Comparison of manual, semi-automated and automated phenotyping techniques for mapping quantitative trait loci for boron/germanium tolerance in barley

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The project

Quantitative trait loci (QTL) for boron and germanium tolerance in barley can be mapped using quantitative non-destructive imaging techniques. Such mapping was originally carried out using conventional subjective end-point phenotyping to identify QTL for boron tolerance by Jefferies et al (1). More recently, a second experiment was independently performed by Julie Hayes and colleagues at the Australian Centre for Plant Functional Genomics (ACPFG) (2) on the same mapping population, using germanium instead of boron, and using LemnaTec scanning systems similar to those at The Plant Accelerator.

Jefferies's experiment identified four boron-tolerance QTL, corresponding to different parameters used to measure the phenotypic response to boron toxicity. A single phenotype was scored for Hayes's toxicity experiment, and the data reconfirmed the presence of both of the previously identified QTL relevant to this phenotypic response. In this report, some of the potential benefits likely be achieved using The Plant Accelerator are described, including much lower labour requirements and the detection of QTL for other, more complex, tolerance responses.

Background

Boron is an essential plant micronutrient (3), but becomes toxic to plants when present in soils at higher concentrations (>1-2 ppm) (4). Levels of boron sufficient to cause significant reduction in cereal crop yields occur in areas of southern Australia (5), as well as parts of west Asia and north Africa (6). Genetic variability for boron tolerance exists in barley, with the two varieties in this mapping project, Sahara 3771 (Sahara) and Clipper, having relatively high and low boron tolerance respectively.

There are multiple aspects of plant physiology which may contribute to boron tolerance; these include tissue exclusion, tissue tolerance and changes in root morphology. In barley the most obvious symptoms of boron toxicity are chlorosis and necrosis extending from leaf tips (1), see Figure 1, although unfortunately these symptoms are not necessarily correlated with tolerance, as characterised by yield of the plant under boron stress (7). There are many interacting agronomic factors that influence crop yield.

Figure 1. *14 day Clipper and Sahara barley seedlings showing leaf symptoms of boron toxicity. The difference in sensitivity of the two varieties is clearly apparent. Germanium leaf toxicity symptoms are not substantially different from the boron symptoms shown here. (Courtesy of Julie Hayes, ACPFG (11)).*

Germanium is chemically similar to boron, as well as to silicon; unlike boron however, it is not considered to be an essential micronutrient for plant growth, nor does it naturally occur in sufficient quantities to inhibit plant growth. However, its chemical properties mean that it is often used as an analogue to silicon in plant silicon uptake studies (8). Germanium is also transported by a known boron transporter protein (HvNIP2;1) in barley (9). This suggested that at phytotoxic concentrations it might cause similar symptoms to boron, and perhaps be associated with the same tolerance QTL originally mapped by Jefferies et al.

The mapping population used for the experiments described here was derived from a cross between the boron-sensitive Australian malt barley cultivar Clipper and the boron-tolerant Algerian landrace Sahara 3771 (10). The report compares three approaches to mapping boron/germanium tolerance in barley using this population: a conventional project using end-point visual assessment as well as destructive phenotyping (1); a semi-automated project using destructive end-point testing with LemnaTec shoot scanning and image analysis (2); and a hypothetical fully-automated project with The Plant Accelerator's LemnaTec system.

Experimental design

The initial mapping project of Jefferies screened for four different phenotypes: solution culture relative root length, necrotic leaf damage, tissue boron concentration and dry matter production. However, even with the advanced imaging systems at The Plant Accelerator, tissue concentration can only be performed destructively, so will not be considered further in this comparison. The other three parameters of root length, leaf damage and biomass production can all be measured, or at least accurately inferred, from imaging data obtainable at The Plant Accelerator. Only leaf symptoms were measured for Hayes's germanium toxicity assay.

Conventional phenotyping

Relative root length

Two replicates of each of the 150 lines in the mapping library were germinated on filter papers soaked in either control or 100mg/l boron and grown upright in the rolled papers covered in foil for 12 days before measuring of the longest root. Relative root length values (RRL) were calculated as the root length at 100 mg/l expressed as a percentage of the root length at B 0 mg/l.

Leaf damage

Two replicates of each line were planted in control and boron-supplemented (100mg/kg) soil. After four weeks each plant was scored for leaf necrosis on a scale of 1 (no visual symptoms) to 6 (>90% necrosis)

Biomass

One week after scoring of leaf damage, plants were harvested 1cm above ground level, dried and weighed.

For full details about the phenotyping assays, refer to Jefferies et al, 1999 (1).

Semi-automated phenotyping

Leaf damage

3-4 seedlings of each line were grown in hydroponics for five days. They were then treated with 40µM GeO_2 and grown for a further nine days. The seedlings were removed, laid horizontally, and a single image taken from above using a LemnaTec Scanalyzer 3D. Image analysis thresholds were set to record the percentage necrotic tissue visible in each image. At the same time as scanning, each seedling was also visually assessed and scored for the degree of necrosis. Since this assessment was performed at the time of imaging, before image analysis when the percentage of necrosis was calculated, there was no potential for confirmation bias in the assigned scores.

Plant Accelerator phenotyping (hypothetical)

Leaf damage, projected shoot area (proxy for biomass)

Two replicates of each of the 150 lines would be planted and treated with boron as per the conventional phenotyping experiment. These plants would be grown in standard greenhouses and imaged once at the end of the project (after 5 weeks). Leaf area would be captured from three directions by the visible light cameras and extrapolated into a prediction of biomass, while percentage necrosis could be recorded either by colour-thresholding of visible light images or by the measurement of chlorophyll health using fluorescence imaging.

Relative root length

Duplication of the root length experiment would require the setting up of additional, non-boron-treated, controls for each line, since this phenotype is a relative rather than an absolute value. Both the controls and the test plants would be planted in The Plant Accelerator's root observation pots (transparent, flat-sided pots 400mm high by 120mm wide and 40mm deep) and visible light imaging used to measure root length which can be seen through the side of the transparent pot. Note that these plants would replace those described above for the biomass and necrosis measurements, and are not additional to

them. However, an additional earlier imaging run may be required to record root lengths before they reach the pot extremities.

N.B. This comparison describes the use of replicates for each line. Statistical analysis of other Plant Accelerator projects has indicated that for some mapping projects, replication may be required for as few as 20% of lines, with all others being grown singly.

Outcomes

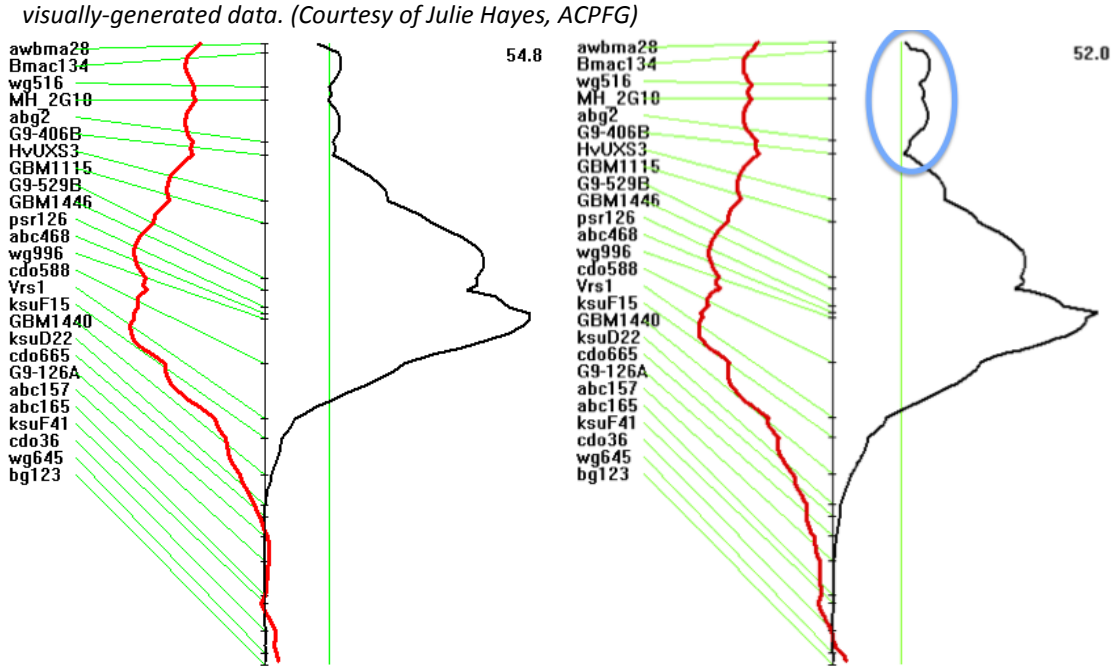
The conventional experiment identified four QTL that were associated with parameters used to measure boron tolerance; one for leaf symptoms on chromosome 2H, one for relative root growth (chromosome 3H), one for boron tissue concentration (6H), and one for all four phenotypes measured (the fourth being biomass) on chromosome 4H. This was mostly consistent with previous data that had suggested three major gene loci contributing to boron tolerance (7).

The manual imaging-based experiment re-discovered two of the QTL that had been detected with the initial boron-tolerance experiment, namely the QTL for leaf symptoms (2H) and the one for boron concentration (6H). Both of these were slightly surprising, although in different ways. The candidate gene for the 6H QTL (the boron transporter *HvNIP2;1*) had been shown by the researchers to also transport germanium; however, this QTL had not been linked to leaf symptoms in the earlier study. On the other hand, the 2H QTL was known to be associated with leaf symptoms, but there was no previous evidence to suggest that it would be important for non-boron stresses. Also interestingly, this project did not find the 4H QTL, the strongest of those identified by Jefferies. The candidate gene for this QTL is a borate transporter, which seems to be entirely boron-specific, unlike the boron/germanium cross-activity of the other two QTL.

There was very good agreement between the QTL traces based on image analysis of the seedlings and the visual scoring. Only one minor difference is apparent, at a small second peak above the major peak (Figure 2). Under this region is a gene controlling flowering time and which may have affected seedling development rate and leaf size/morphology in the lines. The minor 'peak' is slightly larger for the visual scoring analysis, suggesting that visual assessment may have been more influenced by maturity differences that were in reality not affected by germanium treatment.

Of course, no data exists for the hypothetical Plant Accelerator experiment, but there seems every likelihood that it would also identify three of the QTL, and perhaps even the 6H QTL (which was associated with leaf symptoms in the second experiment, but only with tissue concentration requiring destructive testing in the first). However, it is quite possible that additional QTL would be identified in this way; the higher accuracy and fully objective scoring of leaf damage possible with image analysis measurements may help to identify genes of small effect, while the recording of other parameters such as leaf area (a proxy for biomass) may identify other boron-influenced QTL.

Figure 2. *2H QTL for leaf symptoms to germanium using LemnaTec scanned image analysis (left) and visual scoring (right). The circled region highlights the minor secondary QTL, which was more pronounced in the visually-generated data. (Courtesy of Julie Hayes, ACPFG)*



Benefits of The Plant Accelerator

Even for a single time point comparison such as described here, The Plant Accelerator offers distinct advantages. Jefferies describes both the root length and leaf damage assays as 'time-consuming' and the latter as 'highly subjective' while Julie Hayes commented "It took me two days to do the imaging – of course, if I'd used the new automated system you have, it would have been a lot less labour-intensive". It was also Julie herself who pointed out the possible subjectivity of her visual assay resulting in the larger secondary QTL peak. These issues would have been much relieved with the use of The Plant Accelerator; at the same time, the great range of other measurements recorded may well have led to the discovery of further boron-tolerance response QTL.

However, where The Plant Accelerator would come into its own would be the investigation of time-dependent responses to toxic levels of boron or germanium. Root length, tissue damage and leaf area could all be recorded every other day and QTL found which are responsible for changes in aspects of boron/germanium tolerance over time. It is likely that this more accurate and detailed information would also much more readily discover specific differences between germanium and boron tolerance responses.

Other points to note

- For genetically simple traits (as boron tolerance appears to be, with only around four gene loci of major effect), there are still advantages of The Plant Accelerator over conventional visual scoring, but they are perhaps not as pronounced as is the case for more complex traits where a continuum of phenotypic expression might occur.

- Estimated costs for all three experiments described here are very comparable, being in the region of $1,000 for the leaf symptom assay only (not including the mapping population construction nor the statistical analysis after scoring, but taking into account labour, consumables and greenhouse fees). However, adding in biomass using The Plant Accelerator comes at no additional cost, while it perhaps more than doubles the other two protocols. Inclusion of relative root length measurement increases the cost of The Plant Accelerator project by around $1,000, probably a similar amount to performing it manually.

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