal variation.

Abbreviations: BA, 6-Benzyladenine; **CLS,** Corm-like structure; **ISSR,** Inter simple sequence repeat; **KT,** Kinetin (6-furfuryl-amino purine); **MS,** Murashige and Skoog medium (1962); **NAA,** α-Naphthaleneacetic acid; **RAPD,** Randomly amplified polymorphic DNA; **UPGMA,** Unweighted pair group method with arithmetic average.

INTRODUCTION

Amorphophallus species, belonging to the family *Araceae*, are perennial herbaceous plants, adapted to the shady and mountainous areas and mainly distributed over Southeast Asia and Africa (Gandawijaja, 1983). There are 22 species grown in China, of which *Amorphophallus albus* Liu & Wei is a native species and mainly cultivated in southwest China (Long, 1998). *Amorphophallus albus* is a traditional medicinal plant with a large subterranean corm and the ability to lower blood cholesterol and sugar levels, help with weight loss, and promote intestinal activity and immune function (Zhang et al., 2005). The corms of *A. albus* are important source of glucomannan, which serves as a gelling agent, thicker, film former, and emulsifier in industry (Nishinari et al., 1992). Recent years have seen a highly accelerated demand for *A. albus* corms, which led to over-harvesting and depletion of its natural resources (Long et al., 2003). Moreover, natural genetic variation is lacking in *A. albus* due to long-term vegetative propagation (by corm setts) and lack of seeds. To date, no successful breeding of improved cultivars of *A. albus* has been documented, even though this crop has been cultivated for hundreds of years. Callus cultures and subsequent regeneration may result in the generation of useful somaclonal variants not available by conventional methods.

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Plant tissue culture has been successfully used in many valuable medicinal plants for efficient propagation (Rout et al., 2000) or for selection of useful mutants (Sato et al., 1988; Bertin et al., 1997). In *Amorphophallus*, plant regeneration has been achieved in some economically important species using corm segments (Asokan et al., 1984; Irawati et al., 1986), leaf (Kohlenbach and Becht, 1988), inflorescence, and bulbil (Zhuang and Zhou, 1987). Wu and Xie (2001) investigated the effect of growth regulators on corm explant callusing in *A. albus*. Successful induction of adventitious bud from corm-derived callus of *A. albus* and establishment of regenerated plants in soil were reported by Liu et al. (2001) and Yan et al. (2005). To our knowledge, however, there has been no report on genomic stability/instability of *A. albus* regenerated plants.

Recently, several DNA markers have been successfully employed to assess the genomic stability/instability in regenerated plants. Among the markers, the randomly amplified polymorphic DNA (RAPD) and the inter-simple sequence repeat (ISSR) have been favored because of their sensitivity, simplicity, and cost-effectiveness (Yang et al., 1996). In this paper, we aim to develop a simple and efficient tissue culture method for *A. albus* and analyze genomic stability or instability of the regenerated plants using RAPD and ISSR markers. The information obtained on genomic variation will be valuable for the specific purpose of utilizing tissue culture as a means for clonal propagation, or for the selection of useful mutants in *A. albus*.

MATERIALS AND METHODS

Seed corms of *Amorphophallus albus* Liu & Wei, obtained from Hubei Konjac Research Institute, germinated in a greenhouse and were used as source material. The petioles excised from the 2-month-old plants were surface sterilized as described by Hu et al. (2005). The basal medium used in all experiments was MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.7% (w/v) Bacto-agar (Difco Laboratories, USA). The specific concentrations of growth regulators added to MS medium were indicated for different treatments. All components of the medium were mixed and adjusted to 5.8 prior to autoclaving. Forty milliliters of medium were dispensed into polyethylene containers (7 cm × 7 cm × 7 cm) and autoclaved at 121°C (1.04 Kg cm^{-2}) for 15 min.

Callus induction and plant regeneration

The sterilized petioles were chopped into segments (4-6 mm in length) and incubated on MS medium supplemented with NAA (2.69, 5.37, 10.74 μM) alone or in combination with BA (2.22, 4.44 μM) for callus induction and subcultured at an interval of 4 weeks. The compact and nodular calli were selected from those in different types and cultured on MS medium with combinations of NAA (0, 0.54, 1.07, 2.15 μM) and BA (2.22, 4.44, 6.66, 8.88, 11.10 μM) or KT (2.32, 4.65, 6.97, 9.29, 11.62 μM) for corm-like structure (CLS) induction and plant regeneration. All cultures were incubated at 25±1°C in a culture room in darkness or under a 12-h photoperiod (45 μmol m^2 s^{-1}) provided by cool-white fluorescent tubes for callus induction or plant regeneration, respectively. Each treatment was repeated thrice with about 30 petiole segments or callus pieces per replicate. Data were statistically analyzed by analysis of variance (ANOVA) followed by Duncan's Multiple Range Test ($P = 0.05$).

Transplantation

CLS culture would lead to development of both shoot and root. Regenerated plants with well-developed roots along with CLSs were isolated from mother tissues and rinsed under running tap water to remove the gel and then transferred to a soil mixture containing garden soil and peatmoss in the ratio of 1:1 (v/v). The plants were watered every 2-3 days, as needed. A month later, the percentage of surviving plants was investigated.

DNA extraction and PCR amplification

The 20 regenerated plants randomly selected from the >300 soil-established plants along with the single donor mother plant were subjected to RAPD and ISSR analysis. Total genomic DNA was extracted from fresh leaves of each individual plant using a CTAB method described by Porebski et al. (1997). Quantity and quality of DNA was inspected by both gel electrophoresis and spectrometric assays.

A total of 62 RAPD and 26 ISSR primers were initially tested using the donor plant DNA (in two replicates) as template to screen for suitable primers. Both types of amplification were performed in a volume of 15 μl containing 30 ng total DNA, 1×PCR buffer, 2.0 mM $MgCl_2$, 1.0 U *Taq* DNA polymerase (Sangon Biotech, Shanghai, China), 0.25 mM dNTPs, and 0.45 μM or 0.9 μM primer (respectively for RAPD or ISSR). Amplifications were performed in a UNO II Biometra thermocycler. The program for RAPD consisted of an initial denaturation of the DNA at 94°C for 4 min, following by 40 cycles of 50 s at 94°C, 50 s at 36°C, 1.5 min at 72°C, and a final 8-min extension at 72°C. The program for ISSR was: DNA denaturation at 94°C for 4 min; 40 cycles of 30 s at 94°C, 45 s at the annealing temperature, and 2 min at 72°C, and an extension at 72°C for 8 min. The annealing temperature was adjusted according to the Tm of the primer being used in the reaction.

The amplified products of RAPD and ISSR were electrophoresed in a 1% and 2% agarose (Sigma, USA) gel with 1×TAE buffer, stained with ethidium bromide, and photographed under ultraviolet light. PCR reactions were repeated at least twice to establish reproducibility of the results.

Data analysis

Only consistently reproducible bands in the size of 150 bp to 2.0 kb were considered for RAPD and ISSR analysis. Each amplified product was scored 1 for presence and 0

for absence of a band across the 20 regenerated plants and one donor mother plant of *A. albus*. Pairwise similarity matrices were generated using Jaccard's similarity coefficient. Data analysis was performed using NTSYSpc, version 2.10. Dendrograms were created with the unweighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973).

RESULTS

Callus induction

Incubated on callus induction media, most petiole segments swelled and disorganized, followed by development of callus masses in the fourth week. Single NAA containing media stimulated the explant callusing at a low frequency (less than 15%), regardless of NAA concentration. In contrast, addition of both NAA and BA to MS medium resulted in a high-frequency callus formation (Table 1). Of the media tested, MS medium plus 5.37 μM NAA and 4.44 μM BA was most effective, indicating by 76.4±3.2% callus induction. Also, the combination of 2.69 μM NAA and 2.22 μM BA gave a positive response, with an induction rate of 68.8±6.7%. Further increases in NAA concentration led to a declining frequency of callus induction and more explant browning. The induced callus, after being subcultured for 2~3 weeks, developed into three different types: (I) translucent and friable, (II) pale and loose, (III) yellowish in color and nodular and compact in structure (Figure 1A). In our experiment, only the type III calli were used for organogenesis induction because this type of callus multiplied easily and was prone to producing globular structures.

Table 1. Effect of different combinations of NAA and BA on callus induction in *A. albus* after 4 weeks of culture.

NAA (μM)	BA (μM)	Callus induction %[a]	Callus feature
2.69	0	13.2±5.1f	Brown, granular
2.69	2.22	68.8±6.7ab	Yellowish, loose
2.69	4.44	39.7±3.6d	Greenish, nodular, compact
5.37	0	12.4±6.3f	Brown, granular
5.37	2.22	40.5±3.5d	Yellowish, loose
5.37	4.44	76.4±3.2a	Yellowish, nodular, compact
10.74	0	8.7±4.5f	Explant browning
10.74	2.22	27.3±3.7e	Brown, granular
10.74	4.44	53.1±5.7c	Brown, friable

[a]Means followed by the same letters within a column are not significantly different using Duncan's Multiple Range Test ($p<0.05$). Values represent the means of three replicates±SE.

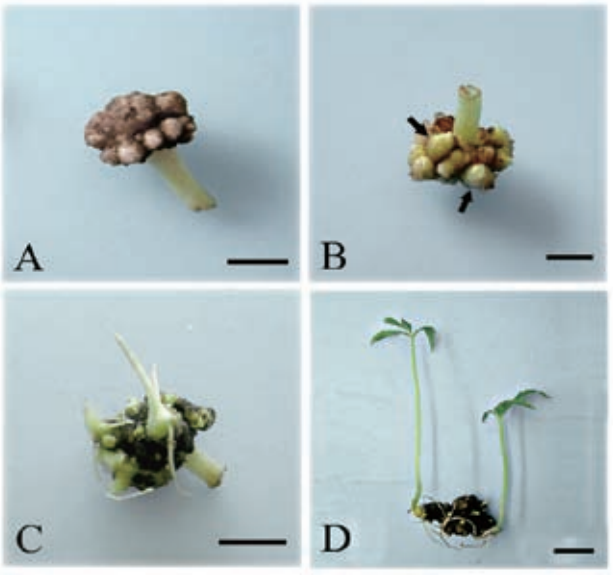

Figure 1. CLS formation and plant regeneration from petiole callus of *A. albus*. (A) Nodular and compact calli (type III) induced from petiole segment on MS medium supplemented with 4.44 μM BA and 5.37 μM NAA. Bar = 5 mm; (B) Numerous CLSs (arrows) formed on the surface of the type III callus incubated on MS medium supplemented with 8.88 μM BA and 2.15 μM NAA. Bar = 5 mm; (C) Apical buds of CLSs geminated and roots developed at base of the buds with the combination of 8.88 μM BA and 2.15 μM NAA. Bar = 10 mm; (D) Complete plantlets regenerated from the CLSs on MS medium plus 8.88 μM BA and 2.15 μM NAA. Bar = 10 mm.

Plant regeneration

Type III calli (approx. 500 mg) were transferred to MS medium with various combinations of NAA and BA or KT for plant regeneration. After 4 weeks of incubation, most calli revealed a special morphogenesis that could be divided into two stages: the formation of CLSs from the calli (Figure 1B) in the first stage, and an almost synchronous development of both shoots and roots from the CLSs (Figure 1C) in the second one. This led to the formation of complete plants with root systems (Figure 1D), and it distinctly differed from the common *in vitro* pathway, which only produced shoots.

The frequency of CLS occurrence varied with BA/NAA or KT/NAA combinations added to MS medium. The use of proper molar ratios of cytokinin to auxin (≈ 4:1) was a key factor promoting CLS formation (Table 2). Among the phytohormone combinations tested, 1.07 μM NAA in combination with 4.44 μM BA (BA/NAA ratio = 4.15:1) proved most effective, giving rise to 78.6±7.4% CLS formation and a mean 7.8±1.2 CLSs per callus. The other phytohormone combinations with a cytokinin/auxin ratio of about 4:1—i.e. 2.22 μM BA + 0.54 μM NAA, 8.88 μM BA + 2.15 μM NAA, 2.32 μM KT + 0.54 μM NAA, 4.65 μM KT + 1.07 μM NAA—also resulted in more than 70% CLS formation and 6~8 CLSs per callus. No statistically significant difference was found among these

Table 2. Effect of different phytohormone combinations on CLS formation from calli of *A. albus* after 4 weeks of culture.

Phytohormone combinations (μM)	Cytokinin/Auxin molar ratios	Shooting response %[a]	Number of shoots per callus[a]
BA + NAA			
2.22 + 0	-	12.2±4.3i	2.6±0.4g
2.22 + 0.54	4.11	70.2±5.4a	6.4±1.1bcd
4.44 + 0.54	8.22	26.5±7.4gh	3.2±0.6fg
2.22 + 1.07	2.07	41.8±7.7def	4.6±0.8def
4.44 + 1.07	4.15	78.6±7.4a	7.8±1.2a
6.66 + 1.07	6.22	32.2±9.3fg	5.0±1.1cde
6.66 + 2.15	3.10	54.5±2.9bcd	6.2±0.9bcd
8.88 + 2.15	4.13	72.1±10.8a	7.2±0.6ab
11.10 + 2.15	5.16	18.7±6.1hi	2.7±0.7g
KT + NAA			
2.32 + 0	-	10.7±4.6i	3.1±0.7fg
2.32 + 0.54	4.29	71.2±9.3a	7.5±1.0a
4.65 + 0.54	8.59	35.3±5.7efg	4.2±1.7efg
2.32 + 1.07	2.16	48.1±7.4cde	5.3±1.3bcde
4.65 + 1.07	4.35	76.2±7.2a	6.5±1.3bc
6.97 + 1.07	6.51	57.1±7.8bc	5.2±0.9cde
6.97 + 2.15	3.24	65.4±7.7ab	6.5±1.1bc
9.29 + 2.15	4.32	56.6±1.0bc	6.2±.10bcd
11.62 + 2.15	5.40	16.2±8.0hi	3.0±0.8fg

[a]Means followed by the same letters within a column are not significantly different using Duncan's Multiple Range Test ($p<0.05$). Values represent the means of three replicates±SE.

combinations. Higher cytokinin/auxin ratios resulted in repression of CLS formation and stimulated adventitious bud differentiation. Lower ratios of BA to NAA—e.g. 2.22 μM BA + 1.07 μM NAA and 2.32 μM KT + 1.07 μM NAA (cytokinin/auxin ratio ≈ 2:1)—repressed CLS formation and promoted callus growth.

Maintained on fresh medium with the same components as the original media for 2 weeks, most CLSs developed into complete plantlets (Figure 1D). The percentage of CLSs converting to plants exceeded 80% in our experiment. These plants along with the basal CLSs were excised from mother tissues and transferred directly to soil after removal of the gel attached to the roots. One month later, more than 90% of the plants survived under *ex vitro* conditions and grew vigorously. Thus, within a time frame of about 4 months, a population of >300 CLS-derived plants, originating from a single mother donor plant, was established in the field. No discernible morphological difference was found between the mother plant and the regenerated plants.

RAPD and ISSR fingerprinting

In order to assess the genetic stability/instability of the regenerated plants, a comparison of the RAPD and ISSR fingerprinting of 20 randomly-selected regenerated plants and their single donor mother plant was carried out. In an initial screening, 21 decamer oligonucleotides were selected from 62 decamer RAPD primers due to the generation of clear and reproducible amplification products (Table 3). Seven of the 21 primers (33.3%) produced monomorphic band patterns while each of the remaining 14 primers generated polymorphic bands in at least one of the 20 individuals relative to the donor plant. Of a total of 154 bands scored, which ranged from 150 bp to 2 kb and averaged 7.3 bands per primer, 32 were polymorphic with 20.8% polymorphism. The highest percentage of polymorphic bands (50%) was possessed by primer S437. The changed banding patterns included both loss of original bands (Figure 2A) and appearance of novel bands (Figure 2B), which occurred at more or less equal frequencies. Cluster analysis was done on the basis of similarity coefficients among the 21 individuals (20 regenerated and one donor plant) generated from RAPD data. The similarity coefficients ranged from 0.85 to 1.00 among the plants with a mean of 0.973. All 21 plants could be clustered into one major group at an 85% similarity level. However, four groups could be obtained at a 93% similarity level (Figure 3).

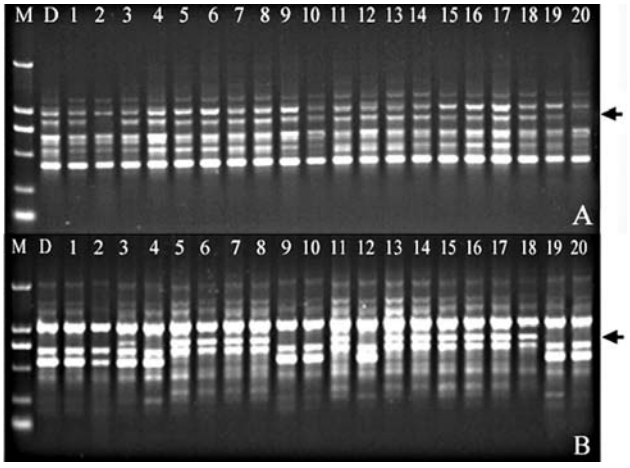

Figure 2. RAPD profiles of 20 regenerated plants (lane 1~20) and their donor mother plant (D) produced using the decamer primers S230 (A) and S437 (B). M, DNA ladder, DL2000 marker (Takara, Dalian, China). Polymorphisms included both absence of original bands (A, arrow) and gain of novel bands (B, arrow).

Table 3. Description of twenty one RAPD primers used for fingerprint analysis of 20 plants regenerated from petiole callus of *A. albus*.

Primers	Sequence (5'-3')	Size of the amplified bands (bp)	Scored bands	Polymorphic bands	
				Number	Frequency (%)
S35	TTCCGAACCC	300-1800	9	4	44.4
S67	GTCCCGACGA	500-2000	7	0	0
S70	TGTCTGGGTG	500-1000	5	2	40.0
S71	TGTCTGGGTG	250-1800	9	4	44.4
S75	GACGGATCAG	250-1500	7	1	14.3
S80	CTACGGAGGA	250-1500	6	0	0
S161	ACCTGGACAC	500-2000	8	2	25.0
S163	CAGAAGCCCA	250-1000	7	0	0
S226	ACGCCCAGGT	250-1500	10	2	20.0
S230	GGACCTGCTG	300-1000	7	1	14.3
S236	ACACCCCACA	300-2000	7	3	42.9
S353	CCACACTACC	250-2000	10	3	30.0
S380	GTGTCGCGAG	150-1000	9	0	0
S387	AGGCGGGAAC	500-2000	9	3	33.3
S425	ACTGAACGCC	250-1500	8	0	0
S427	CAGCCCAGAG	250-1800	7	0	0
S429	TGCCGGCTTG	300-1800	5	1	20.0
S431	TCGCCGCAAA	500-2000	6	2	33.3
S437	CATTGGGGAG	250-2000	6	3	50.0
S504	CCCGTAGCAC	250-1800	6	1	16.7
S507	ACTGGCCTGA	250-1800	6	0	0
Total			154	32	20.8

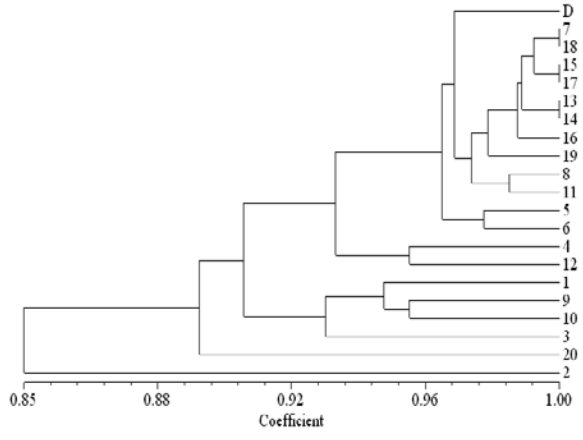

Figure 3. Dendrogram illustrating coefficient similarities among 20 regenerated plants (1~20) and their donor mother plant (D) of *A. albus* based on RAPD data.

In ISSR analysis, 26 anchored microsatellite primers including di-, tri- and tetranucleotide repeat motifs were tested individually to amplify the genomic DNA of the *A. albus* donor plant, out of which 11 primers generated well resolved reproducible band patterns (Table 4). The eleven ISSR primers yielded 136 scorable bands, of which 53 were polymorphic. In comparison to RAPD, ISSR primers produced a higher percentage of polymorphism (39.0%) in the same 20 individuals. An average of 12.4 bands per primer was recorded, and band size varied from 150 bp to 2 kb. The number of bands from each primer varied from 9 bands in UBC840 and UBC873 to 16 bands in UBC866. All the primers were found to be polymorphic and produce different percentages of polymorphism, ranging from the 14.3% produced by UBC818 to the 72.7% by UBC846. As in the RAPD results, the variable bands in the regenerated plants relative to the donor plant also included both the loss of original bands (Figure 4A) and the appearance of

Table 4. Description of eleven ISSR primers used for fingerprint analysis of 20 plants regenerated from petiole callus of *A. albus*.

Primer	Sequences	Size of the amplified bands (bp)	Scored bands	Polymorphism bands	
				Number	Frequency (%)
UBC818	$(CA)_8G$	250-1500	14	2	14.3
UBC825	$(AC)_8T$	250-1800	15	6	40.0
UBC826	$(AC)_8C$	300-1800	13	4	33.3
UBC840	$(GA)_8YT$	150-1500	9	5	55.6
UBC846	$(CA)_8RT$	500-2000	11	8	72.7
UBC850	$(GT)_8YC$	250-2000	11	6	54.5
UBC857	$(AC)_8YG$	250-1500	12	5	41.7
UBC858	$(TG)_8RT$	250-2000	12	4	33.3
UBC864	$(ATG)_6$	200-2000	14	2	14.3
UBC866	$(CTC)_6$	200-1800	16	8	50.0
UBC873	$(GACA)_4$	300-2000	9	3	33.3
Total			136	53	39.0

R = A/G; Y = G/T.

novel bands (Figure 4B). In contrast to the RAPD results, the total number of lost bands in the 20 regenerated plants was much larger than that of novel bands (202 versus 106). The coefficient of similarity in the dendrogram generated by the ISSR data among the 20 regenerated plants and their donor plant ranged from 0.80 to 0.97 with a mean of 0.917. The associations among the 21 plants were similar to those of revealed by the RAPD analysis (a cluster analysis). One major group including all the 21 plants could be obtained with the similarity level set at 80%. However, only at an 85% similarity level could these plants be clustered into four groups (Figure 5).

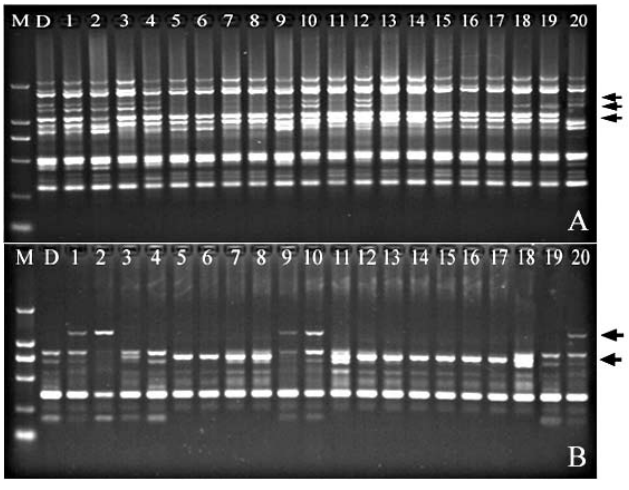

Figure 4. ISSR profiles of 20 regenerated plants (lane 1~20) and their donor mother plant (D) produced using the ISSR primers UBC825 (A) and UBC840 (B). M, DNA ladder, DL2000 marker (Takara, Dalian, China). Polymorphisms included both absence of original bands (A, arrow) and gain of novel bands (B, arrow).

DISCUSSION

Amorphophallus albus is a multipurpose plant in China that has attracted increasing attention due to its economic and medicinal importance. Its propagation has proven difficult because the corms have to grow for at least three years before harvest, and the cormels (usually used as the succeeding year's seed corms) produced by the mother corms are quite few (Long, 1998). That makes it urgent to develop a rapid method of multiplication for commercial cultivation of this important crop. Here, we presented a new method for propagating *A. albus* via corm-like structure (CLS) induction, which differed from previously reported methods that produced only adventitious buds (Liu et al., 2001; Wu and Xie, 2001; Yan et al., 2005). In *A. albus* tissue culture, a callus phase is required for obtaining regeneration, and, to date, no information is available about direct organogenesis from explants. Our results suggest that combinations of NAA and BA at approximately equal concentrations favour callus induction, indicating the need for a proper balance of auxin and cytokinin in petiole explant callusing. Similar findings were described by Yan et al. (2005) in *A. albus* and Liu et al. (2001) in *Amorphophallus konjac* Koch. The type III callus formed in the present experiment was a desirable tissue type characterized by a compact texture and nodular structure and the capacity to produce CLSs (Hu et al., 2004). In some other species, a similar type of callus was also considered an alternative tissue type that could multiply and easily differentiate shoots or other organs (Teng, 1997; Te-Chato and Lim, 2000).

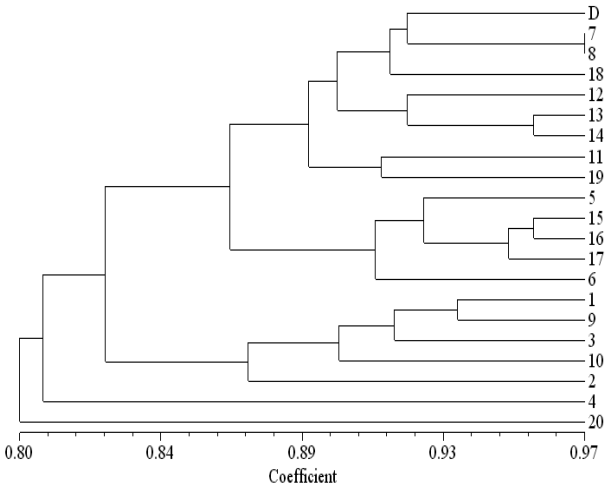

Figure 5. Dendrogram illustrating coefficient similarities among 20 regenerated plants (1~20) and their donor mother plant (D) of *A. albus* based on ISSR data.

As previously observed (Hu et al., 2004), culture of type III callus resulted in formation of numerous CLSs that produced complete plantlets during further culture. Similar morphogenetic events have been reported by Irawati et al. (1986) in tissue culture of *Amorphophallus paeoniifolius* (Dennst.) Nicols., where the globular structures were developed from the long-time conserved calli and could produce shoots and roots if transferred to fresh medium. During *A. konjac* callus culture, we also found that CLSs frequently occurred and exhibited the same morphogenetic pathway as those in present study (Liu et al., 2001; Hu et al., 2005). From these findings, we might conclude that CLS widely exists in the tissue culture of *Amorphophallus* species. Although CLS formation was frequently accompanied by adventitious bud differentiation in *A. albus*, we could adjust the cytokinin/auxin ratio of the medium to promote CLS formation and reduce adventitious bud differentiation. Maintenance of a molar ratio of cytokinin to auxin (≈ 4:1) was a key factor promoting a high-frequency CLS formation. The proper proportion of auxin to cytokinin might support a balance of endogenous growth substances in callus that favoured corm organogenesis. Liu et al. (2001) reported that in *A. konjac* seed-derived callus culture, cormlets frequently occurred in the presence of moderate concentrations of BA and low concentrations of NAA, where the BA/NAA ratio was approximately 5:1. This finding approximated what was observed in the present study.

Based on the findings above, a two-step propagation protocol including callus induction (step 1) and CLS induction and plant regeneration (step 2) can be developed. The obvious advantages of the protocol lie in its: (1) technical simplicity (includes only two steps and excludes rooting culture); (2) shortened time span of plant regeneration (about 3 months from explant incubation to plant regeneration); and (3) development of complete regenerated plants. The strong morphogenetic abilities revealed by the CLSs, i.e., production of both shoots and roots, might be due to the pre-existing bud and root primordial in CLS developed in the morphogenesis induction stage (Hu et al., 2005; unpublished work). It must be noted that, apart from CLS production in our experiment, adventitious buds also differentiated from some calli. These buds, however, were not used for *A. albus* propagation because of the low frequency of conversion to normal shoots and the need for rooting culture.

It is well known that *in vitro* culture conditions act as a stress factor and elicit genetic instability in cultured cells, tissues and organs, a phenomenon known as somaclonal variation (Larkin and Scowcroft, 1981; Karp, 1995). This instability may be a risk associated with the application of *in vitro* techniques to commercial micropropagation and germplasm conservation. Conversely, somaclonal variation may provide another source of novel and useful variability, which can be used in crop improvement, particular for species with a narrow genetic background (Karp, 1995). The introduction of valuable variation through somaclonal variation may help in programmes designed to improve the characteristics of *A. albus*. Though the 20 randomly tagged regenerated plants were phenotypically normal and essentially identical with their mother donor plant, they showed apparent genetic variations when subjected to RAPD and ISSR analysis. Similar findings on genomic variation in phenotypically normal regenerants have been well documented in some other plants (Diwan and Cregan, 1997; Rahman and Rajora, 2001; Kawiak and Lojkowska, 2004). There were two probable reasons for the incongruence of phenotypic versus genomic stability in regenerated plants: (1) the tissue culture-induced genomic changes mainly take place at non-coding regions, which hardly affects gene expression, and hence, phenotype; and (2) even if the changes occur in coding regions, the probability of simultaneous mutation of two alleles in a diploid plant is extremely low and has little effect on phenotypic characteristics.

In our study, the percentage of polymorphic bands among the 20 regenerated plants detected by ISSR was much higher than that detected by RAPD, indicating that the former was more discriminative than the latter of genomic variations in *A. albus*. Meanwhile, our study also shows that *A. albus* genome seems to be rich in dinucleotide repeat motifs like (CA)n and (GA)n and that these repeat regions are prone to change under *in vitro* culture conditions. In a separate experiment, we found that the same set of ISSR primers could produce a high-frequency polymorphism in detection of somaclonal variation in *A. konjac* (Hu et al., 2007). According to Wang et al. (1994), dinucleotide microsatellites are prevalent in plants while mono-, tri-, and tetranucleotide repeats are less common. As observed in band investigation, the ratios of band loss to band gain were distinctly different between RAPD and ISSR, with that of the latter being much higher. This may be because RAPD and ISSR primers target different regions of the *A. albus* genome, and different

regions have different sensitivities to tissue culture conditions, facts demonstrated in several plant species (Xie et al., 1995; Devarumath et al., 2002).

In conclusion, we report herein a simple and efficient tissue culture protocol for obtaining numerous complete regenerated plantlets of *A. albus* in a short period of time. Genomic variations revealed by RAPD and ISSR marker are apparent in regenerated plants, which may be an important source of useful variants that can be used to improve this important crop at the cellar level. The regenerated plants are currently being screened in the field for corm size and glucomannan content.

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白魔芋葉柄愈傷組織植株再生及其無性系變異的 RAPD 和 ISSR分析

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本文概述了一種用於中國藥用植物白魔芋組織培養的簡便方法，即從葉柄愈傷組織誘導植株再生。採用 2 個月苗齡植株的幼葉柄為外植體，接種於附加 5.37 μM NAA 和 4.44 μM BA MS 培養基，愈傷組織誘導頻率高達 76.4±3.2%。所誘導的愈傷組織類型各異，其中 III 型愈傷組織用於形態發生的建立。III 型愈傷組織培養於加有適宜濃度的 NAA 和 BA 或 KT 組合的 MS 培養基上可形成擬球莖結構，繼續培養擬球莖結構便可產生芽和根。最佳形態發生反應出現在細胞分裂素與生長素濃度比約為 4:1 的培養基上，即擬球莖形成頻率約為 70%，每塊愈傷組織長可形成 6~8 個擬球莖。將擬球莖繼代培養於其形成的培養基上，不另需生根培養就可獲得具有完整根系的植株。具有根系的再生植株移栽至大田後的存活率在 90% 以上。採用兩種分子標記技術，即隨機擴增多態性（RAPD）和簡單重複間序列（ISSR），對 20 株再生植株進行變異檢測並發現植株中存在變異。在這 20 株植株中，RAPD 和 ISSR 分別檢測到 20.8% 和 39.0% 多態性頻率。對 RAPD 和 ISSR 資料分別進行聚類分析，結果表明 21 株植株（20 株再生植株和1株母本）的遺傳相似係數分別為 0.973 和 0.917，且能分為不同的族群。白魔芋組織培養中所誘導的高頻無性系變異，可能有助於有用突變體的篩選從而用於其遺傳改良。

關鍵詞：白魔芋；器官發生；分子標記；無性系變異。