

Short Communication

SSR Markers for Verifying Crosses and Assessing Whitefly Resistance in Soybean Breeding Programme

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ABSTRACT

Selection of hand-pollinated seeds is a critical phase in the breeding programme of whitefly-resistant soybean. This study aimed to verify true crosses and assess whitefly resistance in soybean plants derived from crosses between a superior variety and whitefly-resistant germplasm using SSR markers. The whitefly-sensitive variety Anjasmoro was crossed with IAC 100 and G100H germplasm, serving as donors of resistance genes. Whitefly resistance in the three parental lines and 46 F₁ progenies was evaluated in a greenhouse using a free-choice host test. True crosses were verified through molecular selection in the laboratory using the SSR marker Satt045. The results revealed significant differences in the intensity of whitefly-induced leaf damage among the tested genetic materials. The worst leaf damage, up to 84.7%, was shown by Anjasmoro, while germplasm IAC 100 and G100H showed no leaf damage. The intensity of leaf damage in the offspring of Anjasmoro × IAC 100 and Anjasmoro × G100H varied from 0 to 84.7%. It is hypothesised that no successful cross-pollination occurred between IAC 100 or G100H and Anjasmoro in the plants derived from unsuccessful crosses that exhibited severe leaf damage. Selection using SSR markers indicated that plants with high leaf damage intensity had the same banding pattern as Anjasmoro. In contrast, plants with

low leaf damage intensity exhibited a banding pattern that combined those of Anjasmoro with IAC 100 or Anjasmoro with G100H. These findings demonstrate that SSR markers, supported by greenhouse resistance tests, are reliable for verifying true crosses in soybean breeding programmes targeting whitefly resistance.

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INTRODUCTION

The whitefly (*Bemisia tabaci* Genn.), a polyphagous insect, is a major limiting factor in soybean production in Indonesia. Severe infestations during the dry season can lead to yield losses of up to 80%, even resulting in total crop failure. This leaf-sucking pest causes chlorosis on the host plant due to its activity of extracting phloem sap from the leaves (Zaidi et al., 2017). The honeydew excreted during the feeding process provides an ideal growth medium for sooty mould fungi, which indirectly damages the plant by disrupting the photosynthesis process (Oliveira et al., 2001). The damage caused by whitefly is further exacerbated by its role as a vector of various viral diseases (Jones 2003; Navas-Castillo et al., 2011).

One environmentally friendly method for controlling whitefly is by planting resistant varieties. The process of developing resistant varieties requires a lengthy process that begins with finding the source of resistance genes. The soybean lines IAC 100 and G100H are known to possess resistance to whitefly infestations. Pinheiro et al. (2005) mentioned that the IAC 100 line is a soybean germplasm that has resistance to several main pests on soybean. Meanwhile, the G100H line is the offspring of the cross of IAC 100 with the Hymmeshirazu soybean variety (Sari & Sulisty, 2018). In Indonesia, the Anjasmoro soybean variety is widely favoured by farmers due to its large seeds and high productivity, making it a popular choice for cultivation (Krisdiana 2014). However, despite its advantages, Anjasmoro is vulnerable to whitefly infestations, posing a challenge for soybean growers in maintaining its yield potential (Sulistyo & Inayati, 2016). Therefore, to enhance its resistance, hybridising Anjasmoro with IAC 100 and G100H could be a strategic approach, combining the strengths of high yield and large seeds with improved pest resistance.

The next stage is to cross the soybean variety with the resistance gene donor line. The main problem in hand pollination of soybean is the low percentage of successful crossing due to the small size of soybean flowers. In addition, the crossed seeds do not necessarily receive the resistance genes, as soybean is a self-pollinating plant. The expression of resistance genes in the crossed seeds will only be seen after the plants are planted and attacked by the target pest. Therefore, a reliable method is needed to assist soybean breeders in distinguishing between crossed seeds that have received the resistance gene and those that have not. One effective method to assist in distinguishing crossed seeds is the use of molecular markers.

Molecular markers are DNA sequences that are evenly distributed across an organism's genome and are essential for identifying genetic variations among individuals or species (Oliveira & Azevedo, 2022). Among the different types of molecular markers, Simple Sequence Repeat (SSR) markers are widely utilised. SSR markers have proven to be effective tools for analysing genetic diversity, particularly in soybean research (Tasma et al., 2018).

Shakil et al. (2015) highlighted the efficiency of SSR in distinguishing soybean accessions with high genetic similarity. These markers are valued for their codominant inheritance, high polymorphism, reproducibility, widespread distribution across the plant genome, and compatibility with standard Polymerase Chain Reaction (PCR) techniques (Amiteye, 2021; Singh & Singh, 2018). Although no specific SSR primers have yet been reported to be directly associated with whitefly resistance in soybean, previous studies have shown that the SSR marker Satt045 displays high polymorphism and is frequently used to assess genetic distance among soybean germplasm (Chotiyarnwong et al., 2007; Dyah et al., 2024; Meesang et al., 2001) and to support selection processes in breeding programmes (Tasma et al., 2018). The objective of this study was to verify true crosses and assess whitefly resistance in F₁ plants derived from crosses between a high-yielding soybean variety and whitefly-resistant germplasm using the SSR marker Satt045.

METHODS

Plant Genetic Material

The soybean seeds tested were F₁ progenies from a cross between the Anjasmoro variety and the IAC 100 and G100H lines. Anjasmoro is a superior variety widely cultivated by farmers in Indonesia. Released by the Ministry of Agriculture of the Republic of Indonesia in 2001, this variety boasts a high yield potential of up to 2.25 tons per hectare and large seed size (15.3 g per 100 seeds). However, Anjasmoro has a weakness, which is its susceptibility to whitefly attacks (Sulistyo & Inayati, 2016). Meanwhile, IAC 100 and G100H are soybean germplasms that exhibit resistance to whitefly (Pinheiro et al., 2005; Sari & Sulistyo, 2018).

Greenhouse Experiment

The study was conducted in the greenhouse of Indonesian Legume and Tuber Crops Research Institute (ILETRI), East Java, Indonesia. The experiment involved three groups of plant materials consisting of parental lines and their F₁ progenies. The parental lines (Anjasmoro, IAC 100, and G100H) served as reference controls, while the F₁ progenies were derived from two different crosses: Anjasmoro × IAC 100 and Anjasmoro × G100H. This grouping allowed clear comparison and ensured consistent evaluation of whitefly resistance among genotypes.

All soybean seeds were planted in polybags containing a 1:1 mixture of soil and manure. Each seed from one pod was planted in one polybag. Urea, SP36, and KCl fertilisers were applied at planting time at rates equivalent to 50, 100, and 100 kg per hectare, respectively. Resistance testing against whitefly was conducted using a free-choice test method. The whitefly infestation followed the method used by Mansaray and Sundufu (2009), in which 10 adult insects were placed on the leaf surface of each individual soybean plant at two weeks of age.

Damage to the fifth trifoliolate leaf from the growing point due to whitefly attack was assessed when the plants were seven weeks old. Leaf damage was scored visually using the method of Sari and Sulisty (2018), applying a 0-4 rating scale as described in Table 1.

The percentage of leaf damage intensity (LDI) was then calculated based on the visual scores using the following Equation 1:

$$LDI (\%) = \frac{\sum(n_i \times v_i)}{N \times V} \times 100\% \tag{1}$$

When n_i is the number of leaves assigned a score v_i , N is the total number of leaves observed, and V is the maximum score value. This formula converts the qualitative scoring data into quantitative values representing the degree of whitefly damage. Plant resistance levels were then categorised based on the method of Chiang and Talekar (1980), as presented in Table 2.

Table 1
Scoring scale and description of leaf damage symptoms due to whitefly infestation

Score	Description of Symptoms	Severity Level
0	No visible symptoms; leaves appear healthy without curling or sooty mould.	No damage
1	Mild symptoms: leaf curling and/or sooty mould on $\leq 25\%$ of leaf surface.	Slight
2	Moderate symptoms; leaf curling and/or sooty mould on 25-50% of leaf surface.	Moderate
3	Severe symptoms: leaf curling and/or sooty mould covering 50-75% of leaf surface; pod and seed development are abnormal.	Severe
4	Very severe symptoms; leaf curling and/or sooty mould on $>75\%$ of leaf surface; pod and seed development failure.	Very severe

Table 2
Classification criteria for determining resistance levels to whitefly based on mean (X) and standard deviation (SD)

Resistance Category	Criteria
Highly Resistant (HR)	$X_i \leq (X-2SD)$
Resistant (R)	$(X-2SD) \leq X_i \leq (X-SD)$
Moderately Susceptible (MS)	$(X-SD) \leq X_i \leq X$
Susceptible (S)	$X \leq X_i \leq (X+2SD)$
Highly Susceptible (HS)	$X_i \geq (X+2SD)$

Note. X is the population mean; X_i is the individual observed value; and SD is the standard deviation of the population

Genomic DNA Isolation

Soybean genomic DNA was isolated from 0.5 g of leaf samples from three-week-old plants using a modified Doyle and Doyle (1990) method, which included the addition of 2% (w/v) PVP. The resulting DNA pellets were dissolved in 100 μ L of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and treated with 2 μ L of 10 mg/mL RNase (Invitrogen, USA), then incubated at 37°C for 1 hour. The quality of the genomic DNA was assessed through electrophoresis on a 1% agarose gel, and the results were observed under UV light using a UV Transilluminator (UVP, UK). Quantitative analysis of the genomic DNA was performed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA).

SSR Marker Analysis

The SSR marker used in this study was Satt045. The primer sequences were as follows: 5'TGGTTTCTACTTTCTATAATTATTT 3' and 5' ATGCCTCTCCCTCCT 3' for the forward and reverse primers, respectively (Cregan et al., 1999). The repeat type of the SSR marker is (AAT)₁₈, located on chromosome 15, and its PCR product size is 139 bp.

PCR amplification of the SSR marker was performed using a Biometra PCR machine using genomic DNA of the parents and each of the F₁ progenies as DNA templates. The genomic DNA of each hybrid plant was amplified using a 10 μ L volume in a reaction mixture containing 2 μ L of 20 ng template DNA, 5 μ L of Kapa2G Fast Ready Mix (KAPA Biosystems, USA), 0.5 μ L each of 10 μ M forward and reverse primers, and 2 μ L of sterile ddH₂O. The PCR protocol, as described by Tasma et al. (2001; 2018), involved an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of DNA amplification with denaturation at 94°C for 30 seconds, primer annealing at 55°C for 1 minute, DNA extension at 72°C for a minute, post-extension at 60°C for 15 minutes, and a final incubation at 10°C for 4 minutes.

The PCR products were then analysed by electrophoresis using an 8% polyacrylamide gel in a vertical electrophoresis tank filled with 1x Tris Borate EDTA (TBE) buffer, running at 90 volts for 115 minutes. Visualisation of DNA bands was done under a UV Transilluminator Gel Doc (Bio Rad, California, USA) using the ethidium bromide staining method.

RESULTS AND DISCUSSION

Leaf Damage Intensity

The resistance testing against whitefly indicated that the Anjasmoro variety experienced the most severe leaf damage, reaching 84.7% (Table 3). This finding is consistent with the research by Sulistyono and Inayati (2016), which reported leaf damage in the Anjasmoro variety at 76.6% in host choice tests.

Additionally, Krisnawati et al. (2023) stated that Anjasmoro remains classified as a soybean variety susceptible to whitefly, regardless of insecticide treatment. In contrast, the IAC 100 and G100H showed no significant leaf damage during testing. This result differs slightly from the findings of Sari and Sulisty (2018), who reported leaf damage in G100H ranging from 10.91% to 18.03%. Krisnawati et al. (2023) also reported that leaf damage in G100H reached 5.69% and 9.87% when treated and untreated with insecticides, respectively. Nevertheless, both germplasms are still categorised as soybean varieties resistant to whitefly.

The resistance evaluation of the Anjasmoro × IAC 100 progeny against whitefly infestation showed significant variations in leaf damage, ranging from 0% to 84.7%, with an average of 18.1% (Table 3). Based on the degree of leaf damage, the Anjasmoro × IAC 100 progeny were classified into four resistance groups: nine highly resistant plants, nine resistant plants, seven susceptible plants, and four highly susceptible plants. The highly resistant group (plants numbered 7, 8, 9, 12, 14, 15, 16, 17, and 18) exhibited no leaf damage. The resistant group (plants numbered 3, 4, 5, 6, 10, 11, 13, 25, and 27) showed leaf damage between 4.5% and 16.7%, which is below the population average. In contrast, the susceptible and highly susceptible groups (plants numbered 1, 2, 19, 20, 21, 22, 23, 24, 26, 28, and 29) experienced more severe leaf damage, ranging from 19.7% to 84.7%.

These findings suggest that the resistant and highly resistant groups may have acquired resistance genes from the IAC 100 germplasm, as evidenced by the relatively low leaf damage. Conversely, the heightened leaf damage observed in the susceptible and highly susceptible groups implies a potential failure in hand pollination, leading to the absence of resistance gene transmission from IAC 100 to the Anjasmoro variety.

The resistance testing results on the Anjasmoro × G100H progeny revealed slight disparities compared to those observed in the Anjasmoro × IAC 100 progeny. The resistance evaluation of Anjasmoro × G100H progeny indicated leaf damage ranging from 0% to 50.3% (Table 3). Although the leaf damage was less severe compared to the Anjasmoro × IAC 100 progeny, only a limited number of plants in the Anjasmoro × G100H progeny exhibited resistance to whitefly infestation. Based on the extent of leaf damage, the Anjasmoro × G100H progeny were categorised into four resistance groups: three highly resistant plants (plant numbered 11, 16 and 17), six moderately susceptible plants (plant numbered 9, 10, 12, 13, 14, and 15), seven susceptible plants (plant numbered 1, 2, 4, 5, 6, 7, and 8) and one highly susceptible plant (plant numbered 3). The moderately susceptible, susceptible, and highly susceptible groups exhibited leaf damage ranging from 10.0% to 50.3%. The predominance of the latter three groups in the tested Anjasmoro × G100H progeny suggests that hand pollination in the crossbreeding of Anjasmoro with G100H might be more challenging, indicating a difficulty in transmitting resistance genes from G100H to Anjasmoro.

Table 3

Leaf damage assessment and resistance categories of Anjasmoro, IAC 100, G100H and their progenies in greenhouse conditions

Genotype	Leaf damage intensity (%)	Resistance category	Genotype	Leaf damage intensity (%)	Resistance category
Anj/IAC100-1	20.0	S	Anj/G100H-1	17.9	S
Anj/IAC100-2	33.0	S	Anj/G100H-2	33.3	S
Anj/IAC100-3	10.4	R	Anj/G100H-3	50.3	HS
Anj/IAC100-4	5.0	R	Anj/G100H-4	41.7	S
Anj/IAC100-5	4.2	R	Anj/G100H-5	25.0	S
Anj/IAC100-6	15.0	R	Anj/G100H-6	36.5	S
Anj/IAC100-7	0	HR	Anj/G100H-7	20.0	S
Anj/IAC100-8	0	HR	Anj/G100H-8	20.8	S
Anj/IAC100-9	0	HR	Anj/G100H-9	15.0	MS
Anj/IAC100-10	14.8	R	Anj/G100H-10	10.0	MS
Anj/IAC100-11	4.5	R	Anj/G100H-11	0	HR
Anj/IAC100-12	0	HR	Anj/G100H-12	10.0	MS
Anj/IAC100-13	15.0	R	Anj/G100H-13	8.3	MS
Anj/IAC100-14	0	HR	Anj/G100H-14	15.0	MS
Anj/IAC100-15	0	HR	Anj/G100H-15	10.0	MS
Anj/IAC100-16	0	HR	Anj/G100H-16	0	HR
Anj/IAC100-17	0	HR	Anj/G100H-17	0	HR
Anj/IAC100-18	0	HR	Anjasmoro	84.7	HS
Anj/IAC100-19	84.7	HS	G100H	0	HR
Anj/IAC100-20	62.5	HS	Mean	17.4	
Anj/IAC100-21	63.4	HS	Std. Dev.	15.02	
Anj/IAC100-22	22.1	S			
Anj/IAC100-23	60.9	HS			
Anj/IAC100-24	21.3	S			
Anj/IAC100-25	16.7	R			
Anj/IAC100-26	24.4	S			
Anj/IAC100-27	15.4	R			
Anj/IAC100-28	19.7	S			
Anj/IAC100-29	31.3	S			
Anjasmoro	84.7	HS			
IAC 100	0	HR			
Mean	18.1				
Std. Dev.	22.12				

Note. HR = highly resistant; R = resistant; MS = moderately susceptible; S = susceptible; HS = highly susceptible

SSR Evaluation

The Satt045 primer used in this study generated polymorphic band patterns ranging in size from 125 to 200 base pairs (bp) (Figure 1). These findings are consistent with those reported by Chotiyarnwong et al. (2007), who observed polymorphism using the Satt045 primer in 160 soybean genotypes in Thailand, with band sizes ranging from 122 to 157 bp. Additionally, Dyah et al. (2024) identified that the Satt045 primer could produce 42 alleles with sizes ranging from 138 to 202 bp. In this study, the three soybean parental lines were distinguishable based on their banding patterns. Specifically, the Anjasmoro variety exhibited band patterns ranging from 136 to 178 bp, the IAC 100 germplasm displayed band patterns ranging from 125 to 160 bp, and the G100H germplasm showed band patterns ranging from 127 to 160 bp (Figure 1).

SSR marker analysis on the progeny population from the cross between Anjasmoro and IAC 100 showed that plants classified as susceptible (plants numbered 1, 2, 22, 24, 26, 28, and 29) and highly susceptible (plants numbered 19, 20, 21, and 23) exhibited banding patterns identical to Anjasmoro. This observation supports the hypothesis that hand pollination was unsuccessful, preventing the transfer of resistance genes from IAC 100 to Anjasmoro. In contrast, the resistant plants (numbered 3, 4, 5, 6, 10, 11, and 13) and highly resistant plants (numbered 7, 8, 9, 12, 14, 15, 16, 17, and 18) exhibited combined banding patterns from both IAC 100 and Anjasmoro, indicating successful gene transfer for resistance from IAC 100 to Anjasmoro. Furthermore, the SSR marker Satt045 effectively distinguished true F₁ progeny by identifying plants with combined banding patterns reflective of both parental genotypes.

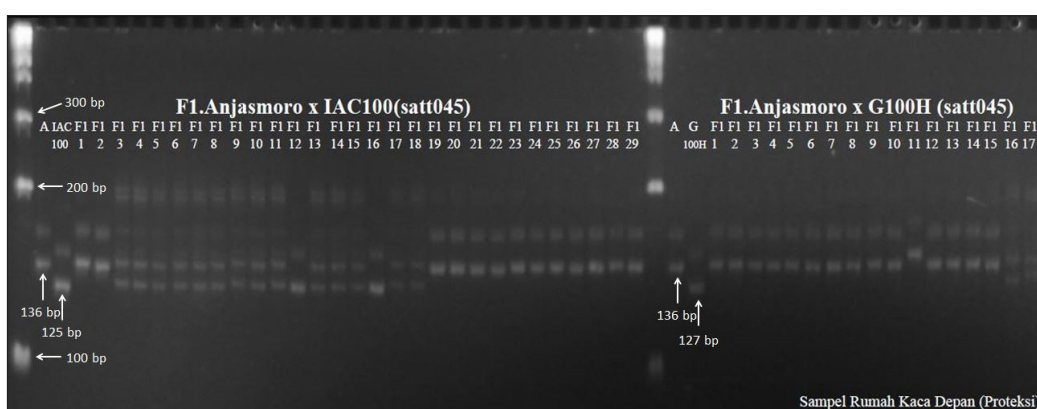


Figure 1. SSR marker banding patterns of Anjasmoro, IAC 100, G100H, and their progenies

Significant findings were observed in plants numbered 25 and 27 from the Anjasmoro × IAC 100 cross. Greenhouse resistance assessments revealed minimal leaf damage caused by whitefly infestation in these plants, indicating that they could be categorised as resistant. However, SSR marker analysis showed that both plants had identical banding patterns to Anjasmoro, suggesting they were selfed progeny rather than true hybrids. This underscores the utility of SSR markers in assisting soybean breeders to effectively screen out undesired progeny resulting from crosses. The ability of SSR markers to produce clear codominant segregation patterns, such as the observed 1:2:1 ratio in the F₂ populations, enables breeders to reliably distinguish true heterozygotes from homozygous individuals (Cregan et al., 1999; Singh & Singh, 2018; Tasma et al., 2018). This precision is crucial for eliminating undesired progeny and accelerating the development of improved soybean lines.

SSR marker analysis of the offspring resulting from the Anjasmoro × G100H cross revealed that all plants categorised as moderately susceptible, susceptible, and highly susceptible exhibited banding patterns identical to those of Anjasmoro. In contrast, plants classified as highly resistant (plants numbered 11, 16, and 17) displayed combined banding patterns from both Anjasmoro and G100H. These findings underscore the effectiveness of SSR markers in reinforcing conventional selection methods conducted under controlled greenhouse conditions. Specifically, the SSR marker Satt045 proved highly reliable in differentiating true F₁ progeny by identifying the presence of resistance alleles contributed by G100H.

CONCLUSION

Greenhouse resistance testing of Anjasmoro, IAC 100, G100H, and their progenies revealed that some hybrid plants derived from these parental lines exhibited resistance to whitefly infestation, as indicated by relatively low levels of leaf damage. Selection using the SSR marker Satt045 confirmed that plants with low leaf damage exhibited banding patterns combining features from Anjasmoro with either IAC 100 or G100H. In contrast, hybrid plants with high levels of leaf damage displayed banding patterns identical to Anjasmoro. These findings highlight the reliability of the SSR marker Satt045 in verifying true F₁ hybrids and its effectiveness as a molecular tool for supporting resistance selection in soybean breeding programmes.

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