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8 Breeding for boron tolerance in lentil (*Lens culinaris* Medik.) using a high-9 throughput phenotypic assay and molecular markers

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21 **Abstract**

22 This study describes the identification of a quantitative trait locus (QTL) in the recombinant inbred line 23 (RIL) population of ILL2024 × ILL6788 and subsequent validation of associated molecular markers. A 24 high quality genetic linkage map was constructed with 758 markers that covers 1,057 cM, with an 25 average inter-marker distance of 2 cM. QTL analysis revealed a single genomic region on Lc2 to be 26 associated with B tolerance and accounted for up to 76% of phenotypic variation (Vp). The best 27 markers for B tolerance were assessed for their utility in routine breeding applications using validation 28 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

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

69 Genetic linkage maps are essential tools for genomic and genetic studies, especially in mapping
70 phenotypic traits. Several genetic linkage maps of lentil have been constructed using a range of
71 molecular marker systems and mapping populations (Eujayl et al. 1998; Gupta et al. 2012a; Gupta et
72 al. 2012b; Rubeena et al. 2003; Taylor et al. 2006), including SSR (Durán et al. 2004; Gupta et al.
73 2012a; Hamwieh et al. 2005; Phan et al. 2007) and SNP markers (Fedoruk et al. 2013; Kaur et al.
74 2011; Kaur et al. 2014; Sharpe et al. 2013; Sudheesh et al. 2016a).

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

87

88 **Materials and Methods**

89 **Plant material**

90 A RIL mapping population was created from a cross between the B tolerant line, ILL2024, with the B
91 intolerant line, ILL6788. Single seed descent was used to develop 178 F_6 lines for mapping.

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

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

108 The soil-based method was a modification of the protocol described by Hobson et al. (2006), with
109 seedlings grown in sealed pots (250 mm square) with 10 kg of a sandy field soil (93% sand, 5% clay,
110 2% silt) amended to 5 mg kg^{-1} soil B (hot $CaCl_2$ extractable). Hobson et al. (2006) used a B
111 concentration of 54 mg kg^{-1} in one of their screening experiments, but also tested 0, 15 and 25 mg
112 kg^{-1} in the more extensive tests. The lentil breeding program currently uses 5-10 mg kg^{-1} B, which has
113 been found to reliably differentiate between tolerant and intolerant phenotypes.

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

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

131 Hydroponic boron screening was conducted using horticultural propagation trays with a peat-based
132 media: Preforma Plug Tray (240 Cell) prefilled with Preforma Plugs with Hole (20 mm x 40 mm) (Jiffy
133 Products International B.V., Hoek van Holland, Netherlands). The manufactured peat blend had the
134 following chemical characteristics: pH 6.2 [1:5 H_2O], CEC 5.81 cmol(+) kg^{-1}, EC 0.15 dS m^{-1} [1:5
135 H_2O], 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
136 weight per plug). Lentil seed were sown directly onto the pre-moistened peat plugs, just under the
137 peat surface, and germinated in the dark for 2-3 d. Trays were then transferred to 40 L plastic
138 containers (L x W x H: 690 x 466 x 178 mm) containing 20 L of tap water. At seven days after sowing,

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

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

153 **Transcriptome sequencing and SNP discovery**

154 Transcriptome sequencing of the lentil genotypes ILL2024 and ILL6788 was performed as described
155 in Sudheesh et al. (2016b). Briefly, lentil plants were grown in a glasshouse (20 °C ± 2 °C under a
156 16/8 hour light/dark photoperiod regime) and various tissue samples were harvested at different
157 growth stages (Sudheesh et al. 2016b). RNA extraction from pooled tissues was performed using the
158 RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) and the RNA-Seq libraries with an approximate
159 insert size of 350 bp were generated using the SureSelect Strand Specific RNA Library Prep Kit, both
160 kits used as per the manufacturer's instructions. Libraries were pair-end sequenced using the HiSeq
161 3000 system (Illumina Inc., San Diego, USA). The raw sequence data was filtered to remove adaptor
162 sequences and to exclude low quality or short reads as described in Sudheesh et al. (2016b).

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

170 **Genotyping, genetic linkage mapping, QTL analysis and validation**

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

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

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176 according to the manufacturer's instructions using 250 ng of template genomic DNA. The genotyping
177 assays were processed by the Illumina iScan reader. Automatic allele calling was achieved using the
178 Illumina Genome Studio software v2011.1. Additional SNP genotyping (200 SNPs) of the population
179 was performed using KASP™ genotyping chemistry (LGC Genomics, Middlesex, UK; Javid et al.
180 2015) using selected SNPs from previously published studies by Kaur et al. (2014) and Sharpe et al.
181 (2013) for map enrichment as well as for linkage group (LG) assignment.

182 A total of 516 EST-SSRs (Kaur et al. 2011) and 30 genomic DNA-derived SSRs (Hamwieh et al.
183 2005) were screened on the parents of the mapping population for polymorphism detection. Primer
184 synthesis and PCR amplifications were performed as described previously (Kaur et al. 2014;
185 Schuelke 2000). PCR products were combined with the ABI GeneScan LIZ500 size standard and
186 analysed using an ABI3730xl (Life Technologies Australia Pty Ltd, Victoria, Australia) capillary
187 electrophoresis platform according to the manufacturer's instructions. Allele sizes were scored using
188 the GeneMapper® 5 software package (Life Technologies Australia Pty Ltd).

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

195 QTL analysis was performed using marker regression, simple interval mapping (SIM) and composite
196 interval mapping (CIM) in QTL Cartographer v2.5 (Wang et al. 2012). An arbitrary LOD threshold of
197 2.5 was used to determine significance for SIM analysis. For CIM analysis, significance levels for LOD
198 thresholds were determined using 1,000 permutations.

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

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

207

208 Results

209 SNP discovery, genotyping and genetic linkage mapping

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

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212 sequences and exclude low quality or short reads, resulting in a high quality set of 42,289,495
213 (ILL2024) and 52,011,343 (ILL7688) paired-end reads, with an average read length of 120 bp. A
214 preliminary set of 26,639 SNPs polymorphic between ILL2024 and ILL6788 were selected from
215 comparison of transcriptome reads against the EST sequence database. Further selection based on a
216 criteria of homozygosity, quality (Q score =>30), depth (=>6 reads) and absence of other known
217 SNPs in close proximity (<35 bp), resulted in a sub-set of 938 high quality SNPs. Of these, 768 SNP
218 loci with a designability rank of >0.8 were selected for the GoldenGate® assay.

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

230 **Phenotypic assays for boron tolerance**

231 There was a high level of correlation (R^2 = 0.78) between tolerance to boron toxicity in soil and
232 hydroponic culture, as well as between repeated assays in hydroponic culture (Table 3). There was
233 homogeneous variation of residuals across the range of scores and so the assays did not always
234 precisely agree on necrosis symptoms of more tolerant lines (i.e. 0-20% necrosis), but it was sufficient
235 to separate tolerant lines of the phenotype derived from ILL2024. Scores for the tolerant check line
236 ILL2024 were closer to 0% necrosis. Phenotypic grouping of cultivars were performed on the following
237 scale: 0-12% foliar necrosis was classed as tolerant, 13-24% necrosis was intermediate tolerance and
238 25-100% necrosis was intolerant (equivalent to susceptible lentil check varieties).

239 **QTL analysis in ILL2024 × ILL6788 mapping population**

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

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

252 **Validation of B tolerance markers in the validation panel**

253 In the validation panel germplasm, 12% of lines exhibited a tolerant phenotype (0-12% foliar
254 necrosis), 41% had an intermediate level of tolerance (13-24% necrosis), and 47% were intolerant
255 (25-75% necrosis). Four flanking markers were used to genotype the validation panel, namely
256 SNP_20000246 and SNP_60000240 (Kaur et al. 2014) and Lc_0002502 and Lc_0002525 (Table 4).
257 Results indicated that the tolerant allele from ILL2024 was not common in the validation panel
258 germplasm, with only 15 and 18 lines having the tolerant allele for marker SNP_20000246 and
259 Lc_0002525, respectively. In addition, these genetic markers were not reliable predictors of the B
260 tolerance phenotype across diverse lentil germplasm.

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

272 **Validation of B tolerance markers in ILL2024 progeny**

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

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

283

284 **Discussion**

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

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

292 **Genetic mapping, QTL analysis and marker validation**

293 High density genetic linkage maps provide an essential tool for plant genetic research, including the
294 anchoring and assembling scaffolds for genome assembly, facilitating QTL identification, marker-
295 assisted selection and map based cloning. The ILL2024 × ILL6788 linkage map exhibited a regular
296 marker distribution with a higher number of map assigned markers, 758 (average inter-locus interval 2
297 cM), in comparison to previously published lentil gene-based linkage maps: 'CDC Robin' × 964a-46,
298 546 markers (Sharpe et al. 2013); ILL2024 × 'Cassab', 318 markers (Kaur et al. 2014); 'Indianhead' ×
299 'Northfield', 460 markers; 'Indianhead' × 'Digger', 329 markers, and 'Northfield' × 'Digger', 330
300 markers (Sudheesh et al. 2016a).

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

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

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324 The whole genome sequencing data available was also used to assist in the identification of potential
325 candidate genes at this locus, which would enable the development of a diagnostic marker. Kaur et al.
326 (2014) discussed the aim to find a candidate B transporter gene like those isolated in *Arabidopsis*
327 (Miwa et al. 2014; Miwa and Fujiwara 2011). Studies in *Arabidopsis*, wheat, rice and *M. truncatula*
328 have revealed genes for efflux-type B transporters and major intrinsic protein (MIP) family controlling
329 B toxicity tolerance (Bogacki et al. 2013; Takano et al. 2002; Takano et al. 2006). At the genomic
330 location on LcChr2, one candidate was a lentil MIP gene (Lc09014) located in proximity (790 Kb) to
331 one of the QTL flanking SNP markers (Lc_0002525). This is the only sequence within the draft
332 genome that could be implicated with having a role in B tolerance, however, given the aforementioned
333 likely chromosome rearrangements, it is possible the landrace donor has different local sequences
334 than the reference genome (KnowPulse 2017).

335 **Application of B markers in lentil breeding**

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

347 The characterisation of the validation panel enabled identification of material for which the association
348 between the B tolerance marker and phenotype was consistent with ILL2024. Hobson et al. (2006)
349 found two landraces to have the highest level of B tolerance from a collection of 310 lentil accessions:
350 ILL2024, the source of B tolerance discussed in this paper, which originated from Ethiopia, and
351 ILL0213A, with a similar level of tolerance that was collected from Afghanistan. Genotyping revealed
352 that there is a likely genetic relationship between these landraces (unpublished data), with both
353 sharing the tolerant allele at all four SNP loci tested. In addition, the breeding line, 05H010L-
354 07HS3010 inherited the tolerant locus from its grandparent, ILL2024, and is an important parent of
355 germplasm in the ILL2024-derived progeny.

356 There were three lines in which the markers Lc_0002525 and SNP_20000246 did not co-segregate,
357 having the Lc_0002525 tolerant allele only. These were, ILL0518 (syn. PI 320951), a virus and rust
358 resistant landrace used as a parent in Australia, USSR-05-05 (syn. PI 577149), a lentil landrace from
359 Tajikistan, and cv. 'Emerald' (syn. PI 508091; Muehlbauer 1987), a USA green lentil. In addition, PI
360 572351 (syn. Ladizinsky 66 or Hoffman #27; Norris 1994) a lentil accession collected in Yugoslavia
361 (southern Bosnia and Herzegovina), was highly tolerant to B and had the SNP_60000240 allele, but
362 genotyping for Lc_0002525 was not performed.

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

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

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

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

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

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

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

416

417 **Competing Interests**

418 The authors declare that they have no competing interests.

419

420 **Author's Contributions**

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

428

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576 **Fig. 1** Scatter-plot of comparison between phenotypic screening in the potted soil experiment and a
577 simple hydroponic assay (2015) for a breeding germplasm set containing a proportion of their genetic
578 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

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

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

580

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 581

	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

582

583

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

584

585

586

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

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

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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

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

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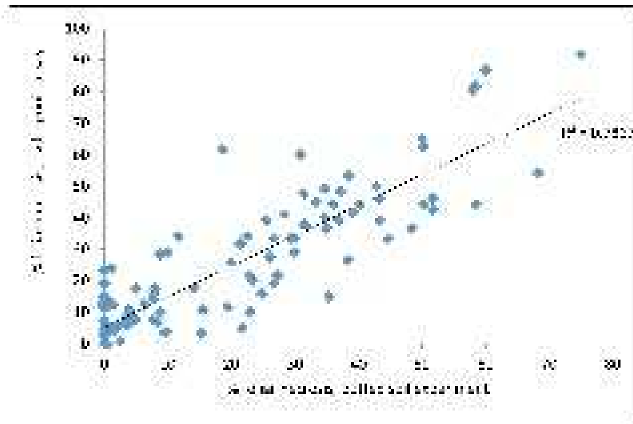

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