Construction of integrated linkage map of a recombinant inbred line population of white lupin (Lupinus albus L.)

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We report the development of a Diversity Arrays Technology (DArT) marker panel and its utilisation in the development of an integrated genetic linkage map of white lupin (Lupinus albus L.) using an F₃ recombinant inbred line population derived from Kiev Mutant/P27174. One hundred and thirty-six DArT markers were merged into the first genetic linkage map composed of 220 amplified fragment length polymorphisms (AFLPs) and 105 genic markers. The integrated map consists of 38 linkage groups of 441 markers and spans a total length of 2,169 cM, with an average interval size of 4.6 cM. The DArT markers exhibited good genome coverage and were associated with previously identified genic and AFLP markers linked with quantitative trait loci for anthracnose resistance, flowering time and alkaloid content. The improved genetic linkage map of white lupin will aid in the identification of markers for traits of interest and future synteny studies.

Key Words: Lupinus albus, genetic linkage mapping, molecular markers, Diversity Array Technology.

Introduction

Of more than 300 species of Lupinus, only L. albus (white lupin), L. angustifolius (blue or narrow-leaved lupin), L. luteus (yellow lupin) and L. mutabilis (pearl lupin) are widely cultivated (Mulayim et al. 2002). White lupin (2n = 50), originated in the Balkan region of the north east Mediterranean is now distributed throughout the Mediterranean region, and from the Azores Islands across North Africa to Ethiopia and Kenya. White lupin is a legume crop of the smooth-seed group known as the Malacospermae. It is principally exhibits self-pollination, although partial out-crossing is often observed. In Australia, white lupin is grown as a crop mainly in Western Australia and New South Wales.

Lupin seeds are valued for their nutritional components, and contain 30–40% protein, 30% carbohydrate, 6% oil, vitamins and fibre (Lagunes-espinoza et al. 2000). They are used as a source of protein for human food and animal feed (Lin et al. 2009). In Australia, lupins are grown as a break crop to reduce disease build-up in mainly cereals-based cropping systems and to maintain soil fertility through the fixation of atmospheric nitrogen (Jones et al. 2003). White lupin is well adapted to soils having low phosphorus due to its proteoid roots (Neumann and Martinho 2002).

Conventional breeding methods have played an important role in the development of improved varieties for yield, resistance to pests and diseases and desired quality characteristics such as ‘sweet’ seed with low alkaloid content. In order to harness increased genetic gains in lupin breeding programs, more efficient tools for the selection of desirable traits are required. Molecular markers offer useful tools to increase selection efficiency through marker-assisted selection. Genome-wide molecular markers and genetic linkage maps are also essential for characterisation of germplasm, assessment of genetic diversity, the study of inheritance of both qualitative and quantitative traits, map-based gene cloning and for comparative genomic studies.

In white lupin, the first genetic linkage map was published with more than 300 genic and Amplified Fragment Length Polymorphism (AFLP) markers and these were further utilised to identify quantitative trait loci (QTL) for anthracnose resistance, flowering time and alkaloid content (Phan et al. 2007). More recently, high resolution melt analysis based Sequence Tagged site (STS) markers were also developed, mapped and utilised for mapping QTL for flowering time, alkaloid synthesis and stem height (Croxford et al. 2008). However, further increased marker density is required to saturate the white lupin genome and provide candidate markers suitable for marker-assisted selection in the breeding programs. Markers based on known genomic sequences, or those from which the sequence can be easily obtained (including Diversity Array Technology—DArT...
markers), allow comparative mapping approaches to be applied to identify additional markers in regions of interest based on colinearity with related species which have more sequence resources.

The current molecular marker technologies such as AFLP, Simple Sequence Repeats (SSR) and STS have limitations that affect the level of uniform genome coverage, reproducibility and technical and time demands. Most of these markers are gel based and have limited capability to rapidly assay large numbers of marker loci. Some of these limitations can be overcome by specialised technology such as high throughput capillary electrophoresis, which can improve allelic discrimination ability, reproducibility and speed. However, the dependence on pre-existing DNA sequence information and high assay costs of the majority of marker technologies remain major limitations for many genetic improvement programs (Wittenberg et al. 2005). DArT has overcome these limitations and has been applied as an alternative to the gel-based marker technologies in various breeding and germplasm development programs and elsewhere (Akbari et al. 2006, Jaccoud et al. 2001, Raman et al. 2012, Wenzl et al. 2004, Wittenberg et al. 2005, Xia et al. 2005, Yang et al. 2006).

This paper reports the (i) development of DArT markers in white lupins and (ii) the dependence on an integrated genetic linkage map based upon newly developed DArT markers together with the existing AFLP and genic markers that were used to generate the first map of white lupins utilising a recombinant inbred line (RIL) population from Kiev Mutant/P27174 (Phan et al. 2007).

Materials and Methods

Mapping population

A subset of 96 F8 RILs was chosen for DArT analysis. These included the parents Kiev Mutant (maternal parent, introduced into Australia by CSIRO in 1982) and P27174 (paternal parent, an Ethiopian landrace) that were used for the construction of the first linkage map and to locate loci linked to anthracnose resistance, flowering time and alkaloid content (Phan et al. 2007).

Development of complexity reduction methods, library creation and array development

The selection of a suitable complexity reduction method is critical in DArT technology. Previously, various combinations of the restriction enzyme (RE) PsI with frequently cutting RE’s such as TaqI, BstNI, AluI, BanII, Msel, HaeIII, MspI, were efficiently used to prepare genomic representation in different plant species including barley, wheat, Arabidopsis and sorghum (Akbari et al. 2006, Mace et al. 2008, Wenzl et al. 2006, Wittenberg et al. 2005). Initially PsI, the primary rare cutting enzyme in conjuction with eight frequently cutting restriction enzymes, TaqI, BstNI, AluI, BanII, Msel, HaeIII, MspI and TaqI+MspI (New England Biolabs; NEB, USA) were tested to find suitable enzyme combinations for the complexity reduction method. Of the different enzyme combinations tested, PsI/AluI andPsI/MspI performed best, as they produced homogenous DNA smears on gels instead of distinct bands. Genomic representations from the parental lines, Kiev Mutant and P27174 were prepared to make the library.

DNA digestion and adapter ligation was performed using 100 ng of genomic DNA in buffer containing 10 mM Tris-OAc, 50 mM KHO₂/CH₃C₂H₅, 10 mM Mg(CH₂COO)_2 and 5 mM DTT. A PsI adapter (5'-'CAC GAT GGA TCC AGT GCA-3' anned with 5'-CTG GAT CCA TCG TGCA-3') was simultaneously ligated to the complimentary overhangs with 3 units of T4 DNA ligase (New England Biolabs, Australia) in a total volume of 7.7 µl. Reactions were incubated at 37°C for 2 hours, followed by 2 hours at 60°C as required by the enzyme combinations. One µl of the digestion ligation reaction product was used as a template for PCR amplification in a 50 µl reaction using DArT PstI+0 primer (5'-CAG TCA AGT TAG ATG GTG CAG-3') using PCR cycles as 94°C for 1 min, 94°C for 20 sec, 58°C for 40 sec, 72°C for 1 min repeat step 2 for another 29 times, 72°C for 7 min and hold at 10°C. PCR products were analysed on 1.2% agarose gel and all targets with an aberrant migration (downshifted targets) or reduced product yield were removed from analysis.

Cloning and library construction for the DArT array

Amplification products (representations) from both the PsI/MspI and PstI/AluI of the parental lines (P27174 and Kiev Mutant) were ligated into the pCR2.1-TOPO vector using the TOPO cloning kit (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA) and transformed into the heat-shock competent TOP10 Escherichia coli strain (Invitrogen) according to manufacturer’s instructions. Transformants were selected on LB (Luria-Bertani) medium containing ampicillin (100 µg/ml) and X-gal (40 µg/ml), incubated at 37°C for 16 hour. Transformated bacterial colonies were tooth picked into 384 well plates containing LB medium supplemented with 4.4% glycerol, 8.21 g/L K₂HPO₄, 1.80 g/L KH₂PO₄, 0.50 g/L Na₃-citrate, 0.10 g/L MgSO₄·7H₂O, 0.90 g/L (NH₄)₂SO₄ and 100 µg/ml ampicillin and were incubated for approximately 22 hours at 37°C. A set of 3,072 L. albus clones was created from each method of PstI/AluI and PstI/MspI combinations and were stored at −80°C in eight 384 well plates. This library was extended with six more plates of L. albus (2,304 clones) and three plates each of L. angustifolius, L. albus and L. luteus (1,152 clones) using PstI/BstNI developed previously by DArT P/L (http://www.diversityarrays.com/) Yarralumla, Australia), making a 20 plate array with a total of 7,680 clones (Table 1).

An aliquot of 0.5 µl of the culture was taken to amplify with 0.2 µM each of the M13 forward and reverse primers using the PCR program 95°C for 4 min, 57°C for 35 sec, 72°C for 1 min followed by 35 cycles of 94°C for 35 sec, 52°C for 35 sec and 72°C for 1 min and the final extension of 72°C for 7 min. The PCR products were then precipitated
with equal volume of isopropanol at room temperature and washed with 100 \( \mu l \) of 77% ethanol. The ethanol was removed and the products were air-dried and dissolved with DArT spotting buffer 50% DM SO, 1.5 M sorbitol, 0.1 M Triethanolamine. HCl, 0.5% (w/v) dextran, 0.02% (w/v) CHAPS (detergent).

**Array development**

Amplicons suspended in the spotting buffer were arrayed with two replicates onto poly-L-lysine coated micro-array slides (Erie Scientific) using a Microgrid II arrayer (Bio-robotics). The arrays were spotted in 50% relative humidity and then dried in the incubator for 24 hours to allow the DNA adhere to the slides. Slides were processed by immersing in milli-Q water at 92\(^{\circ}\)C for 2 min to denature the DNA and further in milli-Q water with 0.1 mM DTT and 0.1 mM EDTA and finally dried by centrifugation at 1,570 rpm for 7 minutes, followed by vacuum-drying for about 20 min.

**Target preparation and labelling of genomic representations**

A pilot test was carried out to test polymorphism among eight genotypes: P27174, Kiev Mutant-I, P25758, Kiev Mutant-II and two RILs from the P27174/Kiev Mutant-I and P25758, Kiev Mutant-II—the parental lines of mapping populations using DArT clones generated with different restriction enzymes (Table 1). The PstI/MspI DArT arrays revealed the highest polymorphism among arrays using different restriction enzymes and therefore selected for the present study. Genomic representations of 96 samples of the RIL population from Kiev Mutant/P27174 including the parents with two replications were prepared as above for library construction, generating ‘targets’ for hybridisation to the arrays. The amplification products were then precipitated using isopropanol, washed with 77% ethanol and dried for 12 hours at room temperature. All targets in the dried form were combined with 5 \( \mu l \) labelling mixture containing 1× NEB buffer 2, 50 \( \mu M \) random decamers and labelling dNTPs (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 0.2 mM dTTP), denatured to 95\(^{\circ}\)C for 3 min and cooled to 25\(^{\circ}\)C before labelling with 5 \( \mu l \) of Cy-dye mix containing 1× NEB buffer 2, 25 \( \mu M \) cy3-dUTP or cy5-dUTP (Amersham Biosciences) and 2.5 units of Klenow exo-fragment of *E. coli* polymerase 1 (NEB). The labelling reactions were performed at 37\(^{\circ}\)C for 3 hours.

**Hybridisation to microarrays**

Cy3 and Cy5 labelled targets were mixed and added with 5 \( \mu l \) of the Inactivation Mix (1× NEB buffer 2, 60 mM

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**Table 1.** Library development and polymorphism revealed using different restriction enzymes for DArT array construction in *Lupinus* species. Number of DArT clones, polymorphic clones and polymorphism rate represent to individual *Lupinus* species/restriction enzyme combination

| Species             | Restriction enzymes | Genotype ID | Country of origin | Accessions type | Number of clones/Lupinus species\(^{a}\) | Number of polymorphic clones\(^{b}\) | Polymorphism (\%)\(^{c}\) |
|---------------------|---------------------|-------------|------------------|----------------|------------------------------------------|----------------------------------|--------------------------|
| *Lupinus albus*     | PstI/MspI           | P27174      | Ethiopia         | Landrace       | 1536                                     | 422                              | 27.5                     |
|                     |                     | P25758      | Greece           | Landrace       |                                          |                                  |                          |
|                     | Kiev-mutant         | P27174      | Ukraine          | Landrace       | 1536                                     | 349                              | 22.7                     |
|                     |                     | P25758      | Greece           | Landrace       |                                          |                                  |                          |
|                     | Kiev-mutant         | 24706       | Unknown          | Landrace       | 2304                                     | 270                              | 11.7                     |
|                     |                     | 27715       | Unknown          | Landrace       |                                          |                                  |                          |
|                     |                     | 28530       | Unknown          | Landrace       |                                          |                                  |                          |
|                     |                     | 28548       | Unknown          | Landrace       |                                          |                                  |                          |
|                     |                     | Amiga       | Chile            | Landrace       |                                          |                                  |                          |
|                     |                     | Andromeda   | Australia        | Landrace       |                                          |                                  |                          |
|                     |                     | Astra       | France           | Landrace       |                                          |                                  |                          |
|                     |                     | ESTA-1      | South Africa     | Landrace       |                                          |                                  |                          |
|                     |                     | Hamburg     | Germany          | Landrace       |                                          |                                  |                          |
|                     |                     | Kiev-mutant | Ukraine          | Landrace       |                                          |                                  |                          |
|                     |                     | Madeira     | Portugal         | Landrace       |                                          |                                  |                          |
|                     |                     | Mini bean   | Australia        | Landrace       |                                          |                                  |                          |
|                     |                     | Minori      | Germany          | Landrace       |                                          |                                  |                          |
|                     |                     | Multolupa-2 | Unknown          | Landrace       |                                          |                                  |                          |
|                     |                     | 2D12        | Germany          | Landrace       |                                          |                                  |                          |
|                     |                     | 2E 12       | Unknown          | Landrace       |                                          |                                  |                          |
| *Lupinus luteus*    | PstI/BstNI          | P27255      | Unknown          | Breeding line  | 1152                                     | 150                              | 13.0                     |
|                     |                     | 83A476      | Unknown          | Breeding line  |                                          |                                  |                          |
| *Lupinus angustifolius* | PstI/BstNI  | P27255      | Unknown          | Breeding line  | 1152                                     | 170                              | 14.8                     |

\(^{a}\)Number of clones represent to the total number of amplicons created for an individual library.

\(^{b}\)Number of polymorphic clones represent to the number of polymorphic DArT markers: polymorphism was calculated in four genotypes P27174, Kiev Mutant (the parents of the population used to generate the map), P25758, Kiev Mutant (mapping population generated to map resistance loci to Pleiochaeta Root Rot).

\(^{c}\)Polymorphism (%) is calculated as the number of polymorphic clones/total number of clones \(\times100\).
Images of the arrays were produced using a TECAN LS300 confocal microarray scanner (Tecan, Austria) at a resolution of 20 μm per pixel with three images on each slide. This included one reference image using a 488 nm laser with a 520 nm filter combination for the measurement of the signal from the polylinker fragment of the cloning vector common to all fragments on the array and one of the two target images displaying hybridised target labelled with Cy3 using 543 nm laser with 590 nm filter and the other target images displaying hybridised target labelled with Cy5 using 633 nm/670 nm laser filter. The resulting TIF images were analysed using DArTSoft version 7.3, a software program developed by DArT P/L. Polymorphic markers were scored as 1’s and 0’s based on the membership probability estimates computed by the clustering algorithm. The clustering algorithm also provided a probability estimate for each individual genotype call. Markers that showed conflicting scores between the replicates or could not be scored in either replicates were recorded as ‘X’ (missing). The segregation ratio at each marker locus was tested for deviation from the expected Mendelian segregation ratio (1:1) by chi-squared tests. DArT analysis includes replication as a part of quality assurance. Every sample is labelled with Cy3 and Cy5 dyes. Reproducibility is calculated automatically by the software and the average reproducibility is taken as the average of all the selected markers of high reproducibility within the threshold.

**Image analysis and data extraction**

Construction of genetic linkage map of white lupin

High quality marker data are critical to the construction of good genetic maps. A combination of marker quality parameters automatically generated from DArTsoft, the software developed in-house was used as quality thresholds for the identification of polymorphic clones such as reproducibility, call rate and P values. Initially, we incorporated 92 DArT markers that had the parameters: call rate > 80%, reproducibility > 95% and P value > 74%. Subsequently, we included 44 more markers as ‘attached markers’ with less stringent thresholds of reproducibility 92%, call rate 73% and P value 64% (Supplemental Table 1). All the markers exhibited a normal segregation ratio of 1 (paternal alleles):1 (maternal alleles) at each DArT locus with the exception of 24 distorted DArT markers (Supplemental Table 1). A total of 136 marker loci with the call rate > 70% were merged with the segregation data of 220 AFLP and 105 gene based markers that were scored in a previous study (Phan et al. 2007). The markers that did not show linkage with other markers below threshold were excluded from the dataset and not further used for linkage map construction. The integrated linkage map based upon 441 DArT, AFLP and gene based markers covered 2,169 cM, with an average interval distance of 4.6 cM where as the average spacing between markers was 12.6 cM, with the recombination fraction of 0.27 for the previous map (Phan et al. 2007).

**Mapping of DArT markers**

In total, 28 linkage groups were identified in the first map of white lupin (Phan et al. 2007). With the addition of DArT markers along with the AFLPs and gene-based markers, the new integrated map comprised 38 linkage groups which varied in length from 2 cM to 153 cM (Table 2). Distribution of DArT markers across the genome in relation to AFLP and DArT markers across the genome in relation to AFLP and the total variance. The DArT toolbox was used for score merge analysis to check the quality of data from replicates.

**Genetic mapping**

Genetic linkage map was constructed using DArT linkage group and marker ordering software (Hudson et al. 2012). The DArT marker ordering system follows a three step process similar to the one described previously (Cheema and Dicks 2009, Raman et al. 2013). The optimum marker order is posed as the travelling salesman path within the group as described by Wu et al. (2008). Finally, the Kosambi function (Kosambi 1944) was used to convert recombination frequencies into genetic distance in centiMorgan (cM). Mapchart software was used for displaying the linkage groups graphically. All the DArT markers in the map were prefixed as ‘lpms’ where ‘l’ stands for lupin, ‘p’ stands for primary restriction enzyme used (PsI), ‘m’s stands for secondary enzyme MsP1 and the numbers correspond to the clone ID.

**Results**

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Table 2. Characteristics of integrated map of an RIL population of Kiev Mutant/P27174 with DArT, genic and AFLP markers

| Linkage groups | Length (cM) | Total markers | No. of AFLP markers | No. of genic markers | No. of DArT markers | Framework markers | Attached markers | Delegate markers |
|----------------|-------------|---------------|---------------------|----------------------|---------------------|-------------------|------------------|-----------------|
| *LG1           | 55.9        | 16            | 10                  | 0                    | 6                   | 13                | 2                | 3               |
| LG1-II         | 130.7       | 20            | 8                   | 7                    | 5                   | 18                | 5                | 2               |
| *LG2-I         | 42.5        | 8             | 5                   | 2                    | 1                   | 7                 | 2                | 1               |
| LG2-II         | 92.7        | 24            | 11                  | 4                    | 9                   | 18                | 11               | 6               |
| *LG3-I         | 33.1        | 15            | 4                   | 0                    | 11                  | 9                 | 8                | 6               |
| LG3-II         | 53          | 16            | 13                  | 3                    | 0                   | 12                | 0                | 4               |
| LG4            | 151.3       | 20            | 13                  | 5                    | 2                   | 20                | 1                | 0               |
| *LG5-I         | 54.2        | 13            | 6                   | 3                    | 4                   | 11                | 4                | 2               |
| LG5-II         | 99.4        | 9             | 5                   | 3                    | 1                   | 9                 | 0                | 0               |
| LG6            | 156         | 18            | 11                  | 6                    | 1                   | 14                | 1                | 4               |
| LG7            | 88          | 18            | 11                  | 4                    | 3                   | 14                | 5                | 4               |
| LG8-I          | 49.7        | 14            | 5                   | 3                    | 6                   | 10                | 6                | 4               |
| LG8-II         | 24.5        | 5             | 5                   | 0                    | 5                   | 0                 | 0                | 0               |
| LG9            | 79.8        | 12            | 12                  | 0                    | 9                   | 3                 | 3                | 3               |
| LG10           | 122.6       | 34            | 11                  | 2                    | 21                  | 17                | 7                | 17              |
| *LG11-I        | 64.9        | 6             | 5                   | 1                    | 0                   | 6                 | 1                | 0               |
| LG11-II        | 18.4        | 5             | 1                   | 4                    | 0                   | 4                 | 0                | 1               |
| LG12           | 50.8        | 13            | 7                   | 4                    | 2                   | 13                | 4                | 0               |
| LG13-I         | 45.2        | 10            | 3                   | 5                    | 2                   | 9                 | 2                | 1               |
| LG13-II        | 5.3         | 2             | 1                   | 1                    | 0                   | 2                 | 0                | 0               |
| LG14           | 36          | 7             | 7                   | 0                    | 7                   | 2                 | 0                | 0               |
| LG15           | 54.4        | 8             | 4                   | 3                    | 1                   | 8                 | 2                | 0               |
| LG16           | 66.1        | 16            | 5                   | 3                    | 8                   | 10                | 0                | 6               |
| LG17           | 69.0        | 20            | 3                   | 6                    | 11                  | 11                | 3                | 9               |
| LG18           | 67.9        | 10            | 5                   | 3                    | 2                   | 10                | 3                | 0               |
| LG19           | 44          | 12            | 4                   | 3                    | 5                   | 8                 | 9                | 4               |
| LG20-I         | 39.8        | 8             | 4                   | 0                    | 4                   | 6                 | 3                | 2               |
| *LG20-II       | 7.5         | 2             | 0                   | 2                    | 0                   | 2                 | 0                | 0               |
| LG21           | 72.2        | 13            | 3                   | 4                    | 6                   | 11                | 1                | 2               |
| LG22           | 30.3        | 6             | 5                   | 1                    | 0                   | 6                 | 2                | 0               |
| LG23           | 33.3        | 6             | 3                   | 3                    | 0                   | 5                 | 3                | 1               |
| LG24           | 46.2        | 7             | 2                   | 3                    | 2                   | 5                 | 2                | 2               |
| LG25           | 2.1         | 2             | 1                   | 1                    | 0                   | 2                 | 0                | 0               |
| LG26           | 14.1        | 4             | 3                   | 0                    | 1                   | 4                 | 1                | 0               |
| LG27           | 22.9        | 3             | 0                   | 3                    | 0                   | 4                 | 1                | 0               |
| LG28           | 13.8        | 3             | 3                   | 0                    | 3                   | 0                 | 3                | 0               |
| LG29           | 11.2        | 3             | 0                   | 0                    | 3                   | 2                 | 0                | 1               |
| LG30           | 16.9        | 5             | 0                   | 1                    | 4                   | 3                 | 2                | 2               |
| LG31           | 8.8         | 5             | 0                   | 0                    | 5                   | 3                 | 0                | 2               |
| LG32           | 6.5         | 4             | 1                   | 0                    | 3                   | 2                 | 0                | 2               |
| LG33           | 19.4        | 4             | 2                   | 0                    | 2                   | 3                 | 0                | 2               |
| LG34           | 25.6        | 7             | 1                   | 1                    | 5                   | 4                 | 0                | 1               |
| LG35           | 8.6         | 2             | 0                   | 2                    | 0                   | 2                 | 0                | 0               |
| LG36           | 7.4         | 2             | 2                   | 0                    | 2                   | 0                 | 0                | 0               |
| LG37           | 13.8        | 2             | 2                   | 0                    | 2                   | 0                 | 0                | 0               |
| LG38           | 12.8        | 2             | 2                   | 0                    | 2                   | 0                 | 0                | 0               |
| Total          | 2169        | 441           | 209                 | 96                   | 136                 | 347               | 96               | 94              |

*Linkage groups (LG) of integrated map were co-mapped/integrated with previous map (Phan et al. 2007) on the basis of a common set of markers.

*Framework markers are those that have consistent map order. Delegate markers map at the same location as the representative framework marker for a specific locus. Attached markers are those that are excluded at the initial stage and later included in the map assigning them to best intervals on the framework map as described previously (Phan et al. 2007).

*Linkage groups identified by Phan et al. (2007) that formed additional groups in this study.

gene based markers is shown in Fig. 1. DArT markers were well distributed throughout the linkage groups and showed close linkage with the AFLP and genic markers. The positions of AFLP and genic markers were consistent with those mapped previously. However, 10 AFLP markers that were not mapped previously due to loose or no linkage could be grouped in the present study (LG30 to LG38). This demonstrates the value of combining different markers systems for the construction of linkage map with good coverage.

DArT markers were mapped in the majority (65.7%) of
Fig. 1. The integrated map based on DArT, AFLP and genic markers of the RIL population derived from Kiev Mutant/P27174. Map distances (on the left) are given in cM (Kosambi 1944). DArT markers are highlighted in bold. Genomic locations and marker loci delimiting QTLs for flowering time (LG1 and LG3) and anthracnose resistance (LG4 and LG17) are marked with solid lines and marked in bold/italics from the previous map (Phan et al. 2007).
Fig. 1. (continued)
the previously-published linkage groups (LGs) (Table 2). However, there were 13 LGs without DArT markers, and eight of these LGs comprised no more than 3 genic/AFLP markers. Two LGs: LG29 and LG31 contained only DArT markers. Although DArT markers were distributed across the genome in a similar way to the non-DArT markers, there are significant differences in the number of markers among LGs. For example, LG3 and LG10 have quite high marker density (31 and 34 markers), whereas LG6 has low marker density (18 markers) with the largest gap spanning more than 55.4 cM (Fig. 1). In total, 8 linkage groups (LG1, LG2, LG3, LG5, LG8, LG11, LG13 and LG20) from the previous map (Phan et al. 2007) were separated to form additional linkage groups due to the poor linkage with DArT markers.

Discussion

We have mapped DArT markers for the first time in white lupin utilising a mapping population derived from Kiev Mutant/P27174 (Phan et al. 2007). These markers were selected based on their high call rate, scoring reproducibility and P values. When DArT markers were compared with microsatellites and other single locus PCR based markers, the DArT technology simultaneously typed thousands of loci in a single assay and provides a cost-effective and sequence-independent tool for whole genome mapping (Wenzl et al. 2004). However, this technology depends on a complex process and requires specialised equipment and therefore can not be used routinely in most laboratories. DArT markers are also dominant and can not detect heterozygous/heterogeneous genotypes within an individual plant. Dominant markers are less informative for constructing linkage maps especially in populations derived from intercrosses (e.g. F2). Furthermore, for a species with low level of sequence diversity, DArT markers may not be the first method of choice for genetic analysis. Nevertheless, our results confirmed that high quality DArT markers can be used for construction of genetic linkage maps in white lupin.

The average reproducibility of all the DArT markers was 98.6% which was similar to that obtained in barley (99.8%) (Wenzl et al. 2004), hop (100%) (Howard et al. 2011) and pigeonpea (99.7%). Call rate being 90.9% and which expresses the reliability of the final scores versus the maximum number of potential scores, was similar to sugarcane (92.5%) (Heller-Uszynska et al. 2010) and banana (91.6%) (Risterucci et al. 2009) was slightly lower than barley (95.0%) (Wenzl et al. 2004), wheat (99.2%) (Akbari et al. 2006), pigeonpea (96.0%) (Yang et al. 2006) and hop (97.6%) (Howard et al. 2011). The P value here was 86.8%, which agreed with the values obtained in hop (89.9%) (Howard et al. 2011) and was slightly higher compared to banana (81.4%) and sugarcane (80.7%) (Heller-Uszynska et al. 2010).

The new integrated genetic map was shorter (2,169 cM) than the map reported previously which spanned a total length of 2,951 cM (Phan et al. 2007). This discrepancy could be due to usage of different algorithms implemented in different mapping software packages, scoring discrepancies of multilocus AFLP markers, and/or the relatively small size of the population used in this study. Interestingly, Phan et al. (2007) utilised MultiPoint software version 1.2 (MultiQTL Ltd., Institute of Evolution, Haifa University, Israel), which utilises a TSP algorithm, similar to the DArT software implemented in this paper. However, there are different implementations of TSP embedded in the two programs, which may account for the differences in the map length. The most likely explanation for the difference in the map distances is the different marker datasets, as we used a subset of the AFLP and genic markers from earlier study. Most importantly, a shorter map using larger number of markers strongly suggests that the quality of marker scores in the new dataset is higher compared to the initial map. Marker density varied across linkage group. This is consistent with other studies where some LGs or chromosomes of a species possess higher levels of polymorphism than others irrespective of marker type (Akbari et al. 2006, Nelson et al. 2006, Wenzl et al. 2006).

It is also interesting that DArT markers showed a distinct distribution across the genome as compared to AFLP markers. This could be attributed due to the use of the EcoRI/MseI enzyme combination used for AFLP analysis. In the present study, we used PstI enzyme, which is a methylation-sensitive restriction enzyme. It is apparent that the ‘methylation-filtration’ effect arising from using PstI partly enriches genomic representations for hypo-methylated ‘gene space’ regions in barley (Wenzl et al. 2006). A similar picture of DArT marker distribution was observed in several other genomes for which high density DArT maps have been already been developed, for example wheat (Akbari et al. 2006) sorghum (Mace et al. 2008) and rye (Bragoszewksa et al. 2009).

Some of DArT markers were mapped within the genomic regions that were associated with anthracnose resistance, flowering time and alkaloid content (Fig. 1) (Phan et al. 2007). For example, a suite of DArT markers were mapped within 15 cM from flanking markers associated with flowering time QTLs on LG1-1 (delimited with AFLP marker M61E32B396) and LG3-1 (delimited with AFLP markers M47E32A266 and M75E38B73) (Phan et al. 2007). Besides segregating for anthracnose, alkaloid content and flowering time, the population in this study also shows segregation for resistance to phomopsis blight (Cowley et al. 2011). Therefore, this integrated map will provide a platform to localise QTLs for resistance to phomopsis blight and the identification of linked molecular markers.

Our results reconfirmed that DArT provides high quality markers that can be used to construct high density genetic linkage maps for plants with no sequence information available. An additional advantage is that useful DArT clones can be readily sequenced to provide information for their conversion into PCR based markers and for linkage group alignment with genomes of other species for which aligned DNA sequence information is available. This can be advantageous in cases when there are not yet any cheap-to-assay markers.
closely flanking a potential target QTL that could be used in foreground selection for the favourable allele.

In conclusion, we have more intensively saturated the first map of white lupin which was based on genic and AFLP markers with DArT markers. This new map can be used (i) as a ‘seed map’ to compare other maps that are based on multilocus AFLP markers, (ii) for various genetic analyses such as genetic diversity, structure and gene flow estimations, and gene mapping, QTL analysis and whole genome assembly studies.

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