Assessing the variation in Manganese Use Efficiency Traits in Scottish Barley Landrace Bere (*Hordeum vulgare* L.)

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- **Background and Aims** Manganese (Mn) deficiency in barley is a global problem. It is difficult to detect in the early stages of symptom development and is commonly pre-emptively corrected by Mn foliar sprays that can be costly. Landraces adapted to marginal lands around the world represent a genetic resource for potential sustainability traits including mineral use efficiency. This research aims to confirm novel Mn use efficiency traits from the Scottish landrace Bere and use an association mapping approach to identify genetic loci associated with the trait.
- **Methods** A hydroponic system was developed to identify and characterise the Mn deficiency tolerance traits in a collection of landraces, including a large number of Scottish Bere barleys, a group of 6-rowed heritage landraces grown in the highlands and islands of Scotland. Measuring chlorophyll fluorescence, the effect of Mn deficiency was identified in the early stages of development. Genotypic data, generated using the 50k Illumina iSelect genotyping array, was coupled with the Mn phenotypic data to create a genome-wide association study (GWAS) identifying candidate loci associated with Mn use efficiency.
- **Key Results** The Bere lines generally had good Mn use efficiency traits. Individual Bere lines showed large efficiencies, with some Bere lines recording almost double chlorophyll fluorescence readings in limited Mn conditions compared to the elite cultivar Scholar. The Mn efficient Bere lines had increased accumulation of Mn in their shoot biomass compared with elite cultivars, which was highly correlated to the chlorophyll fluorescence. Several candidate genes were identified as being associated with Mn use efficiency in the GWAS.

- **Conclusions** Several genomic regions for Mn use efficiency traits originating from the Bere lines were identified. Further examination and validation of these regions should be undertaken to identify candidate genes for future breeding for marginal lands.

Key words: Barley landraces, *Hordeum vulgare*, Bere barley, genetic diversity, micronutrients, nutrient use efficiency, manganese, sustainable agriculture.

INTRODUCTION

To help tackle the challenges of food security (Nelson et al., 2010), more resilient crops that can adapt to increasingly variable environments associated with climate change and the need to use more marginal land need to be developed, they need to be particularly adapted to fluctuations in temperature, light, water, CO_2 and nutrient availability (Sinclair, 1992, Koski, 1996, Newton et al., 2011, Hatfield et al., 2014). A valuable source of resilience traits in crop plants are landraces, distinct but heterogeneous populations that are maintained through continuous selection and multiplication within specific environments, often comprising marginal climates and soils, and alternative cultivation techniques (Fischbeck, 2003). Elite cultivars are unlikely to outperform them in such environments, as they are not adapted to these environments, and thus it is not economically viable to replace them (Abera, 2009, Yahiaoui et al., 2014). This diversity of genetic material offers a substantial genetic resource for breeders, particularly for increased nutrient uptake and efficiency traits, improved human nutrition through accumulation of antioxidants, a range of sources of resistance for combatting both biotic and abiotic stress, and characters useful for low input agriculture. Thus this material is valuable for breeding to increase yield in the harsh agro-ecological and climatic conditions or marginal land (Newton et al., 2010). The landrace of barley with the longest continuous production of any in the UK is the Scottish landrace 'Bere'; which has been grown predominately on marginal land for the last half millennia, and is currently grown in isolated populations on the western and northern islands of Scotland (Jarman, 1996). The soil conditions in the regions that Bere barley grows vary widely, with many areas supporting crops on highly alkaline soils (Martin et al., 2008). This resource from such diverse environments offers a promising genetic resource for breeding micronutrient efficient barley for all environments but especially marginal ones (Schmidt et al., 2019).

Limitation of nutrients in a plant system can often cause permanent damage causing the plant to use other resources less efficiently, resulting in loss of yield (van Maarschalkerweerd and Husted, 2015, Schmidt et al., 2016a). One of the eight essential micronutrients needed for plant growth is Mn (White and Brown, 2010). Whilst barley displays a greater Mn use efficiency than other temperate cereal crops (Marcar and Graham, 1987) lack of available Mn still causes major problems in barley agronomy (Steenbjerg, 1935, Graham et al., 1982, Goldberg et al., 1983, Reuter et al., 1988).

In plant systems Mn plays an important role in the function of multiple enzymes and other proteins. Manganese has a crucial role as a catalytically active metal in the photosystem II (PSII) oxygen evolving complex (OEC) within chlorophyll, where it catalyses the water-splitting reaction (Schmidt et al., 2015). Important roles for Mn are known in approximately 35 enzymes in total (Hänsch and Mendel, 2009, Williams and Pittman, 2010), of which three cannot replace the Mn component (Burnell, 1988), including: Mn superoxide dismutase (Bowler et al., 1991, Poage et al., 2011), oxalate oxidase (Requena and Bornemann, 1999), and the catalytical Mn cluster (Schmidt et al., 2016b) in the OEC of photosystem II mentioned above (Ono et al., 1992, Barber, 2004). Manganese deficiency has been shown to cause a considerable reduction in PSII supercomplex quantity (Schmidt et al., 2015), whilst retaining OEC protein sub-units such as PsbP and PsbQ (Schmidt et al., 2016b).

Manganese deficiency in course textured sandy soils occurs due to limited Mn content caused by leaching; whereas deficiency in alkaline and calcareous soils is caused by reduced Mn availability due to it being present in the bound Mn(III) and Mn(IV) forms as oxides and dioxides. In addition, soils with large organic matter contents have limited Mn availability due to organic chelating agents forming insoluble complexes with the Mn(II) (Tisdale and Nelson, 1956, White and Greenwood, 2013). Chemical correction of Mn deficiency in the soil is limited as Mn fertiliser addition to soil is inefficient due to the conversion of the applied Mn into Mn oxides. Foliar application has been shown to be more effective but has a significant cost that makes it unaffordable to many farmers growing on deficient soils. The best results of fertilisation are seen when both soil and foliar fertilisers are used in combination (Reuter et al., 1973, Pallotta et al., 2000).

Considerable variability between barley genotypes has been shown to occur in high-affinity Mn influx kinetics resulting in differing Mn efficiencies between genotypes (Pedas et al., 2005). To date only one plasma membrane-localised Mn^{2+} transporter protein encoding gene has been identified in barley – Iron Regulated Transporter 1 (*Hv*IRT1). Pedas et al. (2008) demonstrated that the HvIRT1 gene was up-regulated in Mn deficient soils, with up to 40% greater expression than in Fe-deficient soils; thus, it could be an important factor in breeding for Mn efficient barley. Whilst the differences in the high-affinity Mn influx kinetics observed by Pedas et al. (2005) have been shown not to be due to genetic differences in the IRT1, it has been suggested that they could still be due to different isoforms Mn transporters rather than the level of expression (Pedas et al., 2008). Schmidt et al. (2016a) also suggested that plants do not rely on a single mechanism of Mn transport for uptake. Physiological difference may also help account for increased Mn use efficiency as it is possible that root length and architecture, together with rhizosphere processes, have an effect on Mn accumulation. For example, the production of fine root hairs seen in Alfalfa (Gherardi and Rengel, 2004) and production of Mn-deficiency activated phytase exudation in barley (George et al., 2014) have both been suggested to improve Mn-acquisition. Studies by George et al. (2014) and Schmidt et al. (2018) have shown that Bere lines maintain optimal growth in low Mn environments and therefore these and potentially other Bere landraces offer a promising source of Mn use efficiency genes for breeding.

The aim of this investigation was to screen individual lines for Mn-efficiency within a landrace collection to identify those that could provide breeding material to increase Mn use efficiency. Chlorophyll fluorescence was used to measure Mn use efficiency in barley as it has been shown to correlate strongly with Mn-dependant winter survival, grain and leaf Mn concentration, and grain yields (Schmidt et al., 2013) as well as other crops (Adams et al., 1993, Val et al., 1995). This measurement has been used successfully to assess Mn use efficiency in barley (George et al., 2014, Stoltz and Wallenhammar, 2014, Leplat et al., 2016, Schmidt et al., 2018) and other crops (Chatzistathis et al., 2017). A genome-wide association study (GWAS) was used to identify genetic loci associated with these Mn use efficiency traits.

MATERIALS AND METHODS

Landrace bulking

A total of 130 lines (**Error! Reference source not found.**) from The James Hutton Institute (Dundee, UK) Spring Barley Landrace Collection were selected, with seeds predominantly originating from the JIC-GRU (John Innes Centre Germplasm Resources Unit) or SASA (Scottish Agricultural Science Agency) collections. Due to the length of the labelling some lines were given a shorthand (**Error! Reference source not found.**).

The lines were bulked, along with ten elite cultivars (Belgravia, Concerto, KWS Irina, Odyssey, Optic, Propino, RGT Planet, Scholar, Waggon, and Westminster), in universal compost (made using 1200 l of peat, 100 l of sand, 2.5 kg of magnesium limestone, 2.5 kg of calcium limestone, 1.5 kg of Osmocote® Start (11N-4.8P-14.1K+1.2Mg+TE), 3.5 kg of Osmocote® Exact Standard 3-4M (16N-3.9P-10K+1.2Mg+TE), 0.5 kg of Celcote, 100 l of Perlite, 390 g of Intercept insecticide (Active Ingredient: imidacloprid)). Individual plants were grown in a glasshouse in 16 hours light, supplementary lighting was provided as needed when below 150 Wm^{-2} and shading when above 450 Wm^{-2}, and day/night temperatures of 18/15°C until spikelet maturity.

Manganese deficiency screen

The Mn deficiency screen was conducted on all 130 lines landrace lines and ten elite cultivars. For all lines five seeds were germinated in 10 cm Petri dishes, containing approximately 20 ml of distilled water agar (DWA), over 3 days in 18°C and dark conditions.

Hydroponic tanks were filled with nutrient solution, which consisted of NH_4Cl (3 mM), $Ca(NO_3)_2$ (4 mM), KNO_3 (4 mM), $MgSO_4$ (2 mM), KH_2PO_4 (1 mM), Fe-EDTA (100 µM), H_3BO_3 (23 µM), $ZnCl_2$ (6 µM), $CuSO_4$ (1.6 µM), and $CoCl_2$ (1 µM). Differing volumes of $MnCl_2$ solution were added to make a Mn present and Mn absent solution. Hydroponics solutions were aerated by bubbling air through the solution. One seedling for each line/cultivar was transferred to each of the concentrations in a randomised experimental design. Conditions were kept at 16/8h dark/light and 18/15°C average temperature, with the solution replaced twice weekly.

Chlorophyll fluorescence was measured at 3 weeks using a Rapid Screening Chlorophyll Fluorimeter (Pocket PEA, Hansatech Instruments Limited, King's Lynn, UK), set at a light intensity of 2500 µmol m^{-2} s^{-1} and a dark adaptation period of 15 minutes (Schmidt et al., 2013). The experiment was repeated five times to obtain six replicates, with each line/cultivar represented once per replicate. Data from these experiments were collated and analysed using an unbalanced ANOVA in GenStat (Version 19, VSN International Ltd, Hemel Hempstead, UK) – with position within tank within experiment as the blocking factor – to give the means at each of Mn concentration; noting the maximum quantum yield of photosynthesis of 0.83 (Björkman and Demmig, 1987, Murchie and Lawson, 2013).

Genotyping

Three germinated seeds from each of the 130 lines that had been phenotypically analysed were selected out of eight seeds germinated in 10 cm petri dishes on filter paper disk with 4 ml of sterile water. The germination process was undertaken over 7 days in an ambient temperature room, with water reapplied regularly to avoid drying out.

Leaf samples of approximately 5 cm were cut from each line and frozen in liquid nitrogen. Frozen leaf tissue was ground to a powder with a micro-pestle before adding 400 µl of buffer AP1 from the QIAGEN DNeasy Plant Mini Kit, along with 3 µl of RNase A (100 mg/ml). Samples were vortexed (65°C, 10 mins) before adding 130 µl of buffer P3 and being frozen. Samples were then defrosted and eluted using 110 µl of Buffer AE, re-used for repeat elution.

PicoGreen dsDNA Quantitation Reagent was used to quantify the concentration of DNA in the samples, a total of 400 ng of DNA from each line was transferred to a single well on a 96-well plate. Plated samples were genotyped using the 50k Illumina Infinium iSelect genotyping array for barley (Bayer et al., 2017). Analysis of the relatedness of the lines was undertaken by creating a multivariate clustering model with a paired group (UPGMA) algorithm, and a principal coordinate analysis (PCoA) – both using a Hamming/p-distance similarity index – were performed based on the dissimilarity matrix using the program Past3 (Hammer et al., 2001).

The genotypic data was processed by removing the markers that had a low call rate (<80%) or low minor allele frequency (<10%), along with the genotypic lines that had a low rate of marker return (<80%) or high heterozygosity (False Discovery Rate <10%). The statistical program R (R Core Team, 2013), with the GenABEL package (Aulchenko et al., 2007), was used to perform GWAS using a Mixed Linear Model (MLM) approach controlling for population structure and relatedness, as outlined in Yu et al. (2005) and Zhang et al. (2010), with the 0 µM Mn mean data. Quality controls for the GWAS were performed using quantile-quantile (QQ) plots.

Manganese concentration in shoot tissue

Eight Bere and three two-rowed landrace lines (listed in **Error! Reference source not found.**) were selected from the bulked material based on their variance in chlorophyll fluorescence; three elite cultivars were also selected from external seed stocks.

Eight seeds from each line/cultivar were germinated as before. A 60 l hydroponic tank with four chambers was filled with nutrient solution with the concentrations detailed in previous experiments. Differing volumes of $MnCl_2$ solution were added to make alternating chambers 0 and 1 µM Mn. Hydroponics solutions were aerated by bubbling air through the solution. One seedling from each line/cultivar was positioned into each half of the four chambers, making four blocks per $MnCl_2$ concentration. individuals were kept in the nutrient solution for three weeks, with the solution replaced twice weekly, with conditions as outlined in the previous experiments.

At 20 days the chlorophyll fluorescence for each plant was measured as outlined in the previous experiments. The plants were removed from the nutrient solution and were separated into

shoots and roots. The shoots and roots were then placed in separate brown paper bags and dried at 50°C for 3 days in a drying oven before being weighed.

The dried shoot samples were weighed and ground to a powder using a Qiagen TissueLyser II (Retsch). The powdered samples were acid digested as outlined in Brown et al. (2012) using a nitric acid/peroxide digestion procedure.

Elemental analysis was undertaken on digested plant material using an inductively coupled plasma mass spectrometry (ICP-MS; Perkin Elmer ELAN DRC-e), as outlined in White et al. (2012). Data from this experiment was transformed using a $\log_{10}$ transformation to normalize the data, then analysed using a General ANOVA in GenStat for both transformed and untransformed data – with position within block as the blocking factor – to give a list of the means for each of the lines/cultivars at each of the Mn concentrations. This analysis was undertaken for each of other the minerals analysed – Ca, Cs, Cu, Fe, K, Mg, Ni, P, S and Zn.

RESULTS

Fluorescence analysis of barley sub-categories

When the data were grouped into three sub-categories (Bere, elite cultivars, other landraces/old cultivars – the latter being made up from the non-Bere lines of the JHI collection), there was significant differences between the Mn concentrations, the lines/cultivars, and the interaction of the line/cultivar with differing Mn concentrations ($p<0.001$ each). The data showed that certain sub-categories were more affected at 0 µM, using data of the Mn-plus treatment as the baseline (Figure 1). The non-Bere landraces showed greater retained fluorescence than the elite cultivars, approximately 0.03 higher in the Fv/Fm ratio. The Bere lines showed an even greater retained fluorescence than both the elite cultivars and the non-Bere landraces; approximately 0.12 higher than the elite cultivars.

Fluorescence analysis of individual lines

There were significant differences between the Mn concentration, the lines/cultivars, and the interaction of the line/cultivar in differing Mn concentrations ($p<.001$ each). The 0 µM Mn data shows the extent at which the 140 lines/cultivars were affected over these Mn concentrations (Figure 2). The smallest value, showing the smallest Mn use efficiency, was for the elite cultivar Scholar – with a Fv/Fm ratio of 0.36, 43% of the maximum quantum yield of photosynthesis. The elite cultivars had amongst the most affected fluorescence within the lines tested, the largest fluorescence in an elite cultivar was for Waggon at 0.47 – the 69th highest of the 140 tested. The largest fluorescence overall was Bere-24268 at 0.65 – almost twice as much as Scholar. The 14 with the largest fluorescence were all Bere lines – with Fv/Fm ratios over 0.61. The Bere lines with the smallest fluorescence were Bere-3962 and Bere-118, each having a Fv/Fm around 0.44, making them comparable to the elite cultivars. The lines in the other landraces/old cultivars sub-category had the largest range of fluorescence. The line Aramir-M08 had the third smallest fluorescence at 0.37, comparable to the elite cultivar Scholar; the line with the largest fluorescence was Golden Melon-149, with the 15th largest fluorescence at 0.55.

Manganese concentration in tissue

The analysis of mineral concentration of shoot biomass for the 14 lines/cultivars selected from the screen above (identified by arrows in Figure 2) showed significant differences between lines/cultivars for all elements tested ($p<0.005$), with the exception of nickel (**Error! Reference source not found.**). The only element tested that had a significant difference between the different Mn concentrations was Mn ($p=0.003$). This significance was also seen in the interaction of these two variates for Mn ($p<0.001$); two other elements also had interactions of note – K and Mg ($p=0.037$ and 0.053, respectively). P-values for all elements are listed in **Error! Reference source not found.**.

Based on the differences seen in the Mn concentrations – when grown at 0 and 1 µM $MnCl_2$ – and the chlorophyll fluorescence of plants grown in 0 µM $MnCl_2$ (Figure 3), three separate groups can be identified in the subset of lines/cultivars analysed. The group with greatest Mn use efficiency – Bere-24268, Bere-45, Bere-47, and Bere-59 – showed Mn concentration between 175-270 mg kg^{-1} DW when grown in 1 µM $MnCl_2$ hydroponic solution and 15-22 mg kg^{-1} DW when grown in 0 µM $MnCl_2$ hydroponic solution; they also had the greatest chlorophyll fluorescence – retaining more than 95% of the maximum quantum yield of photosynthesis of 0.83. The second group had moderate Mn use efficiency and had Mn concentrations between 130-170 and 9-12 mg kg^{-1} DW when grown in 1 and 0 µM $MnCl_2$ hydroponic solutions, respectively; they maintained at least 75% of the maximum quantum yield of photosynthesis. This group consisted of Bere-155, Bere-25A, Bere-58, and Webbs Burton Malting-216. The last group were Mn inefficient with Mn concentrations between 60-85 and 7-7.5 mg kg^{-1} DW when grown in 1 and 0 µM $MnCl_2$ hydroponic solutions, respectively, and decreased by more than a third of the maximum quantum yield of photosynthesis. This last group contained the elite cultivars, the other landraces/old cultivars and Bere line (Bere-3962) with amongst the least retained chlorophyll fluorescence.

The 14 selected lines/cultivars grown in a 0 µM $MnCl_2$ had low concentrations of Mn in the biomass, with a small but significant difference between the lines/cultivars ($p<0.001$). The data of the shoot Mn concentrations and the chlorophyll fluorescence of plants grown in 0 µM $MnCl_2$ hydroponic solution were highly correlated, with a correlation coefficient of 0.87 (**Error! Reference source not found.**a). This was greater than the correlation of the shoot Mn concentration of plants grown in 1 µM $MnCl_2$ with the chlorophyll fluorescence of plants grown in 0 µM $MnCl_2$ (coefficient of 0.83; **Error! Reference source not found.**b), and the correlation of the shoot Mn concentrations of plants grown in the two $MnCl_2$ concentrations (coefficient of 0.72; **Error! Reference source not found.**c); however, all three were highly positively correlated with each other.

Lines/cultivars grown in a 1 µM $MnCl_2$ concentration showed large and significant ($p<0.001$) differences in concentrations of Mn in the shoot biomass; between 8 and 17 times greater than the concentration when grown in the absence of Mn. The four Bere lines that exhibited greatest Mn use efficiency showed 2.3-3.8 times the concentration of Mn than the elite cultivars, with no sign of Mn toxicity. However, there was no difference between cultivar/lines in the chlorophyll fluorescence of plants grown in 1 µM $MnCl_2$, but there was a negative correlation at this level between Shoot Mn concentration and the chlorophyll fluorescence (coefficient of -0.65; **Error! Reference source not found.**d).

When the shoot Mn concentration for each individual was compared against the corresponding weight of the shoot biomass it can be seen that there was a weak correlation (coefficient of 0.34) of decreasing shoot Mn levels with increasing shoot biomass when grown in adequate Mn concentrations (**Error! Reference source not found.**). Statistical analysis of the data with shoot weight as a co-factor shows that this effect does not change the significance of the genotypic effect. Additionally, the strong correlations seen between the chlorophyll fluorescence when grown in Mn absent conditions and the Mn concentrations when grown in both conditions (**Error! Reference source not found.**a & b) were not seen, with weak or no correlations when comparing against total shoot concentration grown in absent and adequate Mn concentrations respectively (coefficients of 0.43 and 0.07 respectively; **Error! Reference source not found.**).

The root biomass data that was gathered also accounted for an average of 19-33% of the total biomass for individual lines over both Mn concentrations. The three elite lines tested all had the lowest ratio with only 19% of the dried biomass being root. The Bere lines were the only lines with over 25% of the biomass being roots, with six lines exhibiting this ratio or higher. Significant differences were seen between genotype ($p<0.001$) but not between Mn concentrations ($p=0.678$). When these data were compared against the chlorophyll fluorescence data from plants grown in Mn absent conditions it could be seen that there is a clear positive correlation between the variables (coefficient of 0.87; **Error! Reference source not found.**). Additionally, comparisons of the shoot manganese per unit of dried root biomass to the chlorophyll fluorescence in Mn absent conditions shows a correlation when grown in both absent and adequate conditions with coefficients of 0.54 and 0.48, respectively; **Error! Reference source not found.**). This difference in shoot manganese per unit of dried root biomass is shown to be significant between the lines/cultivars, the Mn concentrations, and the interaction of the line/cultivar with differing Mn concentrations ($p<0.001$ each).

Genotyping data

Cluster trees generated (tree for first barley chromosome – H1 – shown in **Error! Reference source not found.**) using the genotypic data showed two main groups of landraces; separating the Scottish lines including Bere – along with other landraces/old cultivars such as Morex, Floye, Gartons Archer, and Tibet37 – from the rest of the landraces. Within the predominantly Bere group four sub-clusters were visually identified, of which one held approximately 75% of the Bere lines. Outside of the main sub-cluster include: 1) Bere-49 and Bere-4828 that grouped with Floye, 2) Bere-2962, Bere-3962, and Bere-8 that grouped with Tibet37, and Tiree Six Row, 3) Bere-122, Bere-52, Bere-7045, Bere-115, and Bere-120 that grouped together separately and 4) Bere-4828A and Bere-112 that grouped with the non-Bere cluster.

This is further supported by the PCoA (graph for H1 shown in Figure 4) which identified that the Bere lines cluster with the lines mentioned above in a group separate from the remainder, and both clusters comprise a mix of two- and six-rowed lines; likewise both Bere-4828A and Bere-112 group with the non-Bere cluster, confirming their mis-labelling. Within the Bere cluster the majority of Bere lines group closely together, but many bere lines are as distant from the main Bere group as different identified lines are from one another – highlighting the great diversity found within lines classified as Bere.

Genome-Wide Association Study (GWAS) analysis

From the 37242 markers used, 10725 were removed as having low (<10%) minor allele frequency and a further 32 because of a low call rate. Of the 142 lines used 13 were excluded because of high heterozygosity, and a further 10 due to being identical by state (IBS).

The QQ plots (**Error! Reference source not found.**) showed that the MLM model for the 0 µM mean had the smallest deviation from the expected null distribution. The Manhattan MLM plot for the 0 µM mean (Figure 5) displayed multiple loci of interest; the most significant association was on the distal end of chromosome 2HL, along with another association on 5HL. Of the top 50 markers with the largest effect, with respect to the Manhattan MLM plot for the 0 µM mean, 11 were in or around the 6HS region identified, and 12 in the 2HL region identified – all with an effect of 0.027-0.032 Fv/Fm.

In the 0 µM mean data 14 significant markers were identified ($p<0.0001$), all with an effect of 0.025-0.032 Fv/Fm (Table 1). Of these 9 were on the locus located distally on 2HL between 687.83-725.12 Mb. Within this there were three localised regions; 687.83 Mb and 724.94-725.12 Mb each with four significant markers, along with one marker at 677.31 Mb. The other markers were in separate locations including the distal end of 7HL, and the centre of 5HL. In the region identified on chromosome 2HL (687.83-725.12 Mb) there are many associated genes, of these a subset of 15 genes were identified as potential candidates based on their likely functions (

Table 2). The 4 sequential markers at 687.83 Mb were found to all be located within a gene encoding for a KS protein with metal-binding Terpene synthase domain (HORVU2Hr1G099480). Other candidate genes in this area encode for; 1) a 3-phosphoglycerate dehydrogenase, 2) a Serine/threonine-protein, 3) a MATE efflux family protein, 4) a yellow stripe-like protein, 5) two heavy metal ATPase', 6) five transporter proteins, for K, Zn, Sulphate, or amino acids, and 7) three serial Photosystem I P700 chlorophyll a apoprotein. Lone markers positioned on 5HL and 7HL were contained within/next to a Photosystem II protein and a Serine/threonine-protein kinase, respectively.

DISCUSSION

Manganese deficiency is a problem for marginal lands worldwide, reducing the yield and area of effective crop production (Schmidt et al., 2013). One method of improving the sustainability of plant production, on agricultural soils with limited Mn availability, is by incorporating Mn use efficiency traits into elite crop cultivars. This study has identified lines that contain such traits, the chromosomal regions that contain the genes controlling these traits, and potential candidate genes.

Most Bere barley lines tested had increased Mn use efficiency compared to elite lines and other landraces/old cultivars tested. This supports and expands on the work undertaken by George et al. (2014), Leplat (2015), Brown et al. (2017), and Schmidt et al. (2018); further identifying Bere lines of interest with respect to Mn use efficiency. The investigation also indicated that the elite spring barley cultivars that were included had very high concentrations of latent Mn deficiency, thus signifying a need for Mn use efficiency traits within the northern European breeding populations. Other lines have been shown to have Mn use efficiency such as the Australian Amagi Nijo and Weeah barley cultivars (Huang et al., 1994, Huang, 1996, Pallotta et al., 2000). Lines of interest with respect to Mn use efficiency include Bere-24268, Bere-45, Bere-43, and Bere-39. Interestingly lines such as Bere-3962 and Bere-118 are genetically similar to the lines above, but had a comparably reduced Mn use efficiency. This could be due to the environment they have become adapted to, such as an acidic soil where Mn deficiency tolerance would be no advantage (Schmidt et al., 2018).

Analysis of the Mn concentration in the shoot biomass showed that an increased level of accumulation of Mn in the biomass corresponded to an increased Mn use efficiency, and that this occurred even when there is an adequate supply of Mn in the environment. This accumulation reaches a concentration that could be considered above the specified critical toxicity threshold concentration for the highly to moderately Mn efficient lines. The four Bere lines with the greatest Mn use efficiency, along with Bere-25A, rose above the 150 mg kg^{-1} DW critical limit outlined in Reuter et al. (1997), and the remaining moderately Mn efficient lines rose above the 120 mg kg^{-1} DW critical limit for Mn toxicity, outlined in MacNicol and Beckett (1985). However, no lines showed toxic effects at the early stages of growth – thus indicating a decreased sensitivity to toxic Mn concentrations. All lines/cultivars showed large decreases in Mn content when grown in Mn deficient conditions, but Mn efficient Bere lines presented a concentration large enough to avoid the specified critical deficiency threshold concentration of Mn in shoot tissue, which ranges from 11 (Schmidt et al., 2013) to 20 mg kg^{-1} DW, and marked on Figure 3 at 17 mg kg^{-1} DW (Reuter et al., 1997, Husted et al., 2009, Schmidt et al., 2016a). In contrast the elite cultivars had Mn concentrations that were well below this critical value. This variation in Mn-efficiency without Mn

provided is most likely to be related to bioavailable Mn in the grain which is not necessarily associated with total Mn present in the grain, but with the expression of factors which mobilise and transport the element to the growing tissue.

The Mn efficient Bere lines also retained almost all their maximum quantum yield of photosynthesis. Other Bere lines, and the old cultivar Webbs Burton Malting-216, displayed signs of some Mn use efficiency by retaining more of the maximum quantum yield of photosynthesis than the elite lines, but not as much as the Mn efficient Bere lines identified, whilst falling below the specified critical deficiency threshold concentration when grown in Mn deficient conditions. The Bere line selected for its reduced efficiency, as identified by the chlorophyll fluorescence, showed no significant difference in Mn shoot concentration from the elite line – indicating that not all Bere lines have high Mn use efficiency. This suggests that there is a range of Mn use efficiency in Bere lines due to different and/or differentially expressed genes. It also indicates that the trait of increased Mn accumulation is not solely responsible for the increases the Mn use efficiency, highlighting the complexity of pathway with multiple methods of transport (Socha and Guerinot, 2014) – differing from boron tolerance that relies mainly on aquaporin transporters (Hayes et al., 2015, Tombuloglu et al., 2016). Additionally, it was noted that this increase tolerance to Mn deficiency and the increased accumulation of Mn in the tissue is correlated with an increased in the ratio of root to shoot tissue. Increased comparative root biomass could explain, in part, the increased accumulation of Mn due to the increase in surface area for ion absorption, and thus account for some of the increased tolerance to low Mn environments (Jungk and Claassen, 1997, Shankar et al., 2013). Alternatively, the difference in root biomass could be a symptom of the difference in tolerance to Mn deficiency, as it has been shown that Mn deficiency in wheat has an increasingly inhibiting effect on root growth compared with the shoot growth (Sadana et al., 2005). Additionally, there was a correlation of the total shoot Mn per gram of root tissue with the observed Mn use efficiency, indicating that comparative increase in root system size only accounts for some of the increased Mn efficiency.

Between cultivars there was a large genotypic variation in Mn use efficiency, causing differential Mn^{2+} uptake. Little research has been done to isolate genomic regions associated with increased Mn use efficiency in barley. The first identified plasma membrane-localised metal transport protein capable of transporting Mn^{2+} in barley was *HvIRT1* – located on 4H and 6H when the sequence from Pedas et al. (2008) was used in a BLAST search. Two studies have identified loci using RFLP markers from populations crossed with the Mn efficient line Amagi Nijo; the first associated locus labelled *Mel1* was identified by Pallotta et al. (2000) located on the distal end of chromosome 4HS (Pallotta et al., 2003). It is of interest to note that this study did not find any association with Mn use efficiency and the region on 4HS, indicating a differential genetic control. The second locus - *Xwg645*, controlling shoot Mn concentration, was on chromosome 2HL (Lloyd, 2000). The locus of most interest in this study was located at 2HL; this corresponds with the *Xwg645* locus identified. Markers associated with Mn use efficiency were identified in chromosome 2HL in winter barley by Leplat et al. (2016), but these do not correspond with the location identified in this study. However one significant marker on 7HL in this study does correspond with the same location identified by Leplat et al. (2016). It is important to note, however, that the Mn use efficiency has multiple genes associated and can be greatly affected by environmental variation (Leplat et al.,

2016) indicating that work on assessing these genes in multiple different field trials is needed to assess what environment the Mn use efficiency is expressed.

The candidate genes identified in this study had a range of different roles that could contribute to Mn use efficiency and were selected based on; 1) terpene synthase - produces terpene compounds that act as antioxidants in response to oxidative stress (Rodziewicz et al., 2014), and have been shown to be activated by Mn; Mn has also been shown to induce ROS production that is neutralised with antioxidants (Farzadfar et al., 2016), 2) 3-phosphoglycerate dehydrogenase – found to be associated with the serine biosynthesis in photosynthetic cells (Okamura and Hirai, 2017), 3) serine/threonine-protein kinase – known for their roles in stress signalling (País et al., 2009), 4) MATE efflux family protein – found to be associated with increased Mn uptake in the shoot of *Arabidopsis thaliana* (Rogers and Guerinot, 2002), 5) yellow stripe-like protein – shown to be involved in increased Mn uptake in *A. thaliana* (Waters et al., 2006), rice (Socha and Guerinot, 2014), and thought to be in barley (Zheng et al., 2011), 6) heavy metal ATPase', with Cu-transporting shown to be involved in transport of heavy metals such as Mn (Hall and Williams, 2003, Dučić and Polle, 2005) and Cu-ATPase shown to be involved in the transport of other heavy metals into the chloroplast (Seigneurin-Berny et al., 2006), 7) transporter proteins, for; a) K – shown to play an adverse role in Mn uptake in barley (Alam et al., 2005), b) Zn – identified as a ZnT (found in animal) the plant homologue would be in the CDF transporter family that are associated with metal tolerance (Manara, 2012), and Zn transporters in mammalian cells have been shown to be involved in Mn transport (Kambe, 2012), c) sulphate – which have been shown to be involved in the transport of heavy metals such as Molybdenum (Fitzpatrick et al., 2008) and in abiotic stress response (Gallardo et al., 2014), and d) amino acid – due to the chelation of metals with amino acids that can be transported (Haydon and Cobbett, 2007, Rentsch et al., 2007, Zemanová et al., 2014), and 8) Photosystem I P700 chlorophyll a apoprotein – as PSI interacts with PSII, but can also operate independently (Allen, 2002).

The increase of Mn use efficiency in an elite background without compromising the yield quantity or quality would allow the growth of elite barley in marginal lands that could previously not economically support elite cultivars. Furthermore, it would provide a buffer to changing environments; preventing deficiencies without the need for routine blanket spraying of Mn foliar fertilizer, thus saving money on purchase and deployment of the chemical. Finally, it will reduce the cases of hidden deficiency that could lead to increased disease (Wilhelm et al., 1988, Marschner et al., 1991, Brennan, 1992), increase susceptibility to drought (Hebbern et al., 2009), and sub-optimal use of other minerals such as phosphorus (Allen et al., 2007, Schmidt et al., 2016a). Together this will help; satisfy the increasing demand for food, maintain yields in an increasingly changing climate, and reduce pollution due to chemical runoff.

CONCLUSION

The work in this project has identified multiple Bere lines – and genetic regions within – that were associated with an increased Mn use efficiency, that corresponds with an increase in Mn accumulation in the shoot biomass and maintenance of maximum quantum yield of photosynthesis. The key region highlighted in this study is at 2HL, with multiple genes of interest that could be involved in Mn transport or utilisation. Candidate genes and regions have been proposed from this

that could be isolated in a non-Mn efficient elite background to identify any increases in Mn use efficiency of elite lines that do not compromise the yield or quality of the grain. Further analysis to compare these regions with regions already tested and incorporated into elite cultivars, such as the Australian Amagi Nijo and Weeah barley cultivars mentioned previously, to identify which provide a truly novel form of Mn use efficiency.

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FIGURE CAPTIONS

Figure 1 Chlorophyll fluorescence at 0 µM $MnCl_2$ for 140 lines/cultivars of barley divided into three groups (Bere, Landrace, and Elite), to compare the relative Mn deficiency in each. Error bars represent one standard error either side of the mean. The number of lines/cultivars collated is noted at the base of each bar.

Figure 2 Chlorophyll Fluorescence at 0 µM $MnCl_2$ for 140 lines/cultivars of barley, colour coded for the three groups (Bere = blue, Landrace = green, Elite = red). The arrows indicate the lines selected to be used to measure the Mn concentration in the leaf tissue below. Error bars represent one standard error either side of the mean.

Figure 3 A subset of the population representing Bere, landrace, and elite lines/cultivars over a range in chlorophyll fluorescence. The columns (primary y-axis) represent the mean Mn concentrations in shoot biomass for plants grown in a hydroponic solution of 0 µM $MnCl_2$ (dark) and 1 µM $MnCl_2$ (light); the black dotted line indicate the specified critical deficiency threshold concentration of Mn in leaf tissue of 17 mg kg^{-1} DW as outlined by Reuter et al. (1997). The data points (secondary axis) display the mean chlorophyll fluorescence of the plants grown in a hydroponic solution of 0 µM $MnCl_2$. Error bars represent one standard error either side of the mean.

Figure 4 Principal Coordinates Analysis of 130 barley cultivars, identifying the division of the population into two distinct sub-groups. The percentage variation represented is 37% for coordinate 1, and 7% for coordinate 2. The lines originally labelled as Bere lines are marked in red, other six-row landraces are in blue, with the rest in black.

Figure 5 Manhattan plot of a Genome-Wide Association Study undertaken using a Mixed Linear Model on the 0 µM Mn mean data. The x-axis is the chromosome number, arranged from the short to long end of the chromosome – chromosome 0 is the unmapped markers. The y-axis is the "$-\log_{10}$ (p-value)", a value of 4 equating to a p-value of <0.0001 – marked with a black line.

TABLES

Table 1 The statistically significant markers found in the GWAS of the 0 µM Mn data, with the chromosome number, position on the physical map, statistical significance, and the effect of the marker (increase in the Fv/Fm ratio) listed.

Marker Name	Chromosome	Position (Mb)	P-value	Effect
JHI_Hv50k_2016_110885	2H	677.31	1.55E-05	0.0296
JHI_Hv50k_2016_113750	2H	687.83	5.07E-05	0.0274
JHI_Hv50k_2016_113753	2H	687.83	5.07E-05	0.0274
JHI_Hv50k_2016_113754	2H	687.83	5.07E-05	0.0274
JHI_Hv50k_2016_113755	2H	687.83	5.07E-05	0.0274
JHI_Hv50k_2016_128224	2H	724.95	7.72E-05	0.0281
JHI_Hv50k_2016_128255	2H	724.97	2.07E-05	0.0312
JHI_Hv50k_2016_128280	2H	724.97	7.72E-05	0.0281
JHI_Hv50k_2016_128407	2H	725.12	8.22E-05	0.0267
JHI_Hv50k_2016_323762	5H	573.35	4.77E-05	0.0301
JHI_Hv50k_2016_355863	5H	648.01	7.21E-05	0.0253
SCRI_RS_167383	7H	275.46	8.54E-05	0.0265
JHI_Hv50k_2016_518726	7H	654.39	4.29E-05	0.0263
12_30351	U	-	7.72E-05	0.0281

Table 2 A list of candidate genes of interest, along with their chromosome number, position on the physical map, the genetic annotation and any references used in the selection; 1) Hall and Williams (2003), 2) Dučić and Polle (2005), 3) Seigneurin-Berny et al. (2006), 4) Allen (2002), 5) Farzadfar et al. (2016), 6) Zemanová et al. (2014), 7) Haydon and Cobbett (2007), 8) Rentsch et al. (2007), 9) Alam et al. (2005), 10) Socha and Guerinot (2014), 11) Zheng et al. (2011), 12) Waters et al. (2006), 13) País et al. (2009), 14) Rogers and Guerinot (2002), 15) Manara (2012), 16) Kambe (2012), 17) Gallardo et al. (2014), 18) Fitzpatrick et al. (2008), 19) Okamura and Hirai (2017), 20) Schmidt et al. (2015), and 21) Schmidt et al. (2016a).

Gene Name	Chr	Position (Mb)	Annotation	Reference
HORVU2Hr1G096930.1	2HL	677.16	heavy metal atpase 5	1,2
HORVU2Hr1G097010.8	2HL	677.26	Copper-transporting ATPase 1	1,2,3
HORVU2Hr1G099170.1	2HL	686.91	Photosystem I P700 chlorophyll a apoprotein A1	4
HORVU2Hr1G099180.1	2HL	686.91	Photosystem I P700 chlorophyll a apoprotein A1	4
HORVU2Hr1G099190.1	2HL	687.03	Photosystem I P700 chlorophyll a apoprotein A1	4
HORVU2Hr1G099480.13	2HL	687.83	KS protein with a metal-binding Terpene synthase domain	5
HORVU2Hr1G099530.1	2HL	687.96	cationic amino acid transporter 8	6,7,8
HORVU2Hr1G099680.1	2HL	688.06	amino acid transporter 1	6,7,8
HORVU2Hr1G099810.14	2HL	688.52	Potassium transporter family protein	9
HORVU2Hr1G099860.1	2HL	688.60	YELLOW STRIPE like 7	10,11,12
HORVU2Hr1G112090.3	2HL	724.96	Serine/threonine-protein kinase	13
HORVU2Hr1G112150.1	2HL	725.00	MATE efflux family protein	14
HORVU2Hr1G112230.2	2HL	725.23	Zinc transporter 8	15,16
HORVU2Hr1G113050.1	2HL	727.21	sulfate transporter 91	17,18
HORVU2Hr1G113180.3	2HL	727.57	D-3-phosphoglycerate dehydrogenase	19
HORVU5Hr1G084800.1	5HL	573.35	Photosystem II protein N	20,21
HORVU7Hr1G121690.1	7HL	654.38	Protein kinase superfamily protein	13

Figure 1

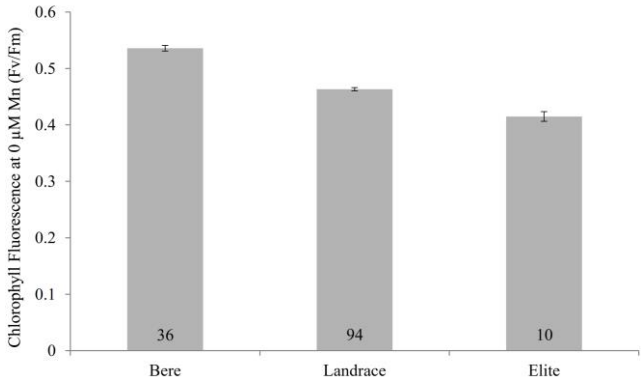

Figure 2

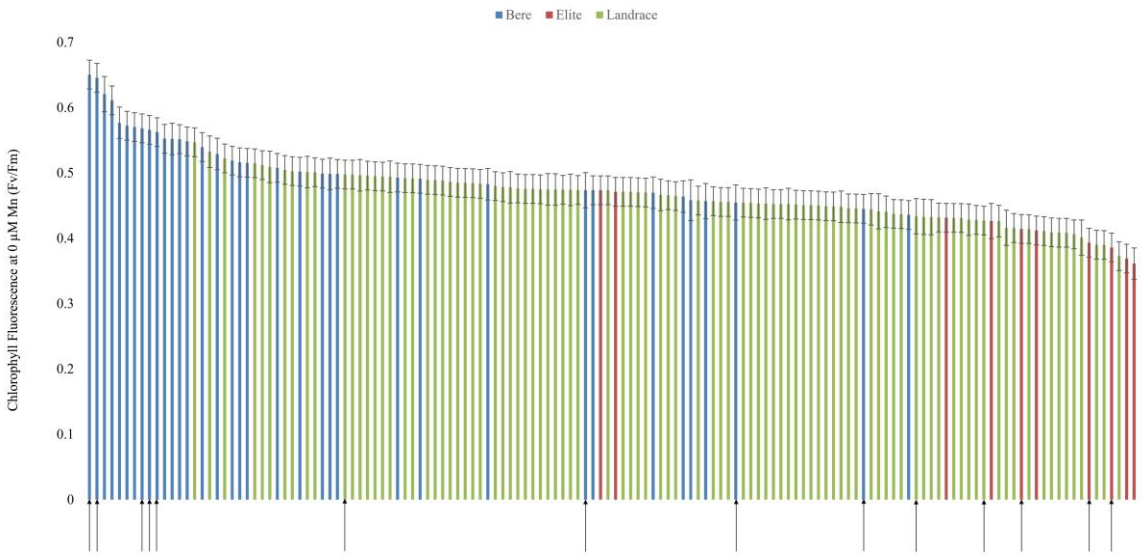

Figure 3

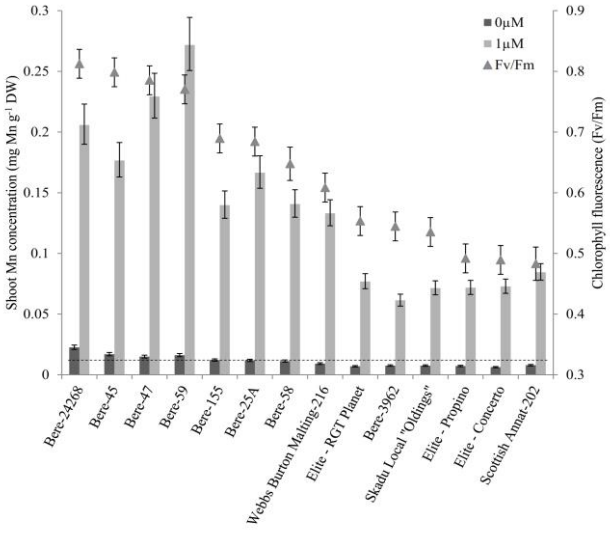

Figure 4

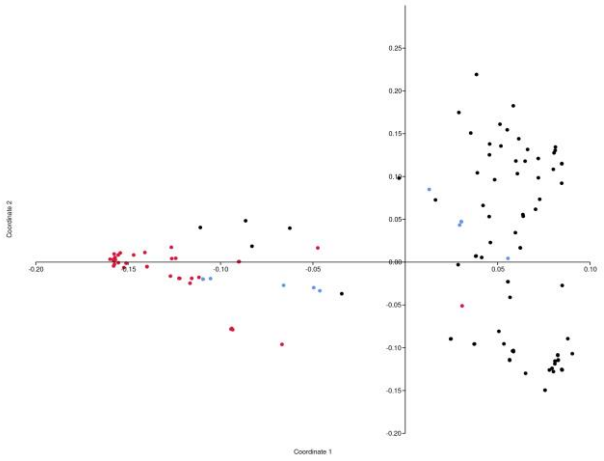

Figure 5