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Full Length Research Paper

Enhancing soybean rust resistance through *Rpp2*, *Rpp3* and *Rpp4* pair wise gene pyramiding

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The threat posed by soybean rust (*Phakopsora pachyrhizi*) on soybean production is worsened by resistance breakdown associated with single gene resistance present in most cultivars. Few studies have however been undertaken to use mapped simple sequence markers for gene pyramiding to enhance rust resistance. This study validated use of identified simple sequence repeat markers for gene pyramiding, and determined the most effective pairwise gene combination for three independent soybean rust resistance genes, R*pp2*, R*pp3* and R*pp4.* Markers Satt460 and AF162283 were polymorphic for the three resistance genes among the parents and were therefore used in selections made in the F_2 and F_3 families. In the F_2 generation, soybean plants (homozygous dominant or heterozygous at both loci) with two gene combinations had relatively lower disease severity and sporulation than the parents, suggesting complementary epistatic gene action for resistance. Similarly, homozygous F_3 families showed lower severity, lesion density and sporulation. Gene R*pp3* contributed positively to resistance with various genetic backgrounds for most parameters measured, compared to R*pp2* and R*pp4* resistance genes. Overall, the results suggest that marker gene pyramiding is feasible and can substantially increase resistance to soybean rust through reduced severity and reduced sporulating lesions.

Key words: Epistasis, genetic background, *Phakopsora pachyrhizi,* simple sequence repeats.

INTRODUCTION

Soybean rust (*Phakopsora pachyrhizi*) is one of the most serious foliar diseases of soybean worldwide. Under heavy infestation, losses of up to 75% have been observed in unprotected fields (Yorinori et al., 2005). The rapid spread of soybean rust, together with the potential of causing severe yield losses, makes it a very important disease of soybean (Miles et al., 2003). Several strategies for controlling soybean rust have been used, such as fungicide application and genetic resistance. The effectiveness of fungicides, however depends on timely application and use of appropriate spraying methods (Yorinori et al., 2005). More so, use of fungicides has cost implications and raises environmental concerns. Genetic resistance is therefore an economic and strategically important means of controlling soybean rust disease (Arias et al., 2008).

In soybean, resistance to rust is manifested phenotypically by red brown lesions (Bromfield, 1984; Bonde et al., 2006), and is conditioned by six major resistance genes R*pp*1, R*pp*2, R*pp*3, R*pp*4, R*pp*5, Rpp_{Hyuuga} which have been mapped to different linkage groups which are R*pp1* linkage group (LG) G (Hyten et al., 2007), R*pp3* LGC2 (Hyten et al., 2009), R*pp2* and R*pp4* LGJ and G, respectively (Silva et al., 2008; Yamanaka et al., 2008), *Hyuuga* LGC2 (Monteros et al., 2007) and R*pp5* LGN (Garcia et al., 2008). Long term utilisation of these race specific resistance genes has prompted the pathogen to mutate and overcome them. Empirical evidence in Africa, Orient and South America has shown that some of the once effective soybean rust resistance genes have been overcome by new rust races (Tschanz et al., 1986;

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Hartman et al., 2004; Laperuta et al., 2008). Despite resistance breakdown associated with race specific genes, they are still effective against a broad range of pathotypes in wheat rust pathosystems (Pfender, 2009). The ability to easily identify specific resistance genes makes it relatively quicker to introgress and use them in combinations to control soybean rust.

Gene pyramiding, which involves assembling multiple desirable genes into a single genotype has been suggested as a method that can overcome resistance instability conferred by single gene resistance to soybean rust (Hartman et al., 2005; Garcia et al., 2008; Yamanaka et al., 2010; Lemos et al., 2011). Our aim for pyramiding rust resistance genes in this study was to enhance soybean rust resistance to field isolates and broaden the genetic base for rust resistance in the available soybean breeding lines. However, incorporating such multiple gene resistance has remained a challenge using conventional methods, due to the requirement of extensive screening using gene specific pathogen races (Sanghai-Maroof et al., 2008). Conventional approaches are not always practically feasible in gene pyramiding given the fact that some genes were identified using foreign races of *P. pachyrhizi* whose access presents logistical and phyto-sanitary challenges. Accordingly, marker assisted selection was the most desirable alternative available for pyramiding resistance genes.

Several methods have been suggested for gene pyramiding when resistance genes are present in different parents, such as production of F_2 and F_3, recombinant inbred lines (RILs) and double haploids (DH) (Servin et al., 2004). Utilisation of each population type depends on the availability of resources, objectives and germplasm available for the study. In soybean, the availability of a dense molecular map comprising of molecular markers such as SSR, RFLP, AFLP and Isozymes (Song et al., 2004) makes marker-assisted selection for specific resistance genes in the early generations feasible. Moreover, several SSR markers tightly linked to known sources of resistance have been mapped, making it possible to trace them during hybridisation, and facilitate their identification through marker assisted selection. Gene pyramiding has been recommended by a number of researchers as a possible way of enhancing single gene resistance for effectiveness against several soybean rust races (Hartman et al., 2005; Yamanaka et al., 2010; Lemos et al., 2011), while breeding for soybean rust resistance. However, no research has been done on the effect of pyramiding soybean resistance genes to enhance resistance trait performance against local Ugandan rust populations. In rice, *Oryza sativum*, pyramiding has been done for three bacterial blight (*Xanthomonas oryzae pv. oryzae*) resistance genes using marker assisted selection, resulting in greater resistance (Singh et al., 2001). Saghai Maroof et al. (2008) also pyramided three resistance genes for (SMV). Similarly, enhanced resistance to the fungus, *Magnaporthe grisea*, which causes rice blast was observed after genes *Pi*1, *Piz*-5 and *Pi*ta were pyramided into one genotype (Hittalmani et al., 2000). The success of gene pyramiding strategies is largely facilitated by availability of molecular markers which are tightly linked to the gene interest. Therefore, the objectives of this study were to validate the use of marker assisted selection in F_2 and F_3 families to pyramid three resistance genes in pair-wise combinations, and determine the most effective gene combinations for enhancing resistance to soybean rust.

MATERIALS AND METHODS

Crosses and progeny development

Gene pyramiding was done through single crosses in a screen house using parental lines: PI 230970, Ankur and PI 459025, having three specific resistance genes R*pp2*, R*pp3* and R*pp4*, respectively. The crosses were done in pairwise combinations at Makerere University Agricultural Research Institute (MUARIK) during 2009 season, and were implemented as follows: PI 230970 (R*pp2*) × Ankur (R*pp3*); PI 230970 (R*pp2*) × PI 459025 (R*pp4*) and Ankur (R*pp3*) × PI 459025 (R*pp4*). Successful F_1 hybrid progeny were determined based on phenotypic marker traits like anthocyanin coloration of the seedlings, flower and pubescence colour to eliminate selfed individuals. F_1 plants were allowed to self, to produce F_2 segregating populations which were screened to identify individuals possessing the two soybean rust resistance genes. Selected F_2 plants were scored for disease severity relative to other individuals with single resistance genes and advanced to obtain F_3 generation. Selected plants were harvested separately to ensure family identity. All individuals within the F_3 generation were screened for the two resistance genes and scored for disease resistance parameters.

Field experimental layout and phenotypic screening procedures

Field experimental plots comprising of 2 m rows with 30 to 35 plants for the parents and progenies were established under natural infestation at a soybean rust host spot (Kabanyolo). Spreader rows of a highly susceptible variety Nam 2 were planted around the test material to ensure sufficient disease inoculums. Hybrids from R*pp2* × R*pp3*, R*pp2* × R*pp4*, R*pp3* × R*pp4* gene combinations were assessed for disease severity compared to parental lines with single genes, starting from the R5 stage (Fehr et al., 1971). Rust severity was determined at weekly intervals using a scale based on the counted lesion density per leaflet, where 1 = no lesions; 2 = 1 to 30; 3 = 31 to 75; 4 = 76 to 150; 5 = 151 to 300; 6 = 301 to 750; 7 = 751 to 500; 8 = 1501 to 3000 and 9 = > 3000 lesions from three trifoliates of the mid-canopy (Miles et al., 2008). Sporulation rate was evaluated based on a 1 to 5 scale (where 1 represents no-sporulation and 5 represents profuse sporulation). Using 20× magnification lenses, soybean lines were evaluated for the number of lesions per square centimetre proportion of sporulating lesions. Numbers of pustules per lesion were also assessed after vacuuming selected leaves with a hand held Liliput® vacuum to dislodge any urediniospores for easy counting.

DNA isolation and marker assisted selection

Genomic DNA was isolated from young soybean leaves using a

Table 1. Simple sequence repeat based markers and their position in relation to three soybean resistance loci on a soybean linkage map.

Marker	Linkage group	Position[1] (cM)	Resistance gene	References
Sat_255	J	43.85	R*pp2*	Silva et al. (2008)
Satt620	J	53.71	R*pp2*	Silva et al. (2008)
Satt460	C2	111.87	R*pp3*	Hyten et al. (2009)
Sat263	C2	118.78	R*pp3*	Hyten et al. (2009)
Satt288	G	76.77	R*pp4*	Yamanaka et al. (2008)
AF162283	G	87.94	R*pp4*	Yamanaka et al.(2008)

[1]Soybean SSR map.

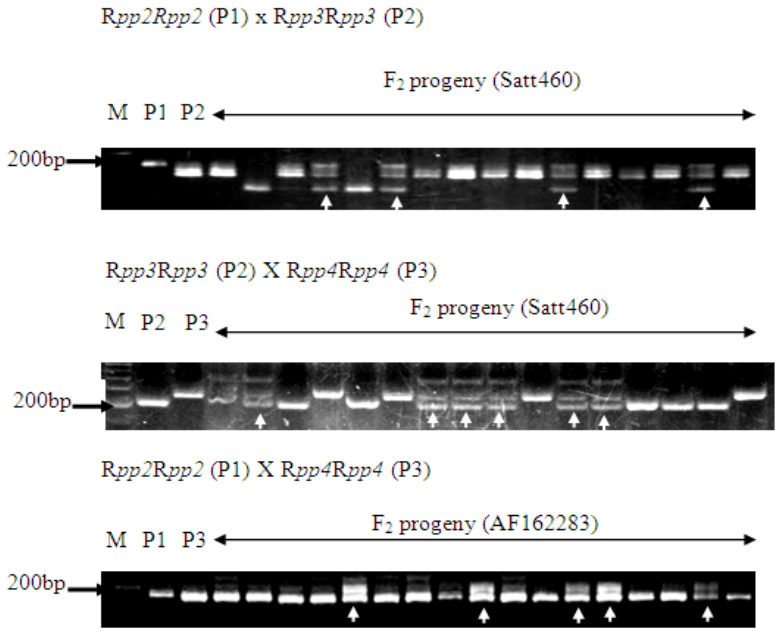

Figure 1. SSR markers for the different parents, crosses for the three resistance genes in pair-wise combinations. The arrows indicate individuals selected on the basis of possessing both parental rust resistance alleles for further F_3 family molecular analysis. M- represents a 100 bp standard molecular weight marker

Wizard Genomic DNA Purification Kit (Promega, USA) in the molecular Laboratory at Makerere University. Simple sequence repeat (SSR) molecular markers (Table 1), flanking the resistance genes (synthesised by University of Cape Town) were optimised according to their primer sequences for marker assisted selection. Parents were assayed for polymorphism using the six SSR primers prior to F_2 and F_3 progeny screening. PCR was performed in a GeneAmp 9700 (Bio-Rad, USA) thermocycler in a 20 µl reaction volume containing 40 ng of template DNA, 0.5 µM of each primer, 0.2 mM dNTPs, 1 U of *Taq* polymerase, 2.5 mM $MgCl_2$ and 1× PCR buffer. Amplification was done with an initial denaturing cycle of 94°C for 2 min, followed by 32 cycles of 94°C for 40 s; annealing at 48°C for 40 s and 72°C extension for 50 s, and a final extension cycle at 72°C for 5 min. The PCR amplicons were fractionated on 3 to 4% metaphor (Lonza Bioscience, Singapore) agarose horizontal gel stained with GelRed™ nucleic acid stain (Biotium, USA). Gel images were taken using a BioDoc-It™ Imaging System (Bio-Rad, USA).

RESULTS

Out of the six SSR markers tested, two markers Satt460 and AF162283 produced polymorphism with significant differences in their amplicon sizes between the parents tested. Therefore, subsequent screening of parents and segregating populations for resistance gene presence was done based on these two markers. The results of SSR amplification of the parents and F_2 offspring segregating for the different genes are presented in Figure 1. For each pair of gene combination, 98 F_2 plants were assayed using the two polymorphic markers during

Table 2. Severity and sporulation rate of genotyped F_2 plants evaluated at two time intervals.

Genotype	No. of plants evaluated	Severity		Sporulation	
		T1	T2	T1	T2
Parents					
R*pp2* R*pp2*	98	3.66±0.26	3.66±0.26	3.33±0.27	2.44±0.34
R*pp*3 R*pp*3	98	3.33±0.28	4.33±0.25	3.16±0.23	3.16±0.23
R*pp*4 R*pp*4	98	4.00±0.35	4.40±0.26	1.40±0.75	3.20±0.61
F_2 plants					
R*pp2_* × R*pp3_*	27	2.88±0.46	3.38±0.28	2.00±0.61	2.44±0.34
R*pp3_* × R*pp4_*	19	2.66±0.35	3.16±0.23	2.33±0.34	2.83±0.24
R*pp2_* × R*pp4_*	11	2.40±0.50	2.60±0.34	1.40±0.75	2.40±0.58
Mean		3.18±0.13	3.59±0.12	2.60±0.15	2.62±0.13

T-Time; at R5 and after one week later, ± standard error; notation R*pp_* implies the alternative allele was either dominant or recessive.

the early stages (V1-R1) of soybean development. Selected individuals were tagged and assessed for disease severity and sporulation. In the F_2 generation, 27 plants were identified to be R*pp2_* × R*pp3_*, 19 plants R*pp3_* × R*pp4_* and 11 plants R*pp2_* × R*pp4_*. Soybean rust severity was generally greater at the second data recoding time (T2) compared to the first recording time (T1) for parents and F_2 plants (Table 2). The parental lines with the R*pp2* gene remained unchanged in severity during the two time intervals. All parents had higher severity compared to the selected plants with two gene combinations. The cross R*pp3_* × R*pp4_* had a significantly lower severity followed by R*pp2_* × R*pp4_* for the two time intervals. Contrary to other genotypes, sporulation decreased from 3.3 to 2.4 for parent with the gene R*pp2*. Progeny from R*pp2_* × R*pp4_* similarly had low sporulation rate followed by R*pp2_* × R*pp3_*.

To ascertain the true genotypes, the selected F_2 plants' seed was planted to produce F_3 families. Marker assisted selection of the parents and F_3 families were done using markers used in the F_2 generation. Ten plants were genotyped in each family to identify segregating families and infer their F_2 genotypes. For each family, three individuals are presented in Figure 2. Molecular marker assay clearly distinguished segregating and non segregating families. In the F_3 generation of R*pp2*R*pp2* × R*pp3*R*pp3,* four families were homozygous while R*pp2*R*pp2* × R*pp4*R*pp4* had two homozygous families. All families from R*pp3*R*pp3* × R*pp4*R*pp4* were segregating and therefore in-depth phenotypic characterisation of disease parameters was not done for this family. The results of rust resistance evaluation of 10 plants per non-segregating F_3 family are presented in Table 3. Significant differences in disease severity, lesion per square centimetre and percentage sporulating with lesions for parents and F_3 families were observed. The F_3 family of R*pp2*R*pp2* × R*pp3*R*pp3* had the least lesions per square centimetre and frequency of lesions with pustules (Table 3). The family derived from R*pp2*R*pp2* × R*pp4*R*pp4* had a severity score lower than all the parents evaluated. However, its sporulation rate was higher than parents R*pp3*R*pp3* and R*pp4*R*pp4*. The numbers of pustules per lesion were not significantly different for all the genotypes evaluated.

DISCUSSION

Based on the molecular data, resistance genes R*pp2*, R*pp3* and R*pp4* were successfully pyramided in pair-wise combinations in the F_2 generation. However, homozygous dominant and heterozygous individuals at both loci could not be readily distinguished. This could be attributed to the apparent dominant nature of the markers or similar sized alleles of the two genes which could not be resolved by the metaphor agarose used to fractionate the amplicons. Nonetheless, results from pyramiding of pairwise gene combinations suggested occurrence of epistatic interactions among the independent dominant genes R*pp2*, R*pp3* and R*pp4* since each gene is at a different locus. Complementary gene action resulted in increase of resistance in all instances across the F_2 and F_3 generations. Such complementary gene action for resistance was reported when resistance genes *Lr*9 and *Lr*24 were pyramided in wheat to enhance resistance to leaf rust (Moullet et al., 2008).

During the evaluation of F_2 generation, R*pp4*R*pp4* had the highest severity compared to other parents and genotypes. However, when it combined with resistance genes R*pp3*R*pp3* and R*pp2*R*pp2*, this resulted in low severity, suggesting epistatic gene interaction of these genes. This corroborates observations by Yamanaka et al. (2010) and De Lucia et al. (2008) that the effectiveness of soybean rust resistance genes depends on the genetic background in which they are introgressed. This could further explain the continued utilisation of some

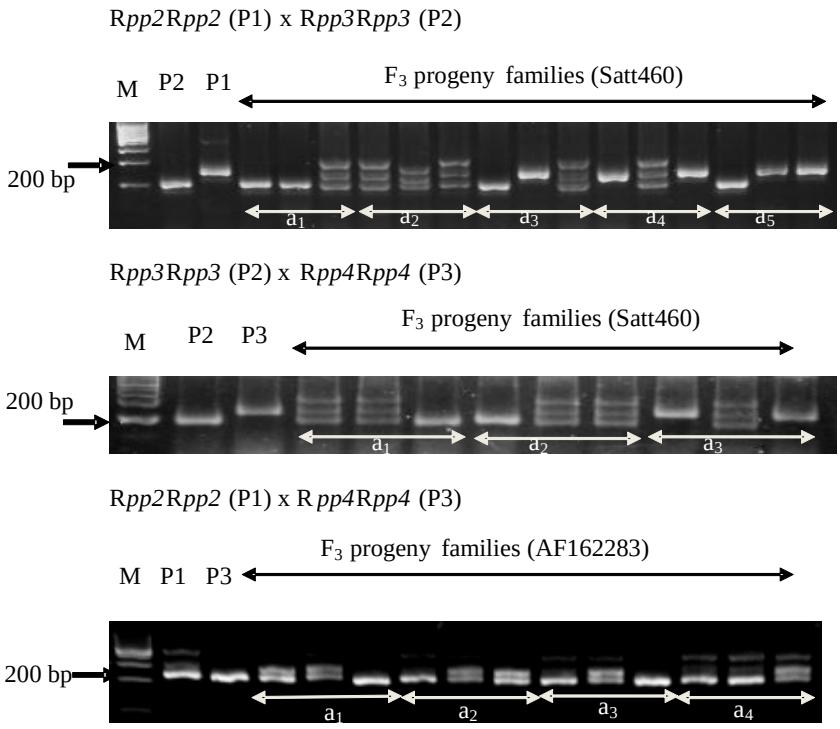

Figure 2. SSR markers for the different parents and F_3 families with two gene combinations. The arrows indicate three individuals per family, a_1-a_5 shows the families selected for genotyping, families with equal number of amplified alleles were selected for all phenotypic characterisation of resistance. M- represents a 100 bp standard molecular weight marker.

Table 3. Disease response parameters for the parents and ten homozygous dominant plants from F_3 families.

Genotype	Disease severity	Lesions /cm^2	Reaction type	Sporulating lesions (%)	Pustules per lesion
Parents					
R*pp2*R*pp2*	4.91±0.48	45.39±5.68	RB	100	2.0
R*pp3*R*pp3*	3.28±0.39	24.52±4.56	RB	18	1.5
R*pp4*R*pp4*	3.20±0.33	24.93±3.90	RB	38	1.1
F_3 families					
R*pp2*R*pp2* × R*pp3*R*pp3*	2.62±0.48	18.41±3.16	RB	16	0.9
R*pp2*R*pp2* × R*pp4*R*pp4*	3.02±0.30	26.21±3.49	RB	15	1.9
Mean	3.14±0.19	24.95±2.62		24	1.2
F probability	≤0.05	≤0.05		≤0.01	ns

± standard error; ns- non-significant.

classical resistance genes that have been overcome by certain soybean rust races. Parent R*pp4*R*pp4*, however, did not contribute to reduced sporulation in the combination R*pp3*_ × R*pp4*_, suggesting differences in genetic control of these resistance parameters. In the F_3 family generation, some selected families were homozygous, and resistance genes were fixed at both loci. Evaluation of F_3 generation for R*pp2*R*pp2* × R*pp3*R*pp3* had the lowest severity, lesions per square centimetre and pustules per lesion despite parent R*pp2*R*pp2*, showing relatively high susceptibility, which supports further presence of complementary gene action for resistance to soybean rust. From our results, it can be deduced that the R*pp3* locus contributed to most of the resistance parameters compared to R*pp2* and R*pp4*. Such disproportionate contribution between resistance

loci for resistance was observed by Lemos et al. (2011) during pyramiding of three soybean rust resistance loci. In their study, R*pp5* was the most important primary factor for resistance to soybean rust. Therefore, we can infer that dominance and complementary epistasis exists among the independent soybean rust resistance genes. The numbers of pustules per lesion were not significantly different among the tested F_3 family genotypes. Thus, we conclude that pustules per lesion are not a good measure of soybean rust resistance under our field experimental conditions.

Though the presence of multiple virulence in soybean rust was seen as the main challenge to the efficacy of gene pyramiding (Shanmugasundaram et al., 2004), our study noted increased resistance in the two gene combinations. Furthermore, our results suggest that the utilisation of marker assisted selection in pyramiding soybean rust resistance genes is possible. Although the number of lines tested was small, the results from our study clearly demonstrate that pyramiding R*pp2* and R*pp3* in homozygous condition increases resistance. All the genes tested contributed complementarily to resistance, though, in a disproportionate manner. Introgression of these double resistance genotypes into farmer preferred cultivars is therefore recommended. This study did not focus on the durability aspect of the resistance genes which is crucial for any resistance breeding programme. Consequently, further research on evaluating soybean resistance genes for durability and using diverse pathogen populations is recommended, as this is important for sustainable soybean production.

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