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Molecular characterization and DNA fingerprinting of some local eggplant genotypes and its wild relatives

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Abstract**—** *Collection and characterization of local genotypes and landraces are prerequisite for any crop improvement program. Molecular diversity and DNA profiling shown exact genetic blue print of any crop. Hence, the experiment was design to establish the molecular diversity and polymorphism among some local eggplant genotypes and its wild relatives for future breeding program. The experiment was carried out at the Biotechnology Laboratory, Sher-e-Bangla Agricultural University, Dhaka, Bangladesh, with twenty-five local and two wild relatives (Solanum sisymbriifolium and S. villosum) of eggplant to study molecular diversity and DNA fingerprinting at those genotypes. Five well-known SSR primers (EPSSR82, smSSR01, EM114, EM120 and smSSR04) were used for the molecular characterization of the genotypes. Quality DNA was isolated with 27 genotypes and PCR amplification was carried out with these primer. The amplified DNA fragment was visualized by 2% agarose gel and data were analyzed by POWERMAKER (version 3.25) and NTSYS-PC (version 2.2). Some total at 10 different alleles were generated with a range of 1 to 3 alleles per locus and an average of 2.0 alleles. The highest number (2) of polymorphic bands was observed in the primers EPSSR82 and smSSR01. The Polymorphism Information Content (PIC) of SSR markers ranged from 0.37 to 0.67 with an average value of PIC = 0.54. Gene diversity ranges from 0.49 (smSSR01) to 0.72 (EPSSR82), with an average value of 0.61. UPGMA method separated the of 27 genotypes into two major clusters (I and II). From the clusters, wild species Solanum villosum belonged to the sub-cluster (IIb), that revealed its distinct variation from the others. On the other hand, wild species Solanum sisymbriifolium showed a close relatedness by forming the same cluster together with thirteen local eggplant genotypes. Molecular diversity and DNA profiling was identified among 25 local eggplant germplasm and its wild relatives. The finding of the experiment could be used for selection of diverse parent for eggplant improvement.*

Keywords**— eggplant, molecular diversity, SSR marker, wild relatives.**

I. INTRODUCTION

Eggplant (*Solanum melongena L.* 2n = 2x = 24) belongs to the plant family of Solanaceae. It is the sixth most important vegetable after tomato, watermelon, onion, cabbage, and cucumber and the most important *Solanum* crop native to Asia [1]. Eggplants have a remarkable demand and are considered as the second important vegetable crop after potato in Bangladesh [2]. As eggplant is a native plant of Indian sub-continent which surely can define its abundance in this region. Though it is cultivated almost all over the country its production is not as good as expected for being an ancient plant of this region. In the year 2014-15, total area devoted to eggplant cultivation was 1,22,014 acres with annual production of 4,50,146 metric tons [2]. Eggplant has a number of health benefits. It is an important source of fiber, potassium, manganese, as well as vitamins C, K, and B6. Phenolic compounds in eggplant contain significant amounts of chlorogenic acid, one of the most powerful free radical scavengers found in plants. Chlorogenic acid has been shown to decrease low-density lipid (LDL) levels, and also serves as an antimicrobial, antiviral, and anti-carcinogenic agent. Despite eggplant's economic importance, its improvement and molecular study of different land races, local genotypes and germplasm characterization was not well studied. The development of new eggplant varieties addressing old and new breeding objectives requires of genetic diversity [3, 4, 5]. Collection and characterization of genetic resources and local cultivars are required for the improvement of new varieties. SSR markers for eggplant have been developed in the recent years and are being mainly used for assessing the genetic diversity and genome

similarity in the related species [6,7]. Co-dominant markers such as simple sequence repeat (SSRs) could generate more information and has high repeatability than other dominant markers like RAPD or AFLP [8,9]. SSRs have proved as a more powerful marker than AFLPs to study the relationships amongst closely related eggplant materials [10]. SSR markers are multi-allelic, highly abundant, well distributed in the genome and are suitable for high throughput PCR which makes them ideal for molecular diversity studies [11]. Genetic diversity assessment is very important to identify groups with similar genotypes and to conserve, evaluate and utilize the genetic resources. The diversity of the germplasm can be used as a potential basis of genes that lead to improved performance of the superior cultivars and can also be used to determine distinctness and uniqueness of the phenotypes and the genotypes. Wild species remain largely unexploited for eggplant breeding. *S. villosum* and *S. sisymbriifolium* are two wild relatives of Solanum *melangena* which showed considerable resistance to bacterial wilt. So, the study was focused on genetic diversity of some local eggplant germplasm through SSR marker to generate more information and to assess relatedness among local landraces and also with their wild relatives.

II. MATERIALS AND METHOD

Collection of material

A sum total of 27 materials were used in the study and among those 25 were local eggplant genotypes and 2 were wild relatives viz. *Solanum villosum and S. sisymbriifolium*). Germplasms were collected from different districts of Bangladesh. A list of local germplasm and their collected area was given in Table 1. Wild relatives were collected from the Gene Bank of Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh. The experiments were carried out at the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka, Bangladesh.

Seedling raising

Good quality, disease free, healthy seed were sown in plastic pots and kept in nets house. All management practices were done for raising quality seedlings from those materials. Fresh leaves were collected at 3-4 leaf stage of plant for isolation of DNA.

Extraction and quantification of DNA

Total genomic DNA from each genotypes was isolated by CTAB method with slight modification [12]. The extracted DNA was purified by propanol and treated with 10µg/ml RNase A for 20-25 minutes at 37°C to remove the RNA. The purified DNA was dissolved in TE buffer and quantification of DNA was done through electrophoresis on 1% agarose gel staining by ethidium bromide. The sample DNA were stored at -20°C freezer for further use.

Primer selection and PCR amplification

Five SSR primers were selected on the basis of previous works to evaluate the molecular polymorphism study the eggplant local genotypes and wild relatives. PCR reaction was performed using BIONEER KIT (Korea). The PCR reaction having 20.0 µl mixture containing with 3.0 µl sterile de-ionized water, 4.0 µl 10X PCR buffer, 4.0 µl enzyme dilution buffer, 3.0 µl 20 mM $MgCl_2$, 1.0 µl dNTPs (10mM), 0.5 µl top DNA polymerase, 2.5 µl primer (forward and reversed) and 2.0 µl sample DNA (approx. 40-50 ng). The reaction mixture was subjected to the following thermal profile for amplification in a thermocycler: 5.00 min at 95°C for initial denaturation, followed by 33 cycle of 1.00 minutes denaturation at 94°C, 1.00 minutes at annealing with various temperature according to primer melting point and 1.30 minutes at 72°C for extention. A final extension step was done at 72°C for 7 minutes. Electrophoresis was done to visualize the PCR amplified product. It was carried out on 2.0% agarose gel and amplified fragments were visualized by staining with ethidium bromide.

Documentation of PCR amplified DNA products and SSR data analysis

The gel was taken out carefully from the gel chamber and was placed on high performance ultra-violet light box (UV trans-illuminator) of gel documentation for checking the DNA band and photographed by a Gel Cam Polaroid camera. The summary statistics including the number of alleles per locus, major allele frequency, gene diversity and Polymorphism Information Content (PIC) values were determined using POWERMARKER (version 3.25) [13]. Molecular weight for each microsatellite products, in basepairs were estimated with AlphaEaseFC (Alpha Innotech Corporation) version 4.0 software. The individual fragments were assigned as alleles of the appropriate microsatellite loci.The allele frequency data from POWERMARKER was used to export the data in binary format (presence of allele as 1 and absence of allele as 0) for analysis with NTSYS-PC (Numerical Taxonomy and Multiware Analysis System) version 2.2 software [14,15]. Unweighted Pair Group Method of Arithmetic Means (UPGMA) dendrogram was constrcured using a computer programme, POPGENE (Version 1.31) based on Nei"s [16] genetic distance.

III. RESULTS

Eggplant (*Solanam melongena*) is an import vegetable in our country which has wild relatives as well as primitive cultivars and landraces. The molecular genetic maps developed in eggplant have been used both for the tagging of simply inherited traits and the localization of the loci underlying complex morphological characters. The assessment of genetic diversity or relatedness is not only

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important for eggplant improvement but also for the conservation and maintenance of germplasm. Highly polymorphic and repeatable PCR based microsatellite markers or Simple Sequence Repeat (SSRs) markers were used here to assess the polymorphism, diversity and similarity within those local and wild relatives.

DNA amplification by SSR markers and its polymorphism

Five SSR primers *viz*., EM114, EM120, smSSR01, amSSR04 and EPSSR82 produced different banding pattern separately with 25 eggplant genotypes and two wild relative. The amplifications of each SSR primers are presented in Table 2 and Fig. 1 to 4.

The SSR primer EM114 produced only one DNA fragment among all the genotypes under study. The approximate fragment size was 225 bp. It was a monomorphic DNA band which was common in all the genotypes. The amplification product is presented in Fig. 1.

Two fragments of DNA amplification were noticed by the SSR primer EM120. The size of amplification ranged from 50 to 180 bp. All the genotypes produced 180 bp fragment which indicated a monomorphic band. Whereas, the genotypes Salta begun, Ashary, Lalmoni local-1, Cricket, Nilphamari local and Dinajpur local were able to produce 80 bp polymorphic band (Fig. 2). The SSR primer smSSR01 was able to amplify three fragments of DNA among all the individuals. The DNA product ranged from 200 to 350 bp. Among them 300 bp fragment was common in all genotypes. The germplasm Khotkhotia, Thakurgaon local, Bogra local and Khulna local-1 showed second amplification of DNA band. It's indicated that the second fragment at 320 bp is polymorphic in nature. Kurigram local, wild species *Solanum sisymbriifolium* and *Solanum villosum* produced third amplification at 250 bp, which was polymorphic (Fig. 3). The SSR primer EPSSR82 has the ability to amplify three fragment of DNA among all the experimental materials. The band size ranged from 50 to 180 bp. It was noticed that 180 bp fragment was common in all the genotypes and was monomorphic for all. The genotypes Salta begun, Ashary, Lalmoni local-1, Kurigram local, Cricket, Rangpur local, Thakurgaon local, Bogra local, Iswardi and Jessore local-3 were able to regenerate two additional DNA bands between the size ranging from 50 to 70 bp. The above finding indicated that, two polymorphic DNA were regenerated by the primer EPSSR82. A 50 bp DNA fragment was amplified by the primer smSSR04 and it was monomorphic for all the genotypes under study (Fig. 04). On an average, five SSR primers were able to generate some total of 10 DNA amplification (10 bands) with an average amplification for each primer was 2.0. Out of them, five DNA fragment were polymorphic among the genotypes under studied.

Allelic frequency, gene diversity and Polymorphism Information Content (PIC)

Allelic frequency, gene diversity and Polymorphism Information Content (PIC) value of experimental genotypes are presented in Table 3. PCR products of five SSR markers were characterized. Some total 10 alleles were detected for the five polymorphic SSR loci, with an average number of alleles/locus is 2.0. The frequency of the major allele ranged between 0.33 to 0.56 with an average value of 0.49. Polymorphic Information Content (PIC) value for the 5 markers ranged from 0.37 (smSSR01) to 0.67 (EPSSR 82) and the average PIC value was 0.54. Highest PIC value (0.67) was observed in the primer EPSSR82 and it was lowest (0.37) in the primer smSSR01. The primer EPSSR82 was considered as the best marker for diversity analysis in eggplant germplasm followed by EM114 and EM120, respectively. The marker smSSR04 was considered as the least powerful marker. Gene diversity ranged between 0.49 (smSSR 01) to 0.72 (EPSSR 82) with an average of 0.61.The results indicate that the 25 local eggplant landraces present a high degree of homozygosity and are closely related to the wild variety *Solanum villosum* and *Solanum sysimbriifolium*, and also considerable intra-varietal group diversity, and a certain degree of genetic differentiation and polymorphism really do exist.

Nei's Genetic Distance and Genetic Identity

The value of pair-wise comparisons of Nei"s (1972) genetic distance (D) among twenty-five local and two wild relatives of eggplant were computed from combined data of the five primers and it was ranged from 0.200 to 1.000 with an average of 0.600.Comparatively higher genetic distance (1.000) was observed between a number of genotypes. Among them Ashary showed highest genetic dissimilarity with maximum number (14) of genotypes *viz*., Bogra local, Comilla local, Dohazari, Jamalpur local, Jessore local-1, Jessore local-2, Jessore local-3, Jessore local-4, Khulna local, Narsingdi local, Sada Khulna, Thakurgaon local and two wild species. The wild species *Solanum villosum* showed highest genetic distance among twelve eggplant genotypes. The highest genetic distance between them indicated that genetically they are diversed. Genotypes pair with higher value (1.000) of genetic distance is more dissimilar than a pair with a lower value. The lowest genetic distance (0.200) was found in a variety of pairs indicating that they are genetically much closer among them. The highest Nei's genetic identity was observed in various genotype pairs. Among them Bogra local showed maximum genetic similarities with maximum number (10) of genotypes viz., Iswardi, Jamalpur local, Jessore local-1, Jessore local-2, Jessore local-4, Khulna local-1, Narsingdi local, Sada Khulna, Thakurgaon local. From Nei's genetic distance and identity value it was

clearly revealed that the 25 eggplant genotypes and 2 wild species had distinct genetic diversity.

UPGMA dendrogram

A dendrogram was constructed based on the Nei"s genetic distance calculated from 25 eggplant genotypes and two wild species. Unweighted Pair Group Method of Arithmetic Means (UPGMA) indicated the segregation of 27 genotypes into two main clusters I & II (Fig.. 5). Ashary, Khotkhotia, Kurigram local, Lalmoni local-1, Lalmoni local-2, Salta begun, Cricket, Dinajpur local, Nilphamari local, Rangpur-1, Rangpur-2, Rangpur-3 were formed cluster-I. On the other hand, Bogra local, Comilla local, Dohazari, Iswardi, Jamalpur local, Jessore local-1, Jessore local-2, Jessore local-3, Jessore local-4, Khulna local-1, Narsingdi local, Sada Khulna, Thakurgaon local, wild species (*Solanum sisymbriifolium*) were fallen in the cluster II(a) and only one wild species (*Solanum villosum*) formed cluster-II (b). The genotypes – Ashary, Khotkhotia, Kurigram local, Lalmoni local-1, Lalmoni local-2, Salta begun were formed cluster I(a) and the germplsm Cricket , Dinajpur local, Nilphamari local,Rangpur-1, Rangpur-2, Rangpur-3 formed cluster-I(b). Based on above result, it may be concluded that, the close relatives of the eggplant germplasm are grouped in the same cluster due to lower genetic distance and the genetically dissimilar germplasms were placed in another cluster due to higher genetic distance. It was clearly observed that wild species (*Solanum villosum*) was very much different from all the genotypes. The result indicates that the low or high level genetic distance exits within the genotypes.

IV. DISCUSSION

Eggplant is an important vegetable crop in Bangladesh. Different local genotypes and wild relatives were found in Indian sub-continent. Morphologically those genotypes showed huge variation. Diversity study through molecular marker expressed the actual genetic make-up of eggplant genotypes. The present observation noticed the polymorphism at DNA level among the twenty-five local and two wild relatives. This finding also supported by various scientist. Some of them were discussed below.

The 22 amplified DNA products using nine SSR primers with an average amplification for each primer of 2.2 were noticed in six eggplant genotypes showed 70% monomorphic and 30% polymorphic band through using 9 primers in eggplant genotypes [17]. Nineteen SSRs markers for the molecular characterization of 30 eggplant accessions were studied. The polymorphism information content (PIC) of SSR markers ranged from 0.07 to 0.77, with an average value of PIC=0.50[18]. The mean observed heterozygosity (Ho) presented a very low value Ho=0.01, while the mean expected heterozygosity (He) had a value of He=0.57. Genomic SSRs that previously proved to be highly polymorphic in eggplant have been found to be of great value for evaluating the genetic diversity and relationships in a collection of eggplants from different cultivar groups[18, 19]. It possesses a number of desirable horticultural traits such as disease resistance [20] and has medicinal uses [21]. This result clearly indicated that different levels of genetic identity and distance present within the eggplant germplasm and shown in the UPMGA dendrogram (Fig. 5). Different levels of cluster analysis was reviewed which was performed by several scientists. Constructed a dendrogram with scale from 0.16 to 0.97 based on Jaccard's similarity coefficient. Separated the 32 accessions into 4 main clusters (*S. Melongena* and 3 small CWR clusters) and 8 sub-clusters (I-VIII) when a line was drawn at similarity coefficient of 0.42[22]. An experiment with 19 SSR markers to analysis genetic diversity among 30 Spanish eggplant genotypes revealed a considerable diversity exists within each of the cultivar groups. Germplasm from different regions shows a wide range of genetic diversity as well as phenotypic diversity indeed.

V. CONCLUSION

Bangladesh has wide range of diverse eggplant landraces. This experiment was carried out to investigate the diversity and relatedness among twenty-five local and two wild species found in Bangladesh using five highly polymorphic Simple Sequence Repeats (SSR) markers. Total ten DNA bands were generated from the five SSR primers *viz.* EM114, EM120, EPSSR82, smSSR01 and smSSR04. Amplified alleles ranged from 1 to 3 per locus with an average 2.0 alleles/locus were detected. SSR primer EPSSR82 and smSSR04 produced two polymorphic bands whereas, primer EM120 produced single polymorphic band. But, rest of two SSR primers such as EM114 and smSSR04 were not able to generate any polymorphic band. The Polymorphism Information Content (PIC) for all the markers ranged from 0.37 to 0.67 with an average value of PIC = 0.54. Gene diversity ranges from 0.49 to 0.72, with an average value of 0.61. SSR markers showed an average gene diversity of 0.61 for all the genotypes. Dendrogram figure revealed that, the 25 local and two wild relatives of eggplant into two major clusters. It is concluded that SSR markers have been proved to be a powerful tool for molecular genetic analysis of eggplant germplasm for plant breeding programs to assess genetic diversity for the improvement of cultivars. Molecular characterization of local eggplant data might be helpful to select the diverse parents for development of a new variety.

SIGNIFICANT STATEMENT

This work able to identify polymorphism among local genotypes through SSR markers. Molecular diversity and genetic distance also estabilshed between cultivated eggplant and its wild relatives viz. *Solanum villosum* and *Solanum sisymbrifullium*. The result may utilized as a source of diverse parent for any hybridization program.

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Diversity at DNA level information will be used to conserved the local germplasm for future use.

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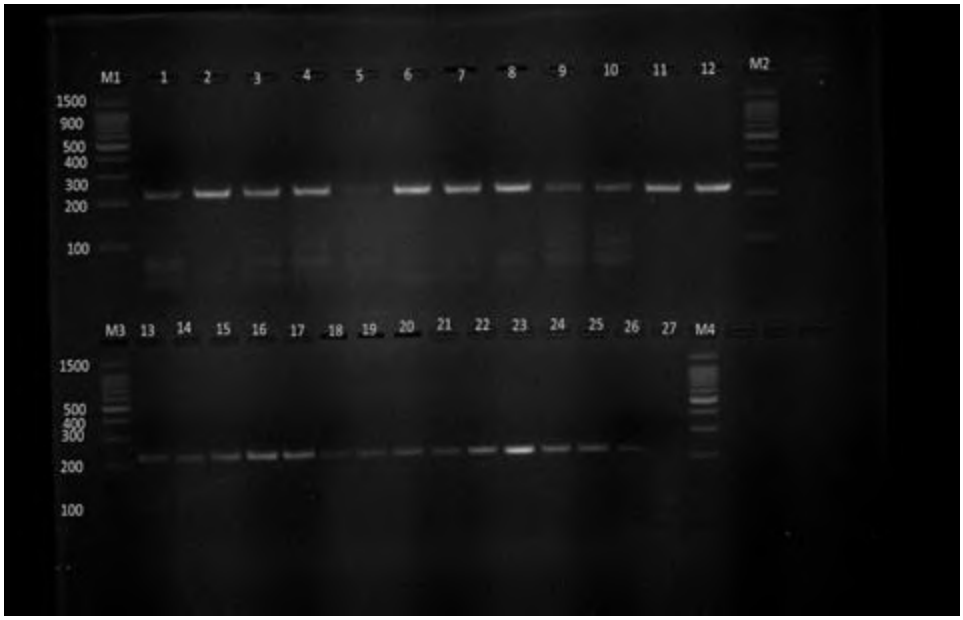

Fig. 1: SSR profile of 27 local and wild eggplant germplasm using primer EM114.

(Lane 1: Salta begun; Lane 2: Ashary; Lane 3: Lalmoni local-1; Lane 4: Lalmoni local-2; Lane 5: Kurigram local; Lane 6: Khotkhotia; Lane 7: Cricket; Lane-8: Rangpurlocal-1; Lane 9: Rangpur local-2; Lane 10: Rangpur local-3; Lane11: Nilphamari local; Lane 12: Dinajpur local ; Lane 13: Thakurgaon local; Lane 14: Bogra local; Lane 15: Iswardi local; Lane 16: Jessore local-1; Lane 17: Jessore local- 2; Lane 18: Jessore local-3; Lane 19: Jessore local-4; Lane 20: Sada khulna; lane 21: Khulna local-1; Lane 22: Jamalpur local; Lane 23: Narsingdi local; Lane 24:Comilla Local; Lane 25: Dohazari; Lane 26 : Wild species Solanum sisymbriifolium; Lane 27: Wild species Solanum villosum and M1=M2=M3=M4=100 bp DNA ladder).

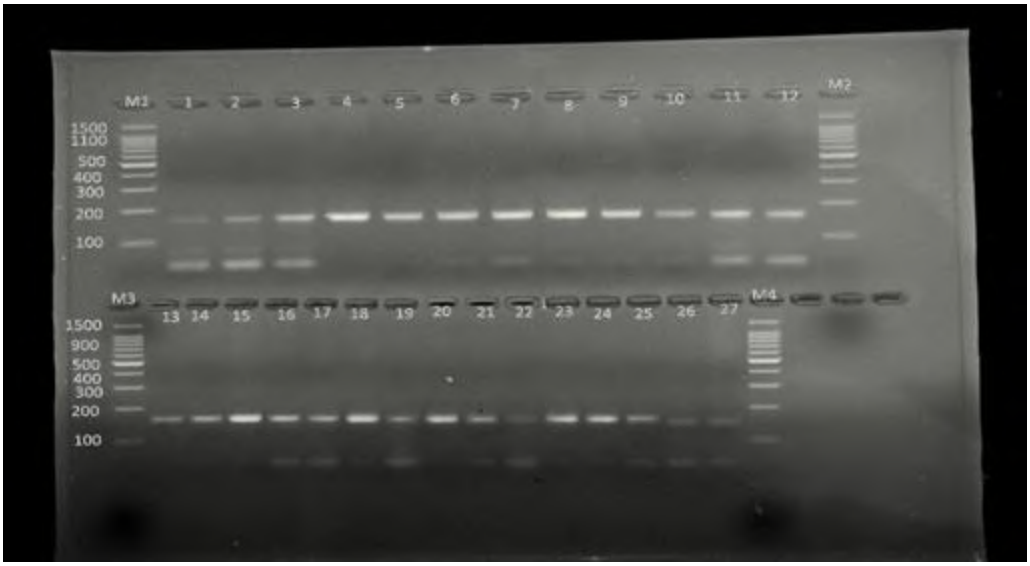

Fig. 2: SSR profile of 27 local and wild eggplant germplasm using primer EM120.

(Lane 1: Salta begun; Lane 2: Ashary; Lane 3: Lalmoni local-1; Lane 4: Lalmoni local-2; Lane 5: Kurigram local; Lane 6: Khotkhotia; Lane 7: Cricket; Lane-8: Rangpur local-1; Lane 9: Rangpur local-2; Lane 10: Rangpur local-3; Lane11: Nilphamari local; Lane 12: Dinajpur local ; Lane 13: Thakurgaon local; Lane 14: Bogra local; Lane 15: Iswardi local; Lane 16: Jessore local-1; Lane 17: Jessore local- 2; Lane 18: Jessore local-3; Lane 19: Jessore local-4; Lane 20: Sada khulna; lane 21: Khulna local-1; Lane 22: Jamalpur local; Lane 23: Narsingdi local; Lane 24:Comilla Local; Lane 25: Dohazari; Lane 26 : Wild species Solanum sisymbriifolium; Lane 27: Wild species Solanum villosum and M1=M2=M3=M4=100 bp DNA ladder).

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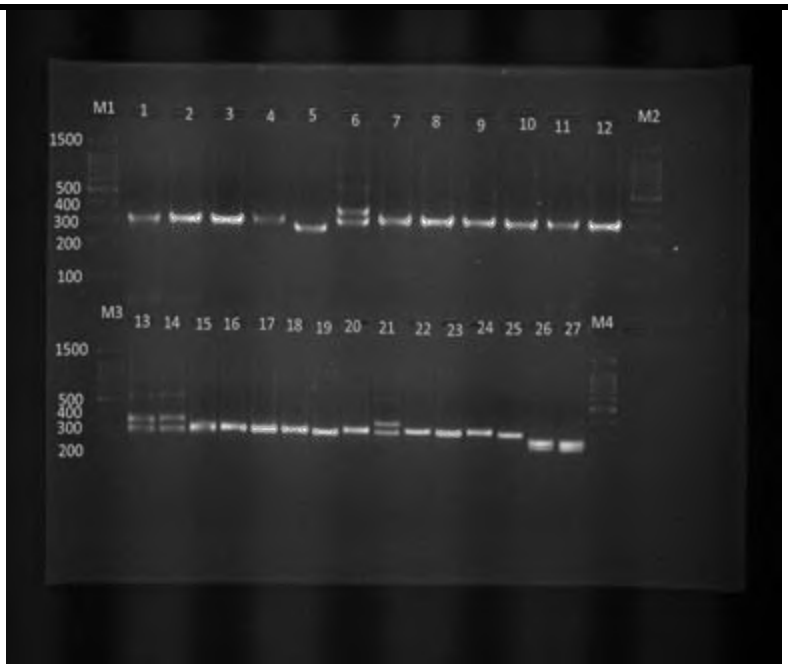

Fig. 3: SSR profile of 27 local and wild eggplant germplasm using primer smSSR01.
(Lane 1: Salta begun; Lane 2: Ashary; Lane 3: Lalmoni local-1; Lane 4: Lalmoni local-2; Lane 5: Kurigram local; Lane 6: Khotkhotia; Lane 7: Cricket; Lane-8: Rangpur local-1; Lane 9: Rangpur local-2; Lane 10: Rangpur local-3; Lane11: Nilphamari local; Lane 12: Dinajpur local ; Lane 13: Thakurgaon local; Lane 14: Bogra local; Lane 15: Iswardi local; Lane 16: Jessore local-1; Lane 17: Jessore local- 2; Lane 18: Jessore local-3; Lane 19: Jessore local-4; Lane 20: Sada khulna; lane 21: Khulna local-1; Lane 22: Jamalpur local; Lane 23: Narsingdi local; Lane 24:Comilla local; Lane 25: Dohazari; Lane 26 : Wild species Solanum sisymbriifolium; Lane 27: Wild species Solanum villosum and M1=M2=M3=M4=100 bp DNA ladder.

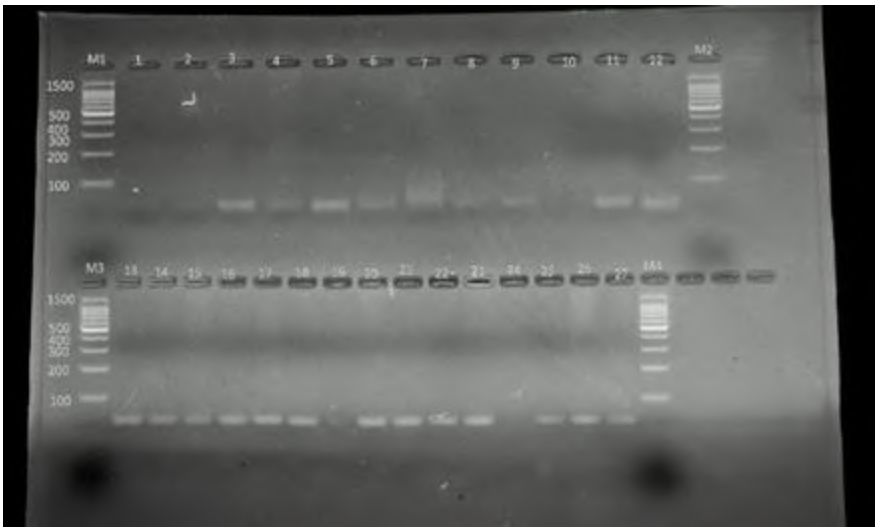

Fig. 4: SSR profile of 27 local and wild eggplant germplasm using primer smSSR04.
(Lane 1: Salta begun; Lane 2: Ashary; Lane 3: Lalmoni local-1; Lane 4: Lalmoni local-2; Lane 5: Kurigram local; Lane 6: Khotkhotia; Lane 7: Cricket; Lane-8:Rangpur local-1; Lane 9: Rangpur local-2; Lane 10: Rangpur local-3; Lane11: Nilphamari local; Lane 12: Dinajpur local ; Lane 13: Thakurgaon local; Lane 14: Bogra local; Lane 15: Iswardi local; Lane 16: Jessore local-1; Lane 17: Jessore local- 2; Lane 18: Jessore local-3; Lane 19: Jessore local-4; Lane 20: Sada khulna; lane 21: Khulna local-1; Lane 22: Jamalpur local; Lane 23: Narsingdi local; Lane 24:Comilla Local; Lane 25: Dohazari; Lane 26 : Wild species Solanum sisymbriifolium; Lane 27: Wild species Solanum villosum and M1=M2=M3=M4=100 bp DNA ladder.

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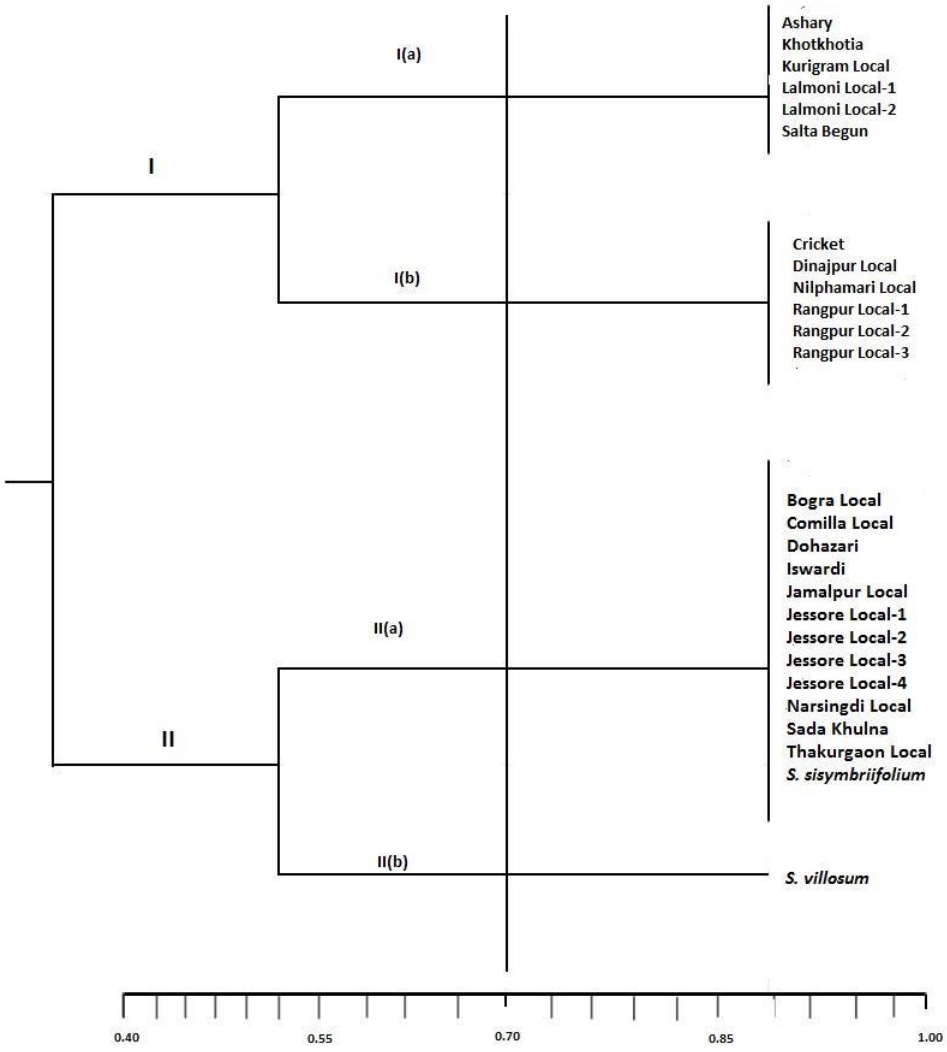

Fig.5: Unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei"s (1972) genetic distance, summarizing data on differentiation for twenty-five local and two wild relatives of eggplant.

Table 1: Name of the local genotypes and their collected area in Bangladesh.

SL. No.	Entry Name	Collected Area
1	Salta Begun	Lalmonirhat District,
2	Ashary	Lalmonirhat District
3	Lalmoni Local-1	Lalmonirhat District
4	Lalmoni Local-2	Lalmonirhat District
5	Kurigram Local	Kurigram District
6	Khotkhotia	Rangpur District
7	Cricket	Rangpur District
8	Rangpur Local-1	Rangpur District
9	Rangpur Local-2	Rangpur District
10	Rangpur Local-3	Rangpur District
11	Nilphamari Local	Nilphamari District
12	Dinajpur Local	Dinajpur District
13	Thakurgaon local	Thakurgaon District
14	Bogra Local	Bogra District

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15	Iswardi	Local	Pabna District
16	Jessore	Local-1	Jessore District
17	Jessore	Local-2	Jessore District
18	Jessore	Local-3	Jessore District
19	Jessore	Local-4	Jessore District
20	Sada Khulna		Khulna District
21	Khulna	Local-1	Khulna District
22	Jamalpur	Local	Jamalpur District
23	Narsingdi	Local	Narsingdi District
24	Comilla	Local	Comilla District
25	Dohazari		Comilla District
26	Wild species (*Solanum sisymbriifolium*)		*BARI, Gazipur
27	Wild species (*Solanum villosum*)		*BARI, Gazipur

*BARI= Bangladesh Agricultural Research Institute

Table.2: PCR amplified DNA fragment size and number of polymorphic band with 27 genotypes

Primer no.	Primers' Name	Primer sequences (5'-3')	(G+C) %	No.of DNA band(s)	No.of polymorphic band(s)	Band size range (bp)
1	EM114	For. AGCCTAAACTTGGTTGGTTTTTGC Rev.GAAGCTTTAAGAGCCTTCTATGCAG	43	1	0	225
2	EM120	For. GGATCAACTGAAGAGCTGGTGGTT Rev.CAGAGCTTCAATGTTCCATTTCAC A	44	2	1	50-180
3	EPSSR82	For. ACATGCCACTCATGTTGGTG Rev. CTTCAGCCATGGACCACATT	50	3	2	50-180
4	smSSR01	For. GTGACTACGGTTTCACTGGT Rev. GATGACGACGACGATAATAGA	46	3	2	200 - 400
5	smSSR04	For. AATGAGTCAGAAACCACGCC Rev. CGTTTAACCTTTGGCTCGGAA	49	1	0	50-80
Total	-	-	-	10	5	-
Mean	-	-	-	2.0	1.0	-

Table.3: Major allelic frequency, gene diversity and PIC value of different eggplant genotypes

Markers	Obs. No.	Availability	Allele no.	Major allele frequency	Gene diversity	PIC value
EM114	27	1.00	1.0	0.52	0.63	0.57
EM120	27	1.00	2.0	0.56	0.61	0.55
EPSSR 82	27	1.00	3.0	0.33	0.72	0.67
smSSR 01	27	1.00	3.0	0.56	0.49	0.37
smSSR 04	27	1.00	1.0	0.48	0.61	0.53
Mean	27	1.00	2.0	0.49	0.61	0.54

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Bacterial wilt resistant gene searching in Eggplant (*Solanum melongena*) and its two wild relatives

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Abstract**—** *Eggplant is an important vegetable in all over the world. Bacterial wilt caused by Ralstonia solanacearum is a major disease of eggplant. Near about 32% crop loss occurred due to this disease. The wild relatives of eggplant viz. Solanum villosum and Solanum sisymbriifolium showed resistant against bacterial wilt disease. Hence, attempted was made to search the resistant gene of bacterial wilt in three Solanum spp. Some total of 16 different bacterial wilt resistant molecular markers RAPD (OPB-17, OPG-05, OPH-07) UBC (176, 205, 317) SSR (STM0007, emh01J23, emb01N07) SCAR (me1, me2, me4, me5) and SRAP (SCU176-534, SCU176-1190F1R1, SCU176-1190F2R2) were screened to identified the resistant DNA fragment. Only one primer UBC#176 showed amplification in target DNA. The primer UBC#176 gave DNA amplification in target position at 350 bp in the species of Solanum villosum. The obtained DNA fragment showed maximum 79% homology with Solanum lycopersicum cultivar I-3 chromosome 8 which has GeneBank Accession No. CP023764.1. Alternative approach was made to identify resistant gene of bacterial wilt disease. Total RNA was extracted and cDNA was synthesized. The synthesized cDNA was used as template to find out the resistant gene. But none of the gene specific primer was amplified using cDNA as template. Further study was needed to find out the bacterial wilt resistant DNA fragment in wild type.*

Keywords**— Bacterial wilt, eggplant, eggplant wild relatives, resistant gene, sequencing.**

I. INTRODUCTION

Eggplant or brinjal (*Solanum melongena* L. 2n = 2x = 24) is an important solanaceous crop grown widely in Asia, Africa, and the subtropical region including the southern USA. It is the second most important solanaceous fruit crop after tomato *(Solanum lycopersicum* L.) [1]. In respect of total acreage production eggplant is the second most important vegetable crop next to potato in Bangladesh [2]. A large number of eggplant cultivars are grown in Bangladesh. In Bangladesh it is cultivated in an area of about 1,25,860 acres with productivity of 5,07,432 MT (BBS 2017). Eggplant has a number of health benefits such as it has ayurvedic medicinal properties and is good for diabetic patients. It has been recommended as an excellent remedy for those suffering from liver complaints [3]. It is an important source of fiber, potassium, manganese, as well as vitamins C, K, and B6. Phenolic compounds in eggplant contain significant amounts of chlorogenic acid, one of the most powerful free radical scavengers found in plants. Chlorogenic acid has been shown to decrease low density lipid (LDL) levels, and also serves as an antimicrobial, antiviral, and anti-carcinogenic agent.

Successful cultivation of eggplant crop has been hindered due to infestation of many insect pests and diseases. Among these, bacterial wilt disease is the most devastating and a limiting factor caused by *Ralstonia solanacearum* throughout the tropical, sub-tropical and temperate regions of the world [4, 5]. Bacterial wilt is an important disease of many plant species especially solanaceae [6]; causing enormous economic losses, which limits eggplant production from 4.24 to 86.14 percent [7]. It perpetuates in the soil, enters the plant through the roots, progressively invades the stem vascular tissues and blocking of the vessels by bacteria, and finally it leads to partial or complete wilting of plant [8]. Understanding the host and pathogen is a pre requisite for devising proper strategies for control of disease. Control of wilt diseases is also complicated by the scarcity of sources of disease-resistant host germplasm, and the soil and vascular habitats of the pathogen [4, 9]. Host resistance appears to be the main strategy for the control of bacterial wilt because of the

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resistant imposed by the lack of effective chemical controls, the soil-borne nature of the pathogens, and the wide range of hosts [10].

In the Solanaceae, most cultivated species (*Solanum melongena* L.) is highly susceptible to bacterial wilt. The wild species *Solanum phureja* [11, 12] and *Solanum stenotomum* [11] were identified as possible sources of resistance for potato (*Solanum tuberosum*), *Lycopersicon pimpinelifolium* for tomato (*L. esculentum*) [13] and *S. villosum, S. torvum, S. sisymbriifolium, S. gilo* and *S. aethiopicum* [14] for eggplant (*S. melongena*). *Solanum villosum* Mill. has been reported to be useful for introgression of disease resistance. *Solanum villosum* is (2n= 48) believed to have originated in Eurasia, and is sometimes considered to be resistant to bacterial wilt. Interestingly *Solanum villosum* has been identified to carry traits of resistance to most serious diseases of eggplant, particularly bacterial and fungal wilts [15].

Despite of its valuable potential for disease resistance, little is known about *S. villosum* regarding variability in morphology, fertility and levels of resistance to both bacterial and fungal wilts, as well as genetic diversity. The molecular technique is applied to identify disease resistant gene from wild relatives of eggplant. *Solanum villosum* and *Solanum sisymbriifolium* are two important wild relatives of eggplant. Sequence-related amplified polymorphism (SRAP) technology has been recognized as a new and useful molecular marker system for mapping and gene tagging in many crops plants [16]. Sequence characterized amplified region (SCAR) markers are more reproducible and easier to manipulate in marker-assisted selection (MAS) programs than other markers. Due to the co-dominant or dominant nature, SCAR marker can provide a valid tool for the accurate assessment of genotype at the linked locus. SCAR can be considered to be an ideal marker for plant breeding programs. It was screened that two SCAR markers linked to *Fusarium* wilt resistance gene in eggplant [16]. Hence, three *Solanum spp*. *viz. Solanum melongena, Solanum villosum* and *Solanum sisymbriifolium* were used for screening of bacterial wilt resistant gene through SCAR marker. In addition, other categories of molecular markers *viz.* RAPD, UBC, SSR and SRAP were used to identify the target amplification. Therefore, the research work was carried out to investigate the source of bacterial wilt resistant gene in three *Solanum spp*. including cultivated eggplant.

II. MATERIALS AND METHODS

One variety (BARI Begun-01) of eggplant (*Solanum melangena*) and two wild relatives *viz*. *Solanum villosum* and *Solanum sisymbriifolium* were used as experimental materials. DNA extractions from young leaves were performed according to a modified Doyle and Doyle (1990) [17] method by using CTAB protocol. On the basis of literature five different set of molecular markers were synthesized which showed linkage with bacterial wilt resistant in some other crops. The types of molecular markers are (a) RAPD (b) UBC (c) SSR (d) SCAR and (e) SRAP. Some total of 16 different primers were synthesized from all groups of primer. The list of primers is given in 'Table- 01'.

1.1. DNA isolation and purification

Genomic DNA was extracted using CTAB method from three species of *Solanum*. The RNase and proteinase treatment was given to the extracted DNA for purification from RNA and protein. The purified DNA was visualized by 2% agarose gel. DNA concentration was measured by DNA Nanodropper. Working DNA sample was prepared (20-25 nm/µl) for PCR reaction on the basis of concentration of main stock.

1.2. PCR amplification and elution of DNA fragment from gel

PCR amplification was carried out with purified genomic DNA of three *Solanum spp*. and the above gene specific primers. Ten microliter (10 µl) reaction mixture (Bio Basic, Canada, GeneON, Taiwan) was used for target amplification. The annealing temperature was adjusted on the basis of Tm of the primer. The PCR product was visualized on 2% agarose gel and save in gel documentation system. For further study the target fragment of DNA was cut from agarose gel with fine blade and taken in an eppondrof tube. Then the gel fragment was eluted by QIAquick Gel Extraction Kit. The eluted DNA fragment was stored at -20ºC and it was rechecked in 2% agarose gel.

1.3. DNA sequencing and alignment of nucleotide

The primer UBC- 176 produced approximate 350bp DNA fragment. The amplified 350bp fragment of DNA was sequenced by Applied Bio-system, DNA. The obtained DNA sequences were used in NCBI, BLAST program to carry out the sequence homology with other organism through computer software. Another molecular approach was carried out to findout the bacterial wilt resistant gene. The methodological steps are given below.

1.4. RNA extraction and cDNA synthesis

The total RNA was extracted from three *Solanum spp*. by QIAGEN RNA extraction kit. Near about 50-100 mg fresh leaf sample were collected and it was soaked in liquid

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nitrogen and then grind with mortar and pestle. In the successive step QIAGEN RNA extraction protocol was used to isolate total RNA from the leaf sample. Finally the RNeasy spin column was used to isolate the total RNA. Approximate 30-40 µl RNase-free water was added directly to the spin column membrane and the RNA elution was done by 10000 rpm for 1 min. The extracted RNA was visualized by 1% agarose gel.

The total isolated RNA was used for cDNA synthesis. cDNA Synthesis was done by QIAGEN Quantitect Reverse Transcription Kit. Template RNA was thaw on ice. gDNA Wipe out Buffer, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix, and RNase-free water were thaw at room temperature (15–25°C). Each solution mixed by flicking the tubes. It was centrifuged briefly to collect residual liquid from the sides of the tubes, and then stored on ice. The genomic DNA elimination reaction was prepared on ice according to QIAGEN Kit mixed and then stored on ice. Incubation was done for 2 min at 42°C. Then immediately placed on ice. The reverse-transcription master mix was prepared on ice according to same protocol mixed and then stored on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA. Template RNA was added to each tube containing reverse-transcription master mix. Mixed and then stored on ice. Incubation was done for 15 min at 42°C. Incubation was done for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase. Reverse-transcription reactions were stored on ice and proceed directly for long-term storage, store reverse-transcription reactions at –20°C temperature.

1.5. PCR amplification using cDNA as a template

All five categories primers ('Table no.1') were used for amplification of target DNA band. Different thermal conditions were used for different primer to get specific DNA fragment.

III. RESULTS AND DISCUSSION

Solanum melangena (eggplant) is highly susceptible to bacterial wilt disease. *Solanum villosum* and *Solanum sisymbriifolium* are two wild relatives of *Solanum melangena* which showed considerable resistance to bacterial wilt. Hence, attempt was made to screen these two *spp*. with different molecular markers to identify any DNA fragments which are linked with bacterial wilt resistance gene. The major finding of these experiments was given in following sub-heading.

1.6. PCR amplification with different gene specific primers

The three species *viz. Solanum melangena* (BARI begun-01), *Solanum villosum, Solanum sisymriifolium* template DNA were used for amplification of DNA fragment which may linked with bacterial wilt resistant gene. Some total of 16 primers ('Table No. 1') were used for the same. It was showed that, only one primer UBC#176 was given amplification at 350 bp DNA fragment in the species *S. villosum* ('Fig. No.1'). It was not amplified in *S. melangena* (BARI begun- 01), *S. sisymriifolium*. It was reported that a total of 800 RAPD primers were screened and only six primers (UBC#176, 205, 287, 317, 350, and 676) showed polymorphism between resistant pool and susceptible pools of bacterial wilt in the Tomato Line Hawaii 7996 [19] . Of these, only two markers UBC#176 and 317 revealed a 100% linkage in the individual plants comprising the contrasting bulks. The marker UBC#176 was converted into a co-dominant SCAR marker and designated as SCU176-534. The marker SCU176-534 was confirmed by genotyping the individual of the resistant pool and susceptible pools and gave the same result as UBC#176 [19]. The above mentioned reference was a good evidence of our research finding. Hence, there is a probability to identify bacterial wilt resistance DNA sequence in this 350 bp DNA fragment. Hence investigation was carried out with the specific 350 bp fragment.

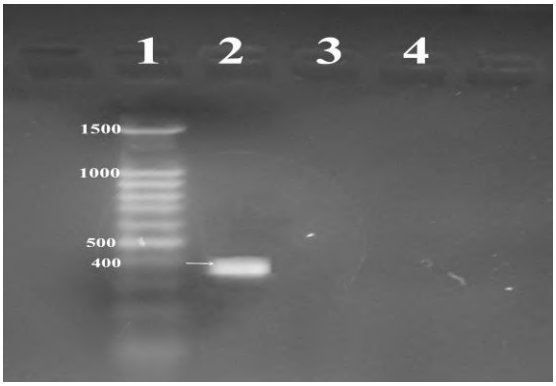

Fig. 1: DNA amplification in Solanum villosum at 350 bp position, Line 1: Ladder, lane 2: Solanum villosum, lane 3: Solanum sisymriifolium,lane 4: Solanum melangena

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Table 1: List of molecular markers which are tightly linked or linked with bacterial wilt resistant gene

Sl No.	Types of Marker	Marker Name	Forward Primer(5′-3′)	Reverse Primer (5′-3′)	Reference List
1.	RAPD	OPB-17	AGGGAACGAG	----	[19]
2.		OPG-05	CTGAGACGGA	----	
3.		OPH-07	CTGCATCGTG	----	
4.	UBC	UBC#176	CAAGGGAGGT		[20]
5.		UBC#205	CGGTTTGGAA		
6.		UBC#317	CTAGGGGCTG		
7.	SSR	STM0007	GACAAGCTGTGAAGTTTATT	AATTGAGAAAGAGTGTGTGTG	[19]
8.		emh01J23	ATGCAGCTCCCATAAACCCTAAAA	GTTTCCAAGACCAGCACTCCAAAC	[21]
9.		emb01N07	TGATAAGAAGGGCAAGCTCAGTCC	GTTTCGAGCTTATGGCTACACTGGACCT	
10.	SRAP	me1	TGAGTCCAAACCGGATA	GACTGCGTACGAATTAAT	[22]
11.		me2	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTTGC	
12.		me4	TGAGTCCAAACCGGACC	GACTGCGTACGAATTTGA	
13.		me5	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTCGA	
14.	SCAR	SCU176-534:	TTGAACCAAGAATCTATTCG	GAACTTGAATGCCTACCAAA	[20]
15.		SCU176-1190F1R1	TGCGGATACTATCGGAAATA	CAACTCATTTCAGTCCGATT	
16.		SCU176-1190F2R2	TCACTCGGTGAGTCAATAGAT	TTTGCCGATGTTATCATGT	

1.7. Gel elution, DNA purification and sequencing of target DNA band

The species *Solanum villosum* produced 350 bp DNA fragment with UBC#176 primer. The amplified fragment was eluted from agarose gel and taken into eppendorf tube. It was purified by QIAGEN DNA purification Kit. The purified DNA fragment was again loaded in the 2% agarose gel for confirmation of purification. It showed exact, DNA band in the same 350 bp position. Hence, it proved that, purification of DNA was successfully completed. The concentration of purified DNA was done by DNA nanodropper. It was 20-25 ng/µl. This amount is sufficient for sequencing works. The required amount of DNA was sent to abroad for sequencing of nucleotide. The sequence of nucleotide was given below. It was 341 bp fragments.

GCAGGAAAAAATGCGGGAATTCCTATTGGCGCCA
GCTCGTTCACGCCGGAAAACCCCTTTTTCAATCGT
GGGAGCTTGACGTACACCTCCCTTGAAAAGTCTG
ATCTATTTTGCTATTGTCCTTACGAATTTTATCGG
AAAATTGATGAAATATGATCGAAGAACCATCCCC
AAAAAAATTATGAAAACTGGGATAATTCCTCCTG
CACCGGGTTGGTTACACTCGAAAACCCCTCCTTAT
ATCGTGTATGCTTGTCCTTTGCCTCCCTTGGGGGG
GTCCGGCCCAGTTTTTGCGAACTCCAACAAATTCC
CCGGAATACCTGACCCCTCCCTGGACAATGGTGT
GGT = 341 bp

1.8. Alignment and homology searching

The obtained sequenced DNA was used for NCBI, BLAST to identify homology with other gene of interest. *Solanum villosum* sequence get 78% homology with *Solanum lycopersicum* cultivar I-3 chromosome 5 which has GeneBank Accession No. CP023761.1. It also showed 78% homology with *Solanum pennellii* chromosome ch05, complete genome which has GeneBank Accession No. HG975444.1 and 79% homology with *Solanum*

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lycopersicum cultivar I-3 chromosome 8 which has GeneBank Accession No. CP023764.1.

Another alternative approach was made to search the bacterial wilt resistance gene in *S. melangena, S. villosum* and *S. sisymriifolium*. The assumption is that, from total RNA we will synthesis cDNA and this cDNA will be used as template for amplification of target bacterial wilt resistant DNA fragment. Previous synthesized all linked gene specific primer will be used for the same. On the basis of this principle the following activities were done.

1.9. Total RNA extraction and cDNA synthesis

Total RNA was extracted from three species *viz. S. melangena* (BARI begun-01), *S. villosum, S. sisymriifolium* were used to check the RNA molecule. It was done on the basis of QIAGEN RNA purification kit. The 2% agarose gel ('Fig. 2'). The high quality RNA band was noticed from the three species. Near about 400-500 µg/µl RNA was generated from leaf tissue. Those total RNA used as a template for cDNA synthesis. QIAgen cDNA synthesis kit was used for the same. The reaction mixture and other protocol were mentioned in the methodology. The produced cDNA was stored at -20°C for further use.

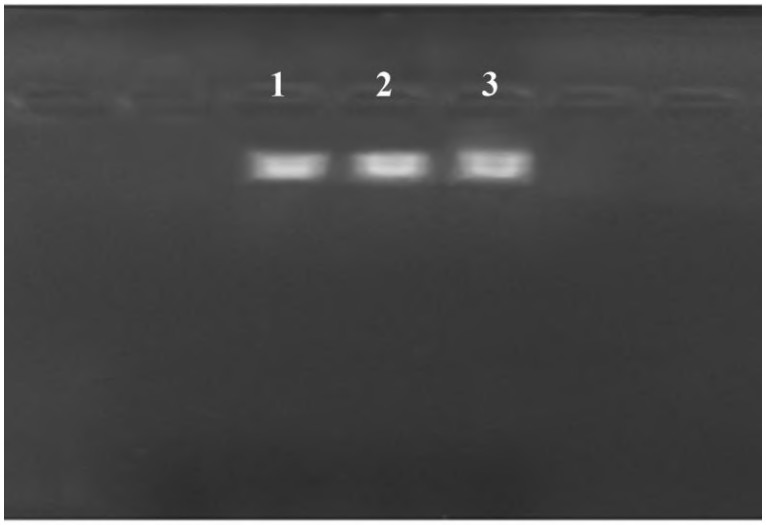

Fig. 2: RNA amplification in Lane 1- BARI Begun 1, Lane 3-Solanum villosum, Lane 4- Solanum sisymbriifolium

1.10. PCR amplification with cDNA as template

The previously synthesized all tightly linked gene specific primers were used for PCR amplification to identify bacterial wilt resistant gene. It was sorry to say that, none of the 16 primers gave amplification in any one of the three *Solanum spp.*

IV. CONCLUSION

Eggplant is an important vegetable in our country. It has multipurpose used in our daily consumption. It's production is seriously hampered due to different biotic diseases. Among them bacterial wilt disease is major one. Wild relatives of eggplants are resistant to bacterial wilt. *Solanum villosum* and *Solanum sisymriifolium* are two common wild relatives of eggplant which showed resistant to bacterial wilt. Hence, attempt was made to identify DNA fragment from wild species which will be resistant to bacterial wilt. Two different approaches was carried out to fulfill the objectives. Some total of 16 different bacterial wilt resistant molecular markers were used for the same. Out of them only one primer UBC#176 was gave DNA amplification in target position (350 bp) in the species *Solanum villosum.* The obtained DNA fragment was sequenced and homology was detected through NCBI, BLAST. It was showed maximum 79% homology with *Solanum lycopersicum* cultivar I-3 chromosome 8 which has GeneBank Accession No. CP023764.1.

Alternatively, Total RNA and cDNA was used to amplify the target link DNA fragment by using of previous primer. None of the primer was able to regenerate any DNA fragment which was linked to bacterial wilt. Hence further CODEHOP method, genome editing or any advanced molecular technique may be applied for searching of target bacterial wilt resistant gene.

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