STRATEGIES IN DEVELOPING MOLECULAR MARKERS FOR MARKER ASSISTED SELECTION IN LUPIN BREEDING IN AUSTRALIA

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ABSTRACT

Marker-assisted selection (MAS) in plant breeding requires that the markers to be linked to genes of industrial significance, be cost-effectively applicable to large number of samples, and a wide range of crosses in a breeding program. We employed the DNA fingerprinting technology 'microsatellite-anchored fragment length polymorphisms (MFLP)' in marker development for lupin, which has the advantage of (1) highly efficiently finding candidate markers, and (2) is easy to convert candidate markers into simple-PCR based markers for routine implementation. To ensure the markers were applicable to wide range of crosses, we developed a strategy of generating multiple candidate markers followed by a validation step to select the best marker before conversion to an implementable form.

KEYWORDS

molecular markers, marker-assisted selection, molecular plant breeding

INTRODUCTION

There are two major challenges in development of molecular markers for marker-assisted selection (MAS). The first challenge is the requirement for the marker to be closely linked to a gene of interest and able to be cost-effectively applied to a large number of samples (Gupta *et al.* 1999). The second challenge comes from the requirement that the marker alleles need to be consistent with phenotype across wide range of germplasm in a breeding program (Holland, 2004). Most molecular markers developed for MAS are not 'perfect' (i.e. not part of the gene itself). As a result, cultivars showing the desirable markers may not necessarily possess the targeted genes, and *vice versa* ('false positive') (Sharp *et al.* 2001; Clements *et al.* 2005). Obviously, the more such 'false positives' in breeding germplasm, the lower the number of crosses the marker can be used to screen the breeding progenies (Clements *et al.* 2005; Sharp *et al.* 2001). It is now well recognised that the ability of a marker to be applicable to a wide range of crosses in a breeding program is a key limiting factor for large scale MAS in molecular plant breeding (Snape, 2004; Holland, 2004).

Phomopsis stem blight is a major disease of lupin caused by fungal pathogen *Diaporthe toxica* (Shankar *et al.* 2002). This report takes phomopsis resistance in cultivar Tanjil of *Lupinus angustifolius* as an example to demonstrate the strategies which we have been using to develop molecular markers for large scale MAS in lupin breeding in Australia.

MATERIALS AND METHODS

PLANT MATERIALS AND ANTHRACNOSE RESISTANCE SCREENING

A cross was made between a single plant of Tanjil (resistant to phomopsis) as pollen donor and a single plant of Unicrop (susceptible) as female. The progeny was advanced to F_8 recombinant inbred lines (RILs). The parent and the 133 RILs were tested for phomopsis disease as describe by Shankar *et al.* (2002).

GENERATING MULTIPLE CANDIDATE MARKERS LINKED TO ANTHRACNOSE RESISTANCE BY MFLP

Six phomopsis resistant RILs and 6 susceptible RILs were used in MFLP fingerprinting to identify candidate markers for phomopsis resistance. Forty-two MFLP gels were conducted containing 336 sets of MFLP fingerprints. Each MFLP gels had 96 samples, which contained 8 sets of MFLP fingerprints on 6 *RR* and 6 *rr* plants. A MFLP marker is considered as a candidate marker linked to phomopsis resistance if its banding pattern matched with the disease phenotyping on the 12 test plants (Yang *et al.* 2002; 2004; 2008).

VALIDATION OF CANDIDATE MARKERS AND SELECTION OF THE BEST ONE

Seventeen Australian cultivars (Table 2) were used to validate each of the candidate markers identified from the MFLP fingerprints. A cultivar other than the parent (Tanjil) and its sister line (Wonga) showing the resistance allele marker banding pattern is considered 'non-specific positive'. The candidate marker showing least number of 'non-specific positives' was selected for conversion into a sequence-specific PCR marker.

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Table 1. List of five candidate markers identified from MFLP gels linked to phomopsis resistance in cultivar Tanjil of *Lupinus angustifolius* L.

Candidate markers	Primer combination in MFLP	MARKER TYPE	Allele linked	Approximate size (bp)
Marker 1	*MseI*-AAC + MF11	Dominant	R-allele	300
Marker 2	*MseI*-AAA + MF23	Co-dominant	R and S alleles	R = 292, S = 290
Marker 3	*MseI*-AGG + MF51	Dominant	R-allele	120
Marker 4	*MseI*-AAC + MF151	Dominant	RS-allele	295
Marker 5	*MseI*-ATG + MF201	Co-dominant	R and S alleles	R = 210, S = 214

Table 2. Validation of five candidate MFLP markers for phomopsis resistance on 17 Australian cultivars of *Lupinus angustifolius* L.

	Marker 1 [1]	Marker 2	Marker 3	Marker 4	Marker 5
Unicrop	S [2]	S	S	S	S
Yandee	S	S	S	S	S
Danja	S	S	S	S	S
Gungurru	S	R* [3]	S	S	S
Yorrel	S	R*	R*	R*	R*
Merrit	S	R*	S	S	S
Myallie	S	S	S	S	S
Kalya	S	S	S	S	S
Wonga	R	R	R	R	R
Belara	S	S	S	S	S
Tallerach	S	S	S	S	S
Tanjil	R	R	R	R	R
Moonah	S	R*	R*	R*	R*
Quilinock	S	R*	R*	S	S
Jindalee	S	R*	R*	S	R*
Mandelup	S	S	S	S	S
Corromup	S	S	S	S	S
Number of non-specific positive[3]	0	6	4	2	3

[1]Marker number refers to the candidate marker number in Table 1.
[2]Marker score R = R-allele marker banding, S = S-allele marker banding.
[3]R* indicates cultivars other than the parent Tanjil and its sister line Wonga which shown phomopsis resistance R-allele marker banding ('non-specific positive').

CONVERSION OF SELECTED CANDIDATE MFLP MARKER INTO SEQUENCE-SPECIFIC PCR MARKER

Methods of DNA sequencing and sequence extension from the selected candidate MFLP marker bands were described earlier (Yang *et al.* 2001; You *et al.* 2004). A pair of sequence-specific primers was designed to flank the DNA addition/deletion sites. Methods of screening for the converted marker, and linkage analysis were described previously (Yang *et al.* 2001; 2004).

RESULTS AND DISCUSSION

In glasshouse phomopsis disease tests, all the parent plants of Tanjil were resistant, while parent Unicrop was susceptible. Among the 133 F_8 RILs, 64 were resistant, and 69 were classified as susceptible. The segregation of resistant:susceptible in the F_8 fits the expected 1:1 ratio (χ^2 = 0.188, P = 0.665), indicating that phomopsis resistance in cultivar Tanjil is controlled by a single dominant gene.

On the 52 MFLP gels, 5 MFLP markers were identified as candidate markers linked to the phomopsis resistance gene in cultivar Tanjil (Table 1). All the 5 candidate markers showed the banding pattern matching perfectly with the disease phenotypes on the 12 plants tested.

The 5 candidate MFLP markers showed profound variations on the 17 cultivars, and the number of 'non-specific positive' ranged from 0 to 6 (Table 2). The most accurate marker, 'Marker 1', had no 'non-specific positive' on any of the tested cultivars (Table 2), which was selected for further investigation to be converted it into simple PCR based marker.

DNA sequencing of selected candidate marker 'Marker 1' and subsequent sequence extension found that the R-allele band was 494-bp; the S-allele was 490 bp. A pair of sequence-specific primers, PhtjMF (5'-CAGAGGACAAAAAGGAGG-3'), and PhtjMR (5'-CAAGACGAAGCGACATTG-3'), were designed to flank the desirable variation sites, which successfully converted the candidate MFLP marker into a co-dominant, sequence-specific marker ideal for routine marker implementation. This marker is designated as 'PhtjM'. Analysis of the marker score data from PhtjM and the glasshouse phomopsis disease score data on the F_8 population containing 133 RILs by computer program MapManager (Manly *et al.* 2001) indicated that marker PhtjM was linked to the phomopsis disease resistance gene in Tanjil, with a genetic distance between marker and R gene of 4.5 centiMorgans.

When marker PhtjM was tested on the 24 Australian cultivars, only two cultivars, Tanjil and its sister line Wonga, showed the resistance allele band, and all the other 22 cultivars had the susceptible-allele marker bands, confirming that marker PhtjM can be used for MAS on any progenies where Tanjil /Wonga is crosses with any other cultivars. Marker PhtjM has been implemented in lupin breeding in Australia (Fig. 1; Fig. 2).

Fig. 1. Large scale marker implementation in Australian national lupin breeding program. F2 plants were individually labelled. DNA was obtained using a rapid extraction protocol for marker testing. Plants showing homozygous disease resistance-allele marker bands were selected and kept in the breeding cycle.

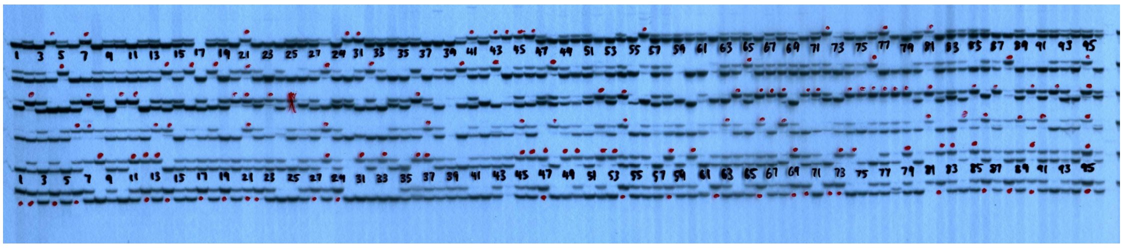

Fig. 2. Anthracnose resistance markers: each set of PCRs contains 96 plants. 576 plants (6 sets x 96 plants) are resolved in one gel for high-throughput.

In the example of developing a marker linked to Anthracnose resistance in Mandelup presented in this paper, we applied a strategy to generate multiple candidate makers first. The candidate markers were then validated on a set of key cultivars, and only the candidate marker with least number of 'non-specific positives' was selected and converted into a sequence-specific marker for routine MAS in lupin breeding. This same strategy was successfully used in our recent research on development of an implementable marker for Anthracnose resistance in cultivar Mandelup of *L. angustifolius* (Yang *et al.* 2008). We conclude that generating multiple candidate markers, followed by application of a 'marker validation' step to select the best candidate marker before marker conversion, is an efficient strategy to develop molecular markers with wide application potential for MAS in molecular plant breeding.

DNA markers can be delineated into two types: DNA hybridisation based markers and PCR based markers. DNA hybridisation based markers (such as RFLP and microarray markers) require high purity of DNA obtained through slow and expensive extraction procedures which plant breeding programs usually cannot afford to implement on large number of plant samples. PCR based markers employing short primers (such as RAPD) are not reliable; markers requiring restriction enzyme (R/E) digestion (such as AFLP and CAPS) bearing additional cost from the R/E making them undesirable for MAS. At present, sequence-specific simple PCR based markers best satisfy the requirements as implementable markers for MAS and these include STMS (sequence-tagged microsatellite site) markers, SCAR (sequence characterised amplified region) markers, STS (sequence-tagged site) markers, and AS-PCR (allele-specific PCR) markers. Sequence-specific simple PCR based markers are obtained either by cloning and sequencing genomic DNA or by converting non-specific markers obtained from generic DNA fingerprinting methods such RAPD, AFLP and MFLP.

MFLP (Yang *et al.* 2001) combines the concept of AFLP (Vos *et al.* 1995) with microsatellite anchor primer technique (Wu *et al.* 1994). Like AFLP makers, the MFLP markers themselves are expensive and undesirable for MAS in plant breeding. However, MFLP has the following advantages: (1) it targets SSRs in the genome and is highly efficient at finding candidate markers; and (2) many MFLP markers can easily be converted into sequence-specific, simple PCR based markers for routine marker implementation (Yang *et al.* 2001; 2002; 2004). By application of MFLP, we have developed a number of markers, including markers for Anthracnose resistance and phomopsis stem blight resistance *L. angustifolius* in narrow-leafed lupin, and for low-alkaloid gene and for Anthracnose resistance genes in *L. albus* which have being implemented in large scale MAS in lupin breeding in Australia. Since 2005, around 20,000 plants are screened annually for MAS in the Australian lupin breeding program.

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