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GENETICS AND PLANT BREEDING

Microsatellite molecular marker-assisted gene pyramiding for resistance to Asian soybean rust (ASR)

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ABSTRACT. The present study aimed at pyramiding ASR-resistance genes through microsatellite (SSR) marker-assisted selection (MAS) and demonstrating the pyramiding steps. To obtain the first generation of gene pyramiding, crosses were made between introduced plants (PI's), which have the genes *Rpp1*, *Rpp2*, *Rpp3*, *Rpp4*, and *Rpp5*. F_1 plants from the initial crosses were intercrossed to obtain plants with the four resistance genes (second pyramiding generation). Plants selected from this second generation were again intercrossed (third pyramiding generation) to increase the number of pyramided genes. For MAS, we used informative SSR markers in each cross. SSR markers were considered informative when the source resistance allele containing the target gene could be followed in the progeny, even in crosses between hybrids that both contained the same allele. Markers published in the ASR genetic mapping studies and in the consensus map of the soybean were used. We obtained plants containing from 2 to 4 genes pyramided per plant. These plants can be used as a source of multiple resistance in breeding programmes for obtaining soybean varieties with more durable resistance to ASR.

Keywords: *Phakopsora pachyrhizi*; gene stacking; marker-assisted breeding; durable resistance.

Piramidação de genes de resistência à ferrugem asiática da soja (FAS) assistida por marcadores moleculares microssatélites

RESUMO. O presente estudo objetivou piramidar genes de resistência à FAS por meio da seleção assistida por marcadores (SAM) microsatélites (SSR), demonstrando os passos para a piramidação. Para obter a primeira geração de piramidação de genes, realizaram-se cruzamentos entre as plantas introduzidas (PI's), que possuem os genes *Rpp1*, *Rpp2*, *Rpp3*, *Rpp4* e *Rpp5*. As plantas F_1 dos cruzamentos iniciais foram cruzadas para obter plantas com os quatro genes de resistência (segunda geração de piramidação). As plantas selecionadas desta segunda geração foram novamente cruzadas (terceira geração de piramidação) para aumentar o número de genes piramidados. Para a SAM, foram utilizados marcadores SSR informativos em cada cruzamento. Marcadores SSR foram considerados informativos quando o alelo de resistência da fonte contendo o gene alvo poderia ser seguido na progênie, mesmo em cruzamentos entre híbridos, ambos contendo o mesmo alelo. Foram utilizados marcadores publicados em estudos de mapeamento genético para a FAS e o mapa consenso da soja. Foram obtidas plantas contendo genes piramidados, de 2 a 4 genes por planta. Essas plantas podem ser usadas como fonte de resistência múltipla em programas de melhoramento para obter variedades de soja com resistência mais durável à FAS.

Palavras-chave: *Phakopsora pachyrhizi*; empilhamento de genes; melhoramento assistido por marcadores; resistência durável.

Introduction

The soybean [*Glycine max* (L.) Merrill] is the most important oilseed for the Brazilian economy, ranking the country as the second largest producer worldwide with a planted area of 33.2 million hectares and production approximately 100.90 million tons in the 2015-2016 harvest year (Conab, 2016). However, a limiting factor for increasing the Brazilian soybean production chain and for improving the international economic position stems from drawbacks faced by farmers with disease occurrence (Arias et al., 2010), such as Asian soybean rust (ASR), in which the aetiological agent is the fungus *Phakopsora pachyrhizi* Sydow & Sydow, due to the high cost of its control and the sharp reduction productivity in the absence of the proper management of crops. In Brazil, ASR was first

detected in 2001 and has been a matter of great concern owing to the high potential for damage and the high cost of its control (Yang, Royer, Tschanz, & Tsai, 1990; Yang, Tschanz, Dowler, & Wang, 1991; Sinclair & Hartman, 1999). The use of cultivars that are tolerant/resistant to disease is still the most effective (Yorinori, 2008) and economical way to minimize losses in grain yield and the most appropriate for the environment, because it greatly reduces fungicide application (Miles, Frederick, & Hartman, 2003; Hartman, Miles, & Frederick, 2005).

Five genes, *Rpp1-Rpp5*, conferred resistance to the ASR isolate identified in Brazil in 2001. Nevertheless, due to the large variability of the pathogen caused by mutation or recombination, a new isolate from the Mato Grosso State, in 2003, caused susceptibility lesions in introduced plant (PI's), carriers of the *Rpp1* and *Rpp3* genes (Arias et al., 2008; Garcia et al., 2008; Silva et al., 2008). The *Rpp2*, *Rpp4* (Arias et al., 2004) and *Rpp5* (Garcia et al., 2008) genes remain resistant to rust in Brazil. For the new locus, *Rpp6*, it has been suggested that its incorporation in breeding soybean cultivars may provide benefits, as PI 567102B once showed resistance to *P. pachyrhizi* isolates from Paraguay and the USA (Li, Smith, Ray, & Frederick, 2012).

It is well known that gene pyramiding is a way to develop cultivars with multiple and long-lasting resistance (Kelly, Miklas, Gepts, & Coyne, 2003; Alzate-Marin, Cervigni, Moreira, & Barros, 2005). In several species, gene pyramiding using MAS has resulted in the successful achievement of resistant cultivars. Parrella, Santos, and Parrella (2008) also pyramided genes conferring resistance to common mosaic virus and to anthracnose in the common bean. In rice crops, Yoshimura et al. (1995), Huang et al. (1997) and Singh et al. (2001) employing RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), PCR (Polymerase Chain Reaction) and STS (Sequence Tagged Site) have pyramided different genes for resistance to the bacterium *Xanthomonas oryzae* pv. *oryzae* into a single genotype. Hittalmani, Parco, Mew, Zeigler, and Huang (2000) observed an increased resistance to the fungus *Magnaporthe grisea*, which causes rice blast disease after pyramiding three genes into a single genotype, with the aid of RFLP and PCR-based markers. In soybean, using SSR markers, Shagai-Maroof et al. (2008) and Shi et al. (2009) pyramided different genes for the resistance of the soybean mosaic virus. Regarding soybean rust, Yamanaka et al. (2008) and Lemos et al. (2011) pyramided the *Rpp2* and *Rpp4* genes into a plant, and *Rpp2*, *Rpp4* and *Rpp5* into another plant, using SSR markers. Through MAS, we can track and identify genes present in each pyramiding generation and verify how these genes are segregating. Research on gene pyramiding usually presents the final results obtained, without showing the pyramiding steps. In this manner, the present study aimed to demonstrate the steps of pyramiding ASR resistance genes using MAS and to obtain plants containing more than one resistance gene to the disease.

Material and method

The study was conducted at the Central Cooperative of Agricultural Research (Coodetec), in a greenhouse at the Laboratory of Biotechnology, in Cascavel, Paraná State, Brazil, during the years 2008 and 2012. To obtain pyramiding generations, plants were grown in a greenhouse with controlled temperature and humidity in 5 L-polyethylene pots using a mixture of ½ soil (dystrophic red latosol), ¼ sand and ¼ organic material. Hybridizations performed were defined according to the presence of the resistance genes in the resistance sources. To obtain the first pyramiding generation, four combinations of crosses were made between the introduced plants (PI's), as shown in Table 1. F_1 plants from the initial generation were intercrossed to obtain the second pyramiding generation, and the combinations made at this phase are listed in Table 1.

Table 1. Crosses made for ASR resistance gene pyramiding, in the three pyramiding generations.

Generation of hybridization	Genealogy	Populations
First generation of hybridization	PI 200492 (*Rpp1*) x PI 459025 (*Rpp4*)	P14
	PI 462312 (*Rpp3*) x PI 230970 (*Rpp2*)	P32
	Kinoshita (*Rpp5*) x Shiranui (*Rpp5*)	P55*
	PI 200492 (*Rpp1*) x PI 230970 (*Rpp2*)	P12
Second generation of hybridization	P32 (PI 462312 x PI 230970) x P14 (PI 200492 x PI 459025)	P3214
	P32 (PI 462312 x PI 230970) x P55 (Kinoshita x Shiranui)	P3255
	P55 (Kinoshita x Shiranui) x P12 (PI 200492 x PI 230970)	P5512
	P55 (Kinoshita x Shiranui) x P32 (PI 462312 x PI 230970)	P5532
Third generation of hybridization	(P3214) x (P3255) = Plant 39** (*Rpp4*) x Plant 21*** (*Rpp2*, *Rpp3*, and *Rpp5*)	P4235
	(P3214) x (P5532) = Plant 55** (*Rpp2*, *Rpp3*, and *Rpp4*) x Plant 15*** (*Rpp2* and *Rpp5*)	P2345

*When crosses of the 1st pyramiding generation were made, *Rpp* genes in Kinoshita and Shiranui were still not known. **Female parent. ***Male parent.

F_1 plants from this generation of crosses were genotyped with SSR markers linked to ASR resistance genes, to select plants with the highest number of *Rpp* genes. From analysis of the genotyped plants with different markers, a new cycle of crosses was carried out (pyramiding generation 3), aiming to combine more genes into the same plant. In this generation, sources of resistance Kinoshita x Shiranui were crossed. Once at this phase, it had not yet been identified that both had the *Rpp5* gene. DNA of the parents used in the crosses was extracted from the seeds. Ten seeds of each parent were ground, and the genomic DNA was isolated according to McDonald, Elliot, and Sweeney (1994) with some modifications (Schuster, Queiroz, Teixeira, Barros, & Moreira, 2004). First, 50 mg of the scrapped seeds were placed into 1.5-mL microtubes containing a 3 mm diameter glass bead. Subsequently, we added 500 μL of extraction buffer [200 mM Tris-HCl pH 7.5; 288 mM NaCl; 25 mM EDTA pH 8.0 and 0.5% (m/v) SDS]. Microtubes were vigorously stirred in a Grinder stirrer for 1 min. Samples were centrifuged at 16,000 g for 10 min. and the supernatant was transferred to new microtubes. The protein was removed by adding 10 µL of Proteinase K (10 mg mL^{-1}), and the mixture was incubated in a water bath at 37°C for 30 min. Then, we added 500 µL of ice-cold isopropanol (-20°C), and the samples were gently homogenized. After two min, microtubes were centrifuged at 16,000 g for 10 min. The supernatant was discarded, and the precipitate was dried for 15 min. at room temperature. RNA was removed by resuspending the precipitate into 300 µL of TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0), containing 40 µg mL^{-1} RNAse A. Microtubes were again placed in a water bath at 37°C for 30 min., inverting every 10 min. We repeated the DNA precipitation with ice-cold isopropanol, and the precipitated DNA was resuspended in 300 µL of TE. DNA samples were quantified on a 0.8% agarose gel by comparison with standards of known concentration. For plants resulting from crosses between resistance sources and between F_1 plants, DNA was obtained from young leaves using the method described by Doyle and Doyle (1990) with modifications (Abdelnoor, Barros, & Moreira, 1995). DNA quantification was performed in the same way as the DNA samples from seeds.

PCR reactions were performed using SSR markers linked to ASR resistance genes (Hyten et al., 2007; Monteros, Missaoui, Phillips, Walker, & Boerma, 2007; Garcia et al., 2008; Silva et al., 2008; Hyten et al., 2009; Ray, Morel, Smith, Frederick, & Miles, 2009) and markers mapped in the same region of these *Rpp* genes derived from the consensus map of the soybean (Cregan et al., 1999; Song et al., 2004). The sequences of the SSR markers are found in detail on the website Soybase (Grant, Nelson, Cannon, & Shoemaker, 2010). Initially, the markers were used to assess the allelic diversity of each marker in the parents (PI's) containing the resistance genes. The markers that showed polymorphism between the parents used in the crosses at the *loci* linked to the resistance genes contained in these parents were used to assess the descendant populations of these crosses. PCR reactions were performed in 0.2-mL microtubes with a total reaction volume of 20 μL, containing 30 ng of DNA, 3 mM of $MgCl_2$, 1X buffer (2 mM Tris and 5 mM KCl), 250 μM of dNTP, 0.4 μM of each forward and reverse primer and one unit of Taq DNA polymerase. The amplifications were performed on a Thermo Hybaid thermocycler (Ashford, Middlesex, UK) programmed for an initial denaturation at 94°C for 3 min.; 35 cycles consisting of a step at 94°C for 30 s, a step at 50°C for 30 seconds and a step at 72°C for 45 seconds. The final extension was performed at 72°C for 20 min. Electrophoresis of the obtained fragments was carried out on 6% denaturing polyacrylamide gels. After completion of electrophoresis, the gels were stained with silver nitrate and digitized for storage and interpretation of the results. For all the generations, alleles were identified in each marker with the letters *a*, *b*, *c*, and *d* in decreasing order by allele size, with *a* being the larger allele. Homozygous plants were identified as *aa*, *bb*, *cc*, and *dd*, and the heterozygous plants were identified with a combination of the present alleles. For example, *ab* identifies heterozygous plants containing alleles *a* and *b*, and so on for the other genotypes/alleles.

For facilitating purposes, since our intention is to show the steps of gene pyramiding using MAS, we disregarded the recombination between the markers and genes, even though, in some cases, these possibilities may be high. Thus, in the results, we considered that the presence of the marker indicates the presence of the gene. The assurance that MAS selected-plants actually contain the selected genes is checked from the progenies of these plants in a later step.

Result and discussion

In the first pyramiding generation, F_1 plants were all heterozygous for two ASR resistance genes (genes present in each parent). Table 2 shows the quantities of seeds obtained in the second pyramiding generation (double hybrids between F_1 plants from crosses between PI's).

Table 2. Microsatellite markers used in polymorphism assessment among ASR resistance sources, and the genotype of each resistance source for the *loci*.

Genes/LG	*Loci*	PI200492 (*Rpp1*)	PI230970 (*Rpp2*)	PI462312 (*Rpp3*)	PI459025 (*Rpp4*)	Shiranui (*Rpp5*)	Kinoshita (*Rpp5*)
	Sct_187	*aa**	*cc*	*bb*	*bb*	*bb*	*bb*
Rpp1/G	Sat_117**	*bb*	*bb*	*ab*	*aa*	*bb*	*bb*
	Sat_372**	*aa*	*dd*	*df*	*cc*	*ef*	*bb*
	Sat_093	*cc*	*bb*	*dd*	*bb*	*aa*	*aa*
	Satt456	*bb*	*aa*	*aa*	*bb*	*aa*	*aa*
	Satt529	*aa*	*bb*	*aa*	*aa*	*bb*	*bb*
	Sct_001	*aa*	*bb*	*bb*	*aa*	*bb*	*bb*
Rpp2/J	Sat_366	*bb*	*bb*	*bb*	*bb*	*aa*	*aa*
	Satt620	*bb*	*aa*	*bb*	*aa*	*bb*	*bb*
	Sat_255**	*bb*	*ab*	*cc*	*cc*	*aa*	*aa*
	Sat_361**	*bb*	*aa*	*cc*	*aa*	*bb*	*bb*
	Sat_621**	*bb*	*bb*	*aa*	*bb*	*aa*	*aa*
	Sctt_011**	*bb*	*aa*	*aa*	*aa*	*aa*	*aa*
	Sat_263	*aa*	*bb*	*cc*	*aa*	*aa*	*aa*
	Sat_251	*bb*	*cc*	*cc*	*aa*	*cc*	*cc*
	Sat_402	*aa*	*bb*	*aa*	*cc*	*bb*	*bb*
	Satt202	*bb*	*bb*	*bb*	*bb*	*aa*	*aa*
	Satt316	*cc*	*aa*	*aa*	*bb*	*aa*	*aa*
Rpp3/C2	Satt708**	*ee*	*ee*	*be*	*ac*	*dd*	*dd*
	Sat_238**	*cc*	*bb*	*dd*	*dd*	*aa*	*aa*
	Satt079**	*bb*	*bb*	*aa*	*cc*	*aa*	*aa*
	Staga001**	*cc*	*dd*	*bd*	*aa*	*bb*	*bb*
	Satt307**	*cc*	*cc*	*dd*	*bb*	*aa*	*aa*
	Sat_142**	*bb*	*bb*	*ab*	*bb*	*aa*	*aa*
	Satt503	*bb*	*aa*	*aa*	*cc*	*aa*	*aa*
	Satt612	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*
	AF162283	*bb*	*aa*	*aa*	*aa*	*bb*	*bb*
	Satt288	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*
Rpp4/G	Satt517**	*aa*	*aa*	*cc*	*bb*	*cc*	*cc*
	Sat_143**	*bb*	*aa*	*aa*	*cc*	*aa*	*aa*
	Sct_199**	*aa*	*bb*	*aa*	*bb*	*aa*	*aa*
	Satt472**	*bb*	*dd*	*cc*	*cc*	*aa*	*aa*
	Satt191**	*cc*	*bb*	*aa*	*cc*	*cc*	*cc*
	Satt080	*bb*	*bb*	*bb*	*aa*	*bb*	*bb*
	Satt125	*aa*	*bb*	*cc*	*cc*	*cc*	*cc*
	Satt485	*aa*	*bb*	*aa*	*bb*	*aa*	*aa*
	Satt387	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*
	Satt584	*aa*	*bb*	*aa*	*bb*	*aa*	*aa*
Rpp5/N	Sat_084	*aa*	*bb*	*aa*	*aa*	*aa*	*aa*
	Sat_266	*cc*	*aa*	*dd*	*ee*	*bb*	*bb*
	Sat_275	*aa*	*bb*	*bb*	*cc*	*dd*	*dd*
	Sat_280	*cc*	*bb*	*cc*	*aa*	*dd*	*dd*
	Satt393**	*aa*	*aa*	*aa*	*aa*	*aa*	*aa*
	Sat_166**	*cc*	*bb*	*ab*	*cc*	*cc*	*cc*

*Letters correspond to alleles of each marker in each ASR resistance source; the letter *a* represents the largest allele and the others in the order of size. **Markers not reported in the literature as associated with ASR resistance, selected based on their position in each gene; LG: refers to the linkage group in which the gene was mapped in soybean (Cregan et al., 1999; Song et al., 2004).

The genotype assessment of these double hybrid plants at the *loci* containing the *Rpp* genes was conducted with the use of polymorphic markers among the four PI's used in the original crosses. The results of the SSR marker analysis were used to distinguish the parent *Rpp* gene donors and are shown in Table 2.

Informative markers were used to select the *Rpp* genes in the second pyramiding generation, which is the first MAS generation. In this study, a marker was considered informative for MAS when it allowed to identify the allele from the resistance source in the progeny of the cross (Table 2). In the P3214 population {[(PI 462312 (*Rpp3*) x (PI 230970 (*Rpp2*)] x [(PI 200492 (*Rpp1*) x (PI 459025 (*Rpp4*)]}, among the 10 markers presenting polymorphism between the resistance sources, six were informative for MAS. In the P3255 population {[(PI 462312 (*Rpp3*) x (PI 230970 (*Rpp2*)] x [(Kinoshita (*Rpp5*) x Shiranui (*Rpp5*)]}, 16 markers were polymorphic, and 11 were informative for MAS. In the P5512 population {[(Kinoshita (*Rpp5*) x Shiranui (*Rpp5*)] x [(PI 200492 (*Rpp1*) x PI 230970 (*Rpp2*)]}, 11 out of 12 polymorphic markers were informative, and in the P5532 population {[(Kinoshita (*Rpp5*) x Shiranui (*Rpp5*)] x [PI 462312 (*Rpp3*) x PI 230970 (*Rpp2*)]}, of the 17 polymorphic markers, 12 were informative for MAS.

Figure 1 illustrates the alleles observed in the Satt620 (linked to the gene *Rpp2* – allele *a*) and Satt503 (linked to the gene *Rpp4* – allele *c*) markers for all the resistance sources used, with the identification of the respective alleles. The use of these markers is only applicable when the selection

target is the gene to which the marker is linked. In this way, when using the Satt620 marker, the target is always the allele from PI 230970. When this parent is not involved in the cross, the marker is not used for selection, although it is possible, from the knowledge of all the parental alleles, to predict all progenies (Figure 2).

When molecular markers are used to identify single hybrids, the use of polymorphic markers between the parents is obvious. It is only required to select polymorphic markers between the parents, and identify the heterozygous descendant. When molecular markers are used to identify the inherited alleles from single hybrids, the use of polymorphic markers is not so obvious. The ideal situation for MAS is when the molecular marker linked to the target gene has a unique allele. In this case, for all generations of MAS, simply select plants containing this allele, and in the absence of recombination, the target gene is being selected (Figure 2). However, this ideal situation is rare. The allele linked to the target gene may occur in other plants without this target gene (alleles identical by state, but not identical by offspring). Nevertheless, this identity does not preclude the use of these markers in MAS.

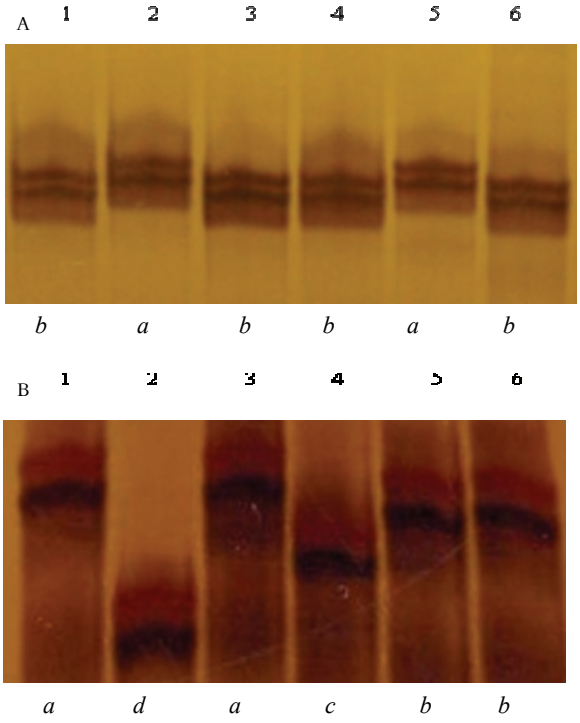

Figure 1. A - Marker Satt620^{aa} linked to the gene *Rpp2* in the PI 230970 and alleles of resistance sources containing other *Rpp* genes. B - Marker Satt503^{cc} linked to the gene *Rpp4* in the PI 459025 alleles of resistance sources containing other *Rpp* genes. 1) PI 462312 (*Rpp3*); 2) PI 459025 (*Rpp4*); 3) Kinoshita (*Rpp5*); 4) PI 200492 (*Rpp1*); 5) PI 230970 (*Rpp2*); 6) Shiranui (*Rpp5*). Letter from *a* to *d* refer to the allele codification. 6% denaturing polyacrylamide gels.

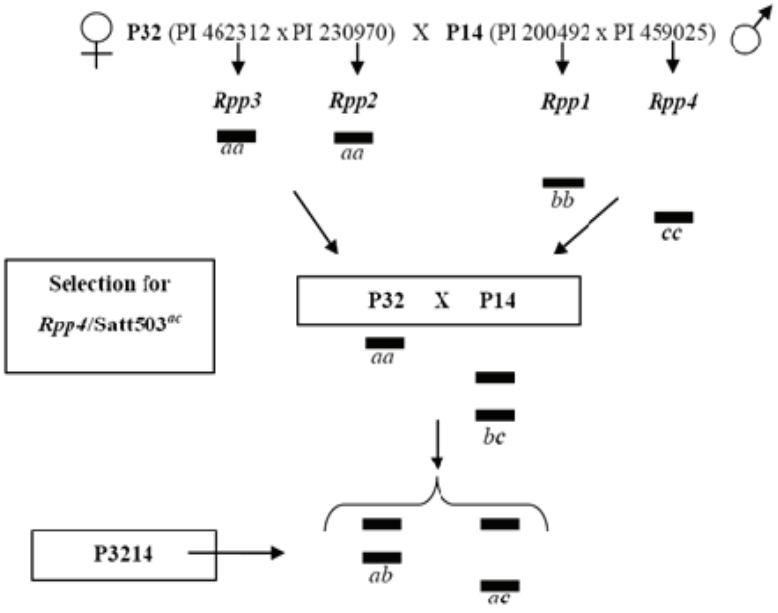

Figure 2. Selection for the *Rpp4* gene in double hybrids in the P3214 population with the Satt503 marker. PI 459025 has the allele *c* of the Satt503 marker, which is not present in any other parent. In any situation, the presence of the allele *c* indicates the presence of the gene, when disregarded recombinations.

In Figure 3A, the selection of the *Rpp2* gene is shown in the P3214 population. For this selection, we used a marker whose resistance allele (*a*) is also in a resistance source that does not contain the *Rpp2* gene. These two resistance sources have identical alleles by state and not necessarily identical by offspring. This possibility of a different origin of the *a* alleles was associated with the possibility of recombination during evolution, which explains why the two SSR fragments are connected to different alleles of the *Rpp2* gene. Single hybrids from the two original crosses [PI 462312 (*Rpp3*) x PI 230970 (*Rpp2*) and PI 200492 (*Rpp1*) x PI 459025 (*Rpp4*)] show the same profile in the molecular marker assessment (genotype *ab* in the nomenclature used in this work). Even so, this marker is considered informative for the *Rpp2* gene, since from the cross of the two single hybrids (both *ab*), the progeny with the *aa* genotype are 100% heterozygous for the locus from PI 230970, and contain the heterozygous gene *Rpp2*. In turn, the *ab* genotype has a 50% probability of allele *a* being derived from PI 230970 (presence of the *Rpp2* gene) and a 50% chance of allele *a* being from PI 459025 (absence of the *Rpp2* gene) (Figure 3A and B).

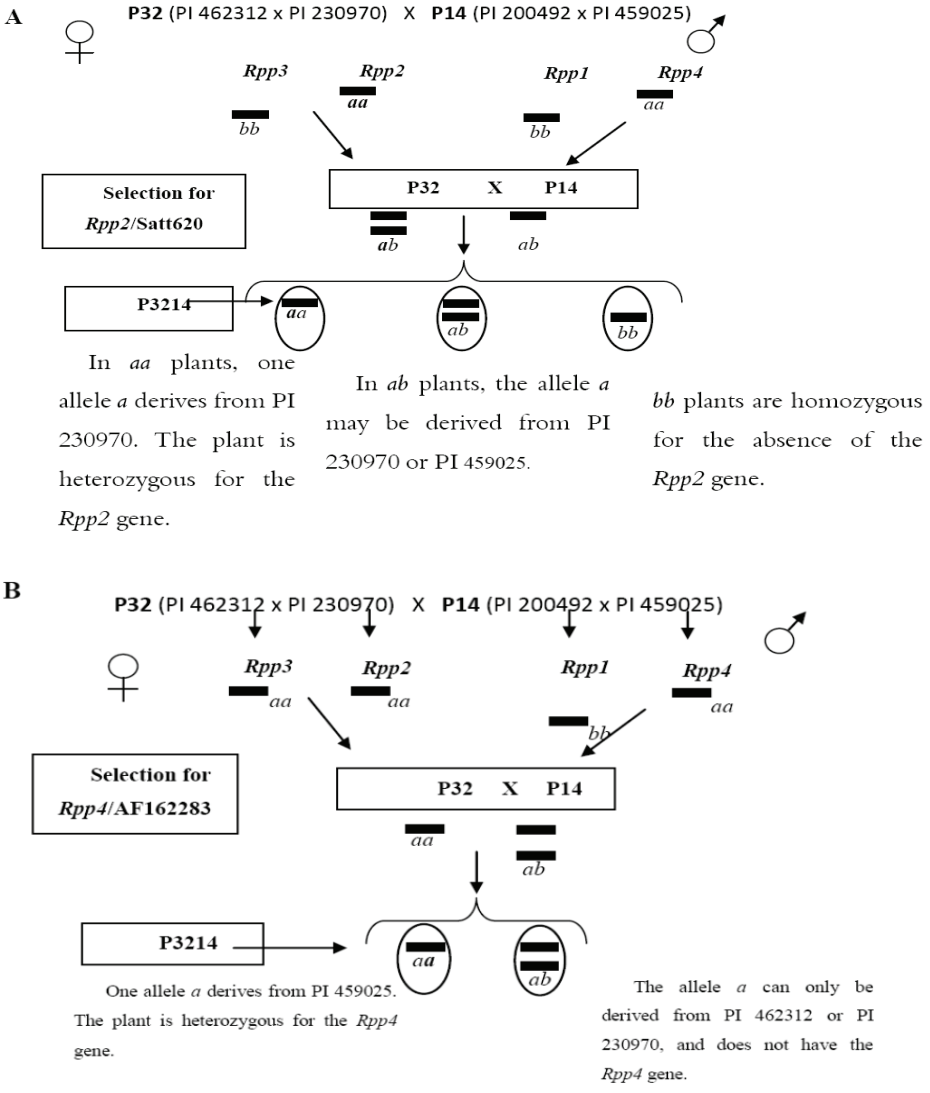

Figure 3. Selection of double hybrids using molecular markers with alleles identical by state. A - The resistance allele for *Rpp2* (*aa*) also appears in a parent that does not have the *Rpp2* gene (PI 459025). The double hybrid P3214 with *aa* genotype is 100% heterozygous for the *Rpp2* gene, and the genotype *ab* is 50% heterozygous for *Rpp2* and 50% without the gene. B - PI 459025 has the allele *a* of the AF162283 marker linked to the *Rpp2* gene. Other two parents, without the *Rpp2* gene (PI 462312 and PI 230970), also have the allele *a*. The double hybrid P3214 may have *aa* (100% heterozygous for *Rpp2*) or *ab* (absence of *Rpp2*) genotype.

A similar situation can occur even if three out of the four resistance sources used in gene pyramiding have the same allele, including the resistance source for the marker target gene. Figure 3B presents the selection for the *Rpp4* gene from PI 459025 using the marker AF162283. The allele *a* of the marker AF162283, which is present in PI 459025, is linked to the resistance gene *Rpp4.* Meanwhile, PI 462312 and PI 230970, resistance sources from other genes used in the pyramiding, have the same PI 459025 allele but do not contain the *Rpp4* gene. In such cases, it is necessary to consider that the resistance source contains the target gene when crossing the initial generation with the source of another gene, which is polymorphic at the target locus, in this case, PI 200492 x PI 459025. The other parental cross should involve the other parent contingent on the same allele of the resistant parent, as illustrated in Figure 3B (PI 462312 x PI 230970). In this way, the simple hybrid containing the gene of interest will be heterozygous for the marker (*ab*), while the other simple hybrid will be homozygous for allele *a.* In the progeny of the cross between the two single hybrids (Figure 3B), genotype *aa* is 100% heterozygous for the *Rpp4* gene (heterozygous by offspring), since one of the *a* alleles must be derived from PI 459025, linked to the *Rpp4* gene, while the other *a* allele can either be from PI 462312 or PI 230970, and none of them contains the *Rpp4* gene. The *ab* genotype should have received the *b* allele from the PI 200492 plant and the *a* allele from PI 462312 or PI 230970. None of these parents have the *Rpp4* gene. Therefore, *ab* plants are 100% absent of *Rpp4*. Meantime, for the progeny of *aa* plants that are heterozygous for the source of the allele *a,* and heterozygous for the *Rpp4* gene, this marker can no longer be used, because it will not be able to identify plants containing the resistance allele. To this end, it is necessary to identify other markers in this region.

The use of markers that are useful only at the first pyramiding generation, as illustrated in Figure 3, is justified if they are closer to the gene of interest than other markers, and they should be replaced by other markers in other generations, which are even farther from the target gene. As such, they continue to be informative in the other generations, especially those with unique alleles (Figure 2). When the pyramiding work started, the *Rpp* genes in Kinoshita and Shiranui were still not known, and therefore, crosses were made between these genotypes. By means of the SSR marker analysis, it was observed that for all the *loci* that were associated with the *Rpp5* gene, the parents Shiranui and Kinoshita showed no polymorphism, indicating possibly that the materials evaluated contain the same gene. For this reason, F_1 plants were derived from crosses between the two materials (1st pyramiding generation, Table 1) and were considered homozygous for *Rpp5*. Thus, all the F_1 plants of the double hybrids, for the P3255, P5512 and P5532 populations (2nd pyramiding generation, Table 2), were considered heterozygous for *Rpp5* and were therefore all selected for this gene. The confirmation that Kinoshita and Shiranui have the *Rpp5* gene was presented after the initial crosses in this work by Garcia et al. (2008), and then the progenies from the cross between Kinoshita and Shiranui definitely were considered homozygous for the *Rpp5* gene, with all the subsequent genetic implications relating to the segregation of the crosses involving these progenies.

In Table 3, the results relative to the number of plants obtained in each population of the second pyramiding generation are listed after MAS. Only the results of the plants containing pyramided genes are presented, disregarding the plants with one or no *Rpp* gene. In the P3214 and P3255 populations, we obtained plants containing two or three *Rpp* genes within a single plant in different combinations. With the P5512 and P5532 populations, we obtained plants containing two *Rpp* genes. In the 4235 population, we obtained plants with two, three and four *Rpp* genes, and in the P2345 population, we obtained plants with three *Rpp* genes.

To ensure that the presence of the markers also meant the presence of the *Rpp* genes, we assessed the progeny of a plant containing three genes in the P3255 population. The P3255 plants obtained from the cross between P32 and P55 and selected by molecular markers for the three genes (*Rpp2*, *Rpp3*, and *Rpp5*) are heterozygous for these three genes and are equivalent to the F_1 generation. We expect to obtain a proportion of 63:1 plants with RB: TAN symptoms in the F_2 generation of this population. In the phenotypic assessment of this F_2 population, we included the parents and observed that PI 462312 (*Rpp3*) was susceptible to the isolate used, whereas PI 230970 (*Rpp2*), Kinoshita (*Rpp5*), and Shiranui (*Rpp5*) were resistant. In this sense, the expected ratio in this F_2 population is 15:1 of RB: TAN lesions, since only the *Rpp2* and *Rpp5* genes maintained resistance to the isolate. Among the 176 F_2 plants evaluated, 165 plants showed an RB lesions, and 11 plants had TAN lesions ($\chi^2 = 0$, P = 100%). This result demonstrates that, at least for the *Rpp2* and *Rpp5* genes, MAS was efficient in selecting the genes. The efficiency of the *Rpp3* gene was not assessed due to the loss of resistance to the isolate used in the evaluation.

In this study, through MAS, we obtained plants that potentially present combinations of genes, including *Rpp3* + *Rpp4* + *Rpp5*; *Rpp2* + *Rpp3* + *Rpp4*; *Rpp2* + *Rpp3* + *Rpp5*; *Rpp2* + *Rpp4* + *Rpp5*; and *Rpp2* + *Rpp3* + *Rpp4* + *Rpp5* (Table 4).

Table 3. Number of plants obtained with each resistance genotype, on the second pyramiding generation for the *loci* of resistance to ASR, assessed with microsatellite markers.

Population (Genealogy)	Markers used in MAS (target gene)	*Rpp* genes present in double hybrids	Number of plants
P3214 {[(PI462312 (*Rpp3*) x (PI230970 (*Rpp2*)] x [(PI200492 (*Rpp1*) x (PI459025 (*Rpp4*)]}	Sct_187 (*Rpp1*) Sat_093 and Satt620 (*Rpp2*) Sat_263 (*Rpp3*) AF162283 and Satt503 (*Rpp4*)	*Rpp1* + *Rpp2*	5
		Rpp1 + *Rpp3*	5
		Rpp2 + *Rpp3*	4
		Rpp2 + *Rpp4*	1
		Rpp3 + *Rpp4*	2
		Rpp1 + *Rpp2* + *Rpp3*	1
		Rpp1 + *Rpp2* + *Rpp4*	1
		Rpp1 + *Rpp3* + *Rpp4*	5
		Rpp2 + *Rpp3* + *Rpp4*	4
		TOTAL	28
P3255 {[(PI 462312 (*Rpp3*) x (PI 230970 (*Rpp2*)] x [(Kinoshita (*Rpp5*) x (Shiranui (*Rpp5*)]}	Sat_093 and Satt620 (*Rpp2*) Sat_263 (*Rpp3*) Sat_084, Sat_266, Sat_275, Sat_280, Satt125, Satt387, Satt485, Satt584 (*Rpp5*)	*Rpp2* + *Rpp5*	3
		Rpp3 + *Rpp5*	8
		Rpp2 + *Rpp3* + *Rpp5*	4
		TOTAL	15
P5512 {[(Kinoshita (*Rpp5*) x (Shiranui (*Rpp5*) x [(PI 200492 (*Rpp1*) x (PI 230970 (*Rpp2*)]}	Sct_187 (*Rpp1*) Sat_093 and Satt620 (*Rpp2*) Sat_084, Sat_266, Sat_275, Sat_280, Satt125, Satt387, Satt485, Satt584 (*Rpp5*)	*Rpp1* + *Rpp5*	4
		TOTAL	4
P5532 {[(Kinoshita (Rpp5) x (Shiranui (Rpp5)] X [(PI462312 (*Rpp3*) x (PI230970 (*Rpp2*)]}	Satt529 and Satt620 (*Rpp2*) Sat_263 (*Rpp3*) Sat_084, Sat_266, Sat_275, Sat_280, Satt125, Satt387 Satt485, Satt584 (*Rpp5*)	*Rpp2* + *Rpp5*	1
		TOTAL	1
P4235 {[P3214 Plant 39 (Rpp4) X P3255 Plant 21 (*Rpp2*, *Rpp3* and Rpp5)]}	Satt431 and Satt547 (*Rpp2*) Sat_263 (*Rpp3*) Satt503 and Satt517 (*Rpp4*) Sat_275 (Rpp5)	*Rpp2* + *Rpp5*	1
		Rpp2 + *Rpp4* + *Rpp5*	1
		Rpp3 + *Rpp4* + *Rpp5*	1
		Rpp2 + *Rpp3* + *Rpp4* + *Rpp5*	1
		TOTAL	4
P2345 {[P3214 Plant 55 (*Rpp2*, *Rpp3* and Rpp4) X P5532 Plant 15 (*Rpp2* and Rpp5)]}	Satt431 and Satt547 (*Rpp2*) Sat_263 (*Rpp3*) Satt503 and Satt517 (*Rpp4*) Sat_275 (*Rpp5*)	*Rpp3* + *Rpp4* + *Rpp5*	1
		Rpp2 + *Rpp3* + *Rpp5*	1
		Rpp2 + *Rpp3* + *Rpp4*	1
		TOTAL	3

Table 4. Genotypes of plants from the P4235 and P2345 populations, derived from crosses between P3214 X P3255 [Plant 39 (*Rpp4*) x Plant 21 (*Rpp2*, *Rpp3*, and *Rpp5*)] and P3214 X P5532 [Plant 55 (*Rpp2*, *Rpp3*, and *Rpp4*) x Plant 15 (*Rpp2* and *Rpp5*)], respectively, evaluated by microsatellite markers on the third pyramiding generation.

Populations	Number of plants	Markers used in MAS (target gene)	Genotypes
P4235	1	Satt431 and Satt547 (*Rpp2*)	*rpp1rpp1 rpp2rpp2 Rpp3rpp3 Rpp4rpp4 Rpp5rpp5*
	1	Sat_263 (*Rpp3*)	*rpp1rpp1 Rpp2rpp2 rpp3rpp3 Rpp4rpp4 Rpp5rpp5*
	1	Satt503 and Satt517 (*Rpp4*)	*rpp1rpp1 Rpp2rpp2 Rpp3rpp3 Rpp4rpp4 Rpp5rpp5*
	1	Sat_275 (*Rpp5*)	*rpp1rpp1 Rpp2rpp2 rpp3rpp3 rpp4rpp4 Rpp5rpp5*
P2345	1	Satt431 and Satt547 (*Rpp2*) Sat_263 (*Rpp3*) Satt503 and Satt517 (*Rpp4*) Sat_275 (*Rpp5*)	*rpp1rpp1 rpp2rpp2 rpp3rpp3 rpp4rpp4 Rpp5rpp5*
	1		*rpp1rpp1 rpp2rpp2 rpp3rpp3 rpp4rpp4 rpp5rpp5*
	1		*rpp1rpp1 Rpp2rpp2 Rpp3rpp3 rpp4rpp4 Rpp5rpp5*
	1		*rpp1rpp1 rpp2rpp2 Rpp3rpp3 rpp4rpp4 rpp5rpp5*
	1		*rpp1rpp1 rpp2rpp2 Rpp3rpp3 Rpp4rpp4 Rpp5rpp5*
	1		*rpp1rpp1 Rpp2rpp2 Rpp3rpp3 Rpp4rpp4 rpp5rpp5*

The attempt to pyramid four genes within the same population requires obtaining two single hybrids containing two genes each, and crossing these hybrids, which results in double hybrids with different numbers of genes conferring resistance, according to the recombination of the four genes that were heterozygous in the two single hybrids. Regarding ASR, there is no phenotypic difference in

the effect of plants containing one or more *Rpp* genes against the examined ASR isolates. Plants containing one or more genes show RB lesions, and plants without any gene present TAN lesions; thus, it is not possible to identify plants that had pyramided genes from the phenotypic analysis. MAS enables the identification of plants containing more than one target gene, directly in F_1 plants from double hybrids, and follows the segregation of these genes in the generations of self-fertilization until reaching homozygosity. It allows reducing the number of self-fertilization generations and the homozygosity of all target genes for pyramiding. For MAS to be effective in selecting plants containing the target genes, it is necessary to identify molecular markers linked to these genes. Hyten et al. (2007), Silva et al. (2008), Garcia et al. (2008), and Hyten et al. (2009) mapped the five ASR resistance genes used in this study in the linkage groups G (*Rpp1* and *Rpp4*), J (*Rpp2*), C2 (*Rpp3*), and N (*Rpp5*). Except for the genes *Rpp1* and *Rpp4*, which are linked to the same group, ASR resistance genes segregate independently in pyramiding generations, which facilitates obtaining the desired allele combinations, since there is no need to obtain recombination between genes. Even with the genetic linkage between *Rpp1* and *Rpp4* genes, we obtained six plants containing these two genes in the P3214 population, indicating that this recombination is common (Table 3).

In the first pyramiding generation, the P3214, P4235, and P2345 populations had four pyramided genes. The P3255, P5512, and P5532 populations had only three possible genes for combination, since only the P55 population had the *Rpp5* gene. In the three populations containing four genes in the parents, we obtained only one plant with the four combined genes in the P4235 population (Table 3). Moreover, in these three populations, we obtained 16 plants containing three combined genes and 18 plants containing two combined genes. In the P3255, P5512, and P5532 populations, we obtained four plants containing three genes, and 16 plants containing two combined genes. In the six populations obtained in this generation, we obtained a plant with four genes, 20 plants containing three genes and 34 plants containing two genes. Considering the combinations containing only the effective genes (*Rpp2*, *Rpp4*, and *Rpp5*), in this generation we obtained a plant containing the genes *Rpp2* + *Rpp4* + *Rpp5*, seven plants containing the genes *Rpp2* + *Rpp4*, 10 plants containing the genes *Rpp2* + *Rpp5* and three plants containing the genes *Rpp4* + *Rpp5*. These plants can be used as parents in crosses to achieve breeding populations containing the pyramided *Rpp* genes. The efficiency of MAS, at least for the *Rpp2* and *Rpp5* genes, which are still resistant to the isolates used in the P3255 population, was demonstrated by the phenotypic analysis of the F_2 population, which has perfectly segregated for two genes.

The preferred molecular markers for MAS are those closest to the target genes. Molecular markers used in the first MAS generation were the markers preferentially reported in the literature as the closest to the mapped *Rpp* genes (Hyten et al., 2007; Silva et al., 2008; Garcia et al., 2008; Hyten et al., 2009), beyond the markers used in other *Rpp* gene pyramiding studies (Yamanaka et al., 2008). However, in some combinations, the closest markers have no polymorphism between the resistance sources. However, in some situations, these markers can be used in the second pyramiding generation (selection of double containing the target genes). Once there is polymorphism between the resistance sources that comprise the single hybrids containing the target marker gene, this can be used in MAS (Figure 3). This is the case even if the resistance sources compounding the other single hybrid used to obtain the double hybrid have the same allele for the marker in question. On the other hand, some of the closest molecular markers cannot be used because they have no polymorphism, or do not achieve good amplification in the PCR reaction. In such cases, the following markers in the linkage group are chosen for MAS, since they are not too distant. Unless there is no other option for markers indicated in mapping studies, the markers located in the region containing the resistance locus from the consensus map of the soybean are selected (Cregan et al., 1999; Song et al., 2004). Tables 3 and 4 show the markers used in each population, for each target gene. The *Rpp1* gene was mapped between the Sct_187 and Sat_064 markers (Hyten et al., 2007), and the Sct_187 marker was informative for the second pyramiding generation. The *Rpp2* gene was mapped in PI 230970 between the Sat_255 and Satt620 markers (Silva et al., 2008). The Satt620 marker was informative for the second pyramiding generation for all populations. Yamanaka et al. (2008) used the Satt529 and Satt620 markers for the selection of *Rpp2*, and the Satt529 markers were also employed in the second pyramiding generation in this study. For the third pyramiding generation, new markers were selected in the genetic map of the soybean (Cregan et al., 1999; Song et al., 2004), because the previous ones were no longer informative for selecting plants containing the *Rpp2* gene. In this generation, we used the Satt431 and Satt547 markers.

The resistance genes *Rpp2*, *Rpp3*, and *Rpp4* were successfully pyramided in pair-wise combinations in the F_2 generation by Maphosa, Talwana, and Tukamuhabwa (2012) based on the molecular data. The Satt460 (*Rpp3*) and AF162283 (*Rpp4*) markers were polymorphic between the parents and thus were used in the selections made in the F_2 and F_3 families. In the present study, the AF16283 marker was only employed in the second pyramiding generation and was no longer informative in the following generations, being replaced by the Satt503 and Satt517 markers. The Satt460 marker was homozygous between the resistance sources used, and therefore non-informative in the selection of the plants. In addition, Lemos et al. (2011) used the markers Satt529 and Satt620 (*Rpp2*); Satt517 and AF162283 (*Rpp4*); and Sat_275 and Sat_280 (*Rpp5*) in a gene pyramiding work. Morceli et al. (2008) used the Sat_275 and Sat_280 markers and achieved total efficiency in the selection of the *Rpp5* gene, concluding that the use of these markers for marker assisted selection is valid, since it identifies the homozygous genotypes and the resistance genes that can be fixed within a few cycles of selection.

The selection of the plants in segregating populations containing appropriate combinations of genes is a critical component of plant breeding (Collard & Mackill, 2008). In this research, we obtained plants that potentially present the following combinations of genes through MAS: *Rpp3* + *Rpp4* + *Rpp5*; *Rpp2* + *Rpp3* + *Rpp4*; *Rpp2* + *Rpp3* + *Rpp5*; *Rpp2* + *Rpp4* + *Rpp5*; and *Rpp2* + *Rpp3* + *Rpp4* + *Rpp5* (Table 4). This is a great advantage to these plants in relation to those without multiple resistance genes in a single plant, as it is believed that the accumulation of multiple race-specific genes in a single plant/variety reduces the probability that a single mutation in the pathogen can overcome all the genetic resistance (Mundt, 1991; Huang et al., 1997; McIntosh & Brown, 1997) provided by the presence of more than one gene. Likewise, Singh et al. (2001) pyramided three genes for resistance to bacterial blight in rice and verified that this technique provided a broad-spectrum resistance to plant populations when compared to the presence of a single gene. The present study, as well as several other studies, aimed at gene pyramiding with the aid of molecular biology techniques in selecting resistant genotypes to the phytopathogens. Parrella et al. (2008), Beraldo, Colombo, Chiorato, Ito, and Carbonell (2009), and Marcondes, Santos, and Pereira (2010) selected families and strains with resistance to anthracnose by pyramiding the *co-4*/*co-5* alleles, using SCAR marker assisted selection.

One of the most important steps in the use of molecular markers is to establish the relationship between a given marker and the *locus* of interest (Alzete-Marin et al., 2005). Due to recombination, the regions surrounding the locus of interest can be different even between related genetic materials. Therefore, a polymorphic marker between parents A and B cannot be polymorphic between A and C. Thus, for each cross, specific markers must be identified. In many cases, the same marker can be useful in different populations derived from different crosses. Herein, we observed that some SSR markers mapped close to the target genes (Hyten et al., 2007; Monteros et al., 2007; Garcia et al., 2008; Silva et al., 2008; Hyten et al., 2009; Ray et al., 2009) and were informative for plant selection. In contrast, there was a need to use new markers located in the same region of the target gene, obtained from the consensus map of the soybean, because, for some crosses, the previously mapped *loci* were informative in the second pyramiding generation and homozygous by state in the third pyramiding generation. There are many studies in the literature that report gene pyramiding in plants, using MAS. However, there is no work illustrating the steps of pyramiding, which discuss its problems and solutions. This study has detailed the steps of gene pyramiding for resistance to ASR through molecular markers, demonstrating the selection of the alleles of interest, and the appropriate choice of the molecular markers. As a result, we obtained plants with different combinations of ASR resistance genes, ranging from two to four pyramided genes. These results can help other gene pyramiding programmes by MAS, following the steps outlined in this work.

Conclusion

Through microsatellite marker-assisted selection, we obtained plants containing a range of 2 to 4 pyramided genes per plant. These plants can be used as a source of multiple resistance in breeding programmes for obtaining soybean varieties with more durable resistance to ASR.

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