Assessment of genetic fidelity of *Fritillaria dagana* (Liliaceae) regenerated plants using ISSR markers

Dinara S. Muraseva[1,*], *Elena V.* Kobozeva[1,2], and *Tatyana I.* Novikova[1]

[1]Central Siberian Botanical Garden, Siberian Branch RAS, 630090 Novosibirsk, Russia
[2]National Research Tomsk State University, 634050, Tomsk, Lenina pr., 36

Abstract. ISSR analysis of *Fritillaria dagana*, endangered ornamental geophyte from the Sayan Mountains, regenerated through direct gemmogenesis from bulb scale tissue was performed. More informative electrophoresis profile with clear and distinct bands was obtained at amplification with $(CAC)_3GC$ primer at 56 ºC annealing. The genetic fidelity of *F.dagana* regenerants to maternal plants was confirmed.

1 Introduction

The genus *Fritillaria* L. (Liliaceae) presents ephemeral bulbous plants and comprises more than 150 species distributed within the Northern Hemisphere with a pronounced center of diversity in southwestern and Himalayan Asia [1]. Twelve of fritillaria species occur in the territory of Russia, therewith five of them are listed in the Red Data Book of the Russian Federation [2] with the status of rare species. Since many natural species of the genus *Fritillaria* are endangered due to irregular gathering the bulbs and flowering shoots, it is necessary to find new effective approaches for their conservation. Previously we developed the protocol of clonal micropropagation of local endemic *Fritillaria sonnikovae* Schaulo and A. Erst from West Sayan for creation of *in vitro Fritillaria* collection [3].

Fritillaria dagana Turcz. ex Trautv. is an endemic species listed in the Red Data Book of the Russian Federation [2] with the status of a rare species (3a) which occurs in certain areas of Siberia, East Sayan and Southern Baikal. An efficient micropropagation protocol of *F. dagana* was developed for the first time [4].

Although clonal propagation should generate plantlets identical to the mother plants, studies have shown that genetic and epigenetic changes called somaclonal variations may occur due to callus formation, use of growth regulators and long period of cultivation. Therefore the risk of somaclonal variation appearance should be assess by using various methods including morphological, biochemical and molecular ones [5, 6]. Molecular methods represent an effective tool for detection of regenerant genetic variability, which is explained by a higher level of DNA changes in comparison with morphological polymorphism. One of the effective methods required in this study is the ISSR analysis (Inter Simple Sequence Repeats). This method is easy to use, low-cost, high reliable,

* Corresponding author: dsmuraseva@csbg.nsc.ru

reproducible, and methodologically less demanding compared to other dominant markers [7, 8, 9]. To date, there is a large number of studies devoted to the detection of somaclonal variation using the ISSR analysis [10, 11, 8].

The present study was undertaken to assess somaclonal variation with the use of ISSR markers in *F. dagana* regenerants obtained through direct gemogenesis from bulb scale tissue.

2 Material and methods

The aseptic scales were cut into the segments of 5 × 5 mm size and used as primary explants. Scale segments (for 4–5 pcs.) were placed by cut-surface down onto induction medium. The cultivation was carried out on solidified B_5 medium [12] supplemented with 5.0-μM 6-benzylaminopurine (BAP) and 2.0-μM α-naphthalene acetic acid (NAA). Microclones of *F. dagana* obtaned through direct regeneration from bulb scale tissue were used for genetic analysis. *In vitro*-raised microbulbs (regenerants) and maternal bulb scale segments were analysed.

DNA was extracted from silica dried bulblets using a Food and Raw materials extraction DNA Kit (BioSilica, Russia) and purified on columns following the manufacturer's protocol. Purity and concentration of the extracted DNA were evaluated using BioSpectrometer kinetic (Eppendorf, Germany) spectrophotometer with μCuvette G1.0 (Eppendorf, Germany) microcuvette.

Fifteen ISSR primers were initially screened: $(CA)_6GT$, $(CA)_6AG$, $(CT)_8TG$, $(AG)_8YC$, $(CAC)_3GC$, $(CTC)_3GC$, $(AC)_8CG$, $(AC)_8YG$, $(GA)_8YC$, $(CT)_8AC$, $(CA)_6AC$, $(CA)_6GG$, $(CA)_6RG$, $(GAA)_6$, $(GACAC)_4$. PCR was performed on C 1000 Thermal Cycler (BioRad Laboratories, USA). The reaction mixture of 15.0 □L contained 2.7 □M $MgCl_2$, 1.25 □M primer, 0.4 □M dNTP, 1× PCR buffer, 1.5 of Taq polymerase (Medigen, Russia) and 5.0 ng of template DNA. The amplification reaction consisted of an initial denaturation step at 94 °C for 1.30 min, followed by 35 cycles of 40 s denaturation at 94 °C, 45 s annealing at 51 °C and 56 °C, 1.30 min extension at 72 °C with a final extension of 72 °C for 5 min. The amplified products were separated by gel electrophoresis on 1.5 % agarose gels in 1×TBE buffer and stained with SYBR-Green (Medigen, Russia). The sizes of the amplification products were estimated with a DNA ladder (Medigen, Russia). DNA fragments were visualized using Gel Doc XR+ and analyzed with Image Lab Software (BioRad Laboratories, USA).

3 Results and discussion

The first changes on the scale surface – the overgrowth of explant tissue – were observed 55–56 days after inoculation on nutrient media. No formation of callus on the *in vitro* culture initiation stage was noted, bulblet regeneration occurred through direct gemmogenesis. The emergence of buds was occurred on the undamaged part of bulb scale protruding above the surface of the medium, but growth of them – close to the wound surface. The frequency of the microshoot regeneration achieved 66 % and 3.6 ±0.4 bulblets were formed, on the average, per one explant. Adventitious bulblets obtained from direct regeneration were separated from the primary explant and used for DNA extraction.

At screening of fifteen ISSR primers, only six were effective (Table). At the stage of the preliminary experiment we tested two annealing temperatures – 51 °C and 56 °C. As a result of DNA-PCR amplification, 5–17 bands ranging in size from 200 bp to 2500 bp depending on the annealing temperature were obtained.

Table The general characteristics of the ISSR primers used in the somaclonal variation tests of *Fritillaria dagana*

Primer sequence (5'-3')	Number of bands, annealing temperature (ºC)		Band size, bp
	51 ºC	56 ºC	
$(CT)_8TG$	8	9	500–1500
$(CAC)_3GC$	14	15	200–2500
$(CTC)_3GC$	14	17	200–2300
$(CA)_6GT$	12	15	200–2500
$(CA)_6AG$	8	14	250–2000
$(AG)_8YC$*	8	14	450–2000

* Y = C or T

Low differentiation of the amplification products was established at 51 ºC of annealing for $(CT)_8TG$, $(CA)_6GT$, $(CA)_6AG$ and $(AG)_8YC$ primers, what complicated the analyze of the amplification profile (Fig. 1 a). Unclear bands were observed with $(CA)_6AG$ and $(AG)_8YC$ at 56 ºC annealing. The maximum number (17) of bands was obtained using $(CTC)_3GC$. More informative amplification profiles with clear and distinct bands were at PCR with $(CAC)_3GC$ at 56 ºC of annealing (Fig. 1 b).

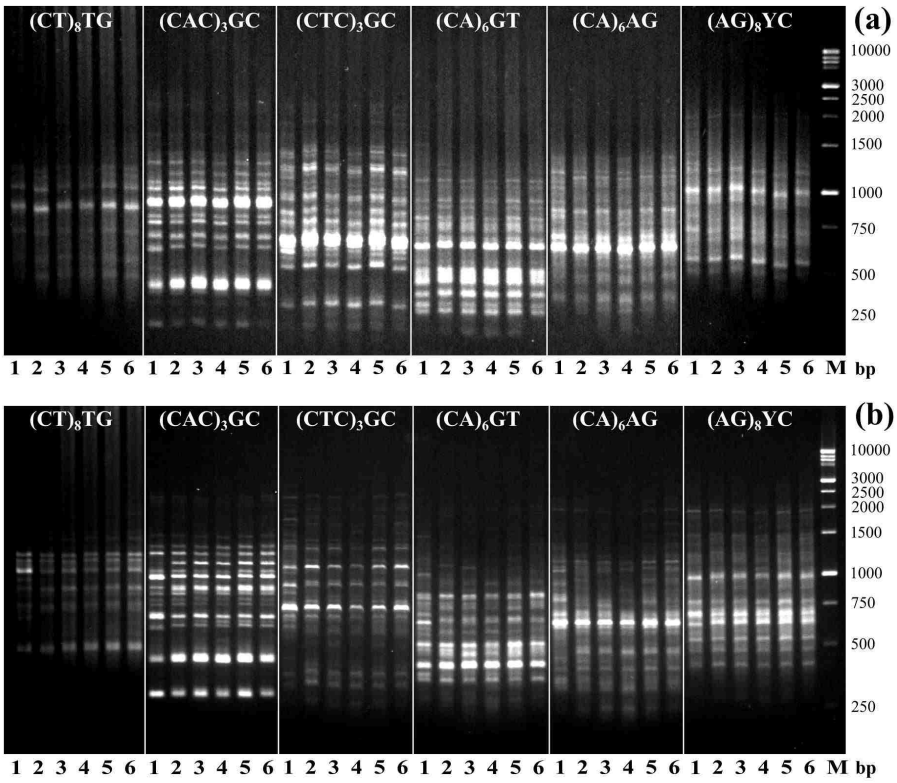

Fig. 1. PCR amplification profiles obtained with ISSR markers of the first generation of *Fritillaria dagana* regenerated plants (2–6 treks) in comparison with the maternal plant (1 trek); primers: $(CT)_8TG$, $(CAC)_3GC$, $(CTC)_3GC$, $(CA)_6GT$, $(CA)_6AG$, $(AG)_8YC$, annealing temperature: **a** – 51 ºC, **b** – 56 ºC, M – a molecular marker; B_5 nutrient medium supplemented with BAP 5.0 µM and NAA 2.0 µM

Generally, the banding profiles from micropropagated plants (2–6 treks) were monomorphic and similar to those of the mother plants (1 trek). In some cases, the differences between the patterns were observed because of different degree of the amplification products during PCR, wich was clarified and checked by repeatability of PCR. As a result, the genetic fidelity of the regenerants to the maternal plant was revealed.

Genetic marking technologies are widely applied methods for monitoring of genetic fidelity of regenerated plants. The diagnostic capabilities of the ISSR markers used by us were successfully illustrated by numerous studies of somaclonal variation in different plant species [13, 14].

In our study, we relied on the work based on assessment of genetic diversity of *F. thunbergii* Miq. [15] and *F. imperialis* L. [16] nature populations by ISSR analysis. We used two primers – $(AG)_8YC$ and $(GAA)_6$ which were offered in the publications. However, in the work with *F. dagana* regenerants the primers didn't show the expected polymorphism and were not considered in further analysis of somaclonal variation. Perhaps the reason is that these species belong to different subgenera – *Fritillaria, Petilium* and *Liliorhiza,* respectively. Previously we used the ISSR markers for identifying somaclonal variation of *F. sonnikovae* Schaulo et A. Erst after *in vitro* slow-growth storage [3]. It was established that the genetic variation process of regenerated plant formed through direct organogenesis did not occur at the first passage following *in vitro* storage for twelve months. We applied the same primer set and achieved the informative PCR amplification profile. The similarity of the effectivness of the ISSR primers can be explained by the position of this plants in one intraspecies group – subgenera *Liliorhiza.*

Thus, the results allow concluding that there is no somaclonal variation of *F. dagana* regenerated plants obtained within the direct gemmogenesis from bulb scale explant tissue. However, these results should be considered preliminary because the ISSR analysis for *F. dagana* was applied for the first time. In future it is planned to optimize the DNA extraction protocol, PCR program, and to carry out a statistical data processing for a large number of primers wich will ensure reliability of the results.

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