

# Identification of an AFLP Fragment Linked to Rust Resistance in Asparagus Bean and Its Conversion to a SCAR Marker

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**Abstract.** Rust disease, incited by the fungus *Uromyces vignae*, adversely affects production and quality of asparagus bean and other types of cowpea in many parts of the world. Genetic resistance to the rust pathogen has been identified in a few accessions, but it is difficult to efficiently transfer the resistance to a broad range of asparagus bean cultivars using traditional breeding approaches. We determined that rust resistance was controlled by a single dominant gene designated *Rr1* in the cross of a highly resistant cultivar ZN016 and highly susceptible cultivar Zhijiang 282. Bulked segregant analysis was applied to an F<sub>2</sub> population derived from these parents, and an AFLP marker (E-AAG/M-CTG), 150 bp in size, was detected in the resistant bulk. The AFLP fragment was then converted to a SCAR marker, named ABRS<sub>AAG/CTG98</sub>, and the genetic distance between the marker and the *Rr1* gene was estimated to be 5.4 cM. This SCAR marker could be used effectively for MAS of *Rr1* in breeding programs to develop rust-resistant asparagus bean cultivars and potentially more widely to breed rust-resistant cultivars of other types of cowpea.

“Asparagus bean” or “yardlong” bean [*Vigna unguiculata* ssp. *sesquipedalis* (L.) Verdc.] is a type of cowpea (*Vigna unguiculata*) that produces very long pods that are harvested at an immature stage and are similar in many respects to “snap beans” or “green beans” from common bean (*Phaseolus vulgaris* L.), except that the pods are much longer, commonly between 0.5 and 1 m in length. Asparagus bean is considered one of the top 10 Asian vegetables, being important throughout southern China and southeast Asia, and its use

is increasing in many other parts of the world. Rust disease, incited by the fungus *Uromyces vignae*, adversely affects production and quality of asparagus bean throughout this region (Wang, 2004) and is an important disease of other types of cowpea (*Vigna unguiculata* ssp. *unguiculata*) grown for fresh or dry grain in Africa and other parts of the world on 13 million ha (Ehlers and Hall, 1997; Emechebe and Florini, 1997; Fery and Singh, 1997). Application of fungicides can control rust disease; however, it increases production costs and is potentially hazardous to the environment, farm workers, and consumers. Use of resistant cultivars is the most effective, economical, and environmentally friendly approach to mitigate losses from the disease. Currently, nearly all asparagus bean cultivars are susceptible to rust, and development of rust-resistant cultivars is a high priority of many asparagus bean breeding programs.

The inheritance of resistance to *U. vignae* in cowpea [*Vigna unguiculata* (L.) Walp.] was studied by Chen and Heath (1993), who reported that the resistance was governed by two partially dominant genes in a cross of cowpea cultivars Dixie Cream and California Blackeye. In asparagus bean, Zhang et al. (1997) studied the segregation patterns of F<sub>1</sub>, F<sub>2</sub>, and BC populations developed from two resistant cultivars Zhijia and Beijia when crossed to two susceptible cultivars Qinfen and Shuijiao 101. Resistance to rust was governed by a single dominant gene in all four of the possible biparental crosses.

Molecular marker-assisted selection (MAS), where molecular markers linked to the target gene facilitate the indirect selection of the linked gene in breeding populations, can significantly reduce the need for costly large-scale bioassays for resistance and greatly increase efficiency in breeding programs (Gupta et al., 2005), especially if there is a need to pyramid multiple resistance genes to a pest that exhibits strain variation and when cultivars with resistance to multiple pathogens need to be developed (Kelly et al., 2003). Development of molecular markers linked to the asparagus bean rust-resistance gene is an essential step toward both MAS and map-based gene cloning of rust-resistance genes in cowpea.

Amplified fragment length polymorphism (AFLP) is a very powerful tool for generating markers for genetic mapping and for generating markers around a specific locus of interest (Thomas et al., 1995; Vos et al., 1995). However, it is difficult to employ the AFLP technique directly in a MAS program or for map-based gene cloning because of its high cost and complicated methodology. Converting AFLP markers into “easy-to-use” markers, such as sequence-characterized amplified region (SCAR) or cleaved amplified polymorphism sequences (CAPS), is critical for both applications. The converted SCARs and CAPS are highly reliable, relatively inexpensive, and can be easily manipulated. Thus, they are valuable in practical breeding programs where large numbers of individuals need to be genotyped at low cost (Kelly et al., 2003), and for map-based gene cloning (Bradeen and Simon 1998; Qu et al., 1998; Xu et al., 2001). We bioassay individuals of parental, F<sub>1</sub>, F<sub>2</sub>, and backcross populations developed from a cross of a rust-resistant and rust-susceptible asparagus bean cultivars for resistance to rust, determine the inheritance of rust resistance, identify an AFLP maker linked to this resistance using bulk segregant analysis (BSA), and then convert this linked marker to a SCAR marker to expand its practical usefulness to breeders for MAS and for gene-based cloning.

## Materials and Methods

*Plant materials.* Two asparagus bean cultivars differing in their resistance to rust, ‘ZN016’ (highly resistant) and ‘Zhijiang 282’ (highly susceptible), and their offspring,

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including F<sub>1</sub>, F<sub>2</sub>, RBC<sub>1</sub> (the population formed by backcrossing with the resistant parent), and SBC<sub>1</sub> (the population formed by backcrossing with susceptible parent), were used in this experiment. Phenotyping for rust resistance were performed on an F<sub>1</sub> population consisting of 49 plants, an F<sub>2</sub> population consisting of 127 plants, an RBC<sub>1</sub> population consisting of 35 plants, and an SBC<sub>1</sub> population consisting of 33 plants.

**Fungus material and disease test.** Conidia of rust were collected from naturally infected asparagus bean plants grown in Hangzhou, China, and the fungus was identified as *U. vignae*. Conidial suspension was prepared by rinsing the source leaves with distilled water, and the suspension was adjusted to have (4~5) × 10<sup>5</sup> conidia per milliliter. At the stage when the third trifoliate leaves had fully expanded, the conidial suspension was sprayed carefully to the whole plant. Then the plants were covered with a polyethylene film tent for 24 h to prevent moisture from evaporating. The disease symptoms were evaluated on the 21st d after inoculation. The first to fifth trifoliate leaves were scored with a rating (*r*) of 0, 1, 2, 3, 4, or 5, standing for average spore balls on one leaf of 0, 1–5, 6–20, 21–50, 51–100, and >100, respectively. Disease index (DI) was calculated according to the equation:

$$\text{Disease index (\%)} = \left[ \frac{\sum (r \times n_r)}{5N_t} \right] \times 100$$

where *r* = rating value, *n<sub>r</sub>* = number of disease leaves with a rating of *r*, and *N<sub>t</sub>* = total number of leaves tested.

Individual plants with DI scores of 0 were classified as Immune (I), scores of 1–10 were considered highly resistant (HR), plants with scores between 11–30 as moderately resistant (MR), plants with scores 31–50 as moderately susceptible (MS). Plants were scored as highly susceptible (HS) if they had a score of 51 or higher.

**DNA extraction and bulked segregant analysis.** Total genomic DNA of each F<sub>2</sub> individual was extracted from ≈250 mg of leaf tissue using a standard cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). BSA was used to identify AFLP markers linked to the rust-resistance gene (Michelmore et al., 1991). A resistant DNA bulk was composed by taking equal amounts of DNA from 10 plants on which there was no spore ball on the first to fifth trifoliate leaves. A susceptible DNA bulk was composed by taking equal amounts of DNA from 10 plants that had an average of >100 spore balls on the first to fifth trifoliate leaves.

**AFLP analysis.** AFLP analysis was performed as described by Vos et al. (1995). To identify AFLP markers, 250 ng of genomic DNA were double-digested with 2.5 U of *EcoRI* and *MseI* for 6 h at 37 °C. The resulting template fragments were ligated to adapters specific for the *EcoRI* and *MseI* restriction sites, and a preamplification reaction was carried out with *EcoRI* + A and *MseI*

+ C primers. The selective amplification was performed with 64 primer pairs having three additional selective nucleotides at the 3' end. PCR-selective amplification was performed in a 20-μL reaction mixture containing 15 ng template DNA, 5 ng *EcoRI* primer, 30 ng *MseI* primer, 0.4 μL 10 mM dNTP, 2.0 μL 10× PCR reaction buffer, and 1 U *Taq* DNA Polymerase. PCR analysis was conducted with the following procedure: initial denaturation for 2 min at 94 °C, followed by 30 s at 94 °C, 30 s at 64 °C, and 60 s at 72 °C for 13 cycles; the annealing temperature was then lowered by 0.7 °C per cycle during the 13 cycles; then 30 s at 94 °C, 30 s at 56 °C, 60 s at 72 °C for 28 cycles; followed by a final extension step of 72 °C for 7 min. The PCR products were separated on a 6% denaturing polyacrylamide gel, stained with a simple and rapid silver staining method described by Xu et al. (2002). All PCR analyses were conducted using a T-gradient Thermoblock (Biometra, Hamburg, Germany).

**SCAR primer design and PCR analysis of sequences.** A polymorphic amplified DNA band corresponding to an AFLP marker, identified by BSA, was excised from the agarose gel, purified by the Vitagene gel purification kit (Axygen Bioscience, Union City, CA) and, as recommended by the manufacturer, cloned by means of the pGEM-T-Easy-Vector into the *Escherichia coli* DH5α strain.

Recombinant plasmid DNA was isolated, and sequence analyses were performed by Sangon Corp. (Shanghai, China). Based on the sequence of the cloned fragment, a pair of oligonucleotide primers was designed with *Primer Premier 5.0* software and synthesized by Sangon Corp. PCR amplification with SCAR primers was performed in a 20-μL reaction mixture containing 0.4 μL of 10 mM dNTPs, (2 μL of 10× PCR buffer, 1.6 μL of 25 mM MgCl<sub>2</sub>, 0.2 μL of 5 U·μL<sup>-1</sup> *TaqE*, 11.3 μL of ddH<sub>2</sub>O, 20 ng of each SCAR primer, and 15 ng of template DNA. The PCR amplification program consisted of initial

denaturation for 4 min at 94 °C, followed by 28 cycles each of 30 s at 94 °C, 30 s at 62 °C, and 60 s at 72 °C, with a final extension step of 72 °C for 7 min. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed on a digital gel documentation system.

**Marker segregation and linkage analysis.** Data were analyzed by the χ<sup>2</sup> test to ascertain the goodness-of-fit between the expected ratio for a single dominant gene and the segregation of the phenotypic data, analysis between the AFLP markers, and the rust-resistance loci. The SCAR marker was tested on 96 resistant F<sub>2</sub> plants and 31 susceptible F<sub>2</sub> plants. Linkage of rust-resistance loci was performed with the software package *MAP-MAKER/EXP*, ver. 3.0 (Lander et al., 1987). Map units were computed by applying the Kosambi function (Kosambi, 1944) with a LOD threshold of 3.0.

## Results

**Genetic analysis for rust resistance.** All plants of the resistant parent were classified as immune or highly resistant, while plants of the susceptible parent were classified as moderate to highly susceptible (Table 1). Nearly all of the F<sub>1</sub> and RBC<sub>1</sub> plants were rated as immune to highly resistant, indicating that resistance to the rust strain employed here was dominant to susceptibility. In the SBC<sub>1</sub> population, 18 out of 33 plants were classified as highly resistant, and 15 plants as moderate to highly susceptible. Of the 127 F<sub>2</sub> individuals, 96 individuals were immune to moderately resistant and 31 individuals were classified as moderately or highly susceptible. These segregations fit a 3:1 ratio (χ<sup>2</sup> = 0.026, *P* = 0.05) and a 1:1 ratio (χ<sup>2</sup> = 0.12, *P* = 0.05) for the F<sub>2</sub> and SBC<sub>1</sub> populations, respectively, indicating that resistance to the strain used in the present work was conferred by a single dominant gene (Table 2). We have

Table 1. Numbers of plants with different disease-severity grade in parental, F<sub>1</sub>, F<sub>2</sub>, and backcross populations following field inoculation with *U. vignae*.<sup>z</sup>

Line or population	Total no. of plants	Resistance class				
		I	HR	MR	MS	HS
ZN016 (HR)	52	47	5	0	0	0
Zhijiang 282 (HS)	46	0	0	0	7	39
F <sub>1</sub>	49	40	2	7	0	0
F <sub>2</sub>	127	16	71	9	13	18
RBC <sub>1</sub>	35	30	5	0	0	0
SBC <sub>1</sub>	33	12	6	0	8	7

<sup>z</sup>ZN016, highly resistant parent; Zhijiang 282, highly susceptible parent; F<sub>1</sub>, population of the hybrid ZN016 × Zhijiang 282; F<sub>2</sub>, the population of the F<sub>2</sub> generation of ZN016 × Zhijiang 282; RBC<sub>1</sub>, population of BC<sub>1</sub> backcrossed with highly resistant parent; SBC<sub>1</sub>, population of BC<sub>1</sub> backcrossed with highly susceptible parent; I, immune; HR, highly resistant; MR, moderately resistant; MS, moderately susceptible; HS, highly susceptible; see text for full description of classification scheme.

Table 2. Observed numbers of resistant or susceptible plants in F<sub>2</sub> and SBC<sub>1</sub> populations and their expected ratios and χ<sup>2</sup> goodness-of-fit test for single major gene controlling resistance to rust.<sup>z</sup>

Population	Total no. of plants	No. of resistant plants	No. of susceptible plants	Ratio of resistant/susceptible plants	Expected ratio	χ <sup>2</sup>	<i>P</i>
F <sub>2</sub>	127	96	31	3.10:1	3:1	0.0026	0.05
SBC <sub>1</sub>	33	18	15	1.2:1	1:1	0.1212	0.05

<sup>z</sup>F<sub>2</sub>, population of the F<sub>2</sub> generation of ZN016 × Zhijiang 282; SBC<sub>1</sub>, population of BC<sub>1</sub> backcrossed with highly susceptible parent.

designated this gene as *Rr1*. These results are consistent with the results obtained by Zhang et al. (1997) with different susceptible and resistant parental lines.

**Identification of an AFLP marker linked to the *Rr1* gene.** The 64 primer combinations used to screen the parental DNA in AFLP analysis amplified 2019 discrete genomic fragments (on the average 31.55 products per primer pair). Fifty-two primer pairs out of 64 (81%) gave 124 polymorphic bands (6%) between the resistant and susceptible parental genotypes. All primer pairs generating polymorphic bands were tested on the two bulks. AFLP bands present in one pool and absent in the other were regarded as candidate markers linked to rust resistance. One primer combination (E-AAG/M-CTG) produced a 150-bp DNA fragment only in the resistant parent, resistant bulk, and resistant  $F_2$  plants (Fig. 1). This result was also confirmed when the analyses were performed on DNA of the  $F_1$  population, indicating that the AFLP marker E-AAG/M-CTG was dominant and was in coupling phase with respect to the *Rr1* gene.

**Sequence analysis.** The AFLP marker E-AAG/M-CTG linked to rust resistance was cloned with the objective of converting it into a simple PCR-based marker. The polymorphic amplified DNA band corresponding to the AFLP marker was reamplified, and a single band with the expected size was observed on an agarose gel. The band was then purified by the Vitagene gel purification kit, cloned by means of the pGEM-T-Easy-Vector into the *E. coli* DH5 $\alpha$  strain. Then it was sequenced and found to be 150 bp long (GenBank accession no. EF524512).

**SCAR primer design and SCAR analysis.** Based on the sequence of the cloned fragment, a pair of oligonucleotide primers was designed with *Primer Premier 5.0* software as follows:

Forward primer, 5'-TCAACAGTGTCA GACCAAC-3'

Reverse primer, 5'-GTTGAAGATTGT GGAAAGCT-3'

These SCAR primers amplified a monomorphic band of 98 bp (named  $ABRS_{AAG/CTG98}$ ), as expected from the sequence data, when tested on parents and  $F_2$  individuals. As shown in Fig. 2, a dominant polymorphism between the parents and the individuals was observed. A 98-bp band was present in the cv. ZN016 (resistant) whereas it was absent in cv. Zhijiang 282 (susceptible).

**Linkage analysis.** This SCAR primer ( $ABRS_{AAG/CTG98}$ ) was tested on 96 resistant  $F_2$  plants and 31 susceptible  $F_2$  plants. The results show that a 98-bp band to be present in 90 out of 96 resistant  $F_2$  plants and absent in all of the 31 susceptible  $F_2$  plants. To estimate the genetic distance of  $ABRS_{AAG/CTG98}$  marker from the *Rr1* gene, linkage analysis was carried out on 127  $F_2$  individuals with *MAPMAKER/EXP*, ver. 3.0, software (Lander et al., 1987), with a LOD threshold of 3.0. It is estimated that the SCAR primer  $ABRS_{AAG/CTG98}$  is 5.4 cM distant from the *Rr1* gene.

## Discussion

The current understanding of the inheritance of rust resistance in asparagus bean is fairly limited. Zhang et al. (1997) reported that resistance to rust in asparagus bean was governed by one dominant gene, and this is also observed in the present study. However, this was not consistent with the results obtained by Chen and Heath (1993) in cowpea, who reported that the resistance was governed by two partially dominant genes. This could be attributed to different rust races being used in the different experiments or the differences in genetic constitutions of the parental lines used in these studies. Further studies outside the scope of the present work are needed to characterize *U. vignae* strain variation and allelism tests are needed to clarify possible strain/resistance gene relationships in asparagus bean and cowpea in general.

AFLP markers have become widely used in making genetic maps, for MAS breeding programs (Kelly et al., 2003), and for saturating genomic regions of interest with markers for the ultimate goal of map-based cloning of target genes. Once AFLP markers linked to target genes are identified, screening efficiency can be improved by converting

the AFLP markers to simple SCAR or CAPs markers (Negi et al., 2000). Such PCR-based markers require small quantities of DNA, are simple to run, and can be considered as the ideal markers for plant breeding programs in which a large number of samples have to be processed. The result from the present experiment, in which a 98-bp band was present in the 90 plants out of 96 resistant  $F_2$  plants but absent in all of the 31 susceptible  $F_2$  plants, confirms the tight linkage of the SCAR marker ( $ABRS_{AAG/CTG98}$ ) to rust resistance and indicates that this marker may be useful for marker-assisted breeding. This marker will facilitate development of rust-resistant asparagus bean cultivars carrying the relevant gene for the resistant trait within a relatively short time span and will be useful for map-based cloning of rust resistance in *V. unguiculata*.

To elucidate the resistance mechanism conferred by *Rr1* in more detail, it is necessary to clone the *Rr1* gene and determine its molecular structure. The genome size of cowpea has been estimated to be 613 megabase pairs (Mbp) (Arumuganathan and Earle, 1991). Based on the linkage map of cowpea published by Menéndez et al. (1997), Chida et al. (2000) estimated that 0.7 cM between the *Cry* gene and RAPD marker *CRGA 5*

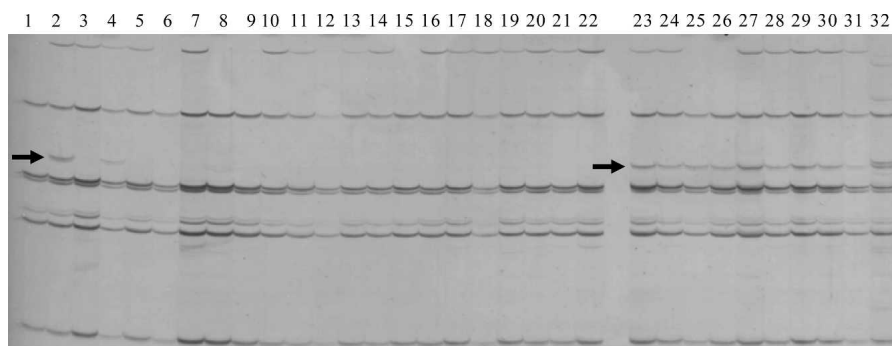


Fig. 1. AFLP amplification profile from genomic DNA of parents (lane 1, highly susceptible parent cv. Zhijiang 282; lane 2, highly resistant parent cv. ZN016), bulks (lane 3, susceptible bulk; lane 4, resistant bulk), susceptible  $F_2$  plants (lanes 5–22), and resistant  $F_2$  plants (lanes 23–32) obtained with the primer combination E-AAG/M-CTG. The arrow indicates the polymorphic band produced in the highly resistant parent, resistant bulk, and resistant  $F_2$  plants.

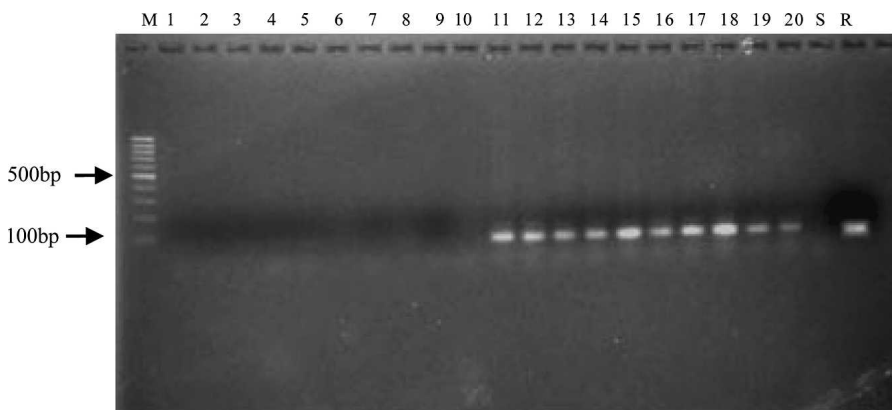


Fig. 2. Electrophoresis patterns of PCR-amplified with genomic DNA of 22 asparagus bean samples, which was used as a template DNA to test candidate SCAR primer pairs ( $ABRS_{AAG/CTG98}$ ): M, molecular marker, 100-bp ladder; lanes 1–10, susceptible plants; lanes 11–20, resistant plants; S, highly susceptible parent 'Zhijiang 282'; R, highly resistant parent 'ZN016'.

corresponds to a physical distance of about 0.44 Mbp. Therefore, larger populations and more primer combinations need to be evaluated to obtain many additional markers that closely flank the *Rr1* gene in asparagus bean.

Bulked segregant analysis (BSA) has been shown to be an efficient way to discover markers linked to important agronomic traits (Michelmore et al., 1991). In this study, we have successfully used a combination of BSA and AFLP techniques to identify markers linked to the rust-resistance gene (*Rr1*) in the asparagus bean.

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