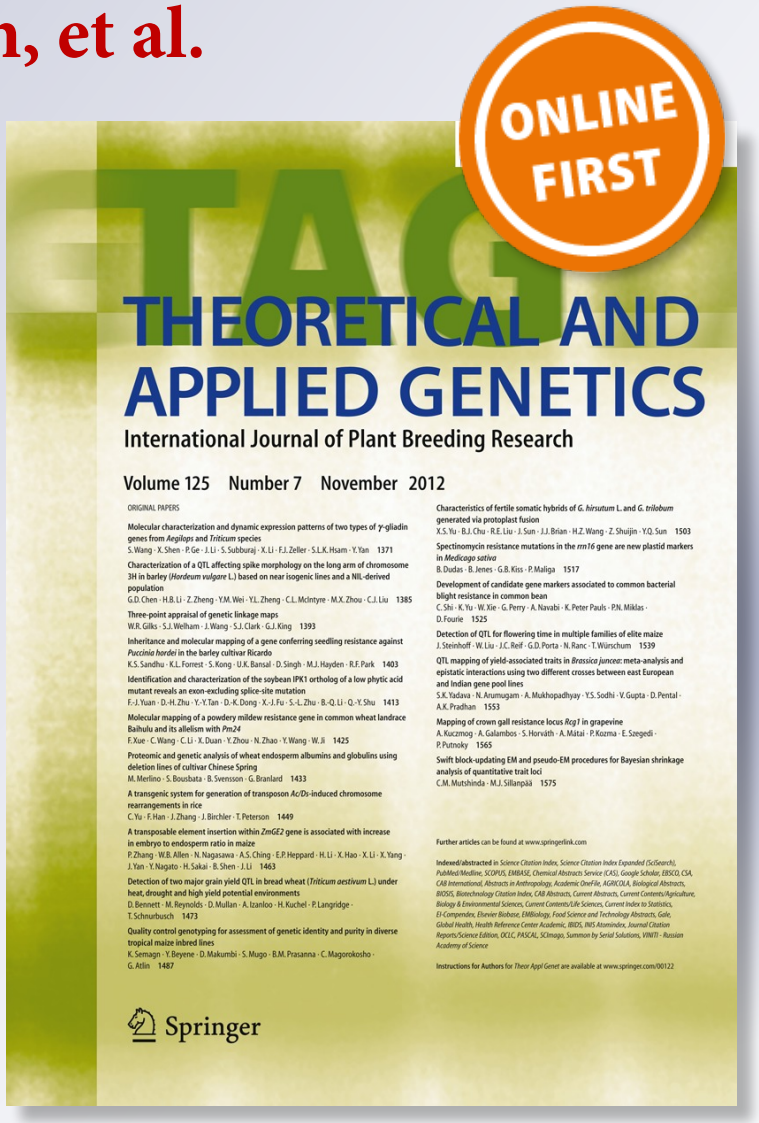
*Rapid development of molecular markers by next-generation sequencing linked to a gene conferring phomopsis stem blight disease resistance for marker-assisted selection in lupin (*Lupinus angustifolius *L.) breeding*

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ORIGINAL PAPER

Rapid development of molecular markers by next-generation sequencing linked to a gene conferring phomopsis stem blight disease resistance for marker-assisted selection in lupin (*Lupinus angustifolius* L.) breeding

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Abstract Selection for phomopsis stem blight disease (PSB) resistance is one of the key objectives in lupin (*Lupinus angustifolius* L.) breeding programs. A cross was made between cultivar Tanjil (resistant to PSB) and Unicrop (susceptible). The progeny was advanced into F_8 recombinant inbred lines (RILs). The RIL population was phenotyped for PSB disease resistance. Twenty plants from the RIL population representing disease resistance and susceptibility was subjected to next-generation sequencing (NGS)-based restriction site-associated DNA sequencing on the NGS platform Solexa HiSeq2000, which generated 7,241 single nucleotide polymorphisms (SNPs). Thirty-three SNP markers showed the correlation between the marker genotypes and the PSB disease phenotype on the 20 representative plants, which were considered as candidate markers linked to a putative R gene for PSB resistance. Seven candidate markers were converted into sequence-specific PCR markers, which were designated as PhtjM1, PhtjM2, PhtjM3, PhtjM4, PhtjM5, PhtjM6 and PhtjM7. Linkage analysis of the disease phenotyping data and marker genotyping data on a F_8 population containing 187 RILs confirmed that all the seven converted markers were associated with the putative R gene within the genetic distance of 2.1 CentiMorgan (cM). One of the PCR markers, PhtjM3, co-segregated with the R gene. The seven established PCR markers were tested in the 26 historical and current commercial cultivars released in Australia. The numbers of "false positives" (showing the resistance marker allele band but lack of the putative R gene) for each of the seven PCR markers ranged from nil to eight. Markers PhtjM4 and PhtjM7 are recommended in marker-assisted selection for PSB resistance in the Australian national lupin breeding program due to its wide applicability on breeding germplasm and close linkage to the putative R gene. The results demonstrated that application of NGS technology is a rapid and cost-effective approach in development of markers for molecular plant breeding.

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Introduction

Narrow-leafed lupin (*Lupinus angustifolius* L.) is a diploid plant species containing (2*n*) 40 chromosomes (Gladstones 1970) which have been given chromosome numbers and aligned with the physical map of a model legume species (Boersma et al. 2005; Nelson et al. 2010).Lupin is an economically important legume crop in southern Australia. The crop is also cultivated in Europe, Chile and South Africa. Phomopsis stem blight (PSB), caused by fungal pathogen *Diaporthe toxica* (formerly called *Phomopsis leptostromiformis*), is a major disease of lupin (Shankar et al. 1996). *Diaporthe toxica* infects young stem of lupin, and remains as latent subcuticular coralloid hyphal structure in green plants (Williamson et al. 1991). Upon plant senescence, the fungus colonizes the stems and develops large lesions. During the saprophytic colonization, the fungus produces the phomopsin mycotoxin, which can

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cause death in animals which graze on lupin stubbles (Van Warmelo et al. 1970). Selection for PSB disease resistance is one of the key objectives in lupin breeding programs. Conventional methods of screening for PSB resistance relies on observing percentage surface area on post-senescent stems covered with lesion of PSB (Cowling et al. 1987) or staining and microscopic examination of subcuticular coralloid structure of infected stems (Williamson et al. 1991), which are tedious and time-consuming. An alternative method is to culture the excised infected cotyledonary internodes on petri plates, and to observe lesion progression and hyphal growth (Shankar et al. 1996). Due to variation of disease progression among individual plants, screening PSB resistance usually requires replicated experiments involving multiple individual plants from each breeding line, which makes selection for homozygous disease-resistant plants from segregating F_2 populations impossible.

Genetic analysis and observation from lupin breeding practice indicated that there were at least three major genes in Australian domesticated lupin lines, and each independently conferring resistance to PSB (Shankar et al. 2002). The two PSB-resistance genes reported by Shankar et al. (2002) were "*Phr1*" in breeding line 75A:258, and "*Phr2*' in cultivar Merrit. The "*Phr1*" gene was tagged by two PCR-based DNA markers "Ph258M1" and Ph258M2" (Yang et al. 2002). However, the *Phr1* gene has not yet been integrated into commercial cultivars in Australia. No marker was reported on any other R genes for PSB-resistance genes in *L. angustifolius*. The Australian cultivar Wonga resistant to PSB disease was derived from a F_5 single plant, which was released in 1996. Cultivar Tanjil was derived from a single seed selection from Wonga. The two cultivars Wonga and Tanjil are very similar genetically and agronomically, except that Tanjil is more homogenous than Wonga. Since its release in 1998, Tanjil has been extensively used as a parental line in the crossing in the Australian national lupin breeding program due to its high yield and resistance to several key diseases including PSB, anthracnose, brown leaf spot, and CMV virus transmission tolerance. Development of a marker linked to the PSB-resistance gene in Tanjil is highly desirable for marker-assisted selection (MAS) for PSB resistance in lupin breeding.

In the last three decades, a number of DNA fingerprinting methods have been used in marker development for molecular plant breeding, including RFLP (Burr et al. 1988), RAPD (Paran and Michelmore 1993), DArT (Wittenberg et al. 2005), AFLP (Brugmans et al. 2003) and MFLP (Yang et al. 2002, 2008). The advent of the next-generation sequencing (NGS) technology in recent years provides great potential for highly efficient marker development for MAS in plant breeding. Miller et al. (2007) reported the "restriction site-associated DNA (RAD)" method based on a microarray platform. Baird et al. (2008) adapted the RAD on the massively parallel NGS platform to efficiently detect DNA polymorphisms adjacent to a particular restriction enzyme recognition sites. RAD sequencing was applied in marker discovery in animals (Baxter et al. 2011), plants (Pfender et al. 2011; Chutimanitsakun et al. 2011) and microorganisms (Lewis et al. 2007). The objective of this study is to apply the NGS-based RAD sequencing for development of molecular markers linked to the PSB-resistance gene in Tanjil of *L. angustifolius* which can be applied for MAS in lupin breeding.

Materials and methods

Plant materials

A cross was made using cultivar Unicrop (susceptible to PSB disease) as female, and cultivar Tanjil (the donor of PSB resistance as pollen donor in *Lupinus angustifolius*. The progeny was advanced into F_8 recombinant inbred lines (RILs) using the single seed decent method without bias. The F_8 RIL population is stored at the Lupin Genetic Collection at the Department of Agriculture and Food Western Australia; the seeds are available on request for scientific research purpose. The two parental lines and RIL population were employed in this study for marker development tagging the putative PSB-resistance gene in Tanjil.

Phenotyping PSB disease resistance

The fungal pathogen *Diaporthe toxica* was cultured on potato-dextrose agar to produce conidia for disease tests. Seeds of the two parental lines and the 146 F_8 derived RILs were sown in 180-mm diameter plastic pots containing river sand. Plants were maintained in a glasshouse with average daily temperature at 15–25 °C. Three-week-old seedling plants were inoculated with pathogen conidial suspension (10^7 conidia per mL). After 72 h incubation in high humidity, plants were returned on bench in the glasshouse. 3 weeks after inoculation, the cotyledonary internodes were excised. After surface sterilisation, the internodes were incubated in petri plates containing moist filter paper. Disease resistance as expressed in lesion progression and pycnidia formation were recorded 2 weeks after incubation. There were at least five plants in each petri plate, and there were six replicate petri plates for each line. Detailed description of the method for PSB disease phenotyping was reported elsewhere (Shankar et al. 1996).

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Identification of candidate markers linked to PSB resistance by RAD sequencing

Selection of test plants for RAD sequencing for identification of candidate markers linked to PSB resistance in this study followed the same principle as in marker development for MAS by MFLP (Yang et al. 2002; Lin et al. 2009; Sadeghzadeh et al. 2010). Twenty plants were used in the RAD analysis. Ten of these plants, including parental line Unicrop and nine RILs, were susceptible to PSB disease. The other ten plants, including parental line Tanjil and nine RILs, were resistant to PSB disease (Table 1, Supplemental Table 1). The use of 20 plants in candidate marker identification stage followed the same principle as for marker development by MFLP (Li et al. 2011), which also resembled the number of plants used in marker development by "bulked segregant analysis" (BSA) (Michelmore et al. 1991), and the number allowed the samples to fit into two lanes on the NGS sequencing platform HiSeq2000 to minimize the cost.

The protocols of RAD sequencing were the same as Chutimanitsakun et al. (2011), except that the restriction enzyme *EcoRI* (recognition site 5′-G/AATTC-3′) was used. Two single-end sequencing libraries (100 bp) were constructed by using the eight-nucleotide multiplex identifiers (MID) (Baird et al. 2008). Each plant was treated separately, and was assigned to a unique MID barcode. Each library contained ten plants. The RAD products from the 20 plants were processed in two lanes on the NGS platform Solexa HiSeq2000 (which contains 16 lanes per run). Sequencing data were segregated into each of the 20 individual plants according to their respective eight-nucleotide MID barcodes (Baird et al. 2008). The length of DNA sequences of RAD reads was 100-bp including the MID barcodes. After the RAD reads were assigned into individual plants, the eight-nucleotide MID barcode sequences were removed. The length of RAD reads used in bioinformatics analysis was 92 bp, which did not include the first nucleotide G of the *EcoRI* recognition site 5′-G/AATTC-3′. The 92-bp RAD reads within each individual plant were clustered into read tags. Namely, RAD reads containing the same DNA sequences were placed into one read tag. Clustered tags containing more than 100 RAD reads were filtered and removed to avoid the detection of SNP markers from repetitive regions (Catchen et al. 2011).

DNA sequences of RAD read tags were compared between the two parental plants. RAD reads with DNA sequences monomorphic between the two parents were filtered and removed. Only the sequence reads containing SNP polymorphic between the two parents were retained. The resultant sequence reads containing SNP markers were compared among the 20 plants. If a SNP marker showed the polymorphic nucleotide genotypes correlating with the disease resistance and susceptibility phenotypes on all the 20 test plants, it was regarded as a candidate marker linked to the disease-resistance gene based on the same principle in candidate marker development using MFLP as DNA fingerprinting (Yang et al. 2004, 2008; Li et al. 2010, 2011, 2012). Any markers with missing data on more than one plant were discarded.

Conversion of candidate SNP markers into sequence-specific PCR markers

Due to the large numbers of candidate markers identified in this study (Table 2), seven candidate markers were selected for conversion into simple PCR-based markers. Selection of candidate markers for conversion was primarily based on RAD reads containing DNA sequences appropriate for primer design. For each selected candidate marker, a pair of sequence-specific primers was designed to flank the SNP site based on the RAD sequence read. The primer near the *EcoRI* restriction site was designated as forward primer; and the primer near the other end of the RAD sequence read was designated as reverse primer (Table 4). The annealing temperature of primers was approximately 54–57 °C calculated using the nearest-neighbour model (http://row.sigma-genosys.eu.com). DNA amplification was conducted in 10 µl PCR mix containing approximate 0.1 µg genomic DNA as template, 0.5 unit of Taq polymerase (Fisher Biotec, Perth), 1 pmol of the forward primer and the reverse primer, 67 mM Tris–HCl (pH 8.8), 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 0.45 % triton X-100, 4 µg gelatin, and 0.2 mM dNTPs. PCR was cycled on a Hybaid DNA Express thermocycler for 30 cycles each of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min. PCR amplification products were resolved as single-stranded conformation polymorphisms (SSCP) (Sunnucks et al. 2000) on 6 % acrylamide gel using a sequi-gen GT sequencing cell (Bio-Rad). Detailed methods of running SSCP gels were described elsewhere (Boersma et al. 2007).

Linkage confirmation between established PCR markers and the PSB-resistance gene

The converted markers were tested on the segregating F_8 RIL population derived from the cross Unicrop × Tanjil. All the 146 RILs employed in the PSB disease tests were tested with the seven sequence-specific PCR markers. In addition, another 41 RILs from the same F_8 population which were not included in the PSB disease test were also tested with the converted PCR markers. These 41 RILs were included in the linkage analysis for which the disease phenotyping was treated as missing

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Table 1 Summary of RAD sequencing on 20 tests plants of *Lupinus angustifolius* obtained from one-eighth of one run on the next-generation sequencing platform Solexa HiSeq2000 for identification of candidate marker-linked phomopsis stem blight (PSB) disease resistance

Plants	Total RAD raw data (bp)	Number of effective RAD reads	Number of RAD tags	Number of RAD reads contributing to 33 candidate markers[a]	Average coverage depth on the 33 candidate markers[b]
PS[c] (Unicrop)	836,713,412	8,456,862	372,549	553	16.8
F_8S1 (UTF$_8$RIL80)	321,903,360	2,632,008	256,531	165	5.0
F_8S2 (UTF$_8$RIL96)	1,073,627,808	10,006,707	428,186	740	22.4
F_8S3 (UTF$_8$RIL156)	293,930,016	2,346,875	237,538	152	4.6
F_8S4 (UTF$_8$RIL23)	568,517,620	5,130,903	349,041	360	10.9
F_8S5 (UTF$_8$RIL102)	572,352,295	5,274,152	345,167	428	13.0
F_8S6 (UTF$_8$RIL178)	751,125,195	7,086,646	379,981	507	15.4
F_8S7 (UTF$_8$RIL233)	1,333,222,416	13,381,793	442,666	1,224	37.1
F_8S8 (UTF$_8$RIL248)	1,074,726,704	10,749,967	408,744	906	27.5
F_8S9 (UTF$_8$RIL249)	776,138,956	7,630,865	373,330	666	20.2
PR[c] (Tanjil)	619,135,068	6,193,264	321,728	381	11.5
F_8R1 (UTF$_8$RIL29)	1,000,139,132	9,540,602	418,264	722	21.9
F_8R2 (UTF$_8$RIL160)	1,225,442,562	11,833,857	448,422	1,047	31.7
F_8R3 (UTF$_8$RIL47)	852,684,904	7,900,950	396,435	537	16.3
F_8R4 (UTF$_8$RIL222)	1,236,571,410	12,083,473	429,711	920	27.9
F_8R5 (UTF$_8$RIL65)	868,124,686	8,210,181	390,961	638	19.3
F_8R6 (UTF$_8$RIL225)	866,888,676	8,406,755	397,107	751	22.8
F_8R7 (UTF$_8$RIL226)	725,474,464	7,179,365	367,607	560	17.0
F_8R8 (UTF$_8$RIL7)	1,098,772,560	10,866,556	428,661	929	28.2
F_8R9 (UTF$_8$RIL8)	1,229,620,848	12,020,931	437,921	982	29.8
Subtotal	17,325,112,092	166,932,712	7,630,550	13,686	19.9[d]

[a] The 33 candidate markers are listed in Tables 2 and 3

[b] Coverage depth expressed as the average number of RAD reads per marker in each plant

[c] *PS* parental cultivar Unicrop which is susceptible to PSB disease, *PR* parental cultivar Tanjil which is resistant to PSB

[d] Presented as the average coverage depth from the 20 test plants

data (Supplemental Table 1). The marker genotyping data and the PSB disease phenotyping data on the 187 RILs were merged and analysed by the software program MapManager QTX (Manly et al. 2001) using Kosambi function to determine the genetic linkage between the markers and the PSB disease-resistance gene. The linkage map (Fig. 2) was initially constructed using MapManager QTX and finalized by RECORD program (Van et al. 2005).

Validation of established PCR markers on commercial varieties

The established seven sequence-specific PCR markers were tested on all the 26 historical and current cultivars (Table 5) of *L. angustifolius* released in Australia. The correlation between the marker genotyping data and the presence of the putative R gene for PSB disease on commercial cultivars was used as the basis to assess the usefulness of the markers for MAS in lupin breeding (Sharp et al. 2001; Yang et al. 2008).

Results

Genetics of PSB disease resistance in cultivar Tanjil of *L. angustifolius*

In the disease tests, all the parental plants of Tanjil were resistant to PSB disease; and all the plants of parental line Unicrop were susceptible. Of the 146 F_8 RILs tested, 70 RILs were resistant to PSB disease; and the other 76 RILs were susceptible. The segregation of resistant: susceptible in the F_8 fit the expected 1:1 ratio ($\chi^2 = 0.2466$, $P = 0.6195$), indicating the presence of a single gene in Tanjil responsible for PSB disease resistance. The PSB

Table 2 Identification of 33 candidate SNP markers linked to phomopsis stem blight disease-resistance gene in cultivar Tanjil of *Lupinus angustifolius* L. by NGS-based RAD sequencing

SNP markers	PS[a]	F_8S1	F_8S2	F_8S3	F_8S4	F_8S5	F_8S6	F_8S7	F_8S8	F_8S9	PR[a]	F_8R1	F_8R2	F_8R3	F_8R4	F_8R5	F_8R6	F_8R7	F_8R8	F_8R9
Candidate marker 1	a[b]	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 2	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 3	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 4	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 5	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 6	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 7	a	–	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 8	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 9	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 10	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 11	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 12	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 13	a	a	a	a	a	a	a	a	–	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 14	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 15	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 16	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 17	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 18	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 19	a	a	a	a	a	a	a	–	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 20	a	a	a	a	a	a	a	a	a	a	b	–	b	b	b	b	b	b	b	b
Candidate marker 21	a	a	–	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 22	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 23	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 24	a	a	a	a	a	a	a	a	a	–	b	b	b	b	b	b	b	b	b	b
Candidate marker 25	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 26	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 27	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 28	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 29	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 30	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 31	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 32	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b
Candidate marker 33	a	a	a	a	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	b

[a] *PS* parental cultivar Unicrop which is susceptible to PSB disease; *PR* parental cultivar Tanjil which is resistant to PSB

[b] The nucleotides of the SNP markers present in parental line Unicrop were recorded as "a"; the nucleotides of the SNP markers present in parental line Tanjil were recorded as "b"; missing data were recorded as "–"

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disease phenotyping data on the parental lines and 146 RILs are presented in the Supplemental Table 1.

Identification of candidate SNP markers linked to PSB disease resistance

From the two lanes of the Solexa sequencing platform HiSeq2000, a total of 17,325,112,092 bp sequence data were collected from the 20 plants subjected to RAD sequencing (Table 1). The average data coverage was 866 Mbp per plants, but there was a significant variation among each plant, ranging from 293 Mbp to 1,333 Mbp per plant (Table 1). A total of 166,932,712 effective RAD reads were obtained from the 20 test plants which were used in marker discovery in this study. These RAD reads were clustered into 7,630,550 read tags; and the number of RAD tags for each of the 20 individual plants ranged from 237,538 to 442,666 (Table 1).

After filtration and removal of sequence reads monomorphic between the two parental plants of Unicrop and Tanjil, a total of 7,241 polymorphic SNP markers were obtained. By comparing polymorphic RAD sequence reads among the 20 plants subjected to RAD analysis, 33 SNP markers were identified as candidate markers linked to the PSB disease-resistance gene in Tanjil (Table 2). For each of these 33 SNP candidate markers, the nine RILs susceptible to PSB disease had the same SNP polymorphic nucleotides as the susceptible parental line Unicrop; while the nine RILs resistant to PSB disease showed SNP nucleotide alleles identical to the disease resistance parental line Tanjil (Table 2). These 33 SNP markers involved a total of 13,686 RAD reads from the 20 plants; and the average coverage depth for these candidate markers on the 20 plants was 19.9X (Table 1). The RAD sequences of the 33 candidate SNP markers linked to PSB resistance are presented in Table 3. The length of RAD sequences presented in Table 3 is 93 bp due to the inclusion of the first nucleotide G of the *EcoRI* recognition site 5′-G/AATTC-3′.

Conversion of candidate SNP markers into simple PCR-based markers

For each of the seven selected candidate markers, a pair of sequence-specific primers was designed to flank the SNP site based on the RAD sequence read (Table 4). The seven SNP markers were successfully converted into PCR markers with these primer pairs. These seven sequence-specific PCR markers were designated as PhtjM1, PhtjM2, PhtjM3, PhtjM4, PhtjM5, PhtjM6 and PhtjM7 (Table 4). Each of the converted PCR markers exhibited as a co-dominant marker on the SSCP gels, which showed the DNA banding pattern correlating to PSB disease phenotypes on the F8 RILs (Fig. 1).

Linkage confirmation between converted markers and the PSB disease-resistance gene

The genotypes of the seven sequence-specific PCR markers on the two parental lines and the 187 F_8 RILs are presented in Supplemental Table 1. All the seven markers segregated in a 1:1 ratio on the F_8 RIL population (Supplemental Table 1). The PSB disease phenotyping data and the marker genotyping data on the 187 F_8 RILs from the cross Unicrop × Tanjil were merged and analysed by software MapManager, which demonstrated that all the seven sequence-specific PCR markers were linked to the R gene in Tanjil for PSB disease resistance (Fig. 2). Marker PhtjM3 co-segregated with the R gene. All the other six PCR markers were linked to the R gene within the genetic distance of 2.1 cM (Fig. 2).

Validation of established marker on commercial cultivars

The seven sequence-specific PCR markers linked to the R gene for PSB disease resistance in Tanjil were tested on the 26 historical and current commercial cultivars, and the genotyping scores are presented in Table 5. Three markers showed the resistance marker allele bands only on the two cultivars Wonga and Tanjil which possess the target R gene for PSB resistance, including PhtjM4 (Fig. 3), PhtjM5 and PhtjM7 (Table 5). Marker PhtjM3, which co-segregated with the target R gene, showed the correct resistance marker allele band on Wonga and Tanjil, but it also exhibited the resistance marker alleles on six cultivars which do not have the R gene (false positives) (Table 5). The number of "false positives" on the 26 cultivars for the other three PCR markers ranged from two to eight (Table 5).

Discussion

In this study, we identified 33 candidate SNP markers linked to a putative disease-resistance gene in one-eighth of one run on the NGS platform Solexa HiSeq2000 by RAD sequencing, and the work of candidate marker discovery was completed in a few days. This is in sharp contrast to traditional DNA fingerprinting methods where only one or a few candidate markers could be found in many weeks or months (Yang et al. 2002; Lin et al. 2009; Sadeghzadeh et al. 2010). The capability of the NGS-based RAD sequencing to rapidly generate large numbers of candidate markers linked to a gene of interest provides a great capacity to select a marker closely linked to the target gene. In theory, the more the candidate markers, the greater is the chance on finding a marker closely linked to the gene of

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Table 3 DNA sequences of the 33 SNP candidate markers linked to phomopsis stem blight disease resistance in cultivar Tanjil of *Lupinus angustifolius* discovered by RAD sequencing on the next-generation sequencing platform Solexa HiSeq2000

Candidate markers	DNA sequences (5′–3′)[a]
Candidate marker 1[b]	GAATTCTAAGTGCCAACATTATCA[**A/C**]TTATCATCAAGATATAACAAAATGGTATTTCATCCATTATGCAAAGACAAAGAAACAATATAAATGAC
Candidate marker 2	AATTCTCTCTCTTCAGAGCAATAATTCTATTGAAGAATGTCCCCTCTTGTACTT[**C/G**]TTCACTTTCCAAATAATATTGTTCTTTCATGAGAACC
Candidate marker 3	GAATTCGAAAATGACTAAGTGACTGATCACTAGTTCCTTTTATGTGATTCTGCCACATAAAATACATT[**A/T**]TATAATATCACTTAATTTATATGT
Candidate marker 4	AATTCTAGAGAAATAACCTTTATTTATGTGAATAACTAAGTGTATGGCACTGTTGCCTCAGTATTGTTT[**T/C**]TAACAAGAAAAACAATCATGTA
Candidate marker 5	GAATTCTCAAAAGATGAATGAGCTCAGTAGTATCACGCCAATGGATGGCGAATTGGATGATACCCTCCTAACTTCCGATCCAGTAAGCC[**A/G**]GAA
Candidate marker 6	GAATTCTCATAGTTAATATTACTTTTTAAGTTATAAAAAAATAGTCAACTAAACTTGATGATAATAAATCACA[**T/C**]TAGTAACATGAGAATCTAT
Candidate marker 7	GAATTCCTTGCTGCTTGGTGACACCATCTTTCCAAGCTTCCCTGGACTCTCTCCCTGTATTT[**A/G**]TTATTATGCATATCAAACTTTCATTCTTTT
Candidate marker 8	GAATTCTACAACTGCATTTATACCATGGAGCTTCCT[**T/G**]GGAGACATGAACAAGGGGAATCAAGCGGAGTCTGTTGCTCAACAAGATCCTCCAAT
Candidate marker 9	GAATTCAACTTCTAATATTTACTTTTATGACATAGTATAGCGTCATCGATGTTAATGATATTCAATGACATTATTATAAGTAGGAAATGTA[**T/A**]T
Candidate marker 10	GAATTCAAAATACAAAATGTATAATGAAAAAGTATACATTCAATATTTTAAAATTCAAAATCTTAT[**A/C**]GTAGCTGTACTTTTTAACACATAATT
Candidate marker 11	GAATTCAATTAATGACTTTCCAATTAAGTTTAAAATAACAAATTTAGTCTTTAATAAAATAGAAAGGAGAAAACTAATCAACATAAG[**C/T**]ATGTT
Candidate marker 12	GAATTCTTTCATTGCTCATATAACTAGATAGTTTCGTTTTATCATACTT[**T/G**]ATGCAAATATATAAGCTTTATGTCTAGATATAGTTTACAACTA
Candidate marker 13	GAATTCAACCAACGTGGGACTTAAATACTTAA[**C/A**]ACTCAGCTCTCACGCCTAGCACAAACTTGAGTGTAAAGTGACAGTGAGGTTGTATCCACC
Candidate marker 14	GAATTCCAAACATATTTTGCAAACAAACATTTACCAAAGCATTAGGTTCAGGTTTTCATTGAATACACGAAGAATACATATCACAA[**T/A**]CAATAC
Candidate marker 15	GAATTCATGATAAGGA[**G/A**]GATGAAGTATTCCAAGAAAGTTGGAGCCAAACCTAGCAGAGAGATTTTTTCACCATTGTTTCACCATTTTTTCAAC
Candidate marker 16	GAATTCCATATGCAATGG[**C/T**]TTTTTTTATTTTTTGGTAAAAAGTGATGGTGTCCCTCTCACGAAATTGCAAATAACAAATTAACAATTAAGATG
Candidate marker 17	GAATTCACATCGAAGCAAAAAGCAAATTAAACTAGCATCCAATTGATGC[**A/T**]TTGTTTAATTACATTCCCCACTCCTCTTGTTTCAAAAATTACA
Candidate marker 18	GAATTCGGGAATTTGTATGAGCTT[**G/A**]GCTTGAACCTTTGAATCCATCCTCCTTCAACATTTTGTGCAACCTTGAACATAAAAGAATTTCCATTA
Candidate marker 19	GAATTCCTTGATAGAGTGCATGCTTCTGAGAAAAAGCTAAGAGAAAAAGGCTTATGGGAAGTTCCTCATCCTTGG[**T/C**]TGAACCTCTTGGTACAA
Candidate marker 20	GAATTCTGAGCTTCGGATACTTAACCTGCACTCTAACCAGCTTGAAGGTCCAATACCAGCAAGC[**A/G**]TTTTTGCTCCAGGGAAGTTGGAAATTCT
Candidate marker 21	GAATTCATTTGAACCATTCATCACAATGAGCATAAAAACATGTAGTCCATCTAAATGTACCAGCAGAAACATTACAAGTCCAAA[**A/G**]TGTAAACA
Candidate marker 22	GAATTCAAGTTGATCCCTA[**T/G**]ATTATTCTTAGAGTGTTCCTGTTTAGTGCTTATTATTTGATTATTAGCTCAGATCAAGATGTGCTGAAAGTTC
Candidate marker 23	GAATTCAAGAAATGAAAGAACATTCCGAGGGAATGTCATATAACCATGCACTTCTG**[C/T**]TCTGGTTTTACCAGGACACCAAAGTGCAAGACCTAA
Candidate marker 24	GAATTCTGTAATATCGCTTACAGGGTTTGCTCTTGTTGGAGGCCCTGCTAGGCAGGATCATCCGAG[**G/A**]GCGATCGAGGCTTTGAGGAAGCTTGA
Candidate marker 25	GAATTCACAATTGATGAATTATGCCTCATTGTCAGTATTGTGCATCAGCTTTC[**T/C**]GTGATGCAAGCATGAACTGCTACATATGTTCTGGTGTGG
Candidate marker 26	GAATTCGGATATTTTGGAGATCTCTTTCACAAGGTTTAATAAGAGTTACATTGTATTCATCTTCCAAATATATAATTGACAGTGAGTT[**C/G**]GTGT
Candidate marker 27	GAATTCCGGTTTTGGTAAGCATTTTAAGTAAGTGCTAGTTCAGAGTGTTTTTTTCAAAGTTAAAAAGAGATTATTTTCACAAGCTTCCTCG[**G/A**]C

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Table 3 continued

Candidate markers	DNA sequences (5′–3′)[a]
Candidate marker 28	GAATTCACTATTCATCCCATTTGCATCTAATATACTTATGTTGGTGTGGGCGAATCGA ACCTAAAAGTTAGTGTGGTAACACAC[**A/G**]TTTTGGAG
Candidate marker 29	GAATTCGAAGAAATTTTAGTAATGTTGAAGAAAGAGAAGGTGAA [**A/T**]TACCTGGAATTGAAGGTGGTGGTTTATTACTGAGTATTGGATCGGTTC
Candidate marker 30	GAATTCATAGTTGTGGGTTCAATCTTTTGTTTGGTCATCGAATGAACAAAACAATGCG CTTGCTGCTGGGCT[**G/A**]TTGGTACATTTAAAATTAAA
Candidate marker 31	GAATTCAGTTGTTCTATGTTGAAAGGTCCAAAGGGCATTATGGAGAGGCTGCATTTGT TTTAGCAAACATTATATCATCATT[**T/C**]CCTTTCCTTC
Candidate marker 32	GAATTCACTTGATGCACCAAAACG[**G/A**]CCTAAATCAAAACGAAGTCGTTTTCAGCAA GGAACTTCAAGTGTTGCACAACAAGCCTCTGATCTAAC
Candidate marker 33	GAATTCTTTTTAGCTTACTTCAATTAGCTTGTCAGAAGACTTCAA [**G/T**]GAAATAAGCTATTTTTAGCTTATTTAAAGAGAAGCTCATTGAATTAG

[a] Polymorphic nucleotides of SNP markers are bold in brackets. The first nucleotides in parentheses were from parent Unicrop, the second nucleotides in the parentheses were from parent Tanjil

[b] Names of candidate markers are consistent with the names in Table 2

Table 4 Seven sequence-specific, simple PCR-based markers linked to the phomopsis stem blight disease resistance in cultivar Tanjil of *Lupinus angustifolius* developed in this study

Marker	Origin	Primer pair	Primer sequences (5′ to 3′)
PhtjM1	Candidate marker 1[a]	PhtjM1F	GAATTCTAAGTGCCAACATT
		PhtjM1R	GTCATTTATATTGTTTCTTTGTC
PhtjM2	Candidate marker 3	PhtjM2F	GAATTCGAAAATGACTAAGTG
		PhtjM2R	ACATATAAATTAAGTGATATTATA
PhtjM3	Candidate marker 7	PhtjM3F	GAATTCCTTGCTGCTTGG
		PhtjM3R	AAGAATGAAAGTTTGATATGC
PhtjM4	Candidate marker 13	PhtjM4F	GAATTCAACCAACGTGG
		PhtjM4R	GTGGATACAACCTCACTGTC
PhtjM5	Candidate marker 16	PhtjM5F	GAATTCCATATGCAATGG
		PhtjM5R	CTTAATTGTTAATTTGTTATTTGC
PhtjM6	Candidate marker 24	PhtjM6F	TCTGTAATATCGCTTACAGG
		PhtjM6R	CAAGCTTCCTCAAAGCC
PhtjM7	Candidate marker 33	PhtjM7F	CTTTTTAGCTTACTTCAATTAGC
		PhtjM7R	CTAATTCAATGAGCTTCTCTT

[a] Candidate marker numbers are consistent with the candidate makers listed in Tables 2 and 3

interest. The close linkage between the marker and gene of interest is a key requirement for MAS in plant breeding (Gupta et al. 1999). One of the seven sequence-specific PCR markers established in this study, PhtjM3, co-segregated with the putative R gene; and all the other six PCR markers were distributed near the R gene within 2.1 cM. The results demonstrated that the NGS technology is a rapid and cost-effective approach to generate a large number of DNA markers closely linked to the target gene of interest in a short period of time for MAS in breeding programs.

A major advantage of application of NGS-based RAD sequencing for marker development is the ease in generating co-dominant markers which can readily be converted into sequence-specific PCR-based markers desirable for MAS in plant breeding. In MAS, co-dominant markers are more informative and useful than dominant markers, because co-dominant markers are capable of differentiating homozygous individual plants from heterozygous plants. Although RAD sequencing produces both dominant markers (originated from DNA variations within the restriction sites) as well as co-dominant markers (from sequence variation adjacent to the restriction sites) (Pfender et al. 2011), we included a step to filter RAD sequencing reads and to retain only markers showing sequence reads both on PSB-resistant plants and on PSB-susceptible plants during candidate marker development stage. This step effectively ensured that only the co-dominant markers are selected. Furthermore, all the SNP markers generated from RAD sequencing have the DNA sequence information. Therefore, conversion of SNP markers resulting from RAD sequencing into simple PCR

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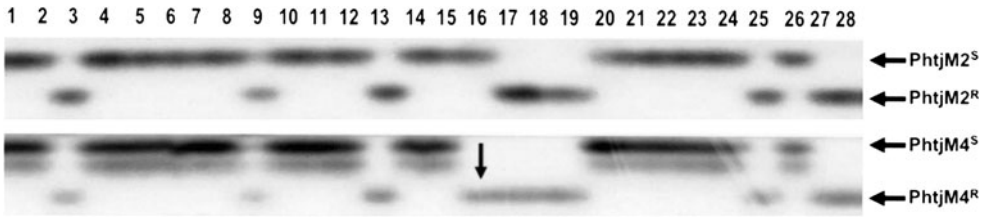


Fig. 1 Screening of molecular markers PhtjM2 and PhtjM4 on 28 F_8 recombinant inbred lines (RILs) from a cross of Unicrop (susceptible to anthracnose disease) × Tanjil (resistant) of lupin (*Lupinus angustifolius* L.). Marker PhtjM2 showed the co-dominant allele bands consistent with the phomopsis stem blight disease phenotypes on all the 28 RILs; while marker PhtjM4 showed a mismatch between marker allele band and disease phenotype on one RIL (*arrowed*) due to genetic recombination

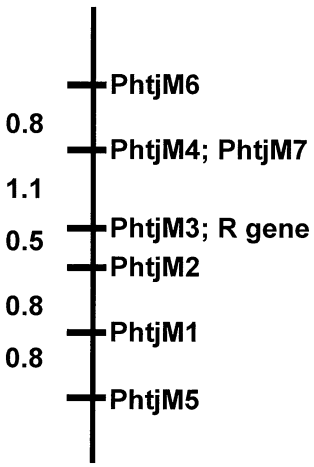


Fig. 2 Genetic linkage (in cM) of seven sequence-specific PCR markers linked to the phomopsis stem blight disease-resistance gene in Tanjil of *Lupinus angustifolius*. The linkage map was initially constructed using MapManager QTX using Kosambi function (Manly et al. 2001), and finalized by RECORD program (Van et al. 2005)

markers does not need DNA cloning and sequencing as required in marker development by traditional DNA fingerprinting methods such as RAPD (Paran and Michelmore 1993), AFLP (Shan et al. 1999) and MFLP (Yang et al. 2002). The length of RAD sequencing reads in our study was about 92 base pairs. Many of SNP mutation sites were in the middle of the RAD sequencing reads, which provided sequence length enough at both ends to design a pair of sequence-specific primers to flank the SNP sites to convert the SNP markers into sequence-specific PCR-based co-dominant markers.

The NGS-based marker development strategy, as demonstrated in this study, provides a solution to deal with the challenge of developing markers applicable to a wide range of breeding germplasm. Developing markers applicable to wide range of breeding germplasm is a major challenge in application of molecular markers on MAS (Sharp et al. 2001). Unless a marker contains the target gene sequence itself, the success of MAS depends on the marker–gene linkage in the breeding populations (Luby and Shaw 2001). Unfortunately, most of molecular markers developed by various DNA fingerprinting methods are near the target genes but not a part of the gene sequence, and certain genetic distance exists between the marker and the gene of interest. The occurrence of "false positives" is a common phenomenon in MAS. For example, the majority of the markers developed and applied in the "Australian winter cereals molecular marker program (AWCMMP)" showed false positives on some breeding germplasm (Sharp et al. 2001). As a consequence, a large part of the resource in the AWCMMP was directed to "marker validation" to define which marker to fit which crosses (Eagles et al. 2001). The "validation" phase greatly slows down the application of molecular markers for MAS in breeding programs (Snape 2004). The most successful markers implemented at the AWCMMP were the two linked to cereal cyst nematode (Ogbonnaya et al. 2001); and the key reason for the success was that the two markers were consistent with CCN-resistance phenotypes across all crosses. Therefore these two markers were "diagnostic" for the trait of interest, which made MAS straightforward because it eliminated the need of "marker validation" process (Sharp et al. 2001; Eagles et al. 2001; Holland 2004). The variation of marker alleles on breeding germplasm can only be found empirically (Sharp et al. 2001; Yang et al. 2008). A molecular marker very closely linked to the target gene may not necessarily be applicable to a wide breeding germplasm. For example, marker PhtjM3 developed in this study, which co-segregated with the putative R gene, had the "false positive" (Yang et al. 2008) marker allele bands on six out of the 26 cultivars. Markers PhtjM1, PhtjM2 and PhtjM6, had eight, six, and two "false positives" on the 26 cultivars, respectively. These markers cannot be used for MAS to screen the F_2 populations resulted from crosses when a lupin line showing a "false positive" marker allele is crossed with Wonga or Tanjil, because all the progeny plants will have the homozygous R-allele marker band, even though the putative R gene for PSB resistance is segregating. *L. angustifolius* was fully domesticated in Australia in the 1960s. The breeding and cultivation of domesticated cultivars of this plant species has a short history of about 50 years. The high rate of "false positive"

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Table 5 Validation of seven sequence-specific PCR markers linked to the R gene conferring resistance to phomopsis stem blight disease on 26 commercial cultivars of *Lupinus angustifolius* released in Australia

Cultivar	Year of release	Presence of target R gene	PhtjM1	PhtjM2	PhtjM3	PhtjM4	PhtjM5	PhtjM6	PhtjM7
Uniwhite	1967	S[a]	S	S	S	S	S	S	S
Uniharvest	1971	S	S	S	S	S	S	S	S
Unicrop	1973	S	S	S	S	S	S	S	S
Marri	1976	S	S	S	S	S	S	S	S
Illyarrie	1979	S	S	S	S	S	S	S	S
Yandee	1980	S	S	S	S	S	S	S	S
Chittick	1982	S	S	S	S	S	S	S	S
Danja	1986	S	S	S	S	S	S	S	S
Geebung	1987	S	S	S	S	S	S	S	S
Gungurru	1988	S	S	S	S	S	S	S	S
Yorrel	1989	S	R*[b]	R*	R*	S	S	R*	S
Warrah	1989	S	R*	R*	R*	S	S	S	S
Merrit	1991	S	R*	R*	R*	S	S	S	S
Myallie	1995	S	S	S	S	S	S	S	S
Kalya	1996	S	S	S	S	S	S	S	S
Wonga	1996	R	R	R	R	R	R	R	R
Belara	1997	S	S	S	S	S	S	S	S
Tallerack	1997	S	S	S	S	S	S	S	S
Tanjil	1998	R	R	R	R	R	R	R	R
Moonah	1998	S	R*	R*	R*	S	S	S	S
Quilinock	1999	S	R*	S	S	S	S	R*	S
Jindalee	2000	S	R*	R*	R*	S	S	S	S
Mandelup	2004	S	S	S	S	S	S	S	S
Coromup	2006	S	S	S	S	S	S	S	S
Jenabillup	2007	S	R*	S	S	S	S	S	S
Gunyidi	2011	S	R*	R*	R*	S	S	S	S
Number of "false positive"[a]			8	6	6	0	0	2	0

[a] *S* indicates the presence of PSB susceptible allele, *R* indicates the presence of PSB-resistance allele

[b] *R** indicates cultivars without the target R gene for PSB resistance but showing the R-allele marker bands (false positive)

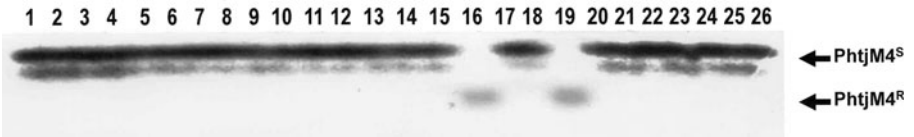


Fig. 3 Genotyping of sequence-specific molecular marker PhtjM4 on 26 Australian historical and current commercial cultivars of *Lupinus angustifolius*. The cultivars are: Uniwhite (*lane 1* susceptible to PSB disease and having no target R gene), Uniharvest (*lane 2, S*), Unicrop (*lane 3, S*), Marri (*lane 4, S*), Illyarrie (*lane 5, S*), Yandee (*lane 6, S*), Chittick (*lane 7, S*), Danja (*lane 8, S*), Geebung (*lane 9, S*), Gungurru (*lane 10, S*), Yorrel (*lane 11, S*), Warrah (*lane 12, S*), Merrit (*lane 13, S*), Myallie (*lane 14, S*), Kalya (*lane 15, S*), Wonga (*lane 16*, Resistant), Belara (*lane 17, S*), Tallerack (*lane 18, S*), Tanjil (*lane 19, R*), Moonah (*lane 20, S*), Quilinock (*lane 21, S*), Jindalee (*lane 22, S*), Mandelup (*lane 23, S*), Coromup (*lane 24, S*), Jenabillup (*lane 25, S*) and Gunyidi (*lane 26, S*). Marker PhtjM4 showed the resistant allele band only on the two cultivars Wonga (*lane 16*) and Tanjil (*lane 19*) which contain the target R gene against PSB

marker alleles among domesticated cultivars possibly reflects the genetic diversity and natural genetic recombination of the wild parents by which the domesticated cultivars were created. Alternatively, the PSB-resistance phenotypes on domesticated cultivars could also involve other R genes for PSB resistance which were not targeted in this study. Fortunately, the ability of NGS-based marker development strategy in discovery of large numbers of candidate markers as demonstrated in this study provided a wealth of choices to test and select the markers applicable

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to wide range of breeding germplasm. Three of the seven established PCR markers, PhtjM4, PhtjM5 and PhtjM7, had no false positives on any of the 26 cultivars, which would be useful for MAS in lupin breeding programs. However, since lupin has a high level of genetic diversity (Li et al. 2010, 2011, 2012), further validation is required when these markers are applied on a wider gene pool, particularly with wild lupin accessions.

Observation from lupin breeding practice and from genetic study (Shankar et al. 2002) indicated that resistance to PSB in lupin is controlled by several major genes in the Australian domesticated lupin lines. The PSB disease phenotyping on cultivars (Yang et al. 2002) is a reflection of the combination effect from these R genes. The putative R gene against PSB in cultivar Tanjil under this study was not reported previously. The molecular markers developed in this study (Table 5) only targeted one single putative R gene contained in cultivar Tanjil. For MAS in lupin breeding, the two markers developed in this study, PhtjM4 and PhtjM7, are equally satisfactory for MAS in lupin breeding. They are sequence-specific simple PCR-based co-dominant markers, which meet the requirement of being cost-effective and amenable to large numbers of samples. They have the resistance marker allele band only specific to the resistant cultivars Wonga and Tanjil, which enables them being applicable to a wide range of crosses. They are closely linked (1.1 cM) to the target R gene for PSB disease resistance, which provide good accuracy (approximately 99 %) in MAS for identification and selection of breeding plants with the target R gene. In lupin breeding, we routinely use one marker in MAS for each major gene of interest to keep the cost down (Yang et al. 2008). Marker PhtjM4 is now being implemented in MAS for selection of PSB resistance in Australian lupin breeding program.

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