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Optimization of PCR Parameters for Molecular Characterization of Gladiolus Genotypes Using ISSR Markers

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ABSTRACT *Gladiolus (Gladiolus x hybridus Hort.), the queen of the bulbous ornamentals, is the leading geophyte grown worldwide for cut flower trade and garden displays. Molecular characterization of plants relies on high yields of pure DNA samples and optimized PCR protocol. In the present study, optimization of PCR conditions for ISSR analysis of Gladiolus genotypes was done at Indian Institute of Horticultural Research, Bengaluru during 2011 - 2012. ISSR protocol was optimized for template DNA, dNTP, primer, and Taq polymerase concentration and annealing temperature. Template DNA of 80 ng, 1mM of dNTP, 0.33 U of Taq DNA polymerase, 1 µM primer gave highly reproducible PCR products. Reproducible amplifiable products were observed in all PCR reactions. The present optimized protocol for ISSR-PCR is suitable for molecular diversity analysis of Gladiolus genotypes.*

INTRODUCTION

Gladiolus is an important bulbous ornamental prized for the beauty of its spikes as well as long vase-life. The modern cultivars are derived from interspecific crosses among several species. Hence, wide variation is exhibited by gladiolus cultivars for their growth habit, size, shape and colour of spikes, and florets. The assessment of natural genetic variation is important not only for ethical and aesthetic reasons but also to ensure that genetic resources may be used even more efficiently and sustainably in horticulture and other industries. Thus, there is a need to assess the variation that already exists and to conserve and utilize effectively. This variation can be accessed by morphological and molecular markers.

PCR-based molecular markers have largely been employed in recent times for various purposes (Hao et al., 2002; Fu et al., 2003). The polymerase chain reaction (PCR) technique has revolutionized molecular biology since it was first described in 1985 (Saiki et al., 1985). After the introduction of thermostable Taq DNA polymerase from Thermus aquaticus and the development of automated oligonucleotide synthesis and thermocyclers, PCR-based techniques have been used in various biological studies ranging from the identification of novel genes and pathogens to the quantification of characterized nucleotide sequences, and provided insights into the intricacies of single cells as well as the evolution of species. In this regard, it is envisaged to carry out the molecular profiling using ISSR markers in the present study. ISSRs are a modification of the SSR approach that use a single primer based on SSR (microsatellites) that are common in the genome. ISSRs polymorphism occurs whenever one genome is missing in one of the SSRs or has a deletion or insertion that modifies the distance between the repeats. Compared with the RAPD method (random amplified polymorphic DNA), the longer primer (16 - 20 bp) can precisely target the template DNA and improve reliability and reproducibility. In addition, ISSRs are cost effective than AFLP (amplified fragment length polymorphism). Thus, ISSRs are better choice which can be used to reveal the genetic variation of ornamental plants. The main objective of present study was to optimize PCR protocol for ISSR analysis of gladiolus genotypes.

MATERIALS AND METHODS

In the present study four gladiolus genotypes were taken for optimization of PCR parameters for molecular profiling by ISSR technique. The leaf samples of Arka Amar, IIHRG-11, IIHRG-12 and Pink Friendship were collected. Standardized DNA extraction protocol was used to isolate genomic DNA. Two gram of young leaves was used for DNA isolation. The yield of DNA per gram of leaf tissue isolated was measured using a UV spectrophotometer at 260 nm. The purity of DNA was determined by estimating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 0.8 % Agarose gel depending on the intensities of band when compared with lambda DNA marker.

For the optimization of PCR reaction conditions, parameters including template DNA, Taq DNA polymerase, dNTP, primer concentration, and annealing temperatures were investigated as described in Table 1. Reactions without DNA were used as negative controls. The thermocycler was programmed for an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at different annealing temperature for each primer, extension at 72°C for 1 min and final extension at 72°C for 8 min and a hold temperature of 4°C at the end. PCR products were electrophoresed on 1.4% (w/v) agarose gels, in 0.5X TBE Buffer at 80 V for 2 h and stained with ethidium bromide (1 µg/ml). Gels with amplification fragments were visualized and photographed in UV light by using Gel Documentation System. 1000 bp ladder was used as DNA molecular weight.

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RESULTS AND DISCUSSION

Tested parameters for ISSR- PCR like concentration of template DNA, primer, Taq DNA polymerase, dNTP, MgCl2, annealing temperature were optimized which had an effect on amplification, banding patterns and reproducibility. The optimized conditions for ISSR-PCR protocol are given in (Table 1). Optimized PCR protocol resulted in better amplification, banding patterns and reproducibility (Fig.1). Annealing temperature was optimized for each primer by carrying out PCR at graded levels of temperature based on melting temperature of the each primer. The optimized annealing temperature for each primer is presented in the Table 2. From the preliminary screening, 9 primers out of 29 that could give good, reproducible bands were selected for further analysis (Fig.1 and Fig.2). Consequently, ISSR primers that produced clear, reproducible and relatively high polymorphic bands were selected (Table 2).

It is essential to optimize the PCR protocol without which it is difficult to achieve optimum results. The study presents graded series of PCR parameters and an appropriate concentration of each parameter for optimum results (Table 1). Template DNA is a prerequisite for PCR amplification. Template DNA of 80 ng per reaction gave optimum amplification. Absence of amplification with lower concentration and presence of smear at higher concentration affected the repeatability. Padmalatha and Prasad (2006) optimized DNA concentration for PCR reaction and they found 50 ng have shown good results in Vitex pubescens, Nervilia aragoana, Gymnema sylvestre, Withania somnifera, Origanum majorana , Boswellia serrata ,Saraca asoca and Gloriosa superba.

The concentration of nucleotide concentration has significant influence on PCR. Deoxynucleotide triphosphates (dNTPs) was also optimized by using different concentrations i.e. 0.5,1, 1.5, 2, 2.5 mM however 1mM dNTP gave good PCR results. Increased concentration reduces the free Mg2+, interfering with the enzyme. Low dNTP concentrations minimize mispriming at nontarget sites and reduce the likelihood of extending misincorporated nucleotides (Innis et al., 1988).

The concentration of Taq polymerase is a critical factor in determining the stringency of a PCR. A concentration 0.33 U enzyme was found sufficient to get optimum amplification. Lower concentration of Taq polymerase (units) failed to show proper amplification and high concentration showed decreased specificity. Hence in the present study 0.33 U was found optimum. Fernándeza et al. (2010) optimized 1 unit of BioTaq DNA Polymerase for PCR reaction in chickpea, melon and pea.

The magnesium concentration affect primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity. But in present study addition of Magnesium chloride did not show any effect on PCR reaction. It may be because of the reason that we were using Taq buffer A which already containing Tris with 15 mM Magnesium chloride.

Primer concentrations between 0.1 and 1 µM are generally optimal. Higher primer concentrations may promote mispriming and accumulation of nonspecific product and may increase the probability of generating a template-independent artifact termed as primer-dimer. In the present study among the tested range of primer, 1 µM was found to be optimum. Roy et al. (2006) reported primer concentration of 100 pmol for ISSR - PCR analysis of micropropagated corms of gladiolus variety 'Pacifica'. The primer concentration varied for the same genus, this may be due to other different PCR parameters and thermocycler programme used by these workers.

In the present investigation Template DNA of 80 ng, 1mM of dNTP, 0.33 U of Taq DNA polymerase, and 1 µM primer gave highly reproducible PCR products and the protocol can be effectively used for molecular characterization of gladiolus genotypes.

Table 1. Different PCR parameters tested and their optimum concentration.

Sl. No.	PCR parameter	Tested range	Optimum concentration
1.	DNA template concentration (ng)	10, 20, 30, 40, 50,60, 80, 100	80 ng
2.	Magnesium chloride (mM)	0.0,1.0, 1.5, 2.0,2.5, 3.0, 3.5	No effect
3.	Deoxynucleotide triphosphates (dNTPs) (mM)	0.5,1, 1.5, 2, 2.5	1 mM
4.	Primer concentration (µM)	0.1, 0.5, 1, 1.5, 2, 2.5, 3.0	1µM
5.	Taq DNA polymerase (units)	0.1,0.33,0.5, 1.0, 1.5, 1.75	0.33 U

Table 2. Properties of amplification products obtained with nine selected primers

Primer	Annealing temperature	Total no. of bands	Polymorphic bands	Percent polymorphism
888	54^{0}C	12	7	58.33
889	54^{0}C	09	6	66.66
890	54^{0}C	10	6	60.00
891	54^{0}C	11	6	54.54
818	56^{0}C	08	5	62.50
823	56^{0}C	08	7	87.50
829	56^{0}C	07	6	85.71
861	63^{0}C	07	3	42.85
862	63^{0}C	04	3	75.00

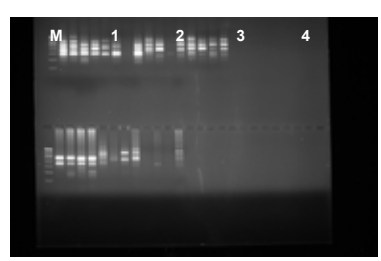

Fig 1. DNA profile generated using optimized PCR protocol using ISSR Primer 888 at 540 C.

M = 1000 bp ladder, 1 = Arka Amar, 2 = IIHRG-11, 3 = Pi ıl. Friend:hip, 4 = I:HRG-12

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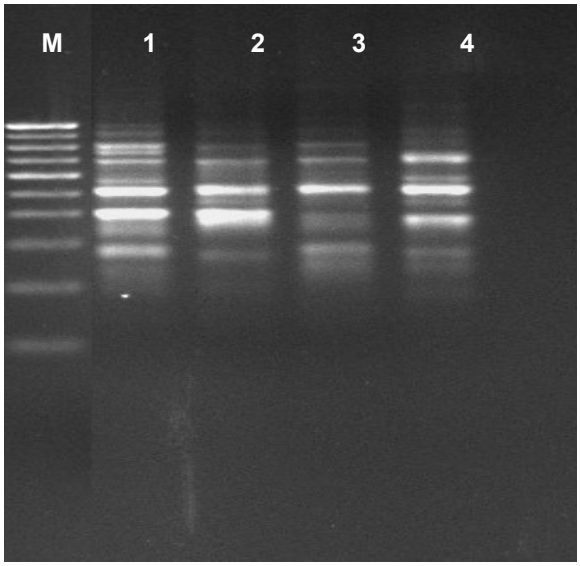

Fig 2. DNA profile generated using optimized PCR protocol using ISSR Primer 891 at 540 C.

M = 1000 bp ladder, 1 = Arka Amar, 2 = IIHRG-11, 3 = Pink Friendship, 4 = IIHRG-12

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