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Research Article

HERBICIDE RESISTANCE IN SRI LANKAN RICE (*Oryza sativa* L.) VARIETIES: A MOLECULAR APPROACH

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Abstract: Application of herbicides at various stages of rice fields with varying frequencies is a common practice in controlling rice weeds. Introduction of Herbicide Resistant (HR) rice enabled implementation of weed management with reduced cost, labor and efficacy while minimizing the environment pollutions. A broad-spectrum herbicide, N-(phosphonomethyl) glycine commercially known as glyphosate, targets 5-pyruvyl shikimate 3-phosphate synthase (EPSPS), an enzyme involved in the biosynthesis of aromatic amino acids through shikimic acid pathway in plants. The plant mutagen, Ethyl Methyl Sulfonate (EMS) causes mutants in EPSPS which occur in glyphosate tolerant crops. The present study focused on the development of HR-rice varieties through chemical mutagens, which adds another dimension into effective weed management in rice cropping systems in Sri Lanka paving the way to secure the food supply to the nation. HR-rice lines were developed using EMS and Fluorescent Amplified Fragment Length Polymorphism (FAFLP) analysis was carried out to identify molecular markers for HR-induced varieties. Seeds of twenty-five cultivated rice varieties were treated with 0.5gl-1 EMS and resulted mutated survived plants were exposed to glyphosate to assess the herbicide resistance. AFLP analysis was performed on EMS-mutated rice plants with 16 AFLP primer combinations. AFLP maker E11M31 indicated higher discriminative capability for natural herbicide resistant rice lines while E11M32 for HR-induced rice lines produced through EMS mutagenesis.

Keywords: *Ethyl Methyl Sulfonate, FAFLP, Glyphosate, Mutagenesis, Rice*

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Introduction

Rice is the world's most important staple food crop for more than half of the human population [1]. Approximately 134 weed species belong to 32 taxonomic families were identified in the rice field in Sri Lanka and they were categorized as grasses, sedges and broad leaves [2]. Weeds reduce the yield qualitatively and quantitatively leading to considerable yield loss and increased production costs with poor economic turn over [3]. Climate change also influences weeds indirectly by enforcing adaptations of agronomic practice [4]. Therefore, it is imperative to develop effective weed control strategies while maintaining crop yield [5]. Globally, ca. 10% loss of rice yield attributed to weed losses are more or less close to 46 million t based on 1987 world rough rice production. Depending on the predominant weed flora and on the controlling, methods practiced by farmers, loss of yield caused by weeds vary across countries in the world. Weed management techniques are necessary to obtain a quality yield because weeds are ubiquitous in rice cultivation areas. Weed control using herbicides is the most popular method which allows economically viable weed control providing cost-effective method in the production of agricultural crops [6]. The introduction of herbicide resistant (HR) rice is a novel approach to enhance selectivity and crop safety in rice cultivation. HR crops provide better flexibility in weed management and new approach to management of hard-to-control weed. Accordingly, farmers can easily control weeds during the entire growing season and have more freedom in deciding the spraying time. Herbicide resistant crops also facilitate with low or no tillage cultivating practices which is more sustainable [7].

Over the last two decades, mutational techniques have become one of the most important tools available to progressive rice-breeding programs and most of the herbicide-tolerant mutants were developed through chemical mutagenesis specific herbicides [8,9]. Among the chemical mutagens, Ethyl Methyl Sulfonate (EMS) is a chemical mutagen which alters the chromosome structure [10].

Application of molecular markers in plant breeding programs has used to gather information on variation on DNA sequences [11]. Conventional breeding programs often based on morphological markers are highly depend on environmental factors as well as time consuming, labor intensive and require large plant populations [12]. Conventional breeding process, in general, require more than a decade to improve a new variety even the performance of the variety concerned cannot be guaranteed. The molecular marker technology makes this procedure more efficient. Development of molecular markers for the purpose of identification of herbicide resistance provides the easiest way of selection of HR lines/varieties in rice breeding programs. Amplified Fragment Length Polymorphism (AFLP) is a DNA fingerprinting technique [13].

The AFLP methodology has been employed in a greater number of studies, such as diversity, phylogeny [14] genomic linkage mapping [15] and the identification of varieties [16], because of the effectiveness and reliability of the methodology. Furthermore, AFLP is widely accepted as an effective tool for the identification of genomic differences among closely related species [17]. As far as Sri Lanka is concerned, limited work [18-23] is reported in relation to the development of herbicide resistance in rice through mutagenic chemicals. As a continuation of previous investigations conducted [18] the objective of the present study was to identify the HR induced rice varieties via chemical mutagen with the aid of florescent AFLP markers.

Materials and Methods

A total of 19 inbred-developed rice varieties and five traditional varieties were used in the study are listed in the [Table-1]. All rice varieties were collected from Rice Research Development Institute (RRDI) at Bathalagoda, Ambalanthota, Bombuwela and Labuduwa in Sri Lanka.

Table-1 *List of rice varieties used in the study*

Select on Number	Name	Age (Month)	Pedigree	Attributes
1	Bg 94-1	3 1/2	IR 262/Ld 66	High yield WP
2	Bg 250	2 1/2	Selection from farmer	
3	Bg 300	3	Bg 367-7//IR 841/Bg 276-5	Resistant to GM- 1, BL,BB,Bph
4	Bg 304	3	Co 10/IR 50//84- 1587/Bg 731-2 GC	Resistant to BL,BB & GM- 1,Bph
5	Bg 305	3	Bg 1203/Bg 1492	Resistant to GM 1&2, BL, BB, Bpb
6	Bg 352	3 1/2	Bg 380/Bg 367-4	resistant to GM BPH,BL and BLB
7	Bg 357	3 1/2	Bg 797/Bg 300//85- 1580/Senerang	Resistant to GM- 1& 2, BL, BB, Bp
8	Bg 359	3 1/2	Bg 12-1/Bg 1492	Bg 12-1/Bg 1492 Resistant to GM 1 & 2, BL, BB, Bi:
9	Bg 360	3 1/2	88-5089/Bg 379-2	Resistant to GM-1, GM-2, BL, Bph
10	At 362	3 1/2	At 85-2/Bg 380	
11	Bg 364	3 1/2	IR 36/Bw 267-3- 11 M	
12	Ld 365	3 1/2	SEL Ld 355	Resistant to iron toxicity
13	Bg 366	3 1/2	Bg300/94-2236//Bg 300/Bg 304	
14	Bg 369	4 1/2		
15	Bg 379-2	4	Bg 96-3*2/Ptb33	Resistant to Bph and BB
16	Bg 403	4 1/2	83- 1026/Bg 379-2	Resistant to BB, BL and Bph
17	Bg 450	4 1/2	Bg 12-1*2/IR42	resistant GM- 1
18	Bg 454	4 1/2	MR 1523/87-519	
19	Bg H4	4	Murungakayan 302/Mas	Wide adaptability, Red pericarp.
20	*KaluHeenati*	4		
21	*Kuruluthuda*	4	Pure line varieties	
22	*Suwadal*			
23	*Rathhal*			
24	*Madel*	3.5/4		
25	*Pachchaperu mal*	3 1/2		

Table-2 Adapters and Primers used in the AFLP study

Oligo Name	Oligo Sequence
MI3 +EIO	5' TGT AAA ACG ACG GCC AGT GAC TGC GTA CCA ATT CAA 3'
MI3 + EII	5' TGT AAA ACG ACG GCC AGT GAC TGC GTA CCA ATT CAC 3'
MI3 + EI2	5' TGT AAA ACG ACG GCC AGT GAC TGC GTA CCA ATT CAG 3
MI3 + EI3	5' TGT AAA ACG ACG GCC AGT GAC TGC GTA CCA ATT CAT 3'
M31	5' GAT GAG TCC TGA GTA AAA A 3'
M32	5' GAT GAG TCC TGA GTA AAA C 3'
M33	5'GAT GAG TCC TGA GTA AAA G 3'
M34	5' GAT GAG TCC TGA GTA AAA T 3'
EOOS	5'GACTGCGTA CCAATTC3 '
MOOS	5'GATGAGTCCTGAGTAA'
ECORI	EAD 1 - 5 'CTC GTA GAC TGC GTA CC 3'
ADAPTOR	EAD 2 5' AAT TGG TAC GCA GTC TAC 3'
MSE 1	MAD 1 5'GAC GAT GAG TCC TGA G 3'
ADAPTOR	MAD 2 5'TAC TCA GGA CTC AT 3'

BB: Bacterial leaf blight BL: Rice blast disease GM-1: Biotype one of rice gall midge, GM-2: Biotype two of rice gall midge Bph: Brown plant hopper PS: Photo period sensitive

Method 1-Seed mutation

Rice seeds were soaked in distilled water for a day at room temperature and then treated with 4.5mmol-1 EMS for 12 hours. Then treated seeds were washed in running tap water in order to leach the residual chemicals and allowed to germinate. The germinated seedlings were transferred into pots filled with mud and glyphosate (0.5 gL-1) was sprayed at two weeks after sawing. After applying glyphosate, plants of certain varieties survived with a substantial growth which are resistant to the herbicide, and the plants which dead are susceptible to the herbicide. For each rice variety, percentage resistance was calculated using the equation 1. Plants with ≥ 50% resistance to glyphosate (0.5 gL-1) were arbitrary considered as resistant varieties. The morphological and yield data were also collected from the resistant varieties and controls [18-21].

Percentage resistance (%) = (Number of resistant seedlings in a variety)/(Total number of seedlings grown in the same variety) X 100 (1)

Method 2-FAFLP molecular study

FAFLP analysis was carried out for the mutated and non-mutated (control) varieties using 16 AFLP primer combinations. The protocol followed the traditional procedure [13] with several modifications. Genomic DNA was extracted from seven days old leaves using PhytoSpin™ Plant genomic DNA extraction kit (Ceygen Biotech).

Restriction digestion

An aliquot of 1µg of DNA from each sample was digested with 5 units of EcoR1, 5 units of Mse1, 4µl of 5 X RL buffer (50 mM Tris-Hac, 50 mM MgAc, 250 mM Kac, 25 mM DTT, 100 ng/µl BSA) in a final volume of 20 µl. The digestion mixture was incubated at 37°C for 3 ½ h.

Adapter ligation

Specific synthetic double stranded adapters (EcoR1 adapter, Mse1 adapter) for each restriction site were ligated to the double digested DNA. EcoR1 adapters were made by adding equimolar amounts of complementary EcoR1 single stranded oligonucleotides to a tube. The tube was heated to 65°C for 5min, and then allowed to cool slowly to room temperature. Mse1 adapters were made similarly in separate tube. Annealed double stranded adapters were stored in -20°C.The doubling of digested DNA sample was made by taking an aliquot of 4 µl of ligation reaction mixture [(4 µlMse1 adapter (10 pmolµl-1), 2 µlEcoR1 adapter (10 pmolµl-1), 4.8µl ATP (10 mM), 8µl 5 X RL buffer, 5U of T4 DNA ligase and H_2O up to 40µl)] was added and the tubes were incubated overnight (~16h) at 37°C in incubator. An aliquot of 3µl of sample was run on 1% agarose gel and visualized under UV transilluminator to check the ligation.

Pre-amplification

Following the ligation of adapters, 2µl of digested/ligated DNA was pre-amplified in 25µl reaction containing 2µl each of pre-amplification primers (8 pmolµl-1), 0.5 mMdNTPs, 1 unit of TaqDNA polymerase (Genscript, USA) and 1X PCR buffer containing 1.5 mM $MgCl_2$ and sterile water. PCR amplification was performed using the following cycle conditions. Denaturation at 94°C for 30 s, annealing at 56°C for 60s, extension at 72°C for 60 s and cycles were repeated for 30 times. Sample of 3µl was run on 1% agarose gel and visualized under UV transilluminator to check the amplification.

Selective amplification

The pre-amplification product was diluted 20 times with sterile distil water and used as a template for selective amplification. The selective amplification reaction was conducted in a final volume of 25µl containing 2µl of diluted pre-amplification PCR products, 0.6µl of EcoR1 fluorescent (Fam, Ned, Vic, Pet) labelled primer (8 pmolµl-1), 0.6µl of Mse1 primer (8 pmolµl-1), 0.5 mM dNTP, 1 unit of Taq DNA polymerase (Genscript, USA), 1X PCR buffer containing 1.5mM $MgCl_2$ and sterile water.

The first PCR amplification was carried out using the thermal cycle conditions: denaturation at 94°C for 30s, annealing at 65°C for 30s.Followed by the annealing temperature was reduced by 0.7°C per cycle for the next 12 cycles.

Subsequently, the cycles were repeated further 23 times with the annealing temperature at 56°C. Extension was performed at 72°C for 60 s for all cycles. Consequently, 3µl from sample was run on 1% agarose gel and visualized under UV transilluminator to check the amplification.

A fluorescent labelled DNA size strand LIZ 600 with known fragment size was mixed with each 2.5µl amplified PCR products and 5µl of deionized formamide loading solution and 2.5µl of ultra-pure water. In order to detect florescent, the PCR product with formamide and the size standard were mixed properly by quick vortexing and samples were loaded in 96 well-plates (Axygen Biosciences, USA). Samples were denatured 95°C for 2 min and chilled immediately by well plate on ice. The denatured amplified products were separated through capillary electrophoresis using Mega BACE 1000 Automated DNA Sequencer (GE Healthcare Life Sciences, USA). Electrophoresis parameters used were 3kv of sample injection voltage for 45 s, 10kv of running voltage for 75 min. at 44°C. The method was repeated for the reliability. Base sequences of primers are given in [Table-2]. This experiment was repeated for sixteen primer combinations.

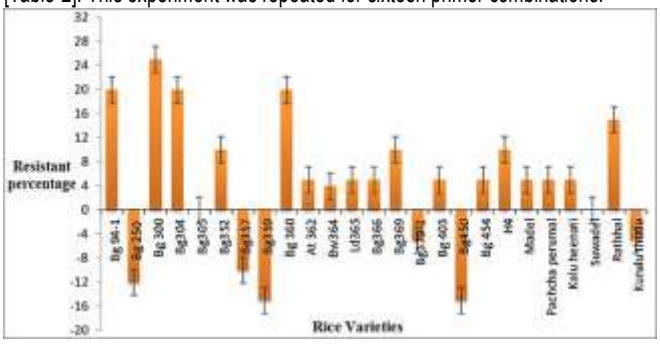

Fig-1 Resistant percentage difference between mutated and non-mutated rice varieties

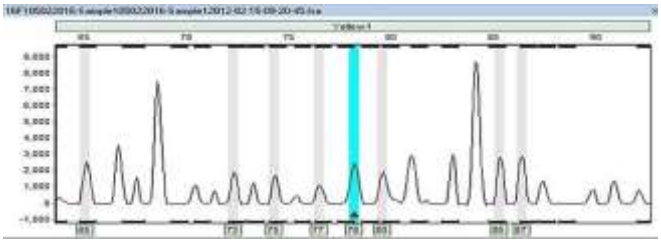

Fig-2 Electropherogram derived from capillary electrophoresis of AFLP analyses for EMS mutated rice lines

Data Analyses

A descriptive statistic was performed on the dataset which obtained from field study. The mean and Standard deviation was computed, and one-way ANOVA was used to compare the means of agro-morphological characters. In molecular data analysis peaks in the range of 30-550 bp of the electropherogram were analyzed and compared by using Mega BACE Genetic Profiler software version 2.2 (GE Healthcare Life Sciences, UK). Each sample was analyzed and compared in the duplicates and unambiguous, reproducible peaks in the electropherogram were scored as AFLP markers. A tolerance limit of 1.0 bases and a minimum peak height of 100 were consistently maintained while scoring the peaks. Each differently sized fragment was treated as a unique character and converted to binary data as presence (1) and absence (0). This binary data was used to compute Jaccard's similarity coefficients [24]and similarity coefficient matrix was generated to assess the genetic resemblances among varieties by using MultiVariate Statistical Package (MVSP 3.1) [25].

Results

The results revealed that most of the rice varieties have increased their resistant to glyphosate after the mutation. (Bg94-1, Bg352, Bg359, Bg360, At362, Bw364, Ld365, Bg366, Bg379-2, Bg403, Bg454, Kaluheenati, Pachchaperumal and Madel) [Fig-1] while six varieties showed reduction. However, a statistically significant differences of plant height, number of panicles/plant, seeds/panicle and 1000 grain weight were observed between non-mutated and EMS-mutated rice plants. However, differences in number of leaves/plant and number of tillers/plant were not statistically significant when comparing mutated rice plants with non-mutated rice plants. AFLP analysis of EMS mutated rice lines indicated variations in several fragments. Out of sixteen primer combinations, five primer sets (E10M31, E10M33, E11M32, E12M32, and E12M33) indicated the possibility to be used in differentiating HR-varieties. Among those the 78 bp fragment of E11+M13 primer (TGT AAA ACG ACG GCC AGT GAC TGC GTA CCA ATT CAC) and M32 primer (GAT GAG TCC TGA GTA AAA) was identified as a specific marker for the resistant lines [Fig-2], which was common to HR induces rice varieties after mutation. All the fragments scored were used in genetic analysis. In the cluster analysis [Fig-3] of all the scored fragments of mutated and non-mutated lines using on UPGMA showed a pattern of clustering in which all the mutated lines were clustered in cluster I while the control (non-mutated) lines were clustered in cluster II. The cluster I further divided into two sub-groups (Group 1A and Group IB).

The group IA included Bg359M, Bw364M, Bg360M, Bg352M, At362M, Bg304M, Bg300M, and Group IB included "MadelM", "PachchaperumalM", H4M, Bg454M, Bg403M, "KaluheenatiM", Bg366M, Ld365M, Bg94-1M Similarly, the Cluster II split into two sub-groups viz. Group IIA including Bg454," Kaluheenati", Bw364 and IIB including Bg366, Bg360, At362, Bg403, Ld365, "Pachchaperumal", "Madel", H4, Bg352, Bg300, Bg359, Bg304 and Bg94-1.

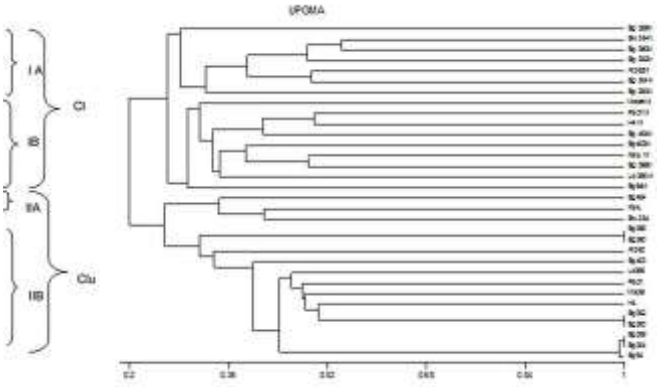

Fig-3 The dendrogram showing genetic diversity among rice varieties (M denotes –S1 varieties, Clu denotes- Cluster) (based on Jaccard's similarity coefficient)

In addition to the cluster analysis, AFLP data on Principal Coordinate analysis (Pco) of mutated and control rice lines were performed with calculating similarity matrix of Gower general coefficient. The pattern of distribution of the mutated and control lines in ordination diagrams are shows in [Fig-4]. The Pco result further confirms the result obtained from the cluster analysis. All the mutated (Denote by M) varieties were clustered in Group A which corresponded to cluster I of the dendrogram and Group B and Group C corresponds to the cluster II o of the dendrogram showing very similar grouping patterns as represented by the cluster analysis.

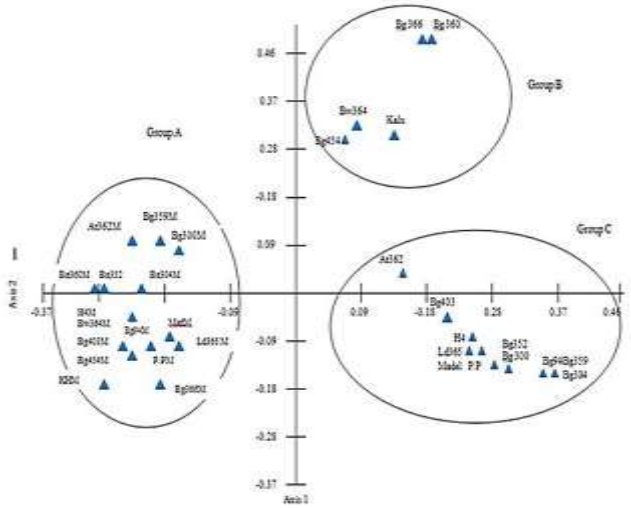

Fig-4 Scattered diagram of principal coordinates (PCo) of Mutated and control rice varieties based on AFLP markers. Oval indicates well-defined groups

Discussion

Induced mutations have been widely used in many other Asian countries for breeding new crop [26]. Varieties such as grain legumes, rice, citrus fruits, mango, papaya etc., which may in turn, contribute to increased food supply and food security [27]. The results of the present study confirmed that the chemical mutagen EMS could be used in breeding programs to develop HR resistant rice lines. Most of the varieties used in the study showed induction of HR after the application of EMS, while six varieties reduced HR and there were two varieties, which showed the same resistant percentage even after the mutation process.

This variation of results revealed that EMS, an alkylating agent, can efficiently induce chemical modification of nucleotides, resulting in various point mutations, including nonsense, missense and silent mutations, among which silent mutations can't generate any modification in phenotype [28]. Among the observed agro-morphological characters, most of the characters were retarded by the mutation process except number of leaves/plant and number of tillers/plants which lead to the yield penalty of mutated plants. However, in a previous report [29] the loss of HR resistant crop yield is much better than the cost for weed control in normal crop cultivars.

In the present study, molecular analysis on the HR induced rice varieties was conducted using fluorescent AFLP markers to confirm the results and to select a molecular marker for the identification of HR rice, which is a continuation of a previous study on inducing herbicide resistance in Sri Lankan rice (*Oryza sativa* L.) varieties by chemical mutagen-EMS [17-20].

The comparison of natural herbicide resistant and susceptible varieties reveals that the E12M32 marker was the appropriate AFLP marker, which can be used in future studies in identifying any natural, herbicide resistant rice varieties. In addition, the E11M32 AFLP marker can also be used for the identification of herbicide resistant induced rice varieties, after mutation.

Conventional breeding processes take 7-10 years to develop a new crop variety and the identification of specific traits make this process more complicated. The identified AFLP markers from the present study make the procedures more efficient, minimizing costs, labor, and time in breeding cycles [30]. However, the present study has used only sixteen rice varieties for the AFLP analysis, and it should be noted that the identified markers are valid only for the selected rice varieties. The reported markers have a potential for use in other varieties to check the occurrence of herbicide resistance. However, repetition of the validity analysis should have to follow before any confirmation of markers.

The dendrogram result from the screening for natural HR rice varieties clearly indicated that glyphosate resistant rice varieties clustered in a separate cluster, while the susceptible lines into another cluster. This confirmed that there are genetic differences between HR and susceptible varieties at the molecular level. Similarly, EMS treated varieties clustered separately, as mutated and non-mutated. The Principal coordinate (Pco) analysis displayed the mutated lines were grouped separately from non-mutated lines. Grouping pattern of the Pco supported the results of the UPGMA cluster analysis. Group A of Pco represents the UPGMA cluster I; group B and C represents the UPGMA cluster II. Therefore, all methods of analyses produced similar results and confirmed the reliability of method of analyzing.

Conclusion

The present study showed that EMS could be used to induce HR in glyphosate susceptible rice varieties, as well as to enhance the existing HR. However, EMS mutant plants showed a slight variation in reduction of agro-morphological characters. Therefore, HR induced varieties may be able use in rice breeding programs for the development of HR rice varieties. This paper reports on genetic analyses of HR induced rice in Sri Lanka by fluorescent AFLP markers.

There is a possibility that maker E11M31 is capable of identification of naturally herbicide resistant lines, while E11M32 indicating a higher discrimination power for HR induced varieties, by chemical mutation. Further, the recognized variations need to be analyzed and sequenced to enable its use as a molecular marker. The introduction of HR rice varieties may add a novel dimension into the effective weed management in rice cultivation.

Future studies are required on the HR rice through several generations, followed by field trials prior to releasing HR rice varieties on a commercial scale. After evaluating HR, these varieties should be subjected to investigations of other important agronomical characters, as well as economic yield constraints

Application of research: The present study focused on the development of HR-rice varieties through chemical mutagens. The findings will enhance effective weed management in rice cropping systems in Sri Lanka and guiding the way to secure the food supply to the nation.

Research Category: Herbicide resistance

Abbreviations: EMS-Ethyl Methyl Sulfonate, HR-Herbicide Resistant
EPSPS-5-pyruvyl shikimate 3-phosphate synthase
FAFLP-Amplified Fragment Length Polymorphism
AFLP-Amplified Fragment Length Polymorphism
RRDI-Rice Research Development Institute
PCR-Polymerase Chain Reaction, ANOVA-Analysis of Variance
UPGMA-Unweighted Pair Group Method with Arithmetic mean

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Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Rice Research Development Institute (RRDI) at Bathalagoda, Ambalanthota, Bombuwela and Labuduwa in Sri Lanka.

Cultivar / Variety / Breed name: Bg 94-1, Bg 250, Bg 300, Bg 304, Bg 305, Bg 352, Bg 357, Bg 359, Bg 360, At 362
Bg 364, Ld 365, Bg 366, Bg 369, Bg 379-2, Bg 403, Bg 450, Bg 454, Bg H4
KaluHeenati, Kuruluthuda, Suwadal, Rathhal, Madel, Pachchaperumal

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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