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Title

Quantitative trait loci for reducing sugar concentration in the barley root tip under boron toxicity

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Introduction

Boron (B) is an essential trace element for higher plants, but when present in the root zone at high concentrations it becomes a limitation for plant growth, leading to a subsequent reduction in crop yield (Cartwright *et al.*, 1986; Nable *et al.*, 1997; Stangoulis and Reid, 2002 and references therein). Tolerant plants, however, have the ability to sustain their growth under high B condition. Their notable responses to B toxicity include maintained root elongation and reduced leaf necrosis (Paull, 1990; Jenkin, 1993; Choi, 2004). To date physiological mechanisms leading to such phenotypic responses have not been fully understood although recent evidence suggests active B efflux as a likely B tolerance mechanism (Hayes and Reid, 2004; Sutton *et al.*, 2007).

The site of B toxicity in plant roots has recently been identified as located in the root tip (Reid *et al.*, 2004), and this is most likely associated with adverse effects on mitosis (Klein and Brown, 1981; Liu *et al.*, 2000). Recently, Choi *et al.* (2007) have observed a low concentration of reducing sugars (RS) within the root tip of the B intolerant barley cultivar Clipper when grown under B toxicity while in the B tolerant genotype Sahara 3771 and its backcross derivative SloopVic, RS concentration increased as part of their tolerance mechanism. This change in carbohydrate metabolism would support root development and thus maintain plant growth under B toxicity. Genome regions associated with this new boron tolerance-related trait have not been reported. In this study, we aimed to detect and map quantitative trait loci (QTL) affecting RS concentration in the root tip of barley at high B supply using a population derived from a cross between Clipper and Sahara 3771.

Material and method

Genetic material

The genetic material used in this mapping experiment was a population of 146 doubled haploid (DH) lines derived from a cross between the B tolerant Algerian landrace Sahara 3771 and the B sensitive Australian cultivar Clipper. The population was produced by the *Hordeum bulbosum* method described by Islam and Shepherd (1981), using embryo culture followed by chromosome doubling through colchicine treatment.

Solution culture

Two parents and DH lines were grown together in the same container. Seeds were washed with a 70% ethanol solution for 1 minute followed by sodium hypochlorite solution (NaClO, 1% Cl) for 5 minutes. After being rinsed with RO water, the seeds were pre-germinated on moist filter paper in glass petri dishes with 4 ml of distilled water at 20°C for 2 days in the dark. Two evenly germinated seeds were transplanted and thinning was then carried out to leave one healthy plant per line.

The plants were grown in aerated quarter-strength Hoagland's solution that consisted of the following nutrients: Ca(NO₃)₂, 1.25 mM; KNO₃, 1.25 mM; MgSO₄, 0.5 mM; KH₂PO₄, 0.25 mM; MnSO₄, 5.9 µM; ZnSO₄, 8.0 µM; CuSO₄, 0.01 µM; NaMoO₄, 0.01 µM; KCl, 0.13 µM; FeEDTA, 20 µM; B(OH)₃, 15 µM. The solution pH was adjusted to 6.0 with KOH and the solution was replaced every 3 days. Boron treatment of 3000 µM B (as boric acid) was applied 1 week after transplanting. Plants were grown in a growth chamber at 20/10°C day/night

temperature and 14/10h day/night photoperiod with light supplied at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR by high pressure sodium lamps (Philips SON-T Agro 400W, Philips, Osnabrück, Germany).

Root tips were harvested on the 4th day of B treatment when the change in the RS concentration occurred (Choi et al., 2007). Leaf symptom was measured at harvesting based on a scale of 1-6, where 1 gave no visual symptom and 6 showed greater than 90% necrotic areas on the oldest leaf. Harvested plants were quickly rinsed in high purity water (18 $\text{M}\Omega \text{cm}^{-1}$ resistivity) for 10 s and quickly blotted dry with tissue paper. Tips of main roots were cut at 1 cm in length, kept in Eppendorf[®] tubes stored in liquid nitrogen during harvesting time, and then dried in a Virtis[®] automatic freeze drier for 24 hours. The experiment was replicated 6 times staggered at 1-day intervals. Root tips from three successive replicates for the same line per treatment were bulked for reducing sugar assay. For data analysis, the experiment was considered as a randomized complete block design with two replications.

Reducing sugar assay

The procedure for RS analysis was described in Choi *et al.* (2007). In brief, freeze-dried samples were weighed and then diluted in a 1.5ml Eppendorf[®] tube with 450 μl nanopure water ($>18 \text{M}\Omega \text{cm}^{-1}$ resistivity), shaken on an agitator platform for 10 minutes and centrifuged at 8000 rpm for another 10 min. All supernatant was collected, placed in a fresh 1.5 ml Eppendorf[®] tube and centrifuged again at 8000 rpm for 10 min, then stored at 4 °C. The top 200 μl of supernatant from the Eppendorf[®] tube was collected and diluted to 300 μl with nanopure water with the addition of 0.5 ml 2% DNS reagent (*3,5-Dinitrosalicylic acid*) in a 0.7 N NaOH solution. All samples were then mixed by vortexing for five seconds and then kept on ice for a further 10 min prior to transferring to a boiling water bath for 5 min, after which samples were immediately transferred to an iced water bath for a further 10 min. Reducing sugars were spectrophotometrically measured at 590 nm with comparison to glucose standards.

Genetic mapping

A genetic map of 420 molecular markers covering seven barley chromosomes was used for mapping. This map was improved based on a framework map of 211 markers reported in Karakousis *et al.* (2003). QTL mapping was performed with QTLNetwork 2.0 (Yang *et al.*, 2008) using mixed linear composite interval mapping (Yang *et al.*, 2007). Significance testing was based on the *F*-test using Henderson method III (Searle *et al.*, 1992), with the threshold ($F = 16.5$) corresponding to the 0.01 probability level defined using 1000 permutations.

Results and discussion

Under B toxicity, the concentration of reducing sugars (RS) in the root tip varied widely within the Clipper/Sahara 3771, ranging from 14 to 141 mg g^{-1} (Fig. 1A). Sahara 3771 had higher RS concentrations than Clipper. Large genotypic variation was also observed for leaf symptom, with Clipper exhibiting more severe leaf necrosis than Sahara 3771 (Fig. 1B), and this trait was negatively correlated with root RS level ($r = -0.30$, $P < 0.001$). Transgressive segregation was observed for both traits, indicating that they might be under complex genetic control.

Four QTLs were detected for RS concentration, located on chromosomes 2H (*QRsc.ClSa-2H*), 3H (*QRsc.ClSa-3H*), 5H (*QRsc.ClSa-5H*) and 6H (*QRsc.ClSa-6H*), explaining 16%, 12%, 12% and 9% of the total phenotypic variance, respectively (Table 1 and Fig. 2). At *QRsc.ClSa-2H*,

QRsc.CISa-5H and *QRsc.CISa-6H*, the (low concentration) alleles contributed from Clipper, while at *QRsc.CISa-3H*, the (low concentration) alleles contributed from Sahara, providing a genetic explanation (transgressive segregation) for the presence of lines with lower RS concentration than Clipper (Figure 1).

Leaf symptom score had two QTLs on 2H and 4H (*QLss.CISa-2H* and *QLss.CISa-4H*), explaining 13% and 12% of total phenotypic variance, respectively, with (low score) alleles contributed from Sahara 3771 (Table 1 and Fig. 2). Both QTLs were located in the same regions as reported previously for a soil-based glasshouse experiment (Jefferies *et al.*, 1999). *QLss.CISa-2H* was also co-located with RS QTL (*QRsc.CISa-2H*) detected here.

Other QTLs for root RS were also co-located with QTLs for other B tolerance traits reported in Jefferies *et al.* (1999). *QRsc.CISa-6H* was located in the 6H region associated with B uptake. *QRsc.CISa-3H* was located in the 3H region associated with relative root length and *QRsc.CISa-5H* was near the 5H markers associated with root elongation per se. Unfortunately, at *QRsc.CISa-3H*, Sahara 3771 carries the unfavorable (i.e., low concentration) allele, while at the adjacent loci for root elongation under boron stress (*awbma15*), Sahara 3771 carries the favorable allele (Jefferies *et al.*, 1999). Marker-assisted selection would enable selection for both favorable alleles.

The presence of multiple QTLs for RS and their association with B tolerance traits provide explanations, at the molecular level, for the role of RS in B tolerance. It is known that the increase in the level of RS in the root tip is a result of more than one physiological mechanism but is strongly associated with source-sink relationships. Enhanced sucrose concentrations were observed in the leaf tissue of the B tolerant Sahara 3771 (Choi *et al.*, 2007) and this may contribute to the supply of extra sugar to the root tip of this genotype. The range of QTLs found for RS may be an indication of differing roles in various physiological pathways. Because the 2H QTLs for root tip RS were co-located with the leaf symptom QTL, it may be associated with maintenance of photosynthesis in the shoot. In contrast, the 3H and 5H QTLs may involve genes expressed in the root because they were co-located with those found in a solution-culture root length assay (Jefferies *et al.* 1999) for relative root length under B stress and root length per se respectively. This screening was carried out in the dark, so root elongation was independent of current photosynthesis and presumably used carbohydrates from the seed reserves. Fine mapping of these regions may identify candidate genes involved in carbohydrate metabolisms associated with such B tolerance pathways.

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Table 1. Quantitative trait loci for root tip reducing sugar concentration and leaf symptom score measured on the Clipper x Sahara 3771 DH population grown on solution at high B supply (3000 μM).

QTL	Chromosome	Nearest marker	Position	Support interval	<i>F</i> -value ¹	Additive main effect ($P < 0.001$) ²	Phenotypic variation explained (%)
Reducing sugar (mg g^{-1})							
<i>QRsc.CISa-2H</i>	2H	<i>awbma27</i>	57.1	48.1 - 66.8	27.8	8.584	15.5
<i>QRsc.CISa-3H</i>	3H	<i>Bmag6</i>	60.9	53.4 - 68.4	24.4	-9.307	11.5
<i>QRsc.CISa-5H</i>	5H	<i>HVPTR15_4</i>	47.6	44.1 - 53.3	21.8	7.902	11.6
<i>QRsc.CISa-6H</i>	6H	<i>Amy1</i>	77.2	70.1 - 82.7	18.5	8.125	9.3
Leaf symptom score (1 - 6)							
<i>QLss.CISa-2H</i>	2H	<i>wg222d</i>	71.0	67.8 - 76.0	27.8	-0.422	12.7
<i>QLss.CISa-4H</i>	4H	<i>cdo1312</i>	98.6	93.5 - 105.5	24.4	-0.412	11.7

¹ *F*-statistics of the peaks, with the significance threshold ($P = 0.01$) is 16.5.

² A positive effect indicates that the allele from Sahara 3771 contributes to higher trait values, while a negative effect indicates that the allele from Clipper contributes to higher trait values.

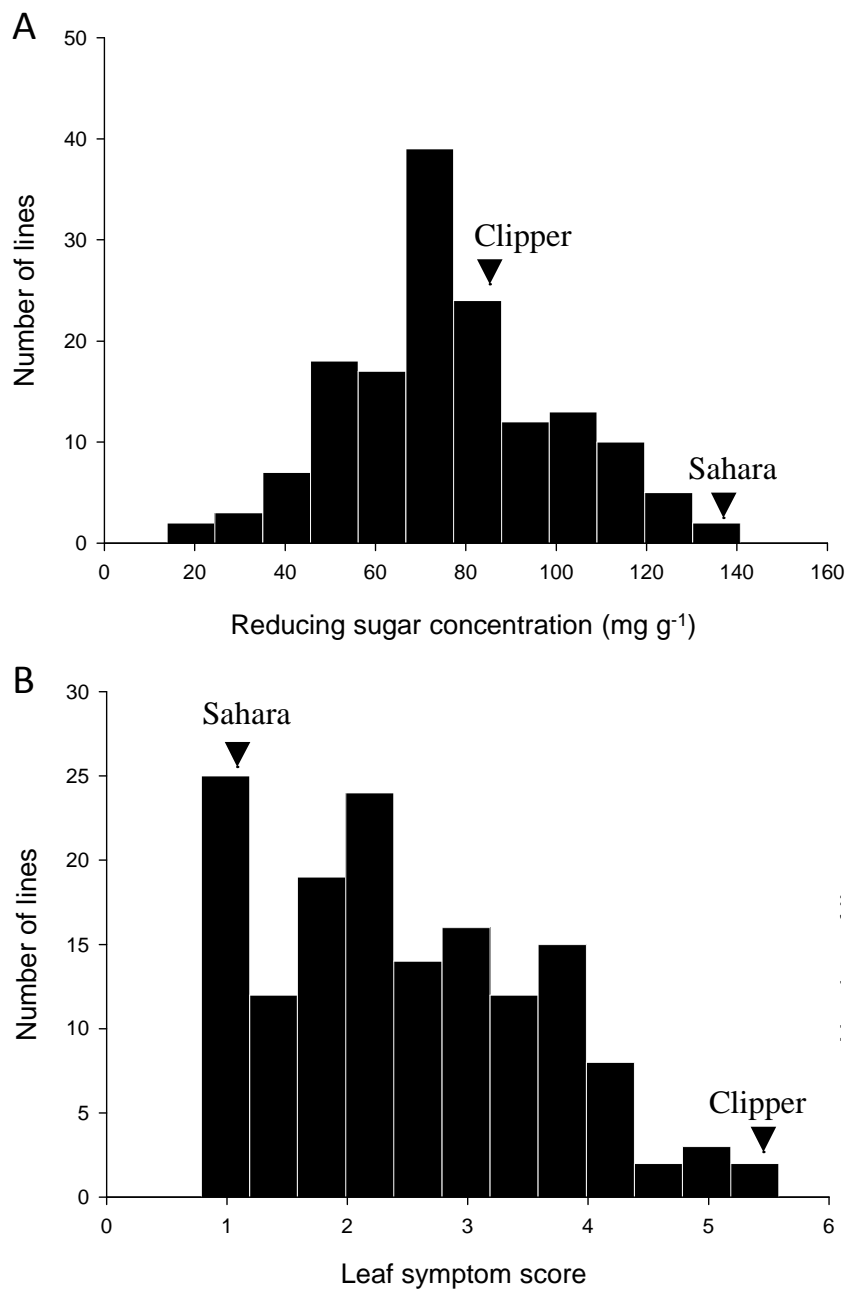


Figure 1. Variation in (A) the reducing sugar concentration in the root tip and (B) leaf symptom score measured on the Clipper x Sahara 3771 DH population grown on solution at high B supply (3000 μ M).

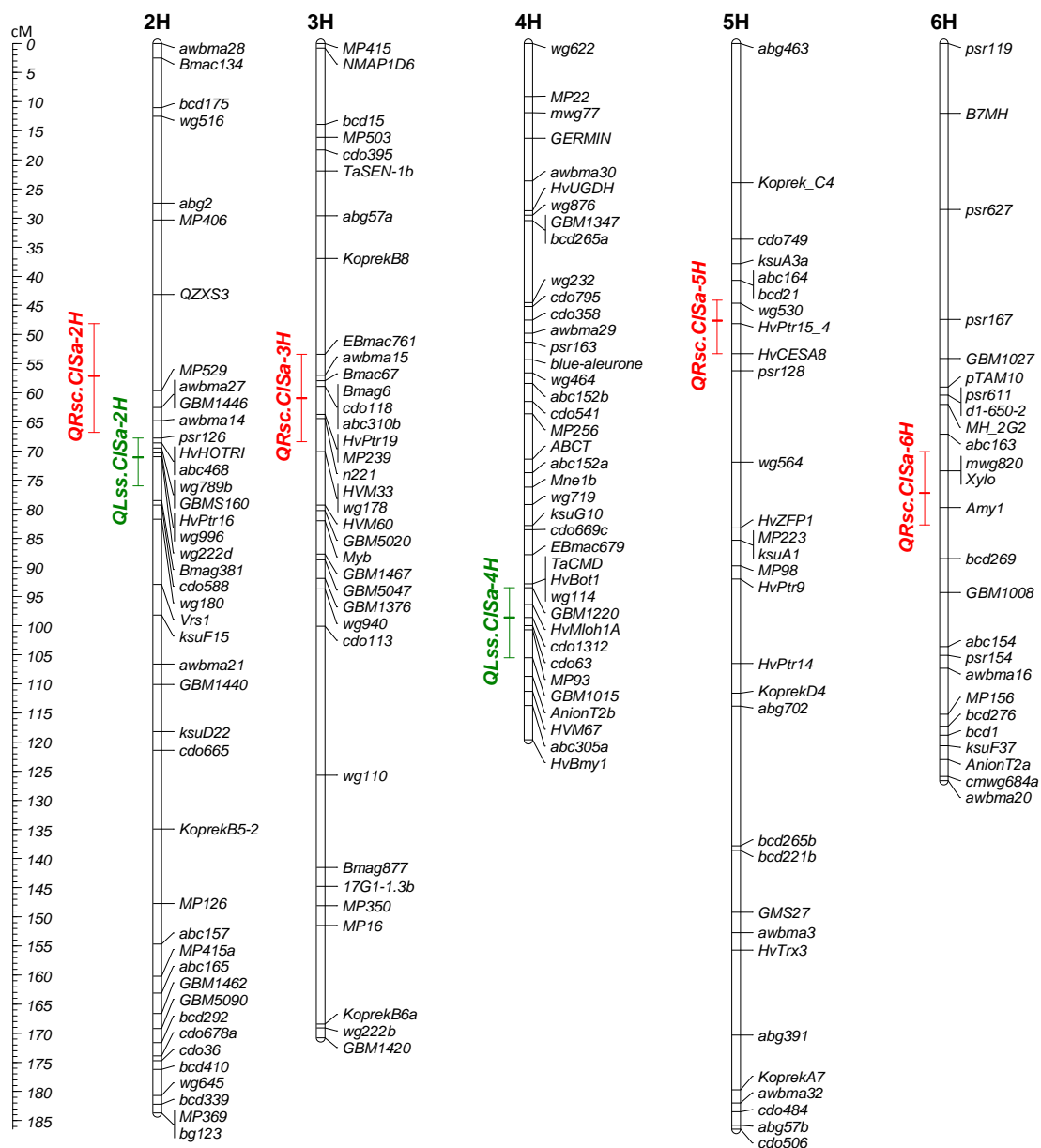


Figure 2. Chromosome locations of regions associated with the reducing sugar concentration in the root tip (in red) and leaf symptom (in green) expressed in the Clipper x Sahara 3771 doubled-haploid population grown on solution at high B supply (3000 μM). Some co-segregating markers were removed to reduce image complexity.