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Characterization of *Fusarium* wilt in resistant and susceptible gladiolus (*Gladiolus* spp) genotypes using DNA markers

POONAM KUMARI[1], T MANJUNATHA RAO[2], ANURADHA SANE[3], RAJIV KUMAR[4] and M V DHANANJAYA[5]

ICAR–Indian Institute of Horticultural Research, Hesaraghatta Lake Post, Bengaluru, Karnataka 560 089

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ABSTRACT

ISSR markers were used for molecular characterization of *Fusarium* wilt in resistant along with susceptible gladiolus (*Gladiolus* spp) genotypes namely Arka Amar, Arka Aayush, IIHRG-12 and Pink Friendship to identify markers for screening population for wilt resistance. Twenty nine ISSR primers were screened for the ability to generate reproducible polymorphic bands. Out of 29 primers, 11 primers produced good polymorphism. Among the selected 11 polymorphic primers, 8 primers produced 100 % polymorphism except three primers, viz. 888 (66.67%), 891 (85.71%) and 818 (85.71%). Primer UBC 861 has produced specific band of size 350 bp for both resistant genotypes, viz. Arka Amar and Arka Aayush. UBC Primer 891 primer produced band of size 250 bp for both susceptible genotypes, i.e. Pink Friendship and IIHRG 12. The dendrogram generated using Ward's method showed that high genetic similarity between genotypes Arka Amar and Pink Friendship at a genetic distance of 8.4. Genotypes Arka Aayush and IIHRG-12 are related to each other at a distance of 8. The results suggest that ISSR markers are useful for detection of resistant bands and also assessing genetic relationship in gladiolus varieties. The identified ISSR marker from the present investigation will help in screening the gladiolus population for *Fusarium* wilt resistance. Cloning and sequencing the desirable polymorphic DNA fragment converting to SCAR, which can be used as marker for detecting resistance in breeding for *Fusarium* wilt resistance mechanism in gladiolus.

Key words: *Fusarium,* Gladiolus, ISSR markers, Polymorphism,

Wilt disease caused by *Fusarium oxysporum* f. sp. *gladioli* causes severe economic losses to the gladiolus (*Gladiolus* spp) growers. The loss caused by this disease in different parts of the world, is estimated 30 to 80% (Maurhofer *et al*. 1995), and 60 to 100% damage depending on varietal response (Pathania and Misra 2000). The commercial cultivars are highly susceptible to disease which is a major bottle neck in the commercial growing (Cerasela *et al*. 2015).

Proper characterization helps in unambiguous discrimination between genotypes, identification of economically important plant genetic resources, detection of redundancies and in monitoring genetic changes during maintenance. During the last two decades, biotechnological approaches were adopted to improve agricultural practices that have opened vistas for plant improvement and utilization (Memon *et al*. 2010). Molecular markers such as DNA and is ozymes are not affected by developmental processes or environmental influences and are used for determination of genetic diversity (Taskin *et al*. 2012 and Zhang *et al*. 2012), and can be used to characterize organisms at the genomic level (Jonah *et al*. 2011).

Characterization and assessment of genetic relationships among diverse accessions allow breeders to select desirable genes from different sources and to accumulate those genes in one cultivar. Development of markers for a new crop or development of additional markers for a crop where markers were developed in the past raises the question of the intended use of the markers. ISSR markers are good for detecting polymorphism, as they reveal variation at multiple loci within the genome. In addition, ISSR molecular markers were widely used in studies of genetic diversity, phylogeny, DNA fingerprinting, gene tagging, genome mapping and evolutionary biology (Tacuatia *et al*. 2012). Thus, ISSR primers were used in the present study. This work is, to our best knowledge, the first attempt to characterize gladiolus genotypes for *Fusarium* wilt resistance by means of ISSR markers.

[1]Ph D Scholar (e mail: poonamjaswalfls@gmail.com), Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute, Pusa, New Delhi 110 012. [2]Principal Scientist (e mail: tmrao@iihr.ernet.in), [4]Senior Scientist (e mail: flori_rajiv@yahoo.co.in), [5]Principal Scientist (e mail: dhananjay@iihr.ernet.in), Division of Ornamental Crops; [3]Principal Scientist (e mail: anuradha@iihr.ernet.in), Division of Plant Genetic Resources, ICAR-Indian Institute of Horticultural Research, Hesaraghatta Lake Post, Bengaluru, Karnataka 560 089.

MATERIALS AND METHODS

In the present study, four gladiolus genotypes resistant and susceptible to Fusarium wilt were used for molecular profiling by ISSR technique. The leaf samples of Arka Amar, Arka Aayush, 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/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.

PCR: PCR amplifications were performed in a thermocycler. The reaction mixture contained reaction buffer 10X 1 µl, 10 µM primer, 1 mM dNTP's, Taq DNA polymerase, 20 ng/µl (4.0 µl) Template DNA. Total reaction volume was 10.0 µl. The thermocycler was programmed for an initial denaturation step of 4 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 0.5 X TBE Buffer at 80 V for 2 hr and stained with ethidium bromide (0.5 mg/ml). Gels were visualized and photographed in UV light by using Gel Documentation System. 1 000 bp ladder was used as DNA molecular weight marker (Fig 1).

The bands were visually scored for the presence (1) or absence (0) in all the genotypes studied and the binary data were used for statistical analysis. Only clear bands were taken into account and the bands were not scored if they were faint or diffused. The band sizes were determined by comparing with 1 kbp DNA ladder, which was run along with the amplified products. The binary data from each study were analyzed with the computer package "STATISTICA". The dissimilarity matrix was developed using Squared Euclidean Distance (SED) that estimated all pair-wise differences in the amplification product (Sneath and Sokal 1973). A dendrogram was computed based on Ward's method of clustering, using minimum variance algorithm (Ward 1963). The present study was carried out at ICAR-Indian Horticultural Research Institute, Hesaraghatta Lake Post, Bengaluru during 2011-2012.

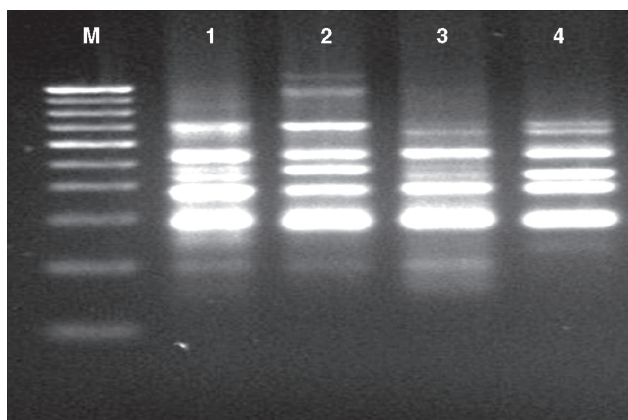

Fig 1 ISSR- PCR amplification of DNA from four genotypes using primer UBC 888 at 54°C. M = 1 000 bp ladder, 1 = Arka Amar, 2 = Arka Aayush, 3 = Pink Friendship, 4 = IIHRG-12.

Table 1 Optimum annealing temperatures for different primers

Primers used (UBC)	Annealing temperature °C (UBC)	Primers used	Annealing temperature (°C)
841	49	854	50
844	49	815	56
847	49	818	56
848	49	823	56
886	54	829	56
887	54	810	53
888	54	906	56
889	54	861	63
890	54	862	63
895	58	866	63
892	58	894	60
903	59	908	65
902	60	896	65
827	55	826	55
891	54		

RESULTS AND DISCUSSION

DNA quantification: Isolated DNA was quantified using nanodrop and agarose gel electrophoresis. Purity (260/280 ratio) of DNA ranged from 1.79 to 1.82. DNA yield was 748.0 ng/µl, 295.0 ng/µl, 276.3 ng/µl and 137.2 ng/µl from genotypes Arka Amar, Arka Aayush, IIHRG-12 and Pink Friendship, respectively.

Molecular characterization using ISSR primers

Twenty nine ISSR primers were screened for the ability to generate reproducible polymorphic bands. The annealing temperature of each primer was optimized for each primer based on Tm value by using gradient PCR. Out of 29 primers, 11 primers produced good polymorphism. The optimum annealing temperature for the 29 primers is presented in Table 1.

PCR analysis using ISSR primers

Among the selected 11 polymorphic primers, 8 primers produced 100% polymorphism except three primers, viz. 888 (66.67 %), 891 (85.71 %) and 818 (85.71 %) (Table 2).

Generation of genotype specific markers

DNA profiles were generated for resistant and susceptible genotypes. Variation in band profiles was observed in the DNA profiles of gladiolus genotypes with different primers (Table 3). Primer UBC 888 has produced

Table 2 Properties of selected ISSR primers used in the study

Primer (UBC)	Sequence (5'-3')	Total number of bands	Monomorphic bands	Polymorphic bands	Percent polymorphism
818	CAC ACA CAC ACA CAC AG	7	1	6	85.71
823	TCT CTC TCT CTC TCT CC	14	0	14	100
827	ACA CAC ACA CAC ACA CG	11	0	11	100
829	TGT GTG TGT GTG TGT GC	9	0	9	100
854	TCT CTC TCT CTC TCT CRG	3	0	3	100
861	ACC ACC ACC ACC ACC ACC	9	0	9	100
862	AGC AGC AGC AGC AGC AGC	11	0	11	100
888	BDB CAC ACA CAC ACA CA	9	3	6	66.67
889	DBD ACA CAC ACA CAC AC	12	0	12	100
890	VHV GTG TGT GTG TGT GT	13	0	13	100
891	HVH TGT GTG TGT GTG TG	14	2	12	85.71

accession specific bands of 1100 and 1000 bp for genotype Arka Aayush (Fig 1). UBC Primer 891 produced genotype specific bands of molecular weight 980, 900, 750 and 550 bp for genotype Arka Amar and also produced genotype specific band of size 700 bp for genotype IIHRG-12. This primer also produced same band of size 250 bp for both susceptible genotypes, i.e. Pink Friendship and IIHRG-12 (Fig 2).

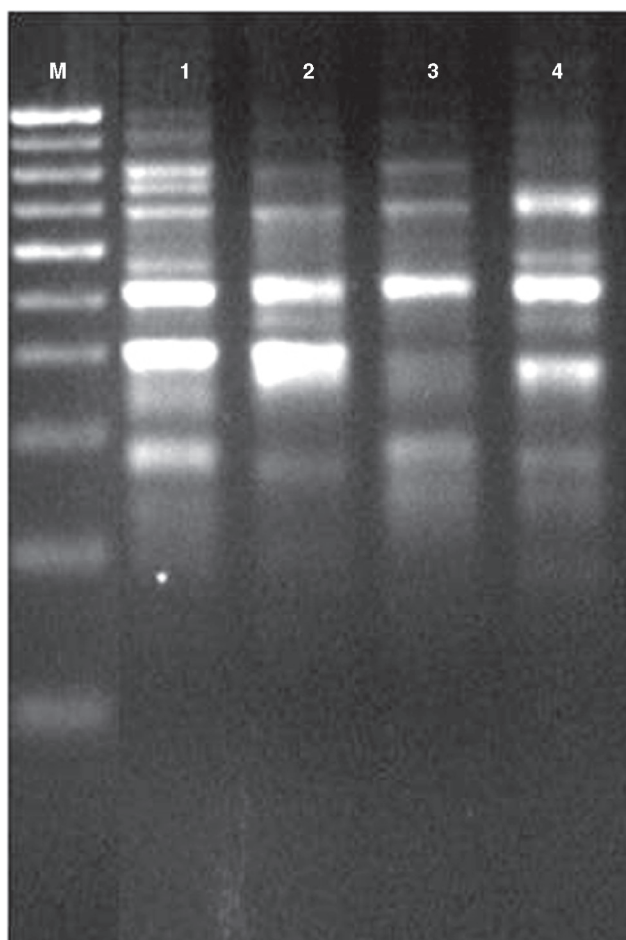

Fig 2 ISSR- PCR amplification of DNA from four genotypes using primer UBC 891 at 54°C. M = 1000 bp ladder, 1 = Arka Amar, 2 = Arka Aayush, 3 = Pink Friendship, 4 = IIHRG-12.

DNA profile generated by using Primer 818 has produced genotype specific bands of size 490 bp and 450 bp for susceptible genotypes Pink Friendship and IIHRG-12, respectively. UBC 823 has produced genotype specific bands of size 1 200 bp and 1 100 bp for susceptible genotypes Pink Friendship and IIHRG-12, respectively, and also produced genotype specific band of molecular weight 950 bp and 700 bp for genotype Arka Amar. Specific band

Table 3 Genotype specific markers with different primers

Primer (UBC)	Base pair (bp)	Genotypes
818	490	Pink Friendship
818	450	IIHRG -12
823	1200	IIHRG-12
823	1100	Pink Friendship
823	950,700	Arka Amar
823	900	Pink Friendship
823	470,350	IIHRG -12
827	580,430	IIHRG- 12
827	420	IIHRG -12
854	700	IIHRG -12
861	600,350	Arka Aayush
861	350	Arka Amar
861	220	Pink Friendship
861	220,180	IIHRG -12
861	180	Pink Friendship
862	980	Pink Friendship
862	950	Arka Amar
862	280	Arka Aayush
888	1100,1000	Arka Aayush
890	650,450	Arka Amar
891	980,900,750	Arka Amar
891	700,250	IIHRG -12
891	550	Arka Amar
891	250	Pink Friendship

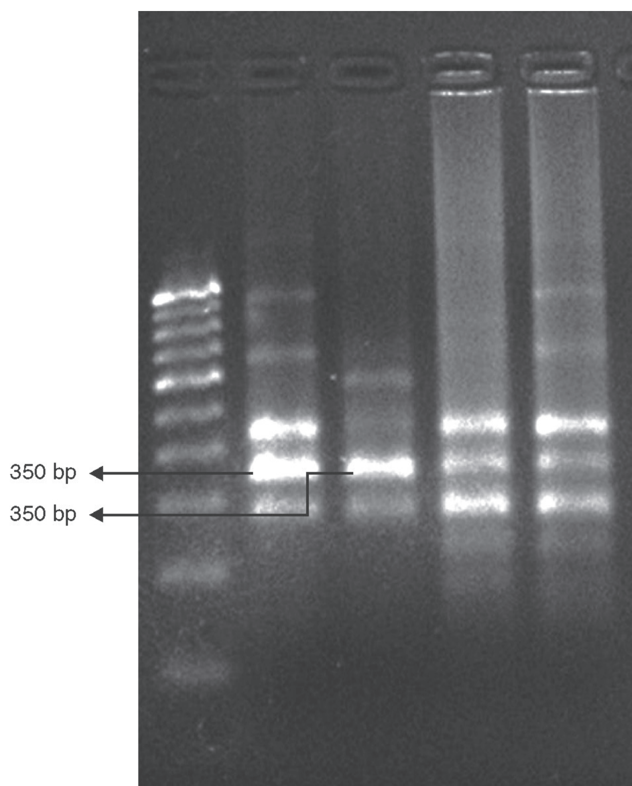

Fig 3 ISSR- PCR amplification of DNA from four genotypes using primer UBC 861 at 63°C. M=1 000 bp ladder, 1=Arka Amar, 2=Arka Aayush, 3=Pink Friendship, 4=IIHRG-12.

of 900 bp was present in IIHRG-12. This primer also produced same band of size 850 bp for both susceptible genotypes i.e. Pink Friendship and IIHRG-12. Primer 854 has produced genotype specific band for IIHRG-12 at 700 bp (Table 3).

Primer 827 has produced genotype specific bands of molecular weight 580, 430, 420, 300, 260, 200 and 130 bp for IIHRG-12. This primer has also produced genotype specific band of molecular weight 120 bp for genotype Pink Friendship. DNA profiles generated by using primer 861 has produced genotype specific band of size 600 bp for genotype Arka Aayush. This primer has produced specific band of size 350 bp for both resistant genotype, viz. Arka Amar and Arka Aayush. Same bands of size 220 and 180 bp were also found in both the susceptible genotypes, i.e. Pink Friendship and IIHRG-12 (Fig 3). Primer 862 produced genotype specific bands for different genotypes. 980 bp band for Pink Friendship, 950 bp band for Arka Amar and 280 bp band for Arka Aayush (Table 3).

Generation of Fusarium *wilt resistant genotype specific markers*

DNA profiles generated by using primer 861 has produced specific band of size 350 bp for both resistant genotype, viz. Arka Amar and Arka Aayush.

Dendrogram

Binary data generated while scoring gel profiles were used to construct dendrogram by using Ward's method (Fig 4). It is divided into two major clusters. High genetic similarity was observed between genotypes Arka Amar and Pink Friendship at a genetic distance of 8.4. Genotypes Arka Aayush and IIHRG-12 are related to each other at a distance of 8.

Molecular characterization of gladiolus genotypes

For this purpose, PCR analysis using ISSR primers was done. Twenty nine ISSR primers were screened. Out of 29 primers, 11 primers produced clear cut ISSR profile for all four gladiolus genotypes and ISSR products ranged from 100-1 300 bp. Among the selected 11 polymorphic primers, 8 primers produced 100% polymorphism except three primers namely 888 (66.67 %), 891 (85.71 %) and 818 (85.71 %). All genotypes were clearly differentiated by their ISSR profiles. Wang *et al.* (2008) detected a total of 185 polymorphic DNA fragments among all the 206 amplified fragments, which accounted for a high level (89.8 %) of the total and could be used for identification of different cultivars. Similarly (Pragya *et al.* 2010b) studied genetic relatedness of 54 gladiolus cultivars using AFLP markers. Jayanthi *et al.* (2013) also evaluated SSRs for detecting genetic variability in oil palm.

Generation of Fusarium *wilt resistant genotype specific markers*

The ISSR primer UBC 861 produced specific band of size 350 bp for both resistant genotype, viz. Arka Amar and Arka Aayush. Dallavalle *et al.* (2002) detected RAPD polymorphisms in gladiolus cultivars with differing sensitivities to *Fusarium oxysporum* f. sp. *gladioli*. Similarly, Dehaan *et al.* (2000) tested 160 arbitrary 10-mer oligonucleotide primers on *Fusarium oxysporum* f. sp. *gladioli* by PCR to find RAPD specific markers. They found RAPD primer G-12 amplified. Wang *et al*. (2013) recognized a Fo366 fragment of the intergenic spacer (IGS) from an *Fo* biological control agent, and designed a new primer, NPIGS-R. This primers was then evaluated for their specificity to amplify DNA from nonpathogenic *Fo* isolates that have biological control potential. The results showed that the modified primer pair, FIGS11/NPIGS-R, amplified a 500-bp DNA fragment from five of seven nonpathogenic *Fo* isolates. Therefore, the primer will prove useful for the identification of *Fo* isolates at that are nonpathogenic to cucumber which can potentially act as biocontrol agents for Fusarium wilt of cucumber. Scovel *et al.,* (2001) reported that *Fusarium oxysporum* f. sp. *dianthi* race 2 caused loss up to 40 % in carnation. They analyzed the inheritance of resistance, using a segregating F_2 population derived from cross between resistant and sensitive parent. Then they extrapolated a genetic model whereby resistance is determined by two major genes. RAPD fragment was sequenced and used to synthesize sequence-specific primers to establish PCR based SCAR marker for resistance.

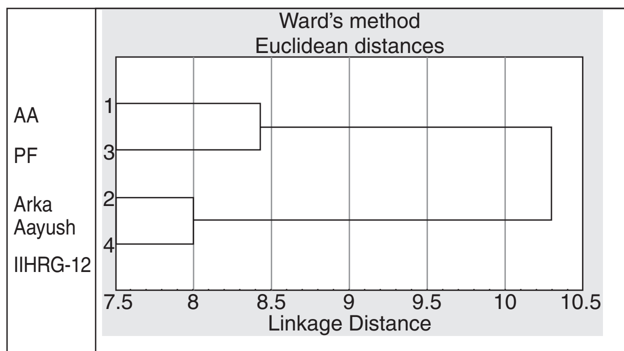

Fig 4 Dendrogram showing molecular genetic relationships among gladiolus genotypes. AA - Arka Amar, PF – Pink Friendship.

Cluster analysis and genetic relationship among genotypes

The Dendrogram based on Ward's method represented the genetic relationship of all gladiolus genotypes (Fig 4). It is clear that all the genotypes used in the present study are related at 10.3 genetic distance. The greatest similarity was found between genotypes Arka Aayush and IIHRG-12, they closely related at a genetic distance of 8, whereas, genotypes Arka Amar and Pink Friendship are related at a genetic distance of 8.4. Pragya *et al.* (2010a) characterized 54 gladiolus cultivars using morphological traits and RAPDs to establish genetic relationships between the selected cultivars by cluster analysis.

The DNA profiles generated by using primer 861 has showed specific band of size 350 bp for both resistant genotypes, viz. Arka Amar and Arka Aayush. This identified ISSR marker can be converted into SCAR marker which will help in screening the population of gladiolus for *Fusarium* wilt resistance in seedling stage which would reduce the time taken for development of wilt resistant cultivars in gladiolus.

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