



ISSR ANALYSIS OF SOMACLONAL VARIATION IN CALLUS-DERIVED PLANTS OF *AMORPHOPHALLUS RIVIERI* DURIEU

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This communication reports detection of somaclonal variation among tissue culture-raised plants of *Amorphophallus rivieri* Durieu, an economically important crop in China, with high content of glucomannan in its corms. A population of regenerated plants was obtained from a single donor plant of *A. rivieri* via corm organogenesis, and 28 plants were randomly selected as a representative sample and subjected to analysis of somaclonal variation using inter-simple sequence repeat (ISSR) markers. Of the 26 ISSR primers screened, 13 gave distinct and reproducible band patterns, yielding 131 bands with an average of 10.1 bands per primer. Ten primers were polymorphic and generated 16 polymorphic bands with 12.2% mean polymorphism. Based on the ISSR data from the regenerated plants and the donor plant, Jaccard's similarity coefficients were calculated; they ranged from 0.961 to 1.000 with a mean of 0.982. A dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA); it showed that a majority of regenerated plants (including the donor plant) clustered closely, with a mean similarity coefficient of 0.987. Low somaclonal variation observed in the regenerated plants indicates that rapid propagation of *A. rivieri* via corm organogenesis is a practicable method with a low risk of genetic instability.

Key words: *Amorphophallus rivieri* Durieu, ISSR marker, regenerated plants, somaclonal variation.

INTRODUCTION

Amorphophallus rivieri Durieu is an economically important crop in China, cultivated mainly for its glucomannan in corms, a high molecular weight carbohydrate widely used in the food, medicine and chemicals industries (Nishinari et al., 1992). Traditionally, propagation of *A. rivieri* is through division of the corms, a procedure requiring more than 20% of the harvested corms as the next season's "seeds". The seed corms often grow in the field for more than three years before harvesting (Zhang et al., 1999). The lack of an efficient propagation system in *A. rivieri* hampers its large-scale cultivation, causing an acute shortage of commercial corms produced in China. The recent discovery of pharmacological functions of glucomannan, for example lowering blood cholesterol and sugar levels, helping with weight loss and promoting intestinal activity and immune function, has accelerated demand for *A. rivieri* corms on both domestic and international markets (Zhang et al., 2005).

Tissue culture of *A. rivieri* has been the subject of several studies focusing on establishment of an in vitro propagation system (Asokan et al., 1984;

Kohlenbach and Becht, 1988; Huang et al., 2003; Hu et al., 2005). In all of these studies, regeneration was achieved based on subculture of calli, which can cause somaclonal variation in the regenerated plants and thereby affect the glucomannan content of the corms produced by regenerated plants. Although the variation of the chromosome ploidy and karyotype of tissue culture-raised plants of *A. rivieri* was reported to be relatively low (Danfeng et al., 1995), stability/instability at the DNA level remains unknown.

The aim of this study was to detect the probable somaclonal variation in callus-derived plants of *A. rivieri* using an efficient molecular marker, inter-simple sequence repeats (ISSR). The information gained on the degree of genomic variation will furnish reference values of use in exploiting tissue culture-raised plants for large-scale cultivation of *A. rivieri*.

MATERIALS AND METHODS

Young petioles were excised from a two-month-old plant of *A. rivieri* and surface-sterilized by successive immersion for 30 s in 75% (v/v) ethanol and 10

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TABLE 1. Description of 13 ISSR primers used for fingerprint analysis of regenerated *A. rivieri* plants. R = A/G; Y = G/T

Primers	Sequence (5'-3')	T_m (°C)	Size of amplified bands (bp)	Scored bands	Polymorphic bands	% Polymorphism
UBC811	(GA) ₈ C	56	1000-100	10	1	10.0
UBC815	(CT) ₈ G	58	1500-100	11	1	9.1
UBC818	(CA) ₈ G	58	1000-200	11	1	9.1
UBC825	(AC) ₈ T	57	1500-100	9	0	0
UBC834	(AG) ₈ YT	55	1000-100	9	1	11.1
UBC840	(GA) ₈ YT	55	1500-200	9	0	0
UBC843	(CT) ₈ RT	54	1000-250	8	0	0
UBC845	(CT) ₈ RC	56	1000-250	10	2	20.0
UBC846	(CA) ₈ RT	57	2000-200	12	4	33.3
UBC857	(AC) ₈ YG	57	1000-100	13	2	15.4
UBC864	(ATG) ₆	53	1500-200	12	2	16.7
UBC873	(GACA) ₄	55	1000-250	9	1	11.1
UBC881	(GGGTG) ₃	61	1000-150	8	1	12.5
Total				131	16	12.2

min in 0.1% (w/v) mercuric chloride (HgCl_2) solution containing a few drops of Tween-20. After four rinses in sterile water, petioles were chopped into segments (2–3 mm thick) and incubated in darkness on MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mg L⁻¹ NAA, 1.0 mg L⁻¹ BA, 3% (w/v) sucrose and 0.7% (w/v) agar (Difco Bacto-agar) (pH 5.8) for callus induction. The induced calli were subcultured at 4-week intervals on callus proliferation medium, which was the same as the induction medium but with the auxin and cytokinin concentrations halved. Nodular compact calli were selected from the cultures and cultured on MS medium supplemented with 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ BA for plant regeneration under a 10 h photoperiod with 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. Culture room temperature was controlled at $25 \pm 1^\circ\text{C}$. Previous observations (Hu et al., 2005) had indicated that complete plantlets with root systems would be obtained through corm organogenesis. At the end of the culture period, well-rooted plantlets were transferred directly to plastic pots containing a 1:1 (v/v) mixture of peatmoss and perlite and kept in shade.

Genomic DNA was extracted from fresh leaves of 28 regenerated plants and the donor plant as described by Porebski et al. (1997). A total of 26 ISSR primers were initially tested using donor plant DNA as template to screen for suitable primers (in two replicates). ISSR-PCR amplifications were carried out in a 25 μL volume containing 15 ng total DNA, 1 PCR buffer, 2.0 mM MgCl_2 , 1.5 units Taq DNA polymerase (Promega U.S.A.), 0.2 mM dNTPs

and 0.9 μM ISSR primer. Amplifications were performed in a PTC-200 thermocycler with the program as follows: initial denaturation for 4 min at 94°C , followed by 40 cycles of 30 s at 94°C , 45 s at specific annealing temperature (T_m) (Tab. 1) and 1.5 min at 72°C , and a final 6 min extension at 72°C . Amplified products were electrophoresed in 2.0% (w/v) agarose (Sigma U.S.A.) gels with $1 \times \text{TAE}$ buffer, stained with ethidium bromide, and photographed under ultraviolet light.

The PCR reaction for each ISSR primer was performed twice in total and only bands reproducible between replicates were used in further analyses. Each amplified product was scored across the 28 regenerated plants and donor plant of *A. rivieri*. The presence (1) or absence (0) of a band was scored for each ISSR primer in a binary matrix and genetic similarity was calculated with Jaccard's coefficients (Jaccard, 1908). The similarity coefficients were then used to construct a dendrogram by UPGMA (unweighted pair-group method with arithmetic mean) using NTSYSpc 2.10e software (Rohlf, 2000).

RESULTS AND DISCUSSION

Calli were initiated with $\sim 75\%$ frequency from petiole explants of *A. rivieri* on MS containing 1.0 mg L⁻¹ NAA and 1.0 mg L⁻¹ BA. After three further rounds of subculture on MS with 0.5 mg L⁻¹ NAA and 0.5 mg L⁻¹ BA, the calli converted to two distinct types: loose calli

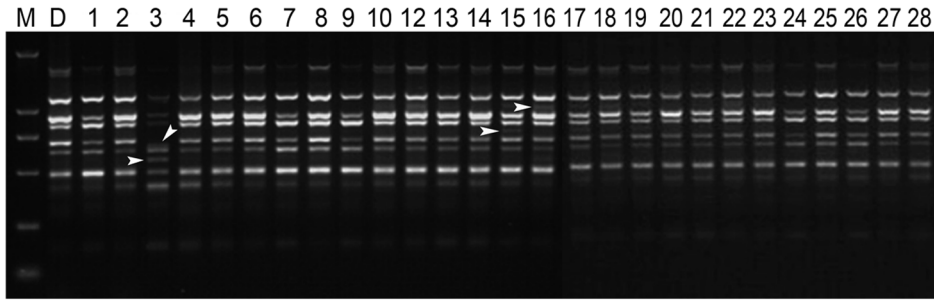


Fig. 1. A band pattern generated by primer UBC846. Line M represents marker DL2000, Line D represents the donor plant, and Lines 1–28 represent callus-derived plants of *A. rivieri*. White arrows indicate polymorphic bands.

and nodular compact calli. Loose calli appeared yellowish, grew fast, and tended to produce multiple tiny buds that were difficult to convert to normal shoots. Nodular compact calli were characterized by dark green "epidermis" and nodular compact texture; they grew slowly but readily produced corm-like structures that could produce complete plants with roots (Hu et al., 2005). The nodular compact calli were selected and transferred to MS medium supplemented with 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} BA for plant regeneration. Two weeks later, as expected, complete plantlets with root systems appeared via corm organogenesis. Omission of the rooting treatment makes this propagation approach quite convenient, and it should produce *A. rivieri* test-tube plantlets on a large scale if somaclonal variation among the plants is minimal. Within five months a population of >200 regenerated plants was obtained from a single donor plant. More than 90% of the plants survived transplantation to the field. No morphological abnormalities versus the donor plant were observed in the regenerated plants.

To estimate the probable somaclonal variation derived from in vitro culture, we randomly selected 28 plants from the population and subjected them to ISSR analysis. Of the 26 ISSR primers initially tested, 13 gave distinct and reproducible band patterns. A total of 131 bands ranging from 100 bp to 2 kb in size were generated across the plant set. The number of bands varied from 8 (UBC843 and UBC881) to 13 (UBC857), with an average of 10.1 per primer (Tab. 1). Ten primers were polymorphic and generated 16 polymorphic bands which were present or absent in the donor plant but absent or present in some of the regenerants. Of the polymorphic primers, UBC846 detected the highest percentage of polymorphic loci (33.3%) (Fig. 1), and the others gave 9.1–20.0% polymorphism. Overall the percentage of polymorphism among the regenerated *A. rivieri* plants averaged 12.2%, much lower than that reported in other micropropagated plants (Guo et al., 2006; Hu et al., 2008; Huang et al., 2009;

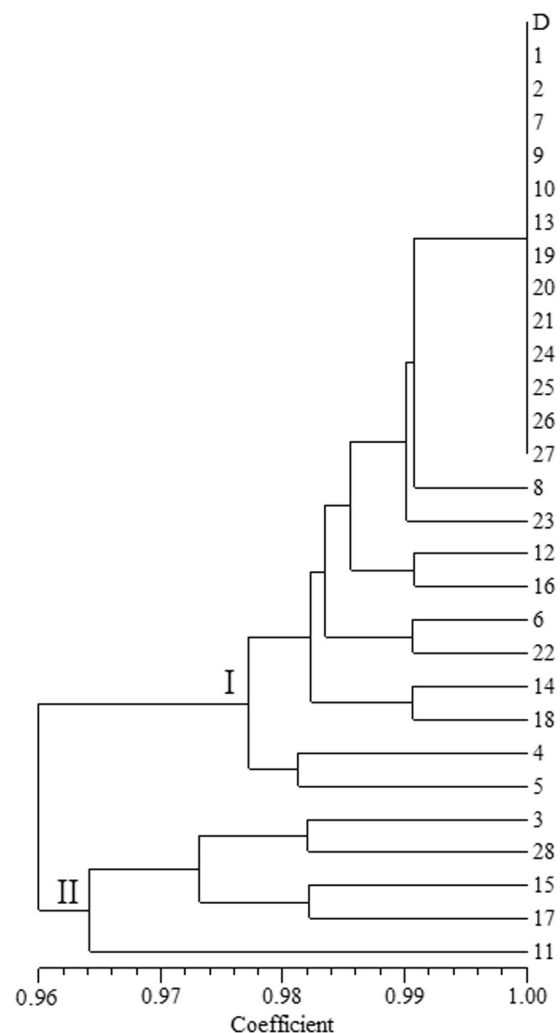


Fig. 2. UPGMA dendrogram derived from Jaccard's similarity coefficients calculated among 28 regenerated plants (1–28) and one donor plant (D), based on amplification profiles generated by ISSR markers.

Bhattacharya et al., 2010) where the frequencies of culture-induced polymorphism detected by RAPD and/or ISSR markers were between 15.7% and 39.0%. There were a limited number of variable loci in the *A. rivieri* genome of plants propagated via corm organogenesis.

The Jaccard's similarity coefficients calculated based on the ISSR data from the 29 plants (28 regenerated plants and donor plant) ranged from 0.961 to 1.000, with a mean of 0.982. Cluster analysis was done on the basis of the similarity coefficients to reveal the associations between the plants. All the plants were divided into two broad groups (I and II) (Fig. 2). Group I contained 24 (82.8%) of the 29 plants and their mean similarity coefficient was 0.987. In this group, 13 regenerated plants (44.8%) showed 100% similarity to the donor plant and the others (38.0%) showed differences from the donor plant at only one or two loci. Group II included the remaining 5 regenerated plants (17.2%), each of which contained three to six variation loci. From this analysis it is clear that a majority of the regenerated *A. rivieri* plants exhibited genetic stability or extremely low variation, and that genetic instability was restricted to a small number of regenerated plants. Our culture of nodular compact calli did not produce extensive DNA variation in *A. rivieri*. It is suitable for rapid propagation of *A. rivieri* and can fill the gap left by the present lack of a good protocol for direct regeneration from *A. rivieri* explants. The low somaclonal variation we observed can be attributed to the choice of material – nodular compact calli – instead of fast-growing calli, which are likely to produce more extensive DNA variation.

ISSR is a useful marker system which has been successfully employed to measure genetic stability/instability in tea (Thomas et al., 2006), *Prunus mume* (Ning et al., 2007), *Swertia chirayita* (Joshi and Dhawan, 2007), *A. albus* (Hu et al., 2008), London plane tree (Huang et al., 2008) and gerbera (Bhatia et al., 2009). It has several obvious advantages over random amplified polymorphic DNA (RAPD), such as higher reproducibility and informativeness (Nagaoka and Ogihara, 1997), which make ISSR more attractive for genotyping and diversity studies. Although no phenotypic variation was observed in the regenerated *A. rivieri* plants, ISSR variation did exist. There are two probable explanations for this incongruity between phenotypic and DNA levels: (1) ISSR variation occurs in non-coding regions of *A. rivieri* genome; and (2) even if the variation occurs at the transcribed sequences, the probability of simultaneous mutation of two alleles in a diploid plant is extremely low. Therefore, these two cases did not affect the phenotypic characters of regenerated *A. rivieri* plants.

To the best of our knowledge this is the first report on assessment of DNA variation in *A. rivieri*

plants regenerated in vitro. Our results showed low frequency (12.2%) of ISSR variation in regenerated plants obtained via corm organogenesis; most of the generated plants had high coefficients of similarity (0.981) to the donor plant. Rapid propagation of *A. rivieri* via corm organogenesis seems practicable, with a low risk of genetic instability.

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