Theor Appl Genet (2002) 105:265–270
DOI 10.1007/s00122-002-0925-1

**H. Yang · M. Shankar · B. J. Buirchell**
**M. W. Sweetingham · C. Caminero · P. M. C. Smith**

# Development of molecular markers using MFLP linked to a gene conferring resistance to *Diaporthe toxica* in narrow-leafed lupin (*Lupinus angustifolius* L.)

Received: 19 June 2001 / Accepted: 14 November 2001 / Published online: 4 June 2002


**Abstract** Phomopsis stem blight (PSB) caused by *Diaporthe toxica* is a major disease in narrow-leafed lupin (*Lupinus angustifolius* L.). The $F_2$ progeny and the parental plants from a cross between a breeding line 75A:258 (containing a single dominant resistance gene *Phr1* against the disease) and a commercial cultivar Unicrop (susceptible to the disease) were used for development of molecular markers linked to the disease resistance gene. Two pairs of co-dominant DNA polymorphisms were detected using the microsatellite-anchored fragment length polymorphism (MFLP) technique. Both pairs of polymorphisms were isolated from the MFLP gels, re-amplified by PCR, sequenced, and converted into co-dominant, sequence-specific and PCR-based markers. Linkage analysis by MAPMAKER suggested that one marker (Ph258M2) was 5.7 centiMorgans (cM) from *Phr1*, and the other marker (Ph258M1) was 2.1 cM from Ph258M2 but further away from *Phr1*. These markers are suitable for marker-assisted selection (MAS) in lupin breeding.

**Keywords** *Lupinus angustifolius* · MFLP · Molecular marker · Resistance · *Diaporthe toxica*

Communicated by H.C. Becker

H. Yang (✉) · B.J. Buirchell · M.W. Sweetingham
Crop Improvement Institute,
Department of Agriculture Western Australia, Locked Bag No. 4,
Bentley Delivery Center 6983, WA 6983, Australia
e-mail: hyang@agric.wa.gov.au
Tel.: +61-8-93683557, Fax: +61-8-94742840

M. Shankar
Centre for Legumes in Mediterranean Agriculture,
The University of Western Australia, Nedlands,
WA 6009, Australia

C. Caminero
Servicio de Investigacion y Tecnologia Agraria,
Junta de Castilla y Leon Ctra. Burgos-Portugal Km 118,
Apdo 172, 47080 Valladolid, Spain

P.M.C. Smith
Department of Botany, The University of Western Australia,
Nedlands, WA 6009, Australia

## Introduction

The fungus *Diaporthe toxica* Williamson et al. [anamorph = *Phomopsis leptostromiformis* (Kühn) Bubák] infects stem tissue of narrow-leafed lupin (*Lupinus angustifolius* L.) forming subcuticular coralloid hyphae as latent infection structures (Williamson and Sivasithamparam 1994). Phomopsis stem-blight (PSB) lesions develop after mycelia grow from coralloid hyphae during stem senescence (Shankar et al. 1998a; Williamson et al. 1991). Mycotoxins produced during saprophytic growth can cause mycotoxicosis in grazing animals, known as lupinosis (Van Warmelo et al. 1970). Selection for phomopsis resistance is one of the major objectives in the lupin breeding program in Western Australia. However, reliable screening for disease resistance requires staining and observing the subcuticular coralloid hyphae under a microscope (Shankar et al. 1996, 1998b), a process which is labour intensive and time-consuming. Shankar et al. (2002) identified two dominant resistance genes against PSB in *L. angustifolius* by traditional genetic analysis. The objective of this study was to develop molecular markers linked to the disease resistance gene (*Phr1*) in 75A:258 for marker-assisted selection (MAS) in lupin breeding.

A number of methods are available for development of molecular markers for MAS (Gupta et al. 1999). The most-widely used methods include restriction fragment length polymorphism (RFLP) (Botstein et al. 1980), random amplified polymorphic DNA (RAPD) (Williams et al. 1990), and the amplified fragment length polymorphism (AFLP) (Vos et al. 1995). Recently, Yang et al. (2001) reported the microsatellite-anchored fragment length polymorphism (MFLP) method. MFLP combines the concept of AFLP with the microsatellite-anchor primer (SSR-anchor primer) technique (Wu et al. 1994; Zietkiewicz et al.1994). Polymorphisms detected in MFLP are SSR-*Mse*I fragments, which contain a microsatellite motif sequence at one end, with an AFLP primer sequence at the other end (Yang et al. 2001). In this study, the MFLP technique was employed for molecular-marker development in *L. angustifolius.*

## Materials and methods

### Plant materials

The parents and progeny from a cross between 75A:258 (resistant to PSB) and Unicrop (susceptible to PSB) of *L. angustifolius* used in this study were from Shankar et al. (2002). A random population consisting of 173 $F_2$ individuals were used for development of molecular markers linked to the disease resistance gene in 75A:258. The parental plants and the $F_2$ individuals were assessed for disease reaction by a non-destructive microscopical test (Shankar et al. 2002). Plants were maintained in a 20 °C glasshouse. Four weeks after sowing, stems of the seedling plants were excised above the second leaf node to encourage the regeneration of two lateral branches. The axillary internode of one of the branches was sprayed to run-off with a conidial suspension ($1 \times 10^7$ conidia per ml) of *D. toxica* using an artist's air brush (Williamson et al. 1991; Shankar et al. 1996). The other branch was protected from infection and maintained for seed production. The inoculated branch was excised 21 days after inoculation at the base, and stored at –20 °C. The epidermal layers of inoculated stem segments were peeled, and stained with alcoholic lactophenol cottton blue (Shankar et al. 1996). Plants were classified as resistant or susceptible based on the size of coralloid hyphae observed under a microscope (Shankar et al. 2002).

Seeds from 21 randomly selected $F_2$ plants were harvested from regenerated and uninfected branches after disease screening. The $F_3$ plants from each of these 21 $F_2$ plants were tested for PSB. A $F_2$ plant was considered as homozygous resistant if all the resultant $F_3$ plants were resistant to PSB. A $F_2$ plant was considered as homozygous susceptible if all the resultant $F_3$ plants were susceptible to PSB. A $F_2$ plant was regarded as heterozygous resistant if the resultant $F_3$ plants segregated in a ratio of 3:1 (resistant:susceptible) (Shankar et al. 2002). Leaf tissues from the parents and from all the $F_2$ individuals were collected and stored at –20 °C for DNA extraction. Commercial cultivars used to test the established markers were from the Department of Agriculture Western Australia.

### DNA extraction and identification of candidate molecular markers

DNA from leaf tissue was extracted as described by Raeder and Broda (1985). Twelve representative plants, consisting of the two parents 75A:258 and Unicrop, four homozygous resistant $F_2$ plants, one heterozygous resistant $F_2$ plant and five homozygous susceptible $F_2$ plants were used to generate MFLP fingerprints as described by Yang et al. (2001). A total of 96 sets of MFLP fingerprints were produced by using six SSR-anchor primers (Table 1) each in combination with 16 AFLP primers (*Mse*I-CNN) (Vos et al. 1995). MFLP products were resolved on sequencing gels (5% acrylamide, 7 M urea) using a Sequi-Gen GT sequencing cell (Bio-Rad). Each gel contained 96 MFLP reactions, which consisted of eight sets of MFLP fingerprints each containing the 12 individual plants. Polymorphic DNA bands in the MFLP fingerprints, showing evidence of correlation to disease resistance or susceptibility, were regarded as candidate markers linked to the disease resistance gene, and were subjected to further investigation.

### Conversion of candidate markers into sequence-specific PCR markers

A piece of dried sequencing gel bearing a DNA polymorphic band of candidate markers was excised from a MFLP fingerprint, and heated in 50 µl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) at 95 °C for 15 min. The DNA fragment was amplified in 50 µl of PCR mix containing 5 µl of the above solution as a template, 20 pmol each of the SSR-anchor primer and the *Mse*I-primer (with which the original SSR-*Mse*I fragment in MFLP was produced), 3 units of *Taq* polymerase (Fisher Biotec, Perth), 67 mM of Tris-HCl (pH 8.8), 2 mM of $MgCl_2$, 16.6 mM of $(NH_4)_2SO_4$, 0.09 µl of Triton X-100, 10 µg of gelatin and 0.2 mM of dNTPs.

**Table 1** Sequences of the six SSR-anchor primers used in MFLP fingerprinting for generating candidate molecular markers linked to PSB resistance in *L. angustifolius*

| Primer name | Sequence (5′–3′) |
|---|---|
| MF128 | DVDTCTCTCTCTCTCTC[a] |
| MF129 | HVHTGTGTGTGTGTG[b] |
| MF51 | GGGAACAACAACAAC |
| MF42 | GTCTAACAACAACAACAAC |
| MF43 | CCTCAAGAAGAAGAAGAAG |
| MF78 | GGCAAGAAGAAGAAGA |

[a] D = A+G+T, V = A+G+C
[b] H = A+C+T

PCR was performed using the same temperature cycles as the selective amplifications in MFLP (Yang et al. 2001). Cloning and sequencing of the amplified fragments followed standard procedures (Ausubel et al. 1998).

If a candidate marker resulted from an insertion/deletion outside the SSR array within the SSR-*Mse*I fragment, the polymorphisms were converted into a sequence-specific PCR-based marker by designing two sequence-specific primers which anneal at either end of, and internal to, the sequenced SSR-*Mse*I fragment (Yang et al. 2001). Primers were designed so that the annealing temperature was approximately 52 °C calculated using the nearest-neighbour model (Breslauer et al. 1986). Screening for the converted marker was performed by PCR (PCR ingredients as above except for the primers) using the two sequence-specific primers with undigested genomic DNA as templates. One of the two primers was labelled with $\gamma$-$^{33}$P as described by Vos et al. (1995). PCR was cycled on a Hybaid DNA Express thermocycler for 25 cycles each of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. The amplified products were resolved on a sequencing gel (as described above) at 55 W for 2 h, and were detected by autoradiography using an X-ray film (Kodak X-Inat Blue XB-1).

If a marker resulted from variation in the number of simple sequence repeat units targeted by the SSR-anchor primer, one sequence-specific primer was designed near the *Mse*I end of the SSR-*Mse*I fragment. Screening for the converted sequence-specific marker was achieved by PCR using undigested genomic DNA as a template with the designed sequence-specific primer in combination with the same SSR-anchor primer, with which the original MFLP polymorphisms were produced. In this case the sequence-specific primer was labelled with $\gamma^{33}$P to avoid the detection and interference of the amplified SSR-SSR fragments (Yang et al. 2001).

### Confirmation of linkage

The converted sequence-specific markers were tested on a random $F_2$ population containing 173 individuals. The marker scoring data in the $F_2$ were merged with the disease scoring data for linkage analysis using the computer program MAPMAKER (Lander et al. 1987). The markers were also tested on 21 cultivars of *L. angustifolius* to verify the correlation of the disease phenotype and the marker scoring data.

## Results

### Phomopsis stem blight resistance in 75A:258

Among the 173 $F_2$ plants from the cross 75A:258/Unicrop used in this study, 132 plants were identified as resistant to PSB, and 41 plants were classified as susceptible. The segregation of resistant:susceptible in the $F_2$ fits

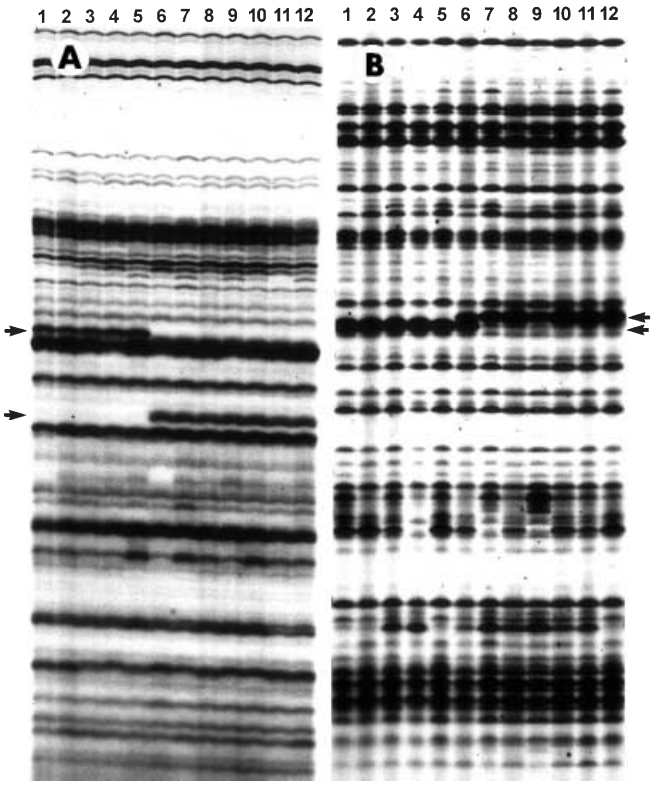


**Fig. 1A, B** Identification of DNA polymorphisms associated with phomopsis stem blight (PBS) resistance in *L. angustifolius* by MFLP fingerprinting. **A** MFLP fingerprint produced by primer combination of MF128 and *Mse*I-CAA. **B** MFLP fingerprint produced by primer combination of MF51 and *Mse*I-CCA. Each MFLP fingerprint contains 12 plants, including the resistance parent 75A:258 (lane 1), four homozygous resistant $F_2$ (lanes 2–5), one heterozygous resistant $F_2$ (lane 6), the susceptible parent Unicrop (lane 7), and five homozygous susceptible $F_2$ plants (lanes 8–12). *Arrows* show the co-dominant polymorphisms associated with PBS resistance gene *Phr1*

the expected 3:1 ratio ($\chi^2 = 0.156$, $p = 0.693$), indicating the presence of a single dominant gene for PSB resistance in 75A:258, designated as *Phr1* (Shankar et al. 2002).

## Identification of candidate molecular markers

Two pairs of polymorphic bands were identified as candidate markers linked to PSB resistance and susceptibility. The first pair was present in the MFLP fingerprint generated by SSR-anchor primer MF128 in combination with *Mse*I-CAA (Fig. 1A). A common band was observed in the resistant parent 75A:258 and the four homozygous resistant $F_2$'s. The susceptible parent Unicrop and the five homozygous susceptible $F_2$ plants showed another common band. The heterozygous resistant $F_2$ plant showed only one band in common with that of the susceptible plants (Fig. 1A). Further investigation indicated that a recombination occurred between this marker and the resistance gene *Phr1* in this particular $F_2$ plant (see below).

The second pair of polymorphic bands was present in the MFLP fingerprint produced by SSR-anchor primer MF51 in combination with *Mse*I-CCA (Fig. 1B). The resistant parent 75A:258 and the four homozygous resistant $F_2$'s shared a common band; whereas the susceptible parent Unicrop and the five homozygous susceptible $F_2$ plants shared another common band. The heterozygous resistant $F_2$ plant showed both bands (Fig. 1B).

## Conversion of candidate markers into sequence-specific PCR markers

Sequencing of the first pair of MFLP polymorphisms revealed that the polymorphisms resulted from an insertion/deletion outside the SSR array within the amplified SSR-*Mse*I fragment. A pair of sequence-specific primers (a forward primer 5′-CAGGCACATATATCTTTATACC-3′, and a reverse primer 5′-TCCAGACTGACTATATTCTTAG-3′) were designed, and successfully converted the MFLP polymorphisms into a sequence-specific PCR-based marker, which we designated as "Ph258M1" (Fig. 2A). Ph258M1 is a co-dominant marker containing two allelic bands: the band $M1^R$ is linked to the disease resistance allele, whereas the band $M1^S$ is linked to the disease susceptibility allele (Fig. 2A).

DNA sequencing confirmed that the second pair of MFLP polymorphisms resulted from variation in the number of repeat units within the microsatellite sequence targeted by the SSR-anchor primer MF51. The MFLP band linked to the susceptibility allele contained six units of the AAC repeat, whereas the MFLP band tagging the resistance allele had only five units of the AAC repeat. A sequence-specific primer (5′-GAACCATTGTAACTAAATCC-3′) was designed. The MFLP polymorphisms were converted into a sequence-specific PCR-based marker by using the designed sequence-specific primer in combination with the SSR-anchor primer MF51 (Fig. 2B). This converted sequence-specific marker was designated as "Ph258M2". Ph258M2 is a co-dominant marker containing two allelic bands. The band $M2^R$ is linked to the disease resistance allele, whereas the band $M2^S$ is linked to the disease susceptibility allele (Fig. 2B). Ph258M2 exhibited some stutter bands (Fig. 2B), which is the typical banding pattern of microsatellite markers amplified by PCR (Chin et al. 1996; Echt et al. 1996).

## Confirmation of linkage

The two allelic bands of the marker Ph258M1 scored 44:88:41 on the random population of 173 $F_2$ individuals from the cross 75A:258/Unicrop. The two allelic bands of marker Ph258M2 were scored 44:91:38. Both Ph258M1 and Ph258M2 segregated to the expected 1:2:1 ratio for a co-dominant marker in the $F_2$ (Table 2).

Linkage analysis by MAPMAKER using the data of marker scoring and the data of disease scoring from the

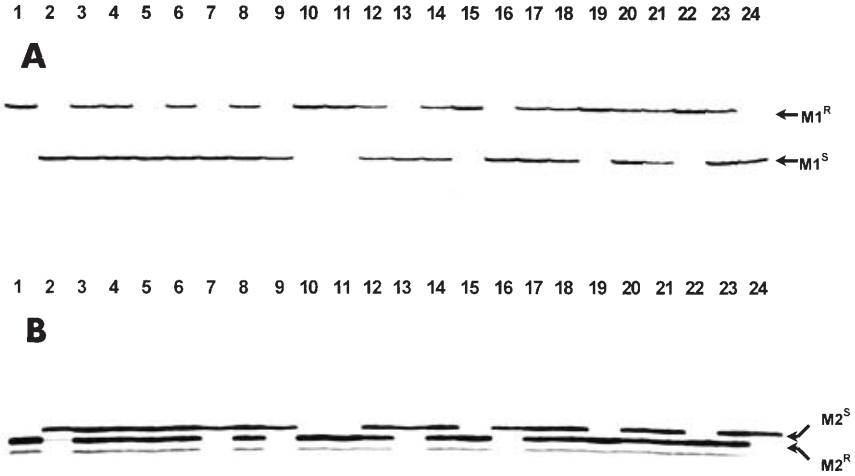


**Fig. 2A, B** Screening of the parents 75A:258 (lane 1) and Unicrop (lane 2), and the resulting 22 $F_2$ individuals (lanes 3–24), using molecular markers linked to PBS resistance gene *Phr1* in *L. angustifolius*. **A** Marker Ph258M1 amplified by PCR with a pair of sequence-specific primers (a forward primer 5′-CAGGCACATATATCTTTATACC-3′, and a reverse primer 5′-TCCAGACTGACTATATTCTTAG-3′). $M1^R$: a DNA band of Ph258M1 linked to the disease resistance allele; $M1^S$: a DNA band of Ph258M1 linked to the susceptiblility allele. **B** Marker Ph258M2 amplified by PCR with a sequence-specific primer (5′-GAACCATTGTAACTAAATCC-3′) in combination with a microsatellite-anchor primer MF51 (5′-GGGAACAACAACAAC3′). $M2^R$: a DNA band of Ph258M2 linked to the disease resistance allele; $M2^S$: a DNA band of Ph258M2 linked to the susceptiblility allele

**Fig. 3** Genetic linkage (in centiMorgans) of the two molecular markers and the disease resistance gene *Phr1* of *L. angustifolius*, analysed by MAPMAKER

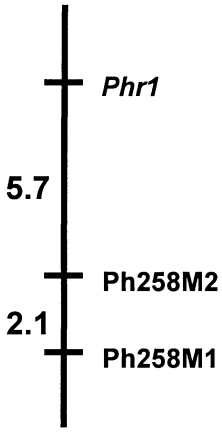


**Table 2** Chi-square test on the segregation ratios of marker scoring on 173 $F_2$ individuals from the cross 75A:258/Unicrop of *L. angustifolius*

| Marker name | Observed segregation | Expected segregation | $\chi^2$ | *P* |
|---|---|---|---|---|
| Ph258M1 | 44:88:41 ($M1^R$:$M1^RM1^S$:$M1^S$)[a] | 43.25:86.50:43.25 | 0.156 | 0.925 |
| Ph258M2 | 44:91:38 ($M2^R$:$M2^RM2^S$:$M2^S$)[b] | 43.25:86.50:43.25 | 0.884 | 0.643 |

[a] $M1^R$ = showing homozygous $M1^R$ band; $M1^RM1^S$ = showing both $M1^R$ and $M1^S$ bands; $M1^S$ = showing homozygous $M1^S$ band
[b] $M2^R$ = showing homozygous $M2^R$ band; $M2^RM2^S$ = showing both $M2^R$ and $M2^S$ bands; $M2^S$ = showing homozygous $M2^S$ band

**Table 3** Correlation of marker screening and disease screening on 173 $F_2$ plants from the cross 75A:258/Unicrop of *L. angustifolius*

| Marker name | No. of $F_2$ plants scored by the markers | No. of $F_2$ plants by disease phenotype (resistant:susceptible) | Matching rate (%) |
|---|---|---|---|
| Ph258M1 | Showing homozygous $M1^R$ band: 44 | 44:0 | 100.0[a] |
| | Showing heterozygous $M1^RM1^S$ bands: 88 | 82:6 | 93.2[b] |
| | Showing homozygous $M1^S$ band: 41 | 6:35 | 85.4[c] |
| Ph258M2 | Showing homozygous $M2^R$ band: 44 | 44:0 | 100.0[a] |
| | Showing heterozygous $M2^RM2^S$ bands: 91 | 85:6 | 93.4[b] |
| | Showing homozygous $M2^S$ band: 38 | 3:35 | 92.1[c] |

[a] Expressed as the number of $F_2$ plants showing the expected disease reaction (resistant) for plants having the homozygous $M1^R$ (or $M2^R$) band/the total number of $F_2$ plants showing homozygous $M1^R$ (or $M2^R$) band × 100%
[b] Expressed as the number of $F_2$ plants showing the expected disease reaction (resistant) for plants having the heterozygous bands $M1^RM1^S$ (or $M2^RM2^S$)/the total number of $F_2$ plants showing heterozygous bands $M1^RM1^S$ (or $M2^RM2^S$) × 100%
[c] Expressed as the number of $F_2$ plants showing the expected disease reaction (susceptible) for plants having the homozygous $M1^S$ (or $M2^S$) band/the total number of $F_2$ plants showing homozygous $M1^S$ (or $M2^S$) band × 100%

**Table 4** Correlation of marker scoring and disease phenotype of 21 commercial cultivars of *L. angustifolius*

| Cultivars | Disease phenotype[a] | Ph258M1[b] | Ph258M2[c] |
|---|---|---|---|
| Unicrop | S | $M1^S$ | $M2^S$ |
| Uniharvest | S | $M1^S$ | $M2^S$ |
| Uniwhite | S | $M1^S$ | $M2^S$ |
| Marri | S | $M1^S$ | $M2^S$ |
| Illyarrie | S | $M1^S$ | $M2^S$ |
| Yandee | S | $M1^S$ | $M2^S$ |
| Chittick | S | $M1^S$ | $M2^S$ |
| Danja | S | $M1^S$ | $M2^S$ |
| Geebung | S | $M1^S$ | $M2^S$ |
| Gungurru | R | $M1^R$ | $M2^R$ |
| Yorrel | R | $M1^R$ | $M2^R$ |
| Warrah | R | $M1^R$ | $M2^R$ |
| Merrit | R | $M1^R$ | $M2^R$ |
| Belara | R | $M1^R$ | $M2^R$ |
| Moonah | R | $M1^R$ | $M2^R$ |
| Quilinock | R | $M1^R$ | $M2^R$ |
| Myallie | R | $M1^S$ | $M2^S$ |
| Kalya | R | $M1^S$ | $M2^S$ |
| Wonga | R | $M1^S$ | $M2^S$ |
| Tallerack | R | $M1^S$ | $M2^S$ |
| Tanjil | R | $M1^S$ | $M2^S$ |

[a] R = resistant to PSB; S = susceptible to PSB
[b] $M1^R$ = showing homozygous $M1^R$ band; $M1^S$ = showing homozygous $M1^S$ band
[c] $M2^R$ = showing homozygous $M2^R$ band; $M2^S$ = showing homozygous $M2^S$

173 $F_2$ plants suggested that Ph258M2 was 5.7 centiMorgans (cM) from the disease resistance gene *Phr1*. Ph258M1 was 2.1 cM from Ph258M2, but further away from *Phr1* (Fig. 3). The log-likelihood for this linkage is –105.32.

The heterozygous resistant $F_2$ individual used in the MFLP fingerprinting (Fig. 1, lanes 6) showed heterozygous $M2^RM2^S$ bands by Ph258M2, but exhibited the homozygous $M1^S$ band by Ph258M1 (Fig. 1A and B, lanes 6; Fig. 2A and B, lanes 5). The results indicated that a recombination occurred in the region between Ph258M1 and Ph258M2 in this particular F2 plant.

For the marker Ph258M1, all the $F_2$ plants showing the homozygous $M1^R$ band were resistant; 93.2% of the $F_2$ plants showing heterozygous $M1^R$ $M1^S$ bands were resistant, and 85.4% of the $F_2$ plants showing homozygous $M1^S$ band were susceptible (Table 3). Marker Ph258M2 showed the same trend as Ph258M1 in that $F_2$ plants showing the homozygous $M2^R$ band had the highest correlation rate (100%) between disease scoring and the marker scoring; while $F_2$ plants showing the homozygous $M2^S$ band had the lowest matching rate (92.1%) between marker scoring and disease scoring (Table 3).

All the nine cultivars susceptible to PSB were homozygous for the $M1^S$ and $M2^S$ bands by Ph258M1and Ph258M2 (Table 4). Among the 12 resistant cultivars, seven showed the homozygous bands $M1^R$ and $M2^R$. The other five cultivars exhibited the homozygous bands $M1^S$ and $M2^S$ (Table 4).

## Discussion

The development of molecular markers linked to the PSB resistance gene *Phr1* in *L. angustifolius* in this study illustrates the advantages of MFLP over some other DNA fingerprinting techniques used for MAS. Similar to AFLP, MFLP produces a much larger number of DNA polymorphisms than RAPD or RFLP. However, each band detected in MFLP contains a microsatellite motif sequence. MFLP generates many co-dominant polymorphisms, and a large proportion of MFLP polymorphisms can easily be converted into sequence-specific PCR markers (Yang et al. 2001). Both Ph258M1 and Ph258M2 reported here were identified as co-dominant polymorphisms in the MFLP fingerprints. Each of them was easily converted into a sequence-specific PCR marker. The ease of conversion of these markers is because all the sequence-specific primers were derived from DNA sequences internal to the SSR-*Mse*I fragments amplified by MFLP (Yang et al. 2001). In contrast, most of AFLP polymorphisms are from variations in restriction sites. When the internal sequences of the AFLP fragments are used to design primers for PCR they rarely identify the polymorphisms (Gupta et al. 1999; Shan et al. 1999).

*L. angustifolius* is a diploid plant species with 20 pairs of chromosomes in a genome. The accuracy of marker screening for predicting the disease resistance phenotype on the $F_2$ plants is greatly influenced by the homozygosity and heterozygosity of the marker alleles, as well as by the genetic distance. All of the $F_2$ plants homozygous for $M1^R$ bands were resistant to PSB. This is because a $F_2$ plant homozygous for $M1^R$ will only be susceptible if recombination occurs at both chromosomes (genotype *phr1phr1*), and the probability of such an event in the $F_2$ population is only 0.6% (7.8% × 7.8%). The percentage of $F_2$ plants showing heterozygous $M1^RM1^S$ bands being susceptible (6.8%) reflects the recombination rate in one chromosome between the $M1^R$ sequence and the *Phr1* gene (equivalent to the genetic distance, 7.8%). In contrast, a $F_2$ plant homozygous for the $M1^S$ band will be resistant if recombination occurs on either or both of the two chromosomes, and the probability of such an event is 15.6% (7.8% + 7.8%).

The commercial cultivars of *L. angustifolius* tested in this study were bred in Western Australia. All nine commercial cultivars susceptible to PSB showed the homozygous $M1^S$ and $M2^S$ bands by Ph258M1 and Ph258M2, which is consistent with the fact that these susceptible cultivars do not possess the resistance gene *Phr1*. In addition to *Phr1*, there are other resistance genes conferring resistance to PSB in *L. angustifolius* (Shankar et al. 2002). Among the 12 resistant cultivars, seven were homozygous for the $M1^R$ and $M2^R$ bands. The other five resistance cultivars were homozygous for the $M1^S$ and $M2^S$ bands, indicating that $M1^R$ and $M2^R$ are specific to the *Phr1* gene. It should be noted that the presence of the $M1^R$ and $M2^R$ bands in a cultivar does not necessarily imply that the cultivar contains the *Phr1* gene. A good

example is cv Merrit, which showed the homozygous $M1^R$ and $M2^R$ bands. However, traditional genetics analysis revealed that Merrit contains a single dominant resistance gene other than *Phr1* against PSB (Shankar et al. 2002). Therefore, it is of paramount importance that the presence of the *Phr1* gene and the linkage to the markers of the resistant parental line in a cross must be verified before these phomopsis resistance markers are used for screening the progeny for MAS in a lupin breeding program.

The two molecular markers linked to PSB resistance gene *Phr1* in *L. angustifolius* established in this study are useful for MAS. Both markers are co-dominant, which offers the benefit for breeders selecting $F_2$ individuals with a high proportion homozygous for the resistance gene (*Phr1Phr1*). This is impossible to achieve by traditional glasshouse screening, which can not distinguish between genotypes *Phr1Phr1* and *Phr1phr1* in the $F_2$. Screening using these markers is less tedious than glasshouse tests. Since both markers are on the same side of the resistance gene *Phr1*, and Ph258M2 is closer to *Phr1* than Ph258M1, we recommend that Ph258M2 be implemented in practical MAS in lupin breeding. However, Ph258M1 has the advantage over Ph258M2 that it does not produce stutter bands and is therefore easier to score.

**Acknowledgements** This research was supported by the Grains Research and Development Corporation, and the Centre for Legumes in Mediterranean Agriculture, The University of Western Australia.

## References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) (1998) Current protocols in molecular biology. John Wiley and Sons Inc, New York, pp 7.4A1–4

Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32:314–331

Breslauer KJ, Frank R, Blöcker H, Marky LA (1986) Predicting DNA duplex stability from the base sequence. Proc Natl Acad Sci USA 83:3746–3750

Chin ECL, Senior ML, Smith JSC (1996) Maize simple repetitive DNA sequences: abundance and allele variation. Genome 39:866–873

Echt CS, May-Marquardt P, Hseih M, Zahorchak R (1996) Characterisation of microsatellite markers in eastern white pine. Genome 39:1102–1108

Gupta PK, Varshney KJ, Sharma PC, Ramesh B (1999) Molecular markers and their applications in wheat breeding. Plant Breed 118:369–390

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE (1987) MAPMAKER: an interactive computer package for constructing genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Raeder U, Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1:17–20

Shan X, Blake TK, Talbert LE (1999) Conversion of AFLP markers to sequence-specific PCR markers in barley and wheat. Theor Appl Genet 98:1072–1078

Shankar M, Cowling WA, Sweetingham MW (1996) The expression of resistance to latent stem infection by *Diaporthe toxica* in narrow-leafed lupin. Phytopathology 86:692–697

Shankar M, Cowling WA, Sweetingham MW (1998a) Histological observations of latent infection and tissue colonisation by *Diaporthe toxica* in resistant and susceptible narrow-leafed lupins. Can J Bot 76:1305–1316

Shankar M, Cowling WA, Sweetingham MW (1998b) A competitive ELISA for detecting resistance to latent stem infection by *Diaporthe toxica* in narrow-leafed lupins. Aus Plant Pathol 27:251–258

Shankar M, Sweetingham MW, Cowling WA (2002) Identification of alleles at two loci controlling resistance to Phomopsis stem blight in narrow-leafed lupin (*Lupinus angustifolius* L.). Euphytica (in press)

Van Warmelo KT, Marasas WFO, Adelaar TF, Kellerman TS, Van Rensburg IJB, Minne JA (1970) Experimental evidence that lupinosis of sheep is a mycotoxicosis caused by the fungus, *Phomopsis leptostromiformis* (Kühn) Bubák. J S Afr Vet Med Assoc 41:235–247

Vos P, Hogers R, Bleeker M, Reijans M, Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Williams JGK, Kubelik ARK, Livak JL, Rafalski JA, Tingey SW (1990) DNA polymorphisms amplified by random primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535

Williamson PM, Sivasithamparam K (1994) Factors influencing the establishment of latent infection of narrow-leafed lupins by *Diaporthe toxica*. Aus J Agric Res 45:1387–1394

Williamson PM, Sivasithamparam K, Cowling WA (1991) Formation of subcuticular coralloid hyphae by *Phomopsis leptostromiformis* upon latent infection of narrow-leafed lupins. Plant Dis 75:1023–1026

Wu K, Jones R, Danneberger L, Scolnik PA (1994) Detection of microsatellite polymorphisms without cloning. Nucleic Acids Res 22:3257–3258

Yang H, Sweetingham MW, Cowling WA, Smith PMC (2001) DNA fingerprinting based on microsatellite-anchored fragment length polymorphisms, and isolation of sequence-specific PCR markers in lupin (*Lupinus angustifolius* L.). Mol Breed 7:203–209

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176–183