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Breeding for boron tolerance in lentil (*Lens culinaris* Medik.) using a high-throughput phenotypic assay and molecular markers

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Abstract

This study describes the identification of a quantitative trait locus (QTL) in the recombinant inbred line (RIL) population of ILL2024 × ILL6788 and subsequent validation of associated molecular markers. A high quality genetic linkage map was constructed with 758 markers that covers 1,057 cM, with an average inter-marker distance of 2 cM. QTL analysis revealed a single genomic region on Lc2 to be associated with B tolerance and accounted for up to 76% of phenotypic variation (Vp). The best markers for B tolerance were assessed for their utility in routine breeding applications using validation panels of diverse lentil germplasm and breeding material derived from ILL2024. A marker generated

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from the dense genetic map of this study was found to be the most accurate of all markers available for B tolerance in lentil, with a success rate of 93% within a large breeding pool derived from ILL2024. However, given the number of the unrelated lines for which the marker-trait association was not conserved, B tolerance screening is still required at later stages to confirm predicted phenotypes.

Keywords: molecular markers - plant breeding – MAS - marker-assisted selection - abiotic stress tolerance

Introduction

Boron toxicity from high B concentrations in cropping soils is a major constraint to intolerant crops, with the potential to adversely affect crop growth and grain yield. These high B soils are particularly common in the alkaline, arid zones of southern Australia, West Asia, India, Iraq, Afghanistan, North Africa, Peru and USA (Ryan et al. 2013; Yau and Erskine 2000). In southern Australia, this has long been recognised as an issue, especially in South Australia, north-western Victoria and the Western Australian Mallee, where high B concentrations (up to 52 mg kg⁻¹) are common, especially in the subsoil, i.e. 40-100 cm in the soil profile (Cartwright et al. 1984; Nuttall et al. 2010; Nuttall et al. 2003). The occurrence of B toxicity symptoms is somewhat variable but is associated with drought conditions and combined abiotic stresses to crops.

In Australia, the effect of B on crop growth has been extensively studied in a range of pulse and cereal crops, with a major focus on barley. This has resulted in the identification of sources of tolerance which have been used within the breeding programs to improve cultivars adapted to these high B soils (Jefferies et al. 1999; Moody et al. 1993; Nuttall et al. 2010; Paull et al. 1992; Sutton et al. 2007). Two molecular mechanisms for B tolerance have been identified in barley, one being increased root efflux of B through an anion transporter (Hayes and Reid 2004; Reid 2007; Sutton et al. 2007), and the other is through reduced expression and/or effectiveness of an aquaporin which functions as a transporter for B influx across plant membranes (Schnurbusch et al. 2010a).

With the introduction and expansion of lentil into southern Australia, B tolerance was recognised as a breeding aim for the crop to expand into lower rainfall areas. The crop generally has low tolerance to elevated soil B, but landrace germplasm from a number of regions has been identified with improved tolerance (Hobson et al. 2006; Yau and Erskine 2000). Given the obvious foliar symptoms associated with B toxicity, i.e. leaf necrosis beginning at the tip, caused by accumulation of high concentrations of B (Reid et al. 2004), screening mechanisms for germplasm are relatively straight-forward (Schnurbusch et al. 2010b). In addition, screening at a seedling stage is able to identify tolerance characteristics that persist to maturity (Yau and Erskine 2000).

Marker-assisted selection (MAS), involving the use of DNA markers to predict trait performance is widely used in crop breeding (He et al. 2014), however its application in lentil has been limited until recently (Kumar et al. 2015). Several types of molecular markers, including simple sequence repeats (SSR) and single nucleotide polymorphisms (SNP), have been identified and effectively used in lentil

genotyping. The transcriptome sequencing approach has generated expressed sequence tag (EST) databases, delivering large numbers of EST-derived SSR and SNP markers (Kaur et al. 2011; Kaur et al. 2014; Sharpe et al. 2013).

Genetic linkage maps are essential tools for genomic and genetic studies, especially in mapping phenotypic traits. Several genetic linkage maps of lentil have been constructed using a range of molecular marker systems and mapping populations (Eujayl et al. 1998; Gupta et al. 2012a; Gupta et al. 2012b; Rubeena et al. 2003; Taylor et al. 2006), including SSR (Durán et al. 2004; Gupta et al. 2012a; Hamwieh et al. 2005; Phan et al. 2007) and SNP markers (Fedoruk et al. 2013; Kaur et al. 2011; Kaur et al. 2014; Sharpe et al. 2013; Sudheesh et al. 2016a).

Over a number of years, work has been conducted to identify and characterise sources of tolerance to elevated soil B in lentil. Hobson et al. (2006) identified the line ILL2024 as a source of tolerance to both salinity and B, which was subsequently crossed widely in the Australian lentil breeding program. In an effort to develop molecular markers for this trait, mapping populations were created from crosses of ILL2024 with cv. 'Cassab' (an intolerant cultivar), as well as ILL6788 (an intolerant landrace). Kaur et al. (2014) mapped a B tolerance locus derived from ILL2024 in the first of those two populations. However, before trait-associated molecular markers can be adopted widely in the breeding program, they need to be validated in breeding populations and in panels of diverse germplasm, as reported for B tolerance in other pulse crops (Javid et al. 2015). This study reports on the continuation of the work of Kaur et al. (2014), identifying the boron tolerance QTL in a second mapping population, ILL2024 × ILL6788, as well as validation and practical implementation of the markers identified in both populations.

Materials and Methods

Plant material

A RIL mapping population was created from a cross between the B tolerant line, ILL2024, with the B intolerant line, ILL6788. Single seed descent was used to develop 178 F₆ lines for mapping.

Two groups of lentil germplasm were selected for B tolerance marker validation. The first was a validation panel composed of Australian breeding lines, as well as diverse germplasm, many with relevance to the Australian breeding program because of their historical use as parents (Table 1 and Supplementary file 1). This diverse validation panel only contained seven breeding lines derived from introgression of the B tolerance donor ILL2024, and only of them had the tolerant phenotype. The second group of germplasm was selected to determine the usefulness of the markers in tracking the tolerant allele within ILL2024-derived populations. A selection of Australian breeding germplasm was used for this purpose, where ILL2024 has been used as a donor parent for introgression of boron tolerance (Table 2 and Supplementary file 2).

Boron tolerance screening

Two different phenotypic assays were used to screen lentil germplasm for tolerance to boron toxicity. In the first method, potted field soil was amended with boron, while in the second, a simple hydroponic assay, plants were grown in trays of peat plugs floating on dilute boric acid solution. The potted soil assay was used to characterise the validation panel of diverse germplasm, and both methods were used to characterise the ILL2024-derived breeding germplasm. The ILL2024 × ILL6788 RIL population was phenotyped using only the hydroponic method.

The soil-based method was a modification of the protocol described by Hobson et al. (2006), with seedlings grown in sealed pots (250 mm square) with 10 kg of a sandy field soil (93% sand, 5% clay, 2% silt) amended to 5 mg kg⁻¹ soil B (hot CaCl₂ extractable). Hobson et al. (2006) used a B concentration of 54 mg kg⁻¹ in one of their screening experiments, but also tested 0, 15 and 25 mg kg⁻¹ in the more extensive tests. The lentil breeding program currently uses 5-10 mg kg⁻¹ B, which has been found to reliably differentiate between tolerant and intolerant phenotypes.

Pots were watered to field capacity (16.5% water content measured by weight) three days prior to planting, with rainwater used to maintain water content, by weight, to reduce build-up of salts compared with using the available tap water. Lentil seeds were sown directly into the soil medium, arranging eight genotypes in every pot (250 mm square), with six plants of each. The experiment used a randomised complete block design with three replicates, with repeating tolerant and intolerant checks in each pot (ILL2024 and 'PBA Blitz', respectively). There were six experimental lines plus two checks in each pot, and a total of 18 plants (3 × 6) per genotype across the experiment.

Instead of the foliar symptom (1-9) scoring system used in previous studies (Hobson et al. 2006), boron toxicity symptoms were scored as estimated percentage of foliar area necrosis (including leaves and stems). This quantitative scoring system was more amenable to statistical analysis. To obtain a detailed assessment of symptom development, the foliar area affected was estimated for each of the six plants per replicate. An average of all six scores was then calculated and used in the analysis. If significant differences occurred between the plants (e.g. standard deviation >9), this was noted as segregation within the line. Using mean scores per replicate, a linear mixed model (REML) analysis in GenStat v14.1 (VSN International 2011) was performed with "box" as an incomplete blocking factor to account for variation between boxes. Predicted means from REML were then used to determine the level of tolerance to boron toxicity.

Hydroponic boron screening was conducted using horticultural propagation trays with a peat-based media: Preforma Plug Tray (240 Cell) prefilled with Preforma Plugs with Hole (20 mm x 40 mm) (Jiffy Products International B.V., Hoek van Holland, Netherlands). The manufactured peat blend had the following chemical characteristics: pH 6.2 [1:5 H₂O], CEC 5.81 cmol(+) kg⁻¹, EC 0.15 dS m⁻¹ [1:5 H₂O], 17 mg kg⁻¹ Al, 74 mg kg⁻¹ Na, 708 mg kg⁻¹ Ca, 163 mg kg⁻¹ Mg, and 156 mg kg⁻¹ K (c. 1.25 g dry weight per plug). Lentil seed were sown directly onto the pre-moistened peat plugs, just under the peat surface, and germinated in the dark for 2-3 d. Trays were then transferred to 40 L plastic containers (L x W x H: 690 x 466 x 178 mm) containing 20 L of tap water. At seven days after sowing,

boric acid stock solution was added to the water to a final concentration of 15 mg L⁻¹ B in solution. Preforma trays were floated on the boron solution and grown for a further 10-15 d to allow symptoms to development. The exact time required for toxicity symptoms to progress depended on temperature and humidity. Intolerant check cultivars ('PBA Blitz' or 'Cassab') enabled determination of the ideal time for assessment: when necrosis on the check cultivar had reached 50-75% of the foliar area. Also included were the tolerant accession, ILL2024, and a moderately intolerant cultivar, 'PBA Jumbo2', with checks repeated at regular intervals between the experimental entries.

The ILL2024 × ILL6788 RIL population was phenotyped with three replicates in a randomised complete block design, with four plants per replicate. Assessment of foliar symptoms was carried out as a percentage estimate as described above for the soil-based method, with average scores calculated across four plants. The ILL2024-derived breeding population was screened using unreplicated designs, with repeating checks, with six plants of each genotype sown together in a row. Three experiments were conducted, two with the entire list of breeding lines (n=109), and a third with a subset (n=37) that were advanced to the next year of evaluation in the breeding program.

Transcriptome sequencing and SNP discovery

Transcriptome sequencing of the lentil genotypes ILL2024 and ILL6788 was performed as described in Sudheesh et al. (2016b). Briefly, lentil plants were grown in a glasshouse (20 °C ± 2 °C under a 16/8 hour light/dark photoperiod regime) and various tissue samples were harvested at different growth stages (Sudheesh et al. 2016b). RNA extraction from pooled tissues was performed using the RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) and the RNA-Seq libraries with an approximate insert size of 350 bp were generated using the SureSelect Strand Specific RNA Library Prep Kit, both kits used as per the manufacturer's instructions. Libraries were pair-end sequenced using the HiSeq 3000 system (Illumina Inc., San Diego, USA). The raw sequence data was filtered to remove adaptor sequences and to exclude low quality or short reads as described in Sudheesh et al. (2016b).

For SNP discovery, quality-trimmed transcriptome sequencing data was aligned to the transcriptome reference dataset (Kaur et al. 2011) using SAMtools mpileup (Li 2011; Li et al. 2009) and VCFtools (Danecek et al. 2011) to identify base variants. These base variants were then filtered on the basis of quality, depth (or coverage) and level of potential polymorphism between the parental genotypes. Regions inclusive of 100 bp from the 5' and 3' ends of the target sequence variant were selected for a sub-set of SNPs that were sent to Illumina for assay design, and the sub-set of finalised 768 SNPs (Supplementary file 3) for mapping purposes was selected based on designability score (>0.8).

Genotyping, genetic linkage mapping, QTL analysis and validation

Genomic DNA was extracted from young leaf tissue using the DNeasy® 96 Plant Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. DNA was resuspended in buffer AE to a concentration of 50 ng µl⁻¹ and stored at -20 °C.

SNP genotyping of the RILs and parents of the ILL2024 × ILL6788 mapping population was performed using GoldenGate™ oligonucleotide pooled assay (OPA; Illumina Inc., San Diego, USA)

according to the manufacturer's instructions using 250 ng of template genomic DNA. The genotyping assays were processed by the Illumina iScan reader. Automatic allele calling was achieved using the Illumina Genome Studio software v2011.1. Additional SNP genotyping (200 SNPs) of the population was performed using KASP™ genotyping chemistry (LGC Genomics, Middlesex, UK; Javid et al. 2015) using selected SNPs from previously published studies by Kaur et al. (2014) and Sharpe et al. (2013) for map enrichment as well as for linkage group (LG) assignment.

A total of 516 EST-SSRs (Kaur et al. 2011) and 30 genomic DNA-derived SSRs (Hamwieh et al. 2005) were screened on the parents of the mapping population for polymorphism detection. Primer synthesis and PCR amplifications were performed as described previously (Kaur et al. 2014; Schuelke 2000). PCR products were combined with the ABI GeneScan LIZ500 size standard and analysed using an ABI3730xl (Life Technologies Australia Pty Ltd, Victoria, Australia) capillary electrophoresis platform according to the manufacturer's instructions. Allele sizes were scored using the GeneMapper® 5 software package (Life Technologies Australia Pty Ltd).

Polymorphic marker data was tested for goodness-of-fit to the expected Mendelian ratio of 1:1 using χ^2 analysis ($P < 0.05$). The genetic linkage map was generated using Map Manager QTX version QTXb19 (Manly et al. 2001). Map distances were calculated using the Kosambi mapping function (Kosambi 1944) at a threshold LOD score of 5. LGs were assigned on the basis of marker loci in common with previously published genetic linkage maps of Kaur et al. (2014), Sharpe et al. (2013) and Sudheesh et al. (2016a). LGs were drawn using Mapchart software v2.2 (Voorrips 2002).

QTL analysis was performed using marker regression, simple interval mapping (SIM) and composite interval mapping (CIM) in QTL Cartographer v2.5 (Wang et al. 2012). An arbitrary LOD threshold of 2.5 was used to determine significance for SIM analysis. For CIM analysis, significance levels for LOD thresholds were determined using 1,000 permutations.

To identify the genomic location of the QTL flanking SNP markers from Kaur et al. (2014) and the current study, the underlying sequences were BLASTN (threshold E-value $< 10^{-10}$) (Altschul et al. 1997) analysed against the draft lentil genome assembly v1.2 (Bett et al. 2016) through the KnowPulse database (<http://knowpulse.usask.ca/portal>; Accessed 24 October 2017).

Genetic markers flanking B tolerance QTL-containing intervals identified in Kaur et al. (2014) and in the current study were used for genotypic analysis of the validation panel and breeding germplasm. Genotyping was performed using KASP™ genotyping chemistry (LGC Genomics) as described in Javid et al. (2015).

Results

SNP discovery, genotyping and genetic linkage mapping

Transcriptome libraries were generated and sequenced from a variety of tissue types from the lentil cultivars ILL2024 and ILL6788. The raw sequence data was then filtered to remove adaptor

sequences and exclude low quality or short reads, resulting in a high quality set of 42,289,495 (ILL2024) and 52,011,343 (ILL6788) paired-end reads, with an average read length of 120 bp. A preliminary set of 26,639 SNPs polymorphic between ILL2024 and ILL6788 were selected from comparison of transcriptome reads against the EST sequence database. Further selection based on a criteria of homozygosity, quality (Q score ≥ 30), depth (≥ 6 reads) and absence of other known SNPs in close proximity (< 35 bp), resulted in a sub-set of 938 high quality SNPs. Of these, 768 SNP loci with a designability rank of > 0.8 were selected for the GoldenGate® assay.

The majority of the SNPs formed two main clusters in Genome Studio representing the homozygous (AA and BB) genotypic classes, however occasionally a third small cluster of heterozygous (AB) genotypes was also observed. A total of 654 SNP markers (85%) generated polymorphic clusters. The mapping population was also screened with 200 additional SNPs, of which 106 markers (71 from Kaur et al. [2014] and 35 from Sharpe et al. [2013]) were polymorphic. A total of 546 publicly available SSR markers were screened and 41 of these exhibited polymorphisms (Supplementary file 4). After the χ^2 analysis (> 10 , $P < 0.05$), a total of 763 loci were used for linkage mapping (Supplementary file 4) in which 758 markers (99%) were assigned to eight LGs and one satellite (Supplementary file 5 & 6) with an estimated cumulative total map length of 1,057 cM with an average inter-locus interval of 2 cM (Supplementary file 5 & 7). The LGs were numbered according to the positions and groupings of previously map assigned markers.

Phenotypic assays for boron tolerance

There was a high level of correlation ($R^2 = 0.78$) between tolerance to boron toxicity in soil and hydroponic culture, as well as between repeated assays in hydroponic culture (Table 3). There was homogeneous variation of residuals across the range of scores and so the assays did not always precisely agree on necrosis symptoms of more tolerant lines (i.e. 0-20% necrosis), but it was sufficient to separate tolerant lines of the phenotype derived from ILL2024. Scores for the tolerant check line ILL2024 were closer to 0% necrosis. Phenotypic grouping of cultivars were performed on the following scale: 0-12% foliar necrosis was classed as tolerant, 13-24% necrosis was intermediate tolerance and 25-100% necrosis was intolerant (equivalent to susceptible lentil check varieties).

QTL analysis in ILL2024 × ILL6788 mapping population

Significant differences in B symptoms were observed between the RILs and parents of the ILL2024 × ILL6788 mapping population. Mean necrosis scores were used to generate frequency distribution histograms (Supplementary file 8). The marker regression, SIM and CIM analyses identified a single genomic region derived from ILL2024 on linkage group 2 (Lc2) that was associated with B tolerance and accounted for up to 76% of the phenotypic variation (Table 4 and Supplementary file 7). BLASTN analysis of sequences underpinning the QTL flanking markers from Kaur et al. (2014; SNP_60000240, SNP_20000246, SNP_20002998) and from the current study (Lc_0002525, Lc_0002502, Lc_0000881, Lc_0001049) to the draft lentil genome assembly v1.2 (KnowPulse 2017) revealed matches locating this QTL on chromosome 2 (LcChr2; Supplementary file 9). In addition, regions of the lentil genome immediately adjacent to and within the QTL intervals were examined for

candidate gene presence. The Lc09014 gene annotated as a major intrinsic protein was located in close proximity to SNP markers Lc_0002525 (Supplementary file 9).

Validation of B tolerance markers in the validation panel

In the validation panel germplasm, 12% of lines exhibited a tolerant phenotype (0-12% foliar necrosis), 41% had an intermediate level of tolerance (13-24% necrosis), and 47% were intolerant (25-75% necrosis). Four flanking markers were used to genotype the validation panel, namely SNP_20000246 and SNP_60000240 (Kaur et al. 2014) and Lc_0002502 and Lc_0002525 (Table 4). Results indicated that the tolerant allele from ILL2024 was not common in the validation panel germplasm, with only 15 and 18 lines having the tolerant allele for marker SNP_20000246 and Lc_0002525, respectively. In addition, these genetic markers were not reliable predictors of the B tolerance phenotype across diverse lentil germplasm.

Overall, markers SNP_20000246 and Lc_0002525 were found to give the best prediction of B tolerance phenotype overall, with the lowest level of false positive predictions (Table 5). However, of the 18 lines containing the tolerant allele for Lc_0002525, with or without Lc_0002502, only six were tolerant to B, while six had an intermediate level of tolerance, and six lines were completely intolerant. Based on the single gene model, “intermediate” tolerance was assumed to be derived from an alternative source of B tolerance not associated with these genetic markers; on that basis, Lc_0002525 was only accurate for 33% of the entries containing the tolerant allele. Marker prediction appeared more accurate for entries carrying the intolerant allele, with correct associations occurring between 89 and 92% of entries; however, the phenotypic ratios for the intolerant allele were very close to the overall average for the panel, so this result is the same as what would have occurred by chance alone.

Validation of B tolerance markers in ILL2024 progeny

Table 6 summarises the marker and phenotyping results for the ILL2024-derived germplasm. B tolerance was determined using data from across the phenotypic screens (Table 3), grouping cultivars as tolerant or intolerant. Only six lines in this population gave intermediate B tolerance, which were grouped with the intolerant phenotype for validation purposes.

As for the validation panel, four SNP markers were tested on this germplasm, SNP_20000246, SNP_60000240, Lc_0002502 and Lc_0002525. Similar results were seen for the markers, but Lc_0002525, from the high density map of ILL2024 × ILL6788, gave the best single marker prediction for the tolerance phenotype, and was accurate for 93% of the ILL2024-derived germplasm. Where evidence of recombination within this locus was seen, Lc_0002525 was found to be more accurate, giving the lowest number of false positive and negative results.

Discussion

This study reports an optimised assay for low-cost, high-throughput assessment of B tolerance in lentil, which was used in conjunction with a new genetic linkage map, that is one of most

comprehensive and high density gene-based linkage maps available for lentil. QTL analysis confirmed a previous finding using an independent mapping population, locating a single genomic region of major effect on Lc2 controlling B tolerance (Kaur et al. 2014). Importantly, using the new map, improved markers for B tolerance in lentil have been developed and will be useful in selecting for B tolerance in populations derived from ILL2024.

Genetic mapping, QTL analysis and marker validation

High density genetic linkage maps provide an essential tool for plant genetic research, including the anchoring and assembling scaffolds for genome assembly, facilitating QTL identification, marker-assisted selection and map based cloning. The ILL2024 × ILL6788 linkage map exhibited a regular marker distribution with a higher number of map assigned markers, 758 (average inter-locus interval 2 cM), in comparison to previously published lentil gene-based linkage maps: 'CDC Robin' × 964a-46, 546 markers (Sharpe et al. 2013); ILL2024 × 'Cassab', 318 markers (Kaur et al. 2014); 'Indianhead' × 'Northfield', 460 markers; 'Indianhead' × 'Digger', 329 markers, and 'Northfield' × 'Digger', 330 markers (Sudheesh et al. 2016a).

The QTL region identified in this study is accurate and confirms the single locus identified in a previous study of B tolerance in lentil (Kaur et al. 2014). This finding is consistent with the outcomes of studies of B tolerance in other legume species such as pea (Sudheesh et al. 2015) and *Medicago truncatula* (Bogacki et al. 2013), which also reported single gene models. Using the dense genetic map developed, a more closely linked marker (Lc_0002525) was found for this B tolerance gene as compared to the markers identified from ILL2024 × 'Cassab'. The best markers identified in that population (SNP_20000246 and SNP_60000240) resulted in more false positive results in the validation panel than Lc_0002525 (i.e. intolerant lines with the tolerant genotype). These included the important parents 'Indianhead' and 'CDC Matador', Canadian cultivars which have been used extensively as sources of resistance to aschochyta blight (*Aschochyta lentis*). In fact, all the other three best markers tested, besides Lc_0002525, gave a false positive result for these two lines. The accuracy of Lc_0002525 in Indianhead and in its progeny makes it considerably more useable in the Australian breeding program, while the other markers would be more problematic for wide-spread application.

Comparison of sequences of the markers flanking the QTL region with the draft lentil genome assembly v1.2 (Bett et al. 2016) revealed a location on LcChr2. Three of the QTL-flanking markers (two from ILL2024 × 'Cassab' and a single marker from ILL2024 × ILL6788) were matched to locations on LcChr2 distant to that of the other SNP loci. However, as both maps presented the marker anomalies, the issues do not appear to have been caused by marker ordering during the linkage mapping process; possible alternative explanation is chromosomal rearrangements in the parental genomes. Of the best markers, SNP_20000246 obtained matches on LcChr2 in between Lc_0002502 and Lc_0002525, which agrees with the validation of the markers in germplasm tested here, as compared with SNP_60000240.

The whole genome sequencing data available was also used to assist in the identification of potential candidate genes at this locus, which would enable the development of a diagnostic marker. Kaur et al. (2014) discussed the aim to find a candidate B transporter gene like those isolated in *Arabidopsis* (Miwa et al. 2014; Miwa and Fujiwara 2011). Studies in *Arabidopsis*, wheat, rice and *M. truncatula* have revealed genes for efflux-type B transporters and major intrinsic protein (MIP) family controlling B toxicity tolerance (Bogacki et al. 2013; Takano et al. 2002; Takano et al. 2006). At the genomic location on LcChr2, one candidate was a lentil MIP gene (Lc09014) located in proximity (790 Kb) to one of the QTL flanking SNP markers (Lc_0002525). This is the only sequence within the draft genome that could be implicated with having a role in B tolerance, however, given the aforementioned likely chromosome rearrangements, it is possible the landrace donor has different local sequences than the reference genome (KnowPulse 2017).

Application of B markers in lentil breeding

Validation of the best markers for B tolerance has assessed the utility of these markers for use in lentil breeding. Unfortunately, there was a large number of lines in the validation panel for which the marker-trait association was not conserved, including more than half the lines with the tolerant allele at the Lc_0002525 and Lc_0002502 loci. Even using the best marker, Lc_0002525, there were 15 lines found to be tolerant to B despite not having the ILL2024 allele (false negatives). This result may point to the presence of other independent sources of B tolerance. These false negatives are not as much of a concern as false positives when the marker is applied in selected populations. Lc_0002525 gave the best result of the four markers for false positives, with only 7% of the validation panel having the tolerant allele without the tolerant phenotype. Therefore, this marker could be readily applied in breeding populations, to track introgressions of the ILL2024 tolerance gene, where the marker is known to be accurate in the parents.

The characterisation of the validation panel enabled identification of material for which the association between the B tolerance marker and phenotype was consistent with ILL2024. Hobson et al. (2006) found two landraces to have the highest level of B tolerance from a collection of 310 lentil accessions: ILL2024, the source of B tolerance discussed in this paper, which originated from Ethiopia, and ILL0213A, with a similar level of tolerance that was collected from Afghanistan. Genotyping revealed that there is a likely genetic relationship between these landraces (unpublished data), with both sharing the tolerant allele at all four SNP loci tested. In addition, the breeding line, 05H010L-07HS3010 inherited the tolerant locus from its grandparent, ILL2024, and is an important parent of germplasm in the ILL2024-derived progeny.

There were three lines in which the markers Lc_0002525 and SNP_20000246 did not co-segregate, having the Lc_0002525 tolerant allele only. These were, ILL0518 (syn. PI 320951), a virus and rust resistant landrace used as a parent in Australia, USSR-05-05 (syn. PI 577149), a lentil landrace from Tajikistan, and cv. 'Emerald' (syn. PI 508091; Muehlbauer 1987), a USA green lentil. In addition, PI 572351 (syn. Ladizinsky 66 or Hoffman #27; Norris 1994) a lentil accession collected in Yugoslavia (southern Bosnia and Herzegovina), was highly tolerant to B and had the SNP_60000240 allele, but genotyping for Lc_0002525 was not performed.

A large number of lines were found to have an intermediate level of B tolerance in the lentil validation panel and this is consistent with results from routine screening in the breeding program and reflects the presence of other genetic sources of tolerance. There are at least two distinct sources of improved tolerance to B toxicity in the Australian lentil breeding pool. The first is ILL2024 which is highly tolerant to both B and soil salinity (Hobson et al. 2006). In addition, there are other lentil cultivars which have a moderate improvement in tolerance to B, such as 'PBA Flash', 'PBA Bolt', 'PBA Giant' and a number of advanced breeding lines, including CIPAL1501 and CIPAL1505. It is likely that this tolerance has been derived from the parent ILL7685, a B tolerant line which does not have the tolerant alleles for any of the markers discussed here. The improved B tolerance in these high yielding cultivars does not match that of ILL2024 in screening experiments, and is rated as "moderately intolerant". Nevertheless, this tolerance has been successfully selected for using these phenotypic screening protocols.

Unfortunately, despite the fourteen years that have passed since ILL2024 was identified, none of its genetic material has yet progressed into an advanced breeding line near to commercial release. The lines identified as ILL2024 progeny and screened in this study were taken from a preliminary yield trial stage (in 2014), and represent a third generation of crosses of ILL2024 genetics; the second generation being crosses of 02-355L-03HS005 and 04-434L-06HS4001 (50% ILL2024), and the third utilising 05H010L-07HS3010 (pedigree: 02-355L-03HS005/CIPAL0413) as well as others. The material tested here also includes reselections for B tolerance from within lines segregating for the trait.

There are two possible explanations for the lack of elite material derived from ILL2024 despite the extensive crossing, reselection and intercrossing that has occurred. The first is that the boron toxicity tolerance mechanism of ILL2024 brings a susceptibility to B deficiency in low B soils, which limits the adaptation of lines containing this locus. The reason for this phenomenon is the very narrow concentration range that exists for most plant species between B deficiency and toxicity. In barley, it was identified that B tolerant lines were susceptible to B deficiency, while intolerant lines had functional mechanisms for increased B uptake when required and so did not suffer B deficiency as easily (Nable et al. 1990). This has also been seen for lentil in the B-deficient soils of Nepal, where cultivars adapted to these regions expressed no deficiency symptoms in the field, whereas cultivars from regions like Syria, where B is at normal to high levels, performed poorly (ICARDA 1998, cited by Yau and Erskine 2000). The second possible explanation is that ILL2024 carries multiple genes of an agronomically unadapted background and these have not been sufficiently removed by breeding. Of particular note is seed shattering, for which there is evidence of strong linkage in early generation lentil breeding trials.

Field validation of the yield advantages from boron tolerance has been difficult to achieve in lentil, as has been seen in barley (Emebiri et al. 2008; McDonald et al. 2010). Some have doubted that breeding for B tolerance will lead to a yield advantage (Reid 2010), and this may be because the majority of the mechanisms employed are associated with B exclusion from plant tissue. Tolerance to

high tissue B concentrations and the ability to cope with other soil constraints, such as salinity and Zn deficiency may be required for adaptation to regions of hostile soils (McDonald et al. 2010).

In the Australian lentil breeding program, tolerance to soil salinity has been selected for alongside B tolerance. Despite poor agronomic background, there is evidence that the program may be achieving ILL2024-derived B tolerance in well-adapted backgrounds. In 2016, there were 10 lines in advanced yield trials (out of 281) that appear to have inherited this tolerance, being derived from second and third generation crosses. Two lines were derived from top-crosses with 05H010L-07HS3010, and have combined B, salt and imidazolinone herbicide tolerance, with yield potential similar to the red lentil cultivar 'PBA Ace'.

Tolerance to soil B will continue to be a breeding objective, and efficient selection methods will be required into the future. The high-throughput phenotypic assay described here will continue to be utilised, and will be complemented by marker analysis for selected populations. The markers identified in this study could be used to accelerate the selection of the next generation of material with this trait, such as progeny of the current best lines, especially when applied at early generation stages before B tolerance screening is possible.

Competing Interests

The authors declare that they have no competing interests.

Author's Contributions

MR, AG and DN developed the high-throughput boron screening method used in the current study. MR, DN and MJ performed and analysed the phenotyping experiments. SS prepared plant materials, performed RNA and DNA extraction, performed sequencing, linkage mapping and QTL analysis. MR and SS interpreted the data and drafted the manuscript. AS and GR contributed to data interpretation and assisted in drafting of the manuscript. SK co-conceptualised the project, participated in experimental design, contributed to data interpretation and assisted in drafting of the manuscript. All authors read and approved the final manuscript.

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Fig. 1 Scatter-plot of comparison between phenotypic screening in the potted soil experiment and a simple hydroponic assay (2015) for a breeding germplasm set containing a proportion of their genetic background from ILL2024

Table 1 Composition of the lentil validation panel used to test the utility of B tolerance molecular markers

Category of lentil germplasm	Number of entries
Australian lentil cultivars	15
Australian breeding lines	122
Canadian breeding lines or cultivars	11
ICARDA sourced germplasm	17
ICRISAT accession	1
USA breeding lines or cultivars	10
Total	176

Table 2 Summary of the genetic background of the selected breeding germplasm derived from crosses with B tolerant donor ILL2024

Proportion of background from ILL2024	No. of lines
1/4	5
1/8	79
1/16	25
Total	109

Table 3 Correlations between phenotypic assays for tolerance to boron toxicity in a breeding germplasm set containing a proportion of their genetic background from ILL2024

	No. of entries	2015 Hydro.	2014 Hydro.	2015 Hydro. (subset)
2015 Soil assay	126	0.89	0.83	0.81
2015 Hydroponic	124	1	0.88	0.92
2014 Hydroponic	122		1	0.82
2015 Hydroponic (subset)	37			1

Table 4 Identification of QTL for B tolerance in ILL2024 × ILL6788 genetic maps based on composite interval mapping

Trait	Linkage group	Flanking markers	Position (cM)	Maximum LOD threshold	% Phenotypic variance	Additive effect
Boron score	Lc2	Lc_0002525, Lc_0002502, Lc_0000881, Lc_0001049	182.8-185.8	65	76	-21.7124

Table 5 Analysis of four markers for the B tolerance QTL coming from ILL2024 in the diverse lentil validation panel summarised in Table 1. The lines with the tolerant allele from ILL2024, and those with the intolerant allele, are separated into the numbers with tolerant and intolerant phenotypes

Phenotype	Tolerant allele		Tolerant allele accuracy ^b	Intolerant allele		Overall accuracy	% False positives ^c
	Tolerant	Intolerant ^a		Intolerant ^a	Tolerant		

SNP_60000240	4	23	15%	132	17	77%	13%
SNP_20000246	3	14	18%	141	18	82%	8%
Lc_0002502	10	22	31%	133	11	81%	13%
Lc_0002525	6	12	33%	143	15	85%	7%

Notes:

- a. Of the 176 entries in the panel, 41% had an intermediate level of tolerance to B, which was assumed to be derived from an alternative source of B tolerance, not associated with the ILL2024 allele, therefore intolerant and intermediate phenotypes have been grouped together as “Intolerant”
 - b. Successful prediction of phenotype is calculated for entries with tolerant allele only
 - c. False positives were lines with tolerant (ILL2024) allele for the marker, but without the tolerant phenotype
- 587
- 588

Table 6 Analysis of B tolerance markers in selected breeding germplasm derived from crosses with donor parent ILL2024, as summarised in Table 2. The lines with tolerant allele, from ILL2024, and those with the intolerant allele, are separated into the numbers with tolerant and intolerant phenotype

Phenotype	Tolerant allele		Tolerant allele accuracy ^a	Intolerant allele		Overall accuracy	% False positives ^c
	Tolerant	Intolerant		Intolerant ^b	Tolerant		
SNP_60000240	54	6	90%	43	6	89%	5.5%
SNP_20000246	53	3	95%	46	7	91%	2.8%
Lc_0002502	52	3	95%	46	8	90%	2.8%
Lc_0002525	54	2	96%	47	6	93%	1.8%

Notes:

- a. Successful prediction of phenotype is calculated for entries with tolerant allele only
 - b. Intermediate tolerance to B was assumed to be derived from an alternative source of B tolerance, not associated with the ILL2024 allele, therefore six lines with intermediate phenotypes were grouped with intolerant lines
 - c. False positives were lines with tolerant (ILL2024) allele for the marker, but without the tolerant phenotype
- 589

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