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Identification and characterization of RAPD–SCAR markers linked to glyphosate-susceptible and -resistant biotypes of *Eleusine indica* (L.) Gaertn

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Abstract *Eleusine indica* is one of the most common weed species found in agricultural land worldwide. Although herbicide-glyphosate provides good control of the weed, its frequent uses has led to abundant reported cases of resistance. Hence, the development of genetic markers for quick detection of glyphosate-resistance in *E. indica* population is imperative for the control and management of the weed. In this study, a total of 14 specific random amplified polymorphic DNA (RAPD) markers were identified and two of the markers, namely S4R727 and S26R₆976 were further sequence characterized. Sequence alignment revealed that marker S4R727 showing a 12-bp nucleotides deletion in resistant biotypes, while marker S26R₆976 contained a 167-bp nucleotides insertion in the resistant biotypes. Based on these sequence differences, three pairs of new sequence characterized amplified region (SCAR) primers were developed. The specificity of these primer pairs were further validated with genomic DNA extracted from ten individual plants of one

glyphosate-susceptible and five glyphosate-resistant (R2, R4, R6, R8 and R11) populations. The resulting RAPD–SCAR markers provided the basis for assessing genetic diversity between glyphosate-susceptible and -resistant *E. indica* biotypes, as well for the identification of genetic locus link to glyphosate-resistance event in the species.

Keywords Goosegrass · Molecular marker · Insertion–deletion · Random amplified polymorphic DNA · Sequence characterized amplified region

Introduction

Eleusine indica (L.) Gaertn. (goosegrass) is an annual herbaceous and self-pollinating grass species that belongs to the family of Poaceae [1]. It is one of the top ten worst weeds in the world that causes major problem in almost all forms of agriculture lands, including orchards, vegetable farms, nurseries and young oil palm plantation in Malaysia [2, 3]. Goosegrass populations have been reported to have evolved resistance towards several groups of herbicides, such as trifluralin [4], glyphosate [5], paraquat [6], and fluzafop butyl [7] and glufosinate [8]. The glyphosate-resistant biotype of *E. indica* was first reported in 1997 at a guava orchard in Teluk Intan, state of Perak, Malaysia. The biotype was found to develop between 8- and 12-fold resistant to glyphosate within 3 years of repeated and over-usage of glyphosate to control the weed [5]. Glyphosate is among the leading herbicides in the market because of its post-emergent, systemic and non-selective property that can effectively control a wide spectrum of weeds that have been widely used all over the world since its introduction in 1974 [9]. Glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is a key enzyme in

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the production of very important aromatic amino acid in plant [10]. The aromatic amino acids function as precursor for an array of secondary metabolites including lignin, flavonoids and alkaloids [11]. Since the first report of evolved glyphosate resistance in *Lolium rigidum* [12] and *E. indica* [5], glyphosate resistance has been detected in 19 weed species that involve 14 countries [3].

Studies on *E. indica* [2, 13, 14] has provided evidence that glyphosate resistance was conferred by a single nucleotide changed from C-319 (susceptible biotype) to T/A-319 (resistance biotype) that substituted the nonpolar Pro-106 with a polar Ser-106 or Thr-106. Similar target-site mutation that replaced the Pro-106 to Thr-106 [15] or Leu-106 [16] had also been identified in *L. rigidum* population in Australia and South Africa, respectively. Several recent studies have confirmed the role of *EPSPS* gene amplification in conferring glyphosate resistance in *Amaranthus palmeri* population originated from Georgia [17, 18].

On the other hand, non-target site mechanisms that endow resistance to glyphosate including reduced or restricted rate of glyphosate translocation have been reported in *L. rigidum* [19], *Conyza bonariensis* [20] and *Conyza Canadensis* [21]. Interestingly, some *Lolium* populations were shown to possess both an *EPSPS* Pro-106 mutation and the restricted glyphosate translocation mechanism [10]. A most recent study conducted by Délye et al. [22] on the diversity of mechanisms conferring resistance to acetolactate synthase (ALS) herbicide in *Papaver rhoeas* L. also revealed the existence of both target-site (mutant *ALS* alleles) and non-target-site resistance (NTSR) mechanisms in the populations investigated. Several gene families such as cytochrome P450, glutathione *S*-transferase (GST) and glycosyltransferases, which involve in plant detoxification process have been shown to play important role in NTSR cases [10, 23]. While study on *Alopecurus myosuroides* has confirmed that the NTSR was a quantitative trait in which accumulation of up to at least three NTSR loci in a single plant could be necessary to confer resistance [24]. There are potentially other herbicide-resistant related genes that are differentially expressed only in resistant plant as previously revealed by mRNA differential display in *E. indica* [25]. These genes may either involve directly in preventing the deleterious effect of herbicides or genes encoded for trans-acting factor that regulate the expression of other herbicide-resistant related genes [24]. However, fewer of these genes have actually been cloned and characterized from weeds. Thus, an integrated functional genomics approach was proposed by Yuan et al. [23] for non-target-site gene discovery in herbicide-resistant weeds in order to further elucidate the complex biochemical processes of the mechanisms at the molecular level.

Genetic variations expressed in the resistant weeds enable them to evolve resistance mechanisms that lead to escape from herbicide control. The existence of genetic variation within and between populations enabled the use of polymorphism-based techniques such as random amplified polymorphic DNA (RAPD) to examine the genetic variation and phylogenetic relationships of triazine-resistant and -susceptible biotypes of *Solanum nigrum* [26]. The amplified fragment length polymorphism (AFLP) was used to investigate the genetic diversity of sulfonyleurea-resistant and -susceptible *Monochoria vaginalis* population in Japan [27] as well as triazine-resistant *Chenopodium album* population in North West Europe [28]. Many of specific RAPD and AFLP markers had been successfully converted into sequence characterize amplified region (SCAR) markers. The SCAR marker was created by cloning and sequencing RAPD or AFLP fragments of interest, and designing longer specific oligonucleotide primers complementary to the ends of the original fragments. When the primers are used in a PCR with original DNA template, single loci was specifically amplified. The SCAR marker is a stable and codominant marker that can be applied in population genetic studies and fingerprintings that overcome some limitations of the RAPD markers [29]. This marker has been widely and successfully used to map various disease-resistant genes in plants such as, Fusarium wilt resistance in banana [30], anthracnose resistance gene in sorghum [31] and bacterial wilt resistance in tomato [32]. RAPD–SCAR markers had also been used for marker-assisted selection of the high flesh/stone ratio in olive [33], for the identification of toxic and non-toxic varieties of *Jatropha curcas* [34] as well as the identification of apospory trait in *Cenchrus ciliaris* [35].

However, there is lack of study on the assessment of genetic variability between herbicide-resistant and -susceptible biotypes of weed species using molecular techniques. Thus, the present work explores the potential of using RAPD technique to assess the genetic variability between glyphosate-resistant and -susceptible biotypes of *E. indica* from several cultivated areas in the state of Perak, Malaysia. Specific RAPD bands with the potential to discriminate between the resistant and susceptible biotypes were identified, sequence characterized and subsequently converted into specific SCAR markers. The characterized SCAR markers may potentially link to NTSR locus in the resistant biotypes of *E. indica* that endow resistance to glyphosate.

Materials and methods

Plant materials

Eleusine indica seeds were randomly collected from ten individuals at five cropping areas (Supplementary Table 1) in the state of Perak, Malaysia due to poor performance of

herbicide glyphosate. The seeds were sown in the glasshouse using commercial potting mix (FLORASCA® 801, TURBA earth and humus GmbH, Papenburg Castle, Germany). The seedlings were sprayed with glyphosate (360 g a.i. L⁻¹ Roundup Transorb®, Monsanto) at double the recommended rate (2.44 kg ai./ha) to screen the susceptible or resistant biotypes of each population. Plants that survive were considered as resistant plants, which were subsequently subjected to dose–response test to determine the resistance level of each population. Glyphosate-susceptible population was originally from Bidor, Perak, Malaysia.

Whole-plant bioassay

The whole-plant bioassay was conducted in the glasshouse using four to five-leaf stage seedlings, measuring 4–5 cm tall. The seedlings were sprayed with glyphosate rates of 0.16, 0.64, 2.56, 10.24 or 40.96 kg a.i. ha⁻¹. The plants were harvested and weighted 3 weeks after the treatments. The shoot fresh weight was plotted against rates of glyphosate in order to determine the glyphosate rate that cause a 50 % reduction in shoot fresh weight (ED₅₀) [36]. Data were fitted to log-logistic regression models: $Y = a/1 + (x/x^0)^b$. Where, Y = shoot fresh weight; a = coefficient corresponding to the upper asymptotes; b = slope of the line; and $x^0 = ED_{50}$.

Regression analysis was conducted using SigmaPlot Version 10.0. Glyphosate rate that inhibited plant growth by 50 % (ED₅₀) were calculated from regression equations. The resistant level was determined by dividing the ED₅₀ value of the each resistant (R) biotype by the ED₅₀ value of the susceptible (S) biotype.

RAPD profile evaluation and specific band identification

Plant genomic DNA was extracted from fresh young leaves using Wizard® Genomic DNA Extraction Kit (Promega). A total of 40 arbitrary primers (S1–S40) purchased from Bio Basic Inc. were screened by gradient PCR to determine optimum annealing temperature of each individual primer. The initial RAPD primers screening were conducted by using genomic DNA from one susceptible plant as template in PCR reactions. The PCR reactions were performed in a total volume of 25 µl containing 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP mix (Vivantis), 0.2 µM primer, 1 unit of *Taq* DNA polymerase (Vivantis), and 50 ng of DNA template. Amplifications were carried out in automated thermal cycler (Mastercycler gradient, Eppendorf) for 45 cycles (denaturation: 94 °C, 30 s; gradient annealing temperature: 35–45 °C, 1 min; elongation: 72 °C, 1 min 30 s). The annealing temperature of each RAPD primer was optimized by gradient PCR. The PCR products were separated

on 1.5 % (w/v) agarose gel by electrophoresis at 90 V for 1 h in 1× TAE buffer and revealed by ethidium bromide (1 µg/ml) staining.

Subsequently, the complete RAPD profiles were generated by using DNA template from three individuals of each population to determine the specificity and reproducibility of the potential specific bands. The PCR reaction mixture and cycling conditions was as above except the optimum annealing temperature of each arbitrary primer was used. The RAPD profiles generated were displayed on 1.5 % (w/v) agarose gel as above. Specific bands which only present in either the glyphosate-susceptible or -resistant biotypes were identified. The RAPD primers that produced the potential specific bands were repeated with ten new individual plants from each population to further verify the specificity of the bands.

Development of SCAR primers

Specific amplified bands obtained from RAPD analysis were excised from the agarose gel and purified with Wizard Plus SV Minipreps DNA Purification System (Promega). The purified bands were cloned into pGEM-T Easy Vector (Promega) and transformed into the *E. coli* strain DH5α host cells. Plasmids extracted from positive recombinant colonies were sent for DNA sequencing (First-Base Laboratories Sdn. Bhd.).

The SCAR primers were designed based on significant differences between nucleotide sequences of the identified RAPD markers from the susceptible and resistant biotypes. The RAPD markers S4R727 and S26R₆976 were successfully converted into SCAR markers with a total of three types of primer pair combinations were produced. These primer pairs were S4SNF/S4R1, S48NF2/S4R1 and S26P6F1/S26P6R. The specificity of these primer pairs were further validated with ten new individual plants from each of SB, R2, R4, R6, R8 and R11 populations. PCR reaction was performed in a total volume of 25 µl containing 1× PCR buffer, 1.5 mM MgCl₂, 0.2 µM dNTP mix (Vivantis), 0.4 µM of each forward and reverse primers, 1 unit of *Taq* DNA polymerase (Vivantis), and 50 ng of DNA template. Amplifications were carried out in automated thermal cycler (Mastercycler gradient, Eppendorf) for 40 cycles (denaturation: 94 °C, 30 s; annealing: 60–65 °C (depend on primer combinations), 1 min; elongation: 72 °C, 1 min 30 s). PCR products were separated in 1.2 % agarose gel electrophoresis.

Results

Dose–response experiment

Seedlings of R2, R4, R6, R8 and R11 populations survived after being sprayed with 2.44 kg a.i./ha glyphosate (twice

the recommended rate) and confirmed as the resistant population. The seedlings of the SB population died, thus it was considered as the susceptible biotype. Whole-plant bioassay revealed that the ED₅₀ values of R2, R4, R6, R8 and R11 populations were 10–41-fold higher than the ED₅₀ value of the susceptible population (SB) (Supplementary Fig. 1). Population R2 had the lowest resistance level of 10-fold, while population R11 had the highest resistance level of 41-fold (Table 1).

Identification of specific marker from RAPD banding pattern

RAPD screening revealed the existence of polymorphisms between the glyphosate-susceptible and -resistant biotypes of goosegrass. The polymorphism patterns generated were used to identify markers for the glyphosate-susceptible or -resistant biotype, resistant biotype with a specific level of resistance and biotype with a range of resistance level. All together, there were 14 specific bands (Table 2) produced by eight out of 40 arbitrary primers (S1–S40) tested in this study. The specific bands were grouped into seven marker categories based on the presence of specific bands in DNA samples. They are Groups (I), susceptible (S); (II) 10–41-fold resistance (10–41 × R); (III) susceptible and 10-fold resistance (S-10 × R); (IV) 16–41-fold resistance (16–41 × R); (V) specific 10-fold resistance (10 × R); (VI) specific 28-fold resistance (28 × R) and (VII) specific 41-fold resistance (41 × R). The result is as summarized in Table 2 and some examples of the obtained RAPD banding patterns are shown in Fig. 1.

However, this paper focuses on the characterization of two interesting specific bands, namely markers S4R727 (727 bp) and S26R₆976 (976 bp), which were amplified with primers S4 and S26, respectively (Fig. 1). Based on the RAPD banding pattern obtained, marker S4R727 was specific to all resistant biotypes (biotypes R2, R4, R6, R8,

R11), while marker S26R₆976 was only present in the 28-fold resistant biotype (population R6). The consistency of the markers were further verified using genomic DNA extracted from ten new, randomly picked individual plants from all biotypes (Supplementary Figs. 3 and 4). Both S4R727 and S26R₆976 DNA RAPD fragments were cloned, sequenced. The homology search in GenBank using BLAST program (<http://www.ncbi.nlm.nih.gov>) showed no significant similarity to any known plant gene or genomic sequence. The search with RepeatMasker (<http://www.repeatmasker.org>) showed that the sequences did not contain any of the known repeat.

Sequence analysis of markers S4R727 and S26R₆976

Firstly, longer (24 bases) forward and reverse SCAR primer pairs were designed complementary to both ends of S4R727 and S26R₆976 fragments based on the nucleotide sequences obtained. However, the designed primers failed to perform specifically, which should only amplify S4R727 marker from all resistant biotypes and S26R₆976 marker from the R6 biotype. Instead, the primers for marker S4R727 also produced a similar size band (727 bp) in the S biotype (Fig. 2a). The primers for marker S26R₆976, which supposedly specific for the R6 (28-fold resistance) biotype also amplified the 976 bp fragment from the other four resistant biotypes (R2, R4, R8, R11) and unexpectedly amplified a smaller size band of ~800 bp from the S biotype (Fig. 2b). Therefore, PCR products from one of the resistant biotype (R8 for marker S4R727 and R6 for marker S26R₆976) were selected for cloning and sequence analysis. Interestingly, the alignment of the nucleotide sequence revealed a 12 nucleotides deletion (5'-GTGTTGCACTCG-3') occurred in the R8 biotype as compared to SB population for marker S4R727 (Fig. 3a). In contrast, a 167 nucleotides insertion (Fig. 3b, sequence highlighted in grey) was detected in the R6 resistant biotype from marker S26R₆976. This large nucleotide sequence insertion explains the visible difference of the PCR products size between the susceptible and resistant biotypes (Fig. 2b).

SCAR primers design and screening of glyphosate-susceptible and resistant biotypes

New SCAR forward primers S4R8NF and S4SNF for marker S4R727 were designed based on the location of the 12 nucleotides deletion in the resistant plant in order to discriminate between the susceptible and resistant plants (Fig. 3a). The forward primer S4R8NF (5'-CGGTATTG-CACG↓ATGAA-3') lack the 12 nucleotides deletion segment as indicated with (↓) in the primer sequence (Fig. 3a). The lack of this 12 nucleotide deletion close to the 3'-end

Table 1 Whole-plant dose–response

Population ^a	ED ₅₀ value ^b (kg a.i/ha)	Level of resistance
SB	0.2008 ± 0.0446	1
R2	2.0505 ± 0.0482	10
R8	3.2825 ± 0.4633	16
R4	4.2784 ± 0.4172	21
R6	5.5717 ± 0.2451	28
R11	8.1388 ± 0.0801	41

Glyphosate rates to reduce the seedling growth by 50 % (ED₅₀) of each population with the resistance level

^a SB is susceptible population; R2, R4, R6, R8 and R11 are resistance population

^b The ED₅₀ values are of mean ± standard deviation

Table 2 The random amplified polymorphic DNA (RAPD) markers for glyphosate-susceptible and -resistant biotypes of goosegrass

Marker's Group	Marker's type	Primer	Marker's name ^a	Size (bp)	Biotype ^{b,c}					
					Susceptible (SB)	Resistant				
						R2 (10×)	R8 (16×)	R4 (21×)	R6 (28×)	R11 (41×)
I	S	S4	S4S814	814	+	–	–	–	–	–
		S19	S19S1245	1,245	+	–	–	–	–	–
		S26	S26S2000	2,000	+	–	–	–	–	–
		S26	S26S900	900	+	–	–	–	–	–
II	10–41 × R	S4	S4R727 *	727	–	+	+	+	+	+
		S15	S15R2000	2,000	–	+	+	+	+	+
		S29	S29R448	448	–	+	+	+	+	+
III	S-10 × R	S4	S4S540	540	+	+	–	–	–	–
		S9	S9S849	849	+	+	–	–	–	–
IV	16–41 × R	S9	S9R340	340	–	–	+	+	+	+
V	10 × R	S20	S20R ₂ 560	560	–	+	–	–	–	–
VI	28 × R	S26	S26R ₆ 976 *	1,000	–	–	–	–	+	–
		S26	S26R ₆ 1500	1,500	–	–	–	–	+	–
VII	41 × R	S21	S21R ₁₁ 1406	1,400	–	–	–	–	–	+

^a The asterisk (*) represent markers selected for further characterization and develop into SCAR primers

^b + present, – not present, *S* susceptible, *R* resistant; × level of resistance

^c The agarose gel pictures of RAPD polymorphic bands is available in Supplementary Fig. 2

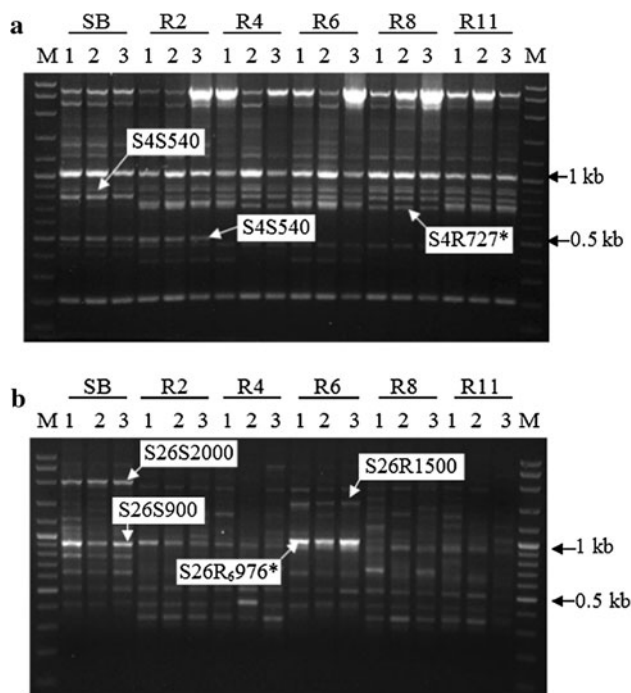


Fig. 1 Examples of RAPD profiles generated using 10-mers oligo primers S4 (a) and S26 (b) showed polymorphisms among susceptible population (SB) and resistant populations (R2, R4, R8 and R11). Two specific bands (markers: S4R727 and S26R₆976) as indicated with (*) were selected for further characterization and converted into SCAR markers. M: DNA ladder; number 1–3 are individual of each population

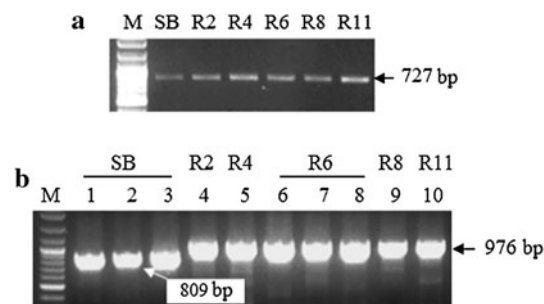


Fig. 2 PCR amplification of marker a S4R727 and b S26R₆976 with SCAR primers designed complementary to both ends of their respective nucleotide sequence. SB, susceptible biotype; R2, R4, R6, R8 and R11 are resistance biotypes. M: DNA ladder; number 1–10 represent individual samples from each population

of primer S4R8NF disrupts the binding of the primer to marker S4R727 in the glyphosate-susceptible biotype. Thus, the primer can only amplify the marker S4R727 from the glyphosate-resistant biotype of *E. indica*. On the other hand, forward primer S4SNF (5'-GGTATTGCACGGT **GTTGCACTCG**-3') contains the 12 nucleotide deletion segment (as highlighted in bold) at the 3'-end of the primer (Fig. 3a), which makes the primer only specific to marker S4R727 in the glyphosate-susceptible biotype of *E. indica*. The reverse SCAR primer, S4R1: 5'-GGACTGGAGTC GGGTTATTAGCAC-3' for the same marker was located

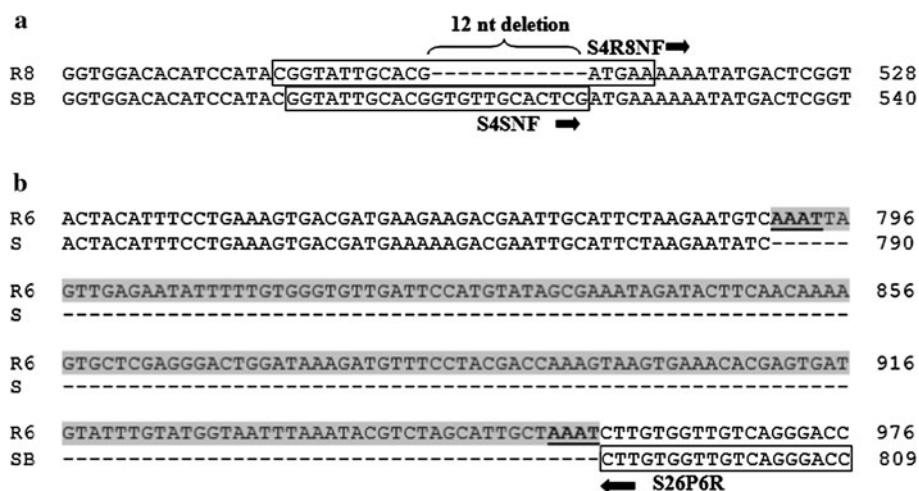


Fig. 3 Nucleotide sequence alignment of **a** marker S4R727 amplified from susceptible (SB) (GenBank ID: JN588750) and resistant (R8) (GenBank ID: JN588749) biotype showing a 12 nucleotides deletion occurred in resistant biotype, while **b** marker S26R₆976 amplified from susceptible (SB) (GenBank ID: JN588752) and resistant (R6)

(GenBank ID: JN588751) biotype showing a 167 bp nucleotides insertion (highlighted in grey) in the resistant biotype. The putative tetranucleotide motifs (AAAT) are *bold-underlined*. *Boxed* sequences are new SCAR forward (S4R8NF and S4SNF) and reverse (S26R6R) primers for their respective markers

at the 3'-end of the sequence. Therefore, SCAR primer combination S4SNF/S4R1 and S4R8NF/S4R1 would amplify 243 bp (from glyphosate-susceptible biotype) and 232 bp (from glyphosate-resistant biotype) fragments of marker S4R727, respectively.

As for marker S26R₆976, there is a significant 167 bp nucleotides insertion in the resistant biotypes (Fig. 3), which is large enough to differentiate between the glyphosate-susceptible and -resistant biotype of *E. indica*. Thus, a new forward primer S26P6F1 (5'-GCGTCATTTTCGATGTTGTTGTC-3') was designed in order to shorten the amplicon size and improve the PCR efficiency. The reverse primer S26P6R (5'-GGTCCCTGACAACCACAAG-3') was used in combination with the new forward primer S26P6F1 (Table 3), which would amplify the 191 bp (from glyphosate-susceptible biotype) and 357 bp (from glyphosate-resistant biotype) fragments of marker S26R₆976, respectively.

The specificity of these primer pairs was further verified with ten new plants from each population. The S4SNF/S4R1 primers combination successfully amplified the 243 bp fragment of marker S4R727 from all 10 individuals of the susceptible biotypes, while no amplification was detected in all 50 individuals from all resistant biotypes (Fig. 4a). On the other hand, the second SCAR primer combination of S4R8NF2/S4R1 successfully amplified the 232 bp fragment of marker S4R727 from all 50 individuals of all resistant biotypes with no amplification in all the 10 individuals from the susceptible biotype (Fig. 4b). The third primer combination of S26P6F1/S26P6R successfully amplified the 191 bp fragment of marker S26R₆976 from all 10 susceptible individuals and at the same time amplified the 357 bp fragment in all 50 resistant individuals from all resistant biotypes (Fig. 5).

Table 3 SCAR primer combinations for the detection of glyphosate-susceptible and -resistant biotype of *E. indica*

SCAR primer combination	Targeted marker	Expected Size (bp)	Biotype specificity
S4SNF/S4R1	S4R727	243	Susceptible
S4R8NF2/S4R1	S4R727	232	Resistant
S26P6F1/S26P6R	S26R ₆ 976	191	Susceptible
S26P6F1/S26P6R	S26R ₆ 976	357	Resistant

Discussions

The glyphosate-susceptible and -resistant biotypes of *E. indica* were confirmed by whole plant dose-response bioassay, which revealed one susceptible and five resistant populations with different level of resistance ranging from 10- to 41-fold (Table 1). These results showed that the resistant biotypes responded differently towards glyphosate, which may be conferred by different resistance mechanisms that naturally exist in the population. The R11 plants, which confer up to 41-fold resistance may have effective defense against the penetration of glyphosate through the cuticle, by having thicker cuticle that prevents glyphosate from entering into living tissue of the plants. Moreover, the R11 plants have smaller and narrower leaves compared to the other populations used in this study. This may result in the reduced contact surface area for glyphosate penetration into the foliar [37]. This study also revealed that the resistance levels of the studied populations were much higher than previously reported in other regions of Malaysia, such as 2.9 fold in Chaah (Johor), 2.1 fold in Lenggeng (Negeri Sembilan), 3.3 fold in Bidor

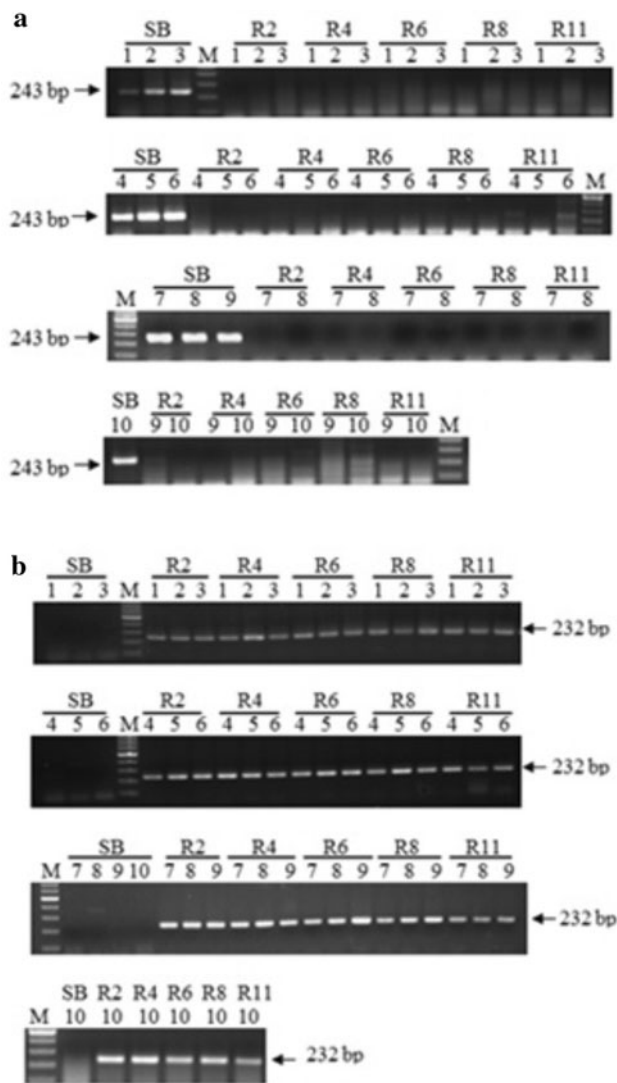


Fig. 4 Screening of susceptible (SB) and resistant (R2, R4, R6, R8, R11) biotypes of *E. indica* with SCAR primer combination **a** S4SNF/S4R1 and **b** S48NF2/S4R1 for the detection of marker S4R727. Ten new individuals from each population were used in this validation. Number 1–10 represents each individual of respective population. M: 100 bp DNA ladder

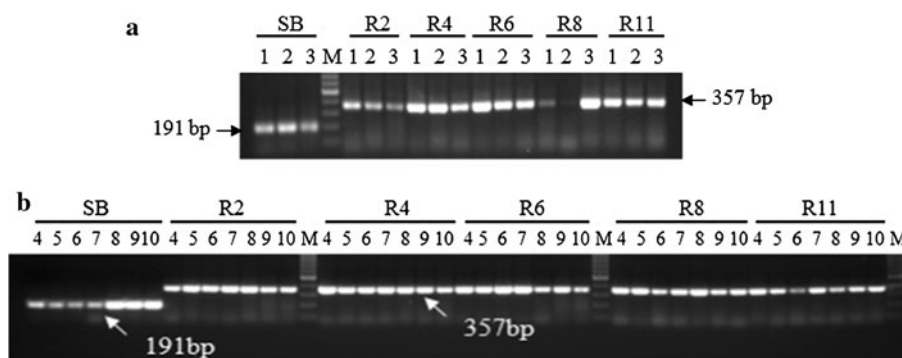
(Perak) and 2.8 fold in Temerloh (Pahang) [14]. The results may imply that the resistance level of some *E. indica* populations in Malaysia has increased tremendously in less than a decade. Previous studies showed that glyphosate-resistant *E. indica* populations from several cropping areas in Malaysia were conferred by target-site mutation of the EPSPS gene [2, 13, 14]. The high level of glyphosate resistance detected in the resistant-biotypes from this study implies that NTSR may also play an important role in conferring resistance against glyphosate in these populations. The resistance may be conferred by the involvement of many genes or polygenes, in which each gene involves, increased the resistance level [38]. Volenberg et al. [39]

suggested that genetic factors other than an altered target-site or metabolism may also contribute to the magnitude of resistance in the imazamox-resistant biotype of *Solanum ptycanthum*. Therefore, further work to investigate the relationship of both target-site and NTSR mechanisms in these populations in endowing high level of resistance are of future research interest in order to understand the resistance mechanisms involved.

The resistant plants used in this study (R2, R4, R6, R8, and R11) were originated from the same state of Malaysia, Perak, where the distance between each population was <50 km. There is a possibility that the plants were genetically linked to each other and from the same ancestor. This could be seen from the RAPD profiles generated, where only eight out of 40 arbitrary primers used manage to produce polymorphic bands. Furthermore, the polymorphic bands were closely related especially between the resistant biotypes. However, RAPD still prove to be a potential technique for the generation of specific marker that linked to the glyphosate-susceptible and -resistant biotypes (Table 2). It is interesting that the technique also produced several specific markers that may link to the glyphosate-resistant biotypes with different levels of resistance as shown by markers in Group V, VI and VII (Table 2). Previous study had also shown that the RAPD technique was able to distinguish the resistant populations of *S. nigrum* from Poland, France and the United Kingdom [26]. *E. indica* is an autogamous species that self-fertilized, where cross-fertilization is rare in the fields [1]. Therefore, most of the individuals in the fields are homogzygous. The robustness of RAPD to generate large number of potentially polymorphic bands, making it a very useful technique to compare the low genetic variability expected between the glyphosate-susceptible and -resistance biotypes of this species. In fact, RAPD has been regarded as an excellent method or marker to track the flow of gene in self-pollinating species [40].

However, there is lack of research using molecular markers to tag genes or locus that linked to herbicide resistance in weeds species. The results obtained from this study clearly showed that molecular markers could be potentially used to identify polymorphic genomic regions (quantitative trait loci, or QTL) associated with herbicide resistance. Once the herbicide-resistant QTL regions have been identified in a particular weed species, the underlying genes and nucleotide polymorphisms associated with the resistant trait can then be determined [41]. Although study on *A. myosuroides* had revealed that up to at least three QTL loci were needed for non-target-site-based resistance to acetyl-coenzyme A carboxylase and acetolactate-synthase herbicides [24], these herbicide-resistant loci and the genes involved had yet to be identified.

Fig. 5 Screening of susceptible (SB) and resistant (R2, R4, R6, R8, R11) biotypes of *E. indica* with SCAR primer combination S26P6F1/S26P6R for the detection of marker S26R₆976. Ten new individuals from each population were used in this validation. Number 1 to 10 represents each individual of respective population. M: 100 bp DNA ladder



In this study, the insertion-deletion (indel) mutations in both S4R727 and S26₆976 markers were consistent in all glyphosate-susceptible and -resistance plants tested as shown in Figs. 4 and 5. This may imply that the markers are closely linked to potential QTL locus that conferred glyphosate resistance in the biotypes of R2, R4, R6, R8 and R11. Thus, the results show the possible existence of similar glyphosate-resistant QTL locus in these populations regardless of the level of resistance. Several previous research had shown that the activity of *EPSPS* gene [20] and other NTSR genes [23] were upregulated in resistant biotypes. The activity of these genes is regulated by specific transcription factors or other herbicide-inducible genes [24]. While Masni Afiza et al. [25] had identified a total of ten potential genes that only expressed in the glyphosate-resistant biotype of *E. indica*. All these studies indicate that herbicide resistance mechanisms in weed species are potentially involved far more ‘regulatory genes’ than the several known genes for target-site and NTSR mechanisms. Thus, molecular techniques such as mRNA differential display or RNA-seq could be applied for the discovery of herbicide-resistant genes in weed species besides molecular markers such as RAPD and SCAR.

On the other hand, the 167-bp insertion fragment (of S26R₆976 marker) in the resistant biotypes is flanked by two perfect direct repeats (AAAT) at both ends (Fig. 3b). The AT-rich tetranucleotide motif (AAAT) is a common type of simple sequence repeats (SSRs) that present in plant genome such as pepper [42] and cucumber [43]. Insertion fragment that flanked by direct repeat (TAA) and inverted repeat that similar to the terminal inverted repeats (TIRs) of transposon had also been characterized from a SCAR marker specific to maize somaclones [44], while other studies showed that the insertion/deletion could locate close to functional genes [45, 46]. Thus, S4R727 and S26R₆976 markers could be used to further locate the presence of any functional gene or transposable element at the 5'- and 3'-regions of the markers.

In conclusion, this study has revealed the potential of using RAPD–SCAR markers for the detection of glyphosate-susceptible and resistant biotypes of *E. indica*. The

discovery of insertion-deletion in both markers implies that these events could be commonly occurred in *E. indica* populations and that can be exploited to develop insertion site-based polymorphism (ISBP) markers to differentiate between the susceptible and resistant biotypes. These markers are imperative for the identification of genetic locus linked to herbicide-resistance event in weeds species in future study.

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