

Ancient barley landraces adapted to marginal soils demonstrate exceptional tolerance to manganese limitation

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- **Background and Aims** Micronutrient deficiency in cereals is a problem of global significance, severely reducing grain yield and quality in marginal soils. Ancient landraces represent, through hundreds of years of local adaptation to adverse soil conditions, a unique reservoir of genes and unexplored traits for enhancing yield and abiotic stress tolerance. Here we explored and compared the genetic variation in a population of Northern European barley landraces and modern elite varieties, and their tolerance to manganese (Mn) limitation.
- **Methods** A total of 135 barley accessions were genotyped and the genetic diversity was explored using Neighbor-Joining clustering. Based on this analysis, a sub-population of genetically diverse landraces and modern elite control lines were evaluated phenotypically for their ability to cope with Mn-deficient conditions, across three different environments increasing in complexity from hydroponics through pot experiments to regional field trials.
- **Key Results** Genetically a group of Scottish barley landraces (Bere barley) were found to cluster according to their island of origin, and accessions adapted to distinct biogeographical zones with reduced soil fertility had particularly larger Mn, but also zinc (Zn) and copper (Cu) concentrations in the shoot. Strikingly, when grown in an alkaline sandy soil in the field, the locally adapted landraces demonstrated an exceptional ability to acquire and translocate Mn to developing leaves, maintain photosynthesis and generate robust grain yields, whereas modern elite varieties totally failed to complete their life cycle.
- **Conclusions** Our results highlight the importance of gene pools of local adaptation and the value of ancient landrace material to identify and characterize genes that control nutrient use efficiency traits in adverse environments to raise future crop production and improve agricultural sustainability in marginal soils. We propose and discuss a model summarizing the physiological mechanisms involved in the complex trait of tolerance to Mn limitation.

Key words: Barley landraces, *Hordeum vulgare*, evolutionary biology, genetic diversity, adaptation, marginal soils, micronutrients, nutrient use efficiency, sustainable agriculture.

INTRODUCTION

It is a key global challenge to increase crop productivity while preserving natural resources, and at the same time facing climate change and degradation of arable lands. Hence, it is time to re-think how we grow plants to meet the United Nations sustainable development goals of zero hunger and responsible crop production (United Nations, 2015). However, this goal is currently quite distant considering that it is contingent on the need to produce on poorly fertile marginal soils with inherent nutrient limitations.

To grow and yield efficiently, plants require 17 essential elements. Of these, several micronutrients are necessary for photosynthesis, where they have key functions in electron transport processes. Light-induced water splitting is catalysed by the manganese (Mn) cluster in the oxygen-evolving complex (OEC) of photosystem II (PSII), and its function is a prerequisite to extract electrons for driving photosynthesis. Iron (Fe) is present as haem and sulphur-Fe proteins and Fe-enzymes functioning in electron transfer and redox reactions within the various photosynthetic

complexes. Iron, copper (Cu) and zinc (Zn) are co-factors of the superoxide dismutases (Fe-SOD and Cu-Zn-SOD), which scavenge reactive oxidative species in the chloroplast, and plastocyanin is a mobile Cu-containing protein involved in electron transport from cytochrome *b₆/f* to photosystem I (PSI) during photosynthesis.

Manganese deficiency is a widespread problem, particularly in dry, calcareous and sandy soils, which favour the oxidation of Mn into plant-unavailable Mn oxides (Schmidt *et al.*, 2016a). Barley is particularly sensitive to Mn deficiency, which significantly reduces crop yields and may even cause complete crop loss in severe winters (Schmidt *et al.*, 2013). Novel genotypes which are able to acquire and utilize Mn and other micronutrients more efficiently are needed to achieve global food security. To achieve this, one strategy is to explore the variation contained in ancient landraces, where barley is an excellent genetic model, because it has been adapted to a wide range of adverse environments during its domestication and subsequent cultivation and expansion (Dawson *et al.*, 2015; Pankin

and von Korff, 2017). For millennia, farmers have selected for specific agricultural traits to maximize yields, including adaptations providing crop robustness and stability under nutrient stress conditions (Dwivedi et al., 2016). The advantage of utilizing ancient landraces for plant genetic improvement has been demonstrated in previous studies investigating the molecular basis of adaptation to, for example, high soil boron in wheat landraces (Pallotta et al., 2014), aluminium tolerance in landraces of barley (Fujii et al., 2012) and phosphorus efficiency in landraces of rice (Gamuyao et al., 2012). These clearly emphasize the impact and power of genetic variation hidden in ancient landraces originating from diverse agronomic zones. In recent research, an unprecedented variation in the ability of Northern European barley landraces and commercial varieties to tolerate Mn deficiency has been observed (George et al., 2014). Apparently these landraces had a greater capacity to acquire Mn, suggesting a wider genetic diversity in tolerance to Mn deficiency compared with modern elite varieties.

Here we explore and compare the genetic variation in a population of Northern European ancient landraces and modern elite varieties grown across three different environments, i.e. hydroponics, pot experiments and field trials under natural conditions. Using chlorophyll *a* (Chl *a*) fluorescence as a well-established proxy for tolerance towards Mn deficiency (Schmidt et al., 2013), we begin to reveal the complex key adaptive traits conserved in the landraces, but lost from elite modern varieties.

MATERIALS AND METHODS

Barley accessions

The accessions used in this study are part of a 'heritage' barley collection which includes landraces and locally adapted cultivars from the UK mainland and Scottish islands, comprising two- and six-row types (Supplementary Data Table S1). This was supplemented with landraces from Denmark, Norway and Sweden, as well as two accessions from the Faeroe Islands. Accessions were sourced from the Germplasm Resources Unit (GRU) at the John Innes Centre in Norwich, Science and Advice for Scottish Agriculture (SASA) in Edinburgh, Nordic Genetic Resource Center previously known as the Nordic Genebank (NGB) in Sweden, the USDA Germplasm Resources Information Network (GRIN) or the N.I. Vavilov All Russian Institute of Plant Genetic Resources (VIR).

Genetic analysis

DNA extraction and 9K select genotyping. Genomic DNA for genotyping was extracted from 7-day-old germinated seedling leaf tissue using Qiagen DNeasy Plant Mini kits (Qiagen, Hilden, Germany). Genotyping using the 9K Illumina SNP chip (Comadran et al., 2012) was performed at TraitGenetics, GmbH in Germany, and single nucleotide polymorphism (SNP) alleles were called using GenomeStudio Genotyping Module v2.0.2 (Illumina, San Diego, CA, USA). The data were filtered to remove >5 % missing SNP data, and any heterozygous calls were scored as missing data. The genetic relationship between these accessions was analysed using Neighbor-Joining clustering by

means of a simple matching distance matrix executed in PAST, version 1.91 (Hammer et al., 2001). STRUCTURE analysis of individuals was undertaken with the STRUCTURE 2.2 software package (Falush et al., 2007), based on 25 000 'burn-in' replications and a further 25 000 Markov chain Monte Carlo (MCMC) steps; other parameters were kept at default settings.

Plant growth conditions and experimental set-up

Hydroponics experiment 1. Barley seeds of 24 selected barley genotypes (Bere and UK landraces, Fig. 1A) were germinated in vermiculite for 6 d in a glasshouse with minimum temperatures of 18 °C:15 °C, light:dark, and a photoperiod of 16 h:8 h, light:dark (minimum 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light intensity). One seedling of each of the 24 different landraces was transferred into each of six 28 L hydroponic containers. Plants were supplied with an aerated nutrient solution (pH 6–6.5) according to Schmidt et al. (2013) except for Mn additions. Control plants received increasing concentrations of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ [100 nM week 1 (split application), 500 nM weeks 2 and 3, 715 nM week 4]. The Mn-deficient plants received only 100 nM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ in week 1 (split application) and then no $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ for the rest of the growth. At 31 d after transplanting, the youngest fully developed leaf (YFDL) from each plant was harvested and freeze-dried for inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis.

Hydroponics experiment 2. In a smaller hydroponic set-up, plants of KWS Irina, Bere Unst Shetland and Bere Clho 8327 were cultivated in 4 L containers under control conditions (as described for experiment 1) or under Mn-deficient conditions, with plants receiving 200 nM $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (split in three) during the first 10 d after transplanting. At 25 d after transplanting, fluorescence was measured and recorded using Imaging PAM, one YFDL per rep was harvested for ICP-OES analysis and the remaining YFDLs were collected and immediately frozen in liquid nitrogen for subsequent thylakoid isolation and analysis.

Pot experiment. Seedlings of three modern elite varieties (KWS Irina, RGT Planet and Graminor Rødhette), and four Bere landraces (Bere North Ronaldsay, Bere West Orkney, Bere Shetland and Bere North Uist) (Table 1) were transplanted to 1 L pots with either Orkney soil (pH 7.8; loamy sand, see field trials for further information) supplemented with fertilizer [Yara Mila 14–3–15–10 (NPKS) + Mg, Cu and B] at a rate of 2 g kg^{-1} soil or into 1 L pots with compost (15 mm dark peat, light peat and sod pea 3:6:1 including 50 kg of clay, 1 kg of aquasorb, 4 kg of lime/dolodust and 0.4 L wetting agent per m^3) supplemented with fertilizers [1.3 kg of base 15–10–20 (NPK) + trace elements and Nutricote 70 day (slow release) 16–10–10 (NPK) per m^3]. Four plants per genotype were planted separately in each of the two soil types, giving a total number of 56 individual plants. The plants were grown in a glasshouse at a photoperiod of 17 h:7 h, light:dark, a minimum light intensity of 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and temperatures between 25 and 15 °C. Soil moisture was maintained by regularly watering the pots with tap water and pots were rotated twice a week. From 14 d of growth, fluorescence measurements were recorded every 3–4 d (the dynamic data are presented in Fig. 4A) until flag leaf stage (BBCH 39). At maturity, plants were left to dry before collection of ears for threshing to determine grain yield.

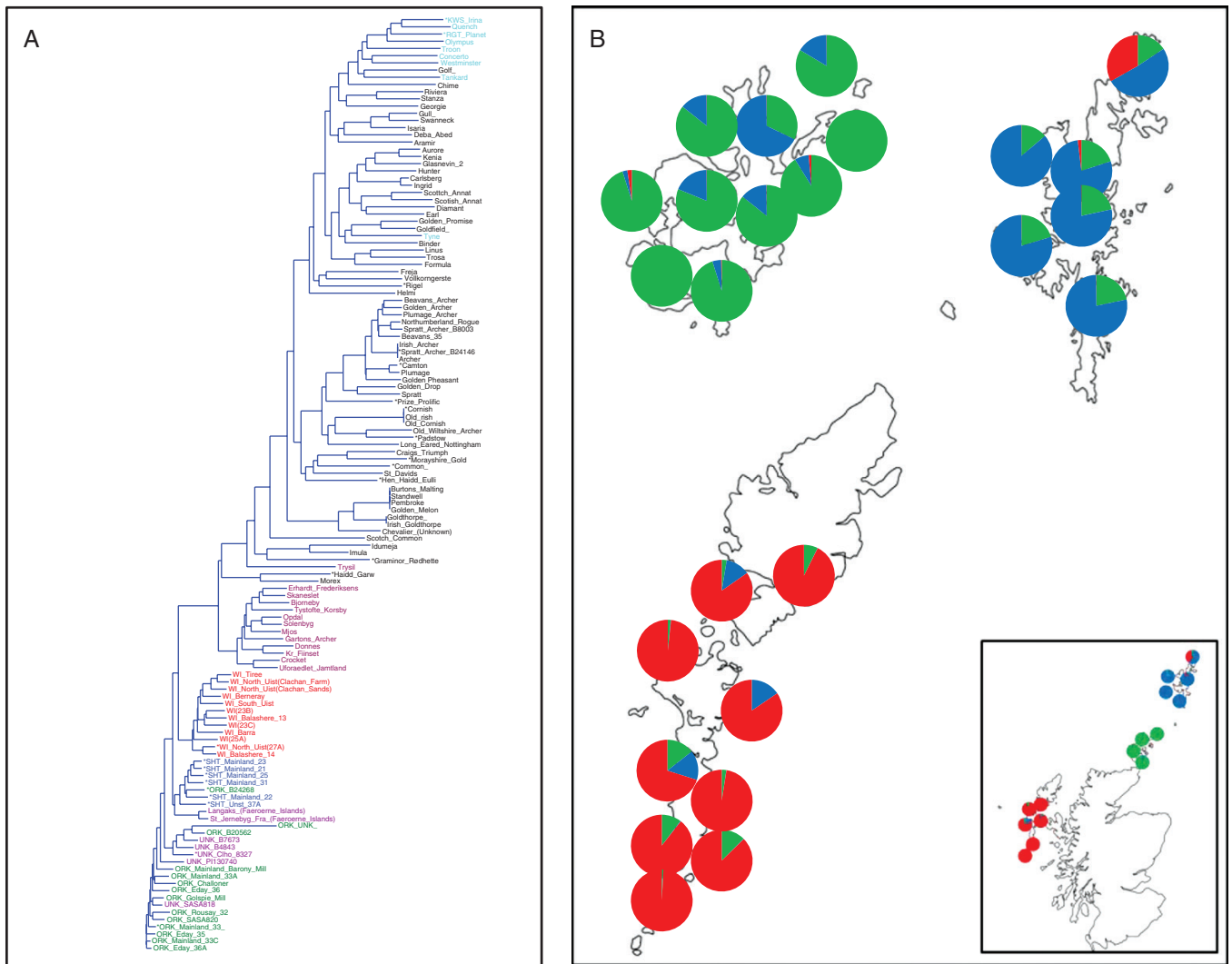


FIG. 1. Biogeographical map: genotypic variation of barley with distribution across geographical zones. (A) Hierarchical clustering using Neighbor-Joining and an association matrix based on simple matching for 130 barley (*Hordeum vulgare*) accessions using 9K iSelect SNP chip (Comadran *et al.*, 2012) (Supplementary Data Table 1). To simplify, two-row landraces UK are coloured black, two-row modern cultivars are light blue, six-row Scandinavian landraces are purple, Bere landraces from Orkney are green, those from the Shetlands are blue, those from the Western Isles are red and those of unknown origin are pink (most group close to Orkney accessions). Two accessions from the Faeroe Islands group closely to Shetlands and Bere North Ronaldsay; the furthestmost of the Orkney Isles is close to most of the Shetland accessions. Accessions with asterisks are used in pot, hydroponics and field experiments. (B) STRUSTRUCTURE results ($K = 3$) for Bere accessions only from Scottish Islands (Western Isles, islands off the West coast of Scotland latitude of 58.217 and longitude -6.37 ; Orkney Isles, North of Scotland with latitude of 58.985 and longitude of -2.96 ; Shetland Isles are 80 km from Orkney at a latitude of 60.347 and the longitude is -1.236); the inset map shows the true position of Western and Orkney Isles in relation to the mainland of Scotland (Shetlands are positioned closer to Orkney for illustration). Q profiles (membership of each of the three groups) are shown as pie charts and located close to collection sites. For example, most accessions from Orkney have similar Q values for membership of that particular K group (green) and range from 0.322 to 0.999. Similar membership profiles were identified for the Western Isles (red) and Shetland Isles (blue).

Field trials. Field experiments were undertaken during the growing season 2017 at Dundee, Scotland (latitude, longitude: 56.484303, -3.118515) and at Burray, Orkney Islands, Scotland (latitude, longitude: 58.848128, -2.915237). In Orkney, the soil was a brown calcareous soil with a textural class of sandy loam, composed of 77 % sand, 11 % silt, 12 % clay, 7.2 % organic matter and a pH of 7.8 (measured in water). Soil-available Mn and Fe, and Cu and Zn (EDTA) were 1.2, 71.1, 10.1 and 1.8 mg L^{-1} , respectively. At the Dundee site, the soil was a Brown Earth with a sandy silt loam texture and a slightly acidic pH of 5.9.

The same seven genotypes as explored in the pot experiment (Table 1) were planted in a randomized complete block

design (Alpha design) with four replicates per genotype (28 plots in total). Plot size was 2.87 m^2 for the Dundee site and 2.31 m^2 for the Orkney site, with a target plant population of 300 plants m^{-2} . Raxil Star was applied as a seed dressing (5 mL kg^{-1} seed). Trial maintenance included fertilization corresponding to 66 kg N ha^{-1} total (split 2:1 at planting and later top dressing) for the Dundee site and 47 kg N ha^{-1} (at planting) for the Orkney site. Chemical pest, fungicide and weed control was applied according to normal practice. The number of days until ear emergence was recorded and, in Orkney, the number of tillers that carried spikes was counted in the middle 25 cm length of the middle two rows of each plot. At maturity, grains

TABLE 1. List of barley genotypes characterized for Mn deficiency tolerance in pot and field trial experiments

	Barley genotype	Row type	Origin	Soil type of adaptation	Soil parent material	Soil pH
Modern elites	KWS Irina	2				
	RGT Planet	2				
Bere landraces	Graminor Rødhetten	6				
	Bere Unst Shetland	6	Shetland	Magnesian gleys rich in organic matter	Drifts derived from ultra-basic igneous rocks	6.2
	Bere North Uist	6	Uist	Brown calcareous soils	Shelly sands	7.2
	Bere North Ronaldsay	6	Orkney	Calcareous gleys	Shelly sands	7.3
	Bere West Orkney	6	Orkney	Saline gleys	Drifts derived from sandstones	4.8

Row type and genotype origin (Supplementary Data Table S1) including the corresponding soil characteristics of the geographical region are given.

Soil data are retrieved from United Kingdom Soil Observatory (UKSO) and Scotland's Soils websites.

were harvested using a small plot combine. At the Orkney site, plants were initially hand cut from each plot and gathered into sheaves, and subsequently combined for the Bere landraces, but for the modern elite genotypes the ears were few and mostly empty, and were therefore threshed using a stationary laboratory thresher (Haldrup LT-15, Haldrup GmbH, Ilshofen, Germany). Grains were dried at 30 °C for 24 h. Thousand grain weight (TGW) was determined using a Marvin digital seed analyser (GTA sensorik GmbH, Germany), and the nitrogen and moisture contents were recorded using a grain analyser (Infratec 1241 grain analyser, Foss Analytics, Denmark).

Leaf and thylakoid analyses

Chlorophyll *a* fluorescence measurements. Chlorophyll *a* fluorescence was recorded with a portable Plant Efficiency Analyzer (HANDY-PEA, Hansatech Instruments Limited, King's Lynn, UK) or Imaging PAM (MAXI measuring head, Walz, Germany). The YFDLs were dark adapted for 25 min before measurements, and PSII photochemical efficiency was calculated as the ratio of F_v to F_m using the PEA Plus software (version 1.10) or visualized using the Imaging-Win software (version 2.41a). Measurement of Chl *a* fluorescence (F_v/F_m values) has been demonstrated to be a powerful tool to reveal the Mn status of the plant and consequently the progression of Mn deficiency (Schmidt et al., 2013; Leplat et al., 2016).

Leaf digestion and analysis of total element concentrations. The YFDLs harvested from each plant (hydroponics and pot experiment) or from 3–5 plants per plot (field experiments) were dried in paper bags in an oven at 60–70 °C for approx. 72 h. The dried leaves were digested in ultrapure acids (70 % HNO₃ and 30 % H₂O₂) at 240 °C and 200 bars for 15 min using a pressurized microwave digestion system (Ultrawave, Milestone Srl, Sorisole, Italy) according to Hansen et al. (2013). Certified reference material [apple (*Malus domestica* Borkh.) leaf, NIST 1515, National Institute of Standards and Technology] was included in each digestion batch to evaluate data quality (the accuracy and precision of the measurements). Following digestion, leaf element concentrations were determined using ICP-OES (Agilent 5100, Agilent Technologies, USA).

Thylakoid isolation and analysis of Mn binding in PSII complexes by SEC-ICP-MS. The YFDLs were used for thylakoid isolation as described previously (Teicher and Scheller, 1998).

Leaves were disrupted in homogenization buffer [0.4 M sucrose, 10 mM NaCl, 5 mM MgCl₂, 20 mM Tricine (pH 7.9), 10 mM L-ascorbate and 10 mM NaF] in a laboratory blender (Waring Laboratory LB20E, Connecticut, USA). The homogenate was filtered through a double layer of Miracloth (pore size 22–25 μm), and centrifuged for 10 min at 6000 g. The supernatant was discarded, and the pellet was resuspended in washing buffer [5 mM Tricine (pH 7.9), 10 mM NaF]. Subsequently, the washed thylakoids were pelleted by centrifugation for 10 min at 11 200 g, and finally re-suspended in a storage buffer [0.4 M sucrose, 10 mM NaCl, 5 mM MgCl₂, 20 mM Tricine (pH 7.9), 10 mM NaF and 20 % glycerol]. The samples were immediately snap-frozen in liquid nitrogen and stored at –80 °C. The protein concentration in each sample was determined using the Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the protocol of the manufacturer. For size-exclusion chromatography (SEC), the thylakoids were solubilized using *n*-dodecyl α -D-maltoside (α -DM) as described in Schmidt et al. (2015), and subsequently passed through a 0.45 μm nylon membrane filter (Q-max RR syringe filters; Frisette, Denmark) and 50 μg of total protein were applied to a size-exclusion column (BioBasic SEC 1000; Thermo Fisher Scientific) using an inert HPLC system (Ultimate® 3000; Dionex, Thermo Fisher Scientific). The metal binding in fractionated photosynthetic complexes was analysed by ICP-mass spectrometry (MS) (Agilent 8800 ICP-QQQ-MS) as previously described (Schmidt et al., 2015).

Data analysis

All data, the mean of three to four replicates and genotypic differences were analysed with one-way or two-way analysis of variance (ANOVA) followed by least significant difference (l.s.d.) test, and *P*-values <0.05 were considered significant. Data were tested for normality and skewness. Mean values (\bar{X}) are listed with their associated s.e values ($\bar{X} \pm \text{s.d.}/\sqrt{n}$). All statistical analyses were performed using SAS (SAS Institute; version 9.4).

RESULTS

Very little is known about the genetic variation within Northern European landraces, and much of the published research on

adaptation has focused on landraces and wild relatives from the Fertile Crescent (Morrell and Clegg, 2007; Dawson et al., 2015; Russell et al., 2016; Pankin and von Korff, 2017). Although these studies have enhanced our understanding of the dynamics of genetic diversity, the impact on contemporary breeding has still to be realized. In this study, a small collection of locally adapted ‘heritage’ accessions from the UK and Scandinavia, most of which have been grown for hundreds of years, surviving both changes in climate and agricultural practice, have been sourced and genotyped with >6000 mapped SNPs (Comadran et al., 2012) (Supplementary Data Table S1).

Genotypic variation in ancient barley landraces according to geographical zones

A simple matching Neighbor-Joining tree (Fig. 1A; for an enlarged version, see Supplementary Data Fig. S1) clearly distinguishes two-row and six-row cultivars and landraces, but more importantly highlights the distinctiveness and highly geographical clustering of the Scottish Island six-row types, known collectively as Bere barley. Each individual is colour coded based on similarity to one another, clearly showing genotypic geographically distinct island patterns (Fig. 1B). Three distinct groups can be identified based on the origin of the individuals evolved on the major island groups of Scotland, being the Western Isles, Orkney Islands and Shetland Islands (Fig. 1B). The colour groupings illustrate the differences and uniqueness amongst island groups and at the same time indicate similarities of individuals within an island (Fig. 1B). Interestingly, the diversity within the Bere six-row collection is less than the small number of six-row types surveyed from Scandinavian countries and the two-row accessions, with 53.6 % of SNPs polymorphic compared with 67.4 and 87.4 %, respectively.

Bere landraces retain superior micronutrient efficiency traits

To exploit the adaptive traits to micronutrient limitation, resulting from a long history of cultivation in adverse environments, a sub-population of 24 contrasting landraces was further characterized in hydroponics under Mn-replete or Mn-deficient conditions. The successful induction of Mn deficiency was verified by leaf Mn concentrations below 11 $\mu\text{g Mn g}^{-1}$ d. wt (Fig. 2A), while plants cultivated under Mn-replete conditions had leaf Mn concentrations well above the critical Mn concentration level, ranging between 39 and 161 $\mu\text{g Mn g}^{-1}$ d. wt (Reuter et al., 1997). The plants did not suffer from any other nutrient deficiencies (Supplementary Data Table S2).

At harvest, 30 days after planting (DAP), clear visual differences between leaves from the two treatments were observed (Fig. 2B). When cultivated under Mn-replete conditions, all plants appeared healthy with dark green leaves and F_v/F_m values above the theoretical maximum of 0.8, indicating optimal PSII efficiency and photosynthesis (Fig. 2C) (Stirbet and Govindjee, 2011). In contrast, the Mn-deficient plants revealed floppy and pale green leaves, in agreement with the well-known characteristics of interveinal chlorosis symptoms in Mn-deficient plants (Hannam and Ohki, 1988; Schmidt et al., 2016a). However, comparing the shoot growth of Mn-deficient plants with that of

control plants (Fig. 2D), it was rather surprising that both leaf size and shoot biomass were increased for Mn-deficient plants as compared with plants grown under Mn-replete conditions; in fact, 75 % of the landraces had greater root and shoot biomass under Mn-limiting conditions as compared with the control.

The barley landraces revealed great diversity in Mn efficiency, as indicated by the large span in F_v/F_m values, ranging from 0.41 to 0.65 (Fig. 2C) under Mn-deficient conditions. This is, to our knowledge, the first documentation showing such large variation in Mn efficiency within genotypes adapted to local environments (Ceccarelli, 1994; Hebborn et al., 2005; Dwivedi et al., 2016). In addition to this, our data show that the most Mn-efficient Bere landraces (landraces displaying the greatest F_v/F_m values in Fig. 2C) were also found to accumulate up to 4.1 times more Mn in the leaf tissue when grown under Mn-replete conditions compared with the Mn-inefficient two-row landraces (Fig. 2A). Furthermore, the Mn-efficient Bere landraces revealed 3.7 and 2.8 times greater Zn and Cu concentrations in the leaf tissue, respectively, compared with other UK landraces (Supplementary Data Table S2). This is remarkable considering that all 24 landraces were co-cultivated under the same conditions, thereby having access to the same amount and composition of nutrients under Mn-deficient and Mn-replete conditions.

In a subsequent hydroponics experiment, two Bere landraces, which contrasted in terms of Mn efficiency (Bere Unst Shetland and Bere Clho 8327, cf. Fig. 2C), were compared against the modern elite genotype KWS Irina (Fig. 3). Under control (Mn-replete) conditions, Bere Unst Shetland had elevated leaf Mn concentrations of >100 $\mu\text{g Mn g}^{-1}$ d. wt (Fig. 3A) and further allocated up to 2.5 times more Zn and Cu in the leaf tissue compared with the modern elite KWS Irina (Supplementary Data Table S3). The recorded biomass data were consistent with the observations described above, with the Bere landraces having larger shoots under Mn deficiency (Fig. 3B in accordance with Fig. 2D) compared with the control. In contrast, a more typical decrease in biomass under Mn deficiency was observed for the modern elite KWS Irina (Fig. 3B). Intact leaves were investigated using imaging Chl *a* fluorescence analysis (Fig. 3C). Under control conditions, the F_v/F_m values were >0.8 for all genotypes. However, under Mn-deficient conditions, distinct differences in PSII functionality appeared, demonstrating much better Mn efficiency in Bere Unst Shetland compared with Bere Clho 8327, but especially compared with KWS Irina (Fig. 3C). Furthermore, a highly specialized allocation of Mn was observed for the Mn-efficient genotype Bere Unst Shetland, as revealed by a prioritized clustering of functional PSII complexes closest to the veins and mid-rib vein of the leaf (Fig. 3C). This is in close agreement with the observable interveinal chlorosis in leaves of plants experiencing severe Mn deficiency. This observation was much more prominent in the Bere landraces compared with the elite KWS Irina. To examine differences in PSII functionality further, Mn loading in isolated and fractionated PSII super- and subcomplexes was investigated according to Schmidt et al. (2015) (Fig. 3D). Under control conditions, more Mn was bound in PSII supercomplexes of the modern elite KWS Irina, compared with Bere Unst Shetland which had a more even distribution of Mn binding between PSII supercomplexes, PSII dimers and PSII monomers (Fig. 3D). Under Mn-deficient conditions, Mn incorporation

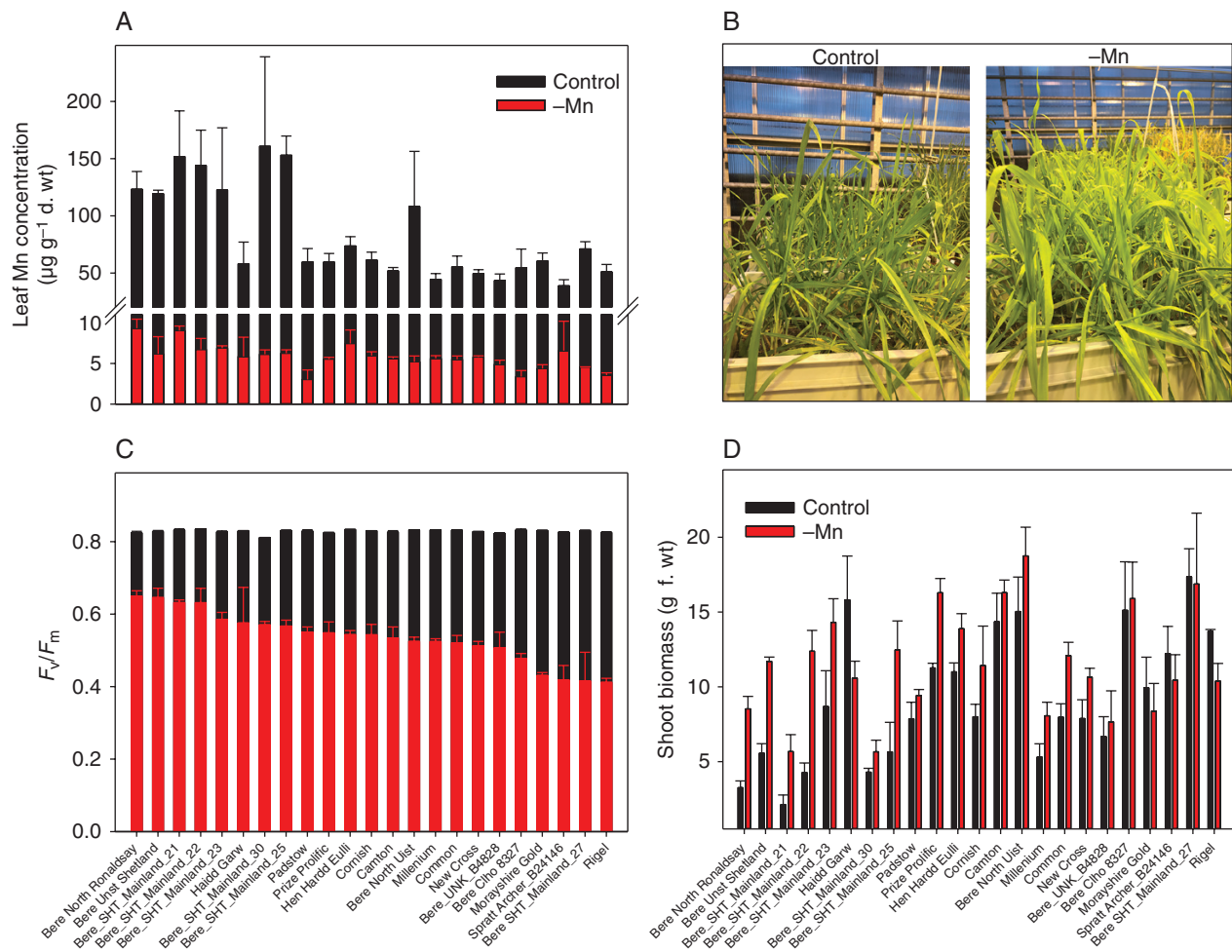


FIG. 2. Tolerance to Mn deficiency in barley landraces in hydroponic growth conditions. Barley landraces were grown in hydroponics under Mn-replete (Control) or Mn-deficient conditions (-Mn). (A) Leaf Mn concentration ($\mu\text{g g}^{-1}$ d. wt) in the youngest fully developed leaf (YFDL). (B) Appearance and vigour of control plants (left photo; full nutrient) and Mn-deficient plants (right photo) at 30 d after transplantation (DAT) to hydroponics. (C) PSII efficiency (F_v/F_m) in the YFDL of plants exposed to control or Mn deficiency conditions. (D) Shoot biomass (g f. wt). In (A), (C) and (D), the values are means \pm s.e. ($n = 3$).

into PSII was severely reduced for both genotypes, but was less pronounced for Bere Unst Shetland compared with the control. Under Mn-deficient conditions, the macro-organization of PSII complexes was similar for both genotypes (Fig. 3D).

Mn efficiency traits have been lost from modern elite varieties

Based on the data obtained from hydroponics (Fig. 2), four Bere landraces (ranking from being Mn efficient to Mn inefficient, all six-row types) were selected together with two modern elite genotypes commonly grown in Northern Europe (KWS Irina and RGT Planet, both two-row types) and a six-row modern elite variety Graminor Rødhette (Table 1). The seven accessions were genotyped, demonstrating that the modern elite genotypes were genetically very distant from the Bere landraces (Fig. 1A).

Plants of the seven genotypes were grown to maturity under semi-controlled glasshouse conditions either in nutrient-replete compost (control conditions) or in soil from Orkney, which has a high pH and limited plant availability of Mn. Already at 11

DAP, for plants grown in Orkney soil, Chl *a* fluorescence measurements enabled separation of the genotypes into two distinct groups (Fig. 4A). Bere Unst Shetland, Bere North Ronaldsay and Bere North Uist were able to retain maximal PSII efficiency (F_v/F_m values ≥ 0.80) up to 14 DAP, then started to decline, reaching F_v/F_m values of 0.60–0.69 at the flag leaf stage (Fig. 4A), but without displaying any pronounced differences in biomass growth compared with control plants. By comparison, the F_v/F_m values of the elite genotypes were reduced to between 0.45 and 0.49 (Fig. 4A), corresponding to severe Mn deficiency (Schmidt *et al.*, 2016b), and plant biomass development was clearly compromised.

Leaf Mn concentrations were $< 6.2 \mu\text{g Mn g}^{-1}$ d. wt for the modern elite varieties and the Bere West Orkney, but $> 13 \mu\text{g Mn g}^{-1}$ d. wt for the three Mn-efficient Bere landraces (Supplementary Data Table S4). The F_v/F_m values of plants grown in compost (Mn sufficient) were > 0.82 for all genotypes throughout the experimental period (data not shown), and leaf Mn concentrations ranged between 45 and $170 \mu\text{g Mn g}^{-1}$ d. wt. The Mn-efficient Bere barley landraces accumulated up to 2.8 and 2.4 times more Zn and Cu, respectively,

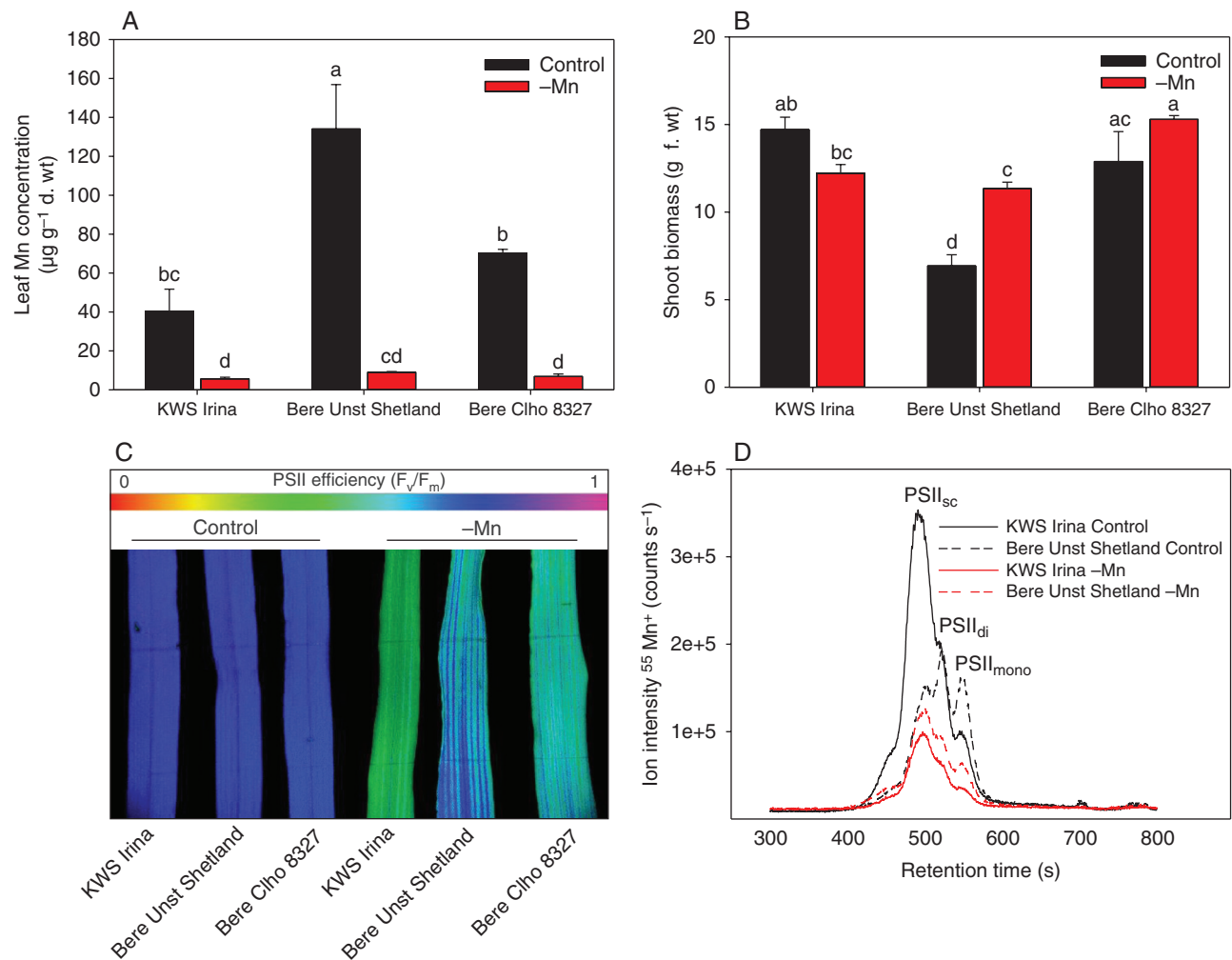


FIG. 3. Comparison of landrace and modern elite genotypes. A modern elite barley genotype (cv. KWS Irina) and two barley landraces contrasting in terms of Mn efficiency (Bere Unst Shetland and Bere Clho 8327; Fig. 2A, C) were cultivated under Mn-replete or Mn-deficient conditions in hydroponics. (A) Leaf Mn concentration ($\mu\text{g g}^{-1}$ d. wt) in the youngest fully developed leaf. (B) Shoot biomass (g f. wt). (C) PSII efficiency (F_v/F_m) for the colour scale indicates the F_v/F_m values. (D) Manganese size-exclusion chromatograms of thylakoids isolated from KWS Irina (solid lines) and Bere Unst Shetland (dashed lines) grown under control (black lines) or Mn-deficient (red lines) conditions (representative chromatograms are shown out of three independent replicates per genotype/treatment). SC, supercomplexes; di, dimers; mono, monomers. In (A) and (B), the values are means \pm s.e. ($n = 3$). Bars with the same letter are not significantly different ($P \geq 0.05$).

in the leaf tissue, compared with the modern elite genotypes (Supplementary Data Table S4). Grain yields were severely reduced in the elite genotypes by 85–93 %, whereas the yield of landraces adapted to high pH soils was limited by 50–67 % (Fig. 4B) when grown in low Mn-available soil compared with compost soil, and TGWs were unaffected (Supplementary Data Table S5).

The Mn-efficient Bere landraces are superior to elite varieties in alkaline marginal agricultural systems

To test the robustness and stability of the pronounced Mn efficiency trait further, the seven genotypes evaluated in the pot experiment were tested under naturally occurring field conditions in Orkney (soil with limited Mn availability) and Dundee (nutrient-replete soil). The observed existence of differential

Mn efficiency among the genotypes was found to be even more dramatic in the field (Fig. 5) than was observed in hydroponics (Figs 2 and 3) and pot experiments (Fig. 4).

In Orkney, at early growth stages (BBCH 20 and onwards), remarkable visual differences in growth, biomass and overall plant vigour became apparent when comparing the Mn-efficient Bere landraces and the modern elite genotypes in the field (Fig. 5A). The Mn-efficient genotypes were thriving, showing no signs of Mn deficiency symptoms (Fig. 5B, right photo), whereas the modern elite genotypes displayed severe Mn deficiency symptoms (Fig. 5B, left and centre photos). These symptoms included floppy and soft leaves easily damaged by high winds (Fig. 5B, centre photo), severe chlorosis in the youngest leaves and multiple necrotic interveinal spots in the older leaves (Fig. 5B, left photo), along with impeded plant growth resulting in delayed growth stages (Fig. 5A) and poor competition against weeds. The dramatic differences between

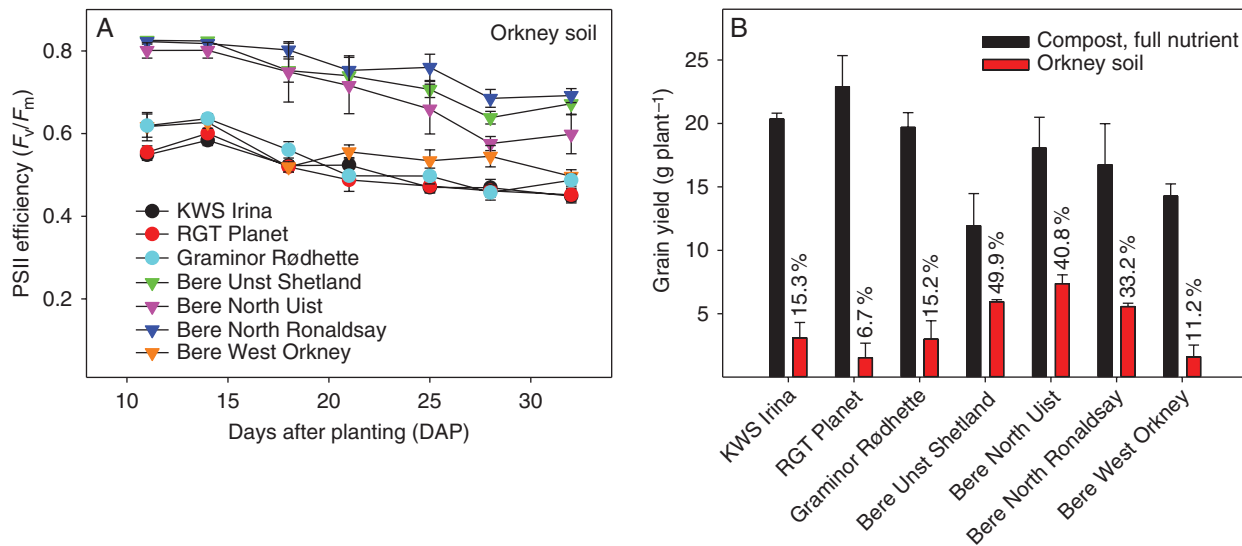


FIG. 4. Pot experiments performed under semi-controlled glasshouse conditions. (A) Three modern elite barley genotypes (circles) and four Bere barley landraces (triangles, originating from the Western Isles, Shetland and Orkney Islands) were cultivated in Orkney soil (high pH sandy soil) or in nutrient-replete compost. PSII efficiency (F_v/F_m) was recorded until the flag leaf stage. (B) Grain yield (g per plant). The values are means \pm s.e. ($n = 4$).

the varieties were unchanged as the season progressed. This was demonstrated by Chl *a* fluorescence measurements at growth stage BBCH 33–37, showing F_v/F_m values <0.37 in the three modern elite varieties, while the three Mn-efficient Beres maintained F_v/F_m values above 0.75 (Fig. 5C).

In the modern elite genotypes, leaf Mn concentrations reached only $4 \mu\text{g Mn g}^{-1}$ d. wt [far below the critical Mn threshold (Reuter *et al.*, 1997)], whereas the Mn-efficient Bere landraces translocated 5- to 6-fold more Mn to the shoot, with sufficient concentrations ranging between 19 and $23 \mu\text{g Mn g}^{-1}$ d. wt (Supplementary Data Table S4). The Mn-efficient Bere landraces further revealed up to 1.7 and 2.0 times greater leaf Zn and Cu concentrations, respectively, compared with the modern elite genotypes (Supplementary Data Table S4). The plants did not suffer from other nutrient deficiencies (Supplementary Data Table S4). The ripening of the modern elite varieties was considerably delayed, so that they were harvested 1 month later than the Bere landraces. Even though the elite genotypes utilized photosynthetic energy to set spikes (Supplementary Data Table S5), the majority of the harvestable ears were empty, comprising mostly husk, as demonstrated by the small TGW (Supplementary Data Table S5), indicating unsuccessful grain filling and resulting in an extremely poor grain yield of $<0.2 \text{ t ha}^{-1}$ (Fig. 5D). It is important to note that these grains were largely empty, without embryo and endosperm. In contrast, the Mn-efficient Bere landraces produced impressive grain yields ranging between 4.6 and 5.0 t ha^{-1} , achieved without Mn fertilization (Fig. 5D).

In the corresponding Mn-replete site in Dundee, growth and plant development were successful for all genotypes, having leaf Mn concentrations ranging between 15 and $25 \mu\text{g Mn g}^{-1}$ d. wt (Supplementary Data Table S4), and optimal concentrations of all other analysed essential elements (Supplementary Data Table S4). Notably, the Mn-efficient Bere landraces accumulated up to 2.6 and 2.8 times more Zn and Cu in the leaf tissue, respectively, compared with the modern elite genotypes. The modern elite genotypes revealed superior brackling and lodging resistance, whereas lodging was a serious issue for the

taller Bere landraces. Even more importantly, the modern elite genotypes generated excellent grain yields (Fig. 5D) up to 7.2 t ha^{-1} , proving their superior growth and high-yielding properties when cultivated under optimal nutrient regimes. In contrast, the Bere landraces achieved grain yields from 3.3 to 3.5 t ha^{-1} (Fig. 5D), which was less than the corresponding yields obtained at the Orkney field site.

Genotypic variation is associated with wider micronutrient efficiency

The Mn-efficient Bere landraces demonstrated exceptional Mn accumulation in the leaf tissue, across all environmental conditions, from hydroponics, through controlled soil pot experiments to the field. Simultaneously, the Mn-efficient Bere landraces accumulated 2.4 to 2.8 times greater Zn and Cu leaf concentrations compared with the modern elite genotypes (Supplementary Data Tables S2–S4). This observation suggests that the landraces may retain traits associated with wider micronutrient efficiencies. However, Fe seems to constitute an important exception as there are no indications that the Mn efficiency trait is associated with increased Fe uptake. In fact, when grown under Mn-deficient conditions in Orkney, leaf Fe concentrations ranged between 49 and $60 \mu\text{g g}^{-1}$ d. wt and between 70 and $80 \mu\text{g g}^{-1}$ d. wt for the Mn-efficient Bere landraces and the modern elite genotypes, respectively.

DISCUSSION

Adaptive traits to Mn deficiency have developed independently from biogeographical zones

During its domestication and subsequent cultivation, barley has adapted to a wide range of environments, and here we have demonstrated the unique ability of Bere barley landraces to

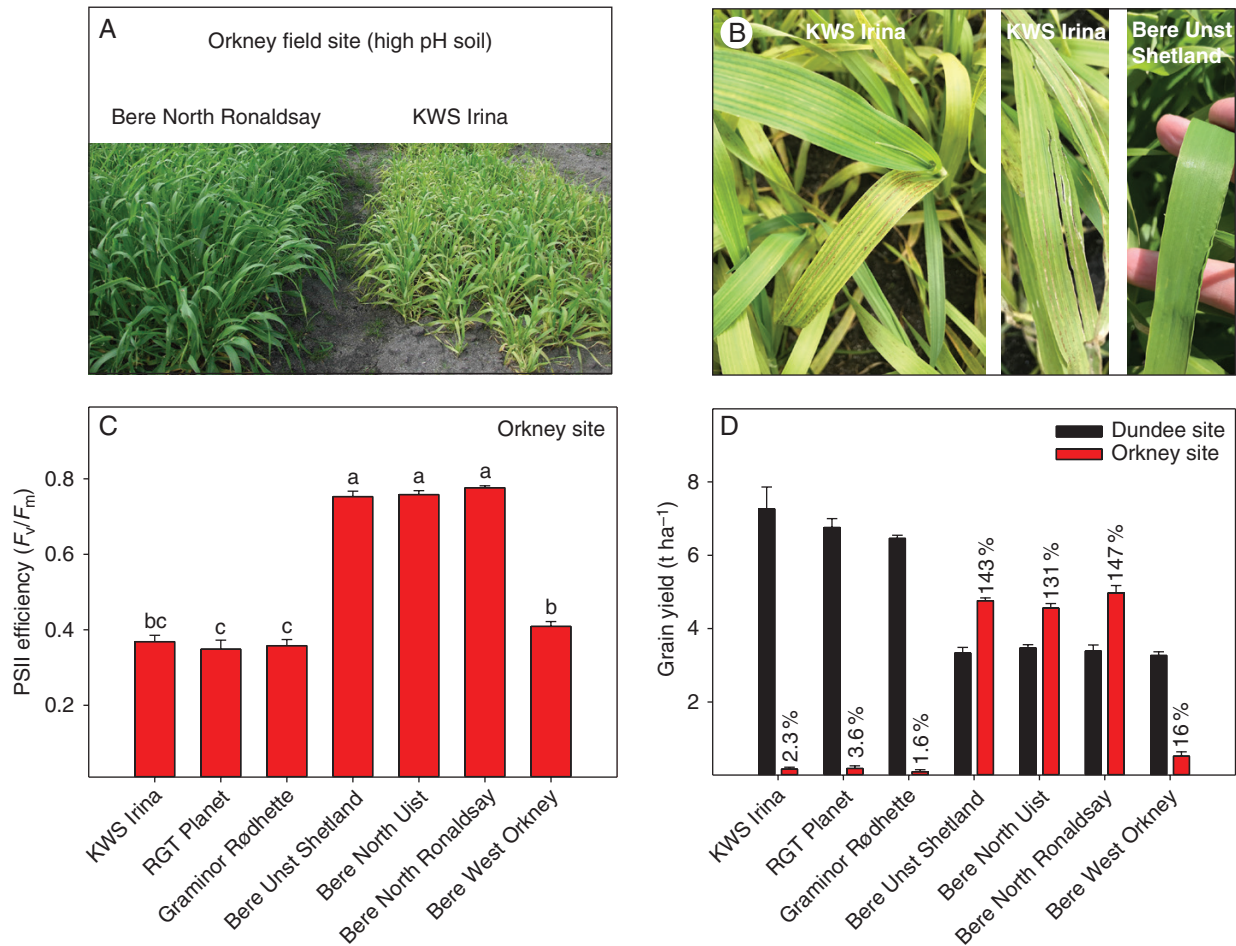


FIG. 5. Field trials conducted in Orkney (calcareous sandy soil, pH 7.8) or mainland Dundee (Mn-replete site). (A) Visual appearance of the Bere barley landrace (Bere North Ronaldsay, left plot) and the modern elite genotype (KWS Irina, right plot) growing in Orkney. (B) Severe Mn deficiency symptoms of interveinal chlorosis (younger leaves) and necrotic aligned interveinal spots (older leaves) in the modern elite genotype KWS Irina (left photo), together with wind-damaged leaves (centre photo). An unaffected leaf of Bere Unst Shetland is shown to the right. (C) PSII efficiency (F_v/F_m) of the youngest fully developed leaves of Orkney-grown plants. Values are means \pm s.e. ($n = 4$). Bars with the same letter are not significantly different ($P \geq 0.05$). (D) Grain yields obtained in the Dundee (black bars) and Orkney (red bars) field sites. The values are means \pm s.e. ($n = 3-4$).

generate robust grain yields under severe Mn-deficient conditions, while modern elite varieties completely failed to set seeds. Although it is well established that varietal differences exist, this is a unique example of the local adaptation of landraces to marginal soils, and is an exemplar of how we may be able to take advantage of these landraces to target genes for specific environmentally limiting conditions. It was particularly striking that the sub-group of Bere barley landraces demonstrating superior tolerance to Mn deficiency were those that have become adapted to alkaline, sandy and high organic matter soils (Table 1), which inherently reduce plant Mn availability (Schmidt *et al.*, 2016a). The Bere West Orkney genotype, which showed no superior Mn efficiency compared with the elite genotypes, has adapted to more acidic soils where Mn deficiency is not likely to be critical. Although the Bere barley landraces were found to cluster genetically according to their islands of origin (Fig. 1B), it appears that Bere barley landraces from distinct biogeographical zones have developed different but equivalent strategies to overcome Mn-deficient conditions (Figs 4 and 5). Under such strong and geographically varied

selection pressure, it seems likely that the adaptation to Mn deficiency has developed independently.

Mn deficiency tolerance is a complex trait

The Mn-efficient Bere landraces had grain yields ranging between 4.6 and 5.0 t ha⁻¹ in the field which was achieved without any supplementation of Mn. This leaves the plants with the only option to mobilize Mn in the root rhizosphere and to increase Mn uptake, Mn translocation and/or internal Mn use efficiency in PSII. Either way, the large effects of Mn deficiency observed for the non-adapted modern elite genotypes, but absent in the adapted Bere landraces, are truly exceptional compared with previous genotypic studies in barley (Hebborn *et al.*, 2005; Husted *et al.*, 2009; Schmidt *et al.*, 2015, 2016b; Leplat *et al.*, 2016). However, although the Bere landraces demonstrated an impressive adaptation in the ability to maintain photosynthesis under Mn-deficient conditions, there was a yield penalty when grown under optimal conditions where

a range of other undesirable traits come into play. The Bere barley landraces accumulated Mn under controlled Mn-replete conditions (Figs 2A and 3A; Supplementary Data Table S4), which together with their decreased biomass (Figs 2D and 3B) implies an impaired ability to regulate Mn uptake and homeostasis under these conditions.

The physiological mechanisms underlying Mn efficiency in plants are still not fully understood. Unique traits in the landraces may involve mechanisms yet to be elucidated or polymorphisms in single genes controlling processes that are already known. Even so, our results suggest that the landrace phenotype is a complex and polygenic trait, and may include a combination of mechanisms ranging from Mn mobilization in the rhizosphere, to uptake, translocation as well as Mn delivery to dependent processes in photosynthesis. These physiological processes are discussed in the following section based on the results obtained in this study.

Responsive root traits confer tolerance to environmental changes in Mn levels

Changing the chemistry of the rhizosphere to mobilize plant-available Mn^{2+} via root exudates, proton release (Rengel and Marschner, 2005; George et al., 2014; Rengel, 2015; Liu et al., 2017) or promoting soil microflora activity (Marschner et al., 2003) may contribute to enhance Mn acquisition under field conditions where Mn availability is limited (Fig. 5). Previous studies with traditional landraces have demonstrated upregulation of root phytase activity under Mn deficiency (George et al., 2014), enabling mobilization of Mn from the phytate-bound pool in soils. However, considering the enormous genotypic variations in tolerance to Mn deficiency observed in the hydroponic experiments (Figs 2 and 3), root traits are unlikely to be the singular responsive mechanism to Mn deficiency. More likely to be involved is the primary uptake of Mn, mediated by high and low affinity root transport proteins. So far, the only known Mn transporters in barley roots are the Natural Resistance Associated Macrophage Protein NRAMP5 (Sasaki et al., 2012; Wu et al., 2016) and the Iron-Regulated Transporter IRT1 (Pedas et al., 2008; Long et al., 2018). The expression of *Nramp5* is unaffected by Mn deficiency, but is slightly upregulated under Fe deficiency (Wu et al., 2016); however, its regulation at the protein level remains unknown. Iron-Regulated Transporter 1 is a broad-spectrum metal transporter that also transports Fe, Zn, Co and Cd, and has previously been associated with genotypic differences in Mn uptake (Pedas et al., 2005, 2008). In this context, both Mn and Fe deficiency have been shown to induce an upregulation of HvIRT1 in genotypes with contrasting Mn efficiency, with the greatest expression levels found in the Mn-efficient genotype (Pedas et al., 2008). In this context, recent research has demonstrated that IRT1 in Arabidopsis acts like a sensor for soil concentration of metals (Dubeaux et al., 2018), by a sophisticated iron-dependent transcriptional network of regulation, controlling IRT1 to ensure optimal metal (including Mn and Zn) absorption by roots. It could be speculated that an adapted (IRT1) transport system exists in Bere barley landraces, where a reduced Fe sensing and/or xylem loading of Fe will increase Mn uptake.

This hypothesis is further supported by the homeostatic interplay between Mn and Fe uptake observed in the Mn-efficient Bere landraces when grown in an alkaline calcareous soil (Supplementary Data Table S4). Studies by Eroglu et al. (2016) have demonstrated Fe deficiency-induced chlorosis phenotypes related to a disturbed Mn homeostasis in Arabidopsis lacking the vacuolar Mn transporter MTP8, indicating that high Mn stress induces Fe deficiency. Given the distinct biogeographical adaptive traits of the Bere barley landraces to high pH soil conditions, where both Mn and Fe availabilities are limited due to their redox sensitivity, such an Fe sensing and uptake strategy for micronutrient in general would be advantageous.

Regulation of Mn translocation and delivery to photosystem II

Manganese uptake by the roots and the translocation of Mn to demanding leaf tissues solely relies on xylem transport, while remobilization is virtually absent due to the phloem immobility of Mn in plants. In graminaceous plant species, upon nutrient uptake by the roots and the subsequent translocation to the shoots, nutrients are preferentially distributed via a well-organized system in the nodes (Shao et al., 2017). Here a junction of vasculatures connects the leaf and the stem, and within nodes, the intervascular transfer of nutrients is mediated by nutrient-specific transporters (Yamaji and Ma, 2017). In rice, a node-switch transport protein, NRAMP3, functions in preferential Mn distribution to active developing tissues under Mn-limiting conditions (Yamaji et al., 2013). However, the mechanisms controlling the intervascular transport and allocation of Mn in leaves, including variations at the genotypic level, remain to be identified. Notably, our data show that the Mn-efficient Bere barley landrace has a distinct Mn allocation pattern along the veins of the leaf (Fig. 3C), which could represent an optimized strategy to ensure immediate access of Mn for PSII repair under Mn limitation. Furthermore, marked differences in Mn loading to PSII of chloroplasts was observed, not only under Mn-deficient conditions but also under control conditions. We have previously revealed differential Mn loading to PSII as a process contributing to Mn efficiency in barley (Schmidt et al., 2015). This increased internal utilization of Mn was further associated with increased photosynthetic protective non-photochemical quenching mechanisms and PSII stability, the latter mediated by the extrinsic proteins PsbP and PsbQ of PSII (Schmidt et al., 2016b). At the mechanistic level, the genotypic differences in Mn delivery to PSII supercomplexes observed between the Mn-efficient landrace and the modern elite genotype under control conditions may reflect adjustments to Mn toxicity given the leaf Mn accumulation of $140 \mu\text{g Mn g}^{-1} \text{ d. wt}$ in the Bere Unst Shetland landrace compared with $40 \mu\text{g g}^{-1} \text{ d. wt}$ in the elite genotype KWS Irina. The greater Mn loading to all PSII complexes observed for Bere Unst Shetland under Mn-deficient conditions compared with KWS Irina, may involve barley homologues of the recently identified Arabidopsis chloroplast high affinity Mn import proteins CMT1 (Eisenhut et al., 2018) and PAM71 (Schneider et al., 2016), which could be present in the Mn-efficient Bere landraces, but lost from the elites. Indeed, an efficient and timely incorporation of Mn in PSII is crucial under low Mn regimes, but could

also involve unidentified Mn chaperones to ensure adequate Mn supply to the thylakoid lumen (Schmidt *et al.*, 2016a; Krieger-Liszkay and Thomine, 2018).

In conclusion, the presented results provide an illustrative case study for the use of traditional landraces as sources of genes and traits to improve agricultural sustainability, and comprise a strong argument to save and maintain extant populations of landraces. We identified a unique Mn-efficient phenotype in Bere landraces that can be used to cope with a timely issue of global significance. The Mn efficiency trait is likely to be polygenic and highly complex, and might include a range of key processes along the entire pathway from Mn mobilization in soil via root exudates, to ion transporter activity and regulation as well as homeostatic responses at the leaf level to regulate photosynthesis and other processes of basic metabolism. In future studies, phenotyping crosses between

Mn-efficient landraces and Mn-inefficient modern elite lines could provide more insight into the genetic basis and help resolve the complexity of the trait. The potential areas of research to unravel the trait are summarized in Fig. 6. Studies into these avenues of research will provide additional information on the molecular basis for micronutrient efficiency and the underlying genetic control to develop novel genotypes matching site-specific soils and climatic conditions, and thereby unlock the production potential of marginal soils.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Table S1: details for accessions presented in Fig. 1. Table S2: leaf element concentrations of UK landraces grown in hydroponics. Table S3: leaf

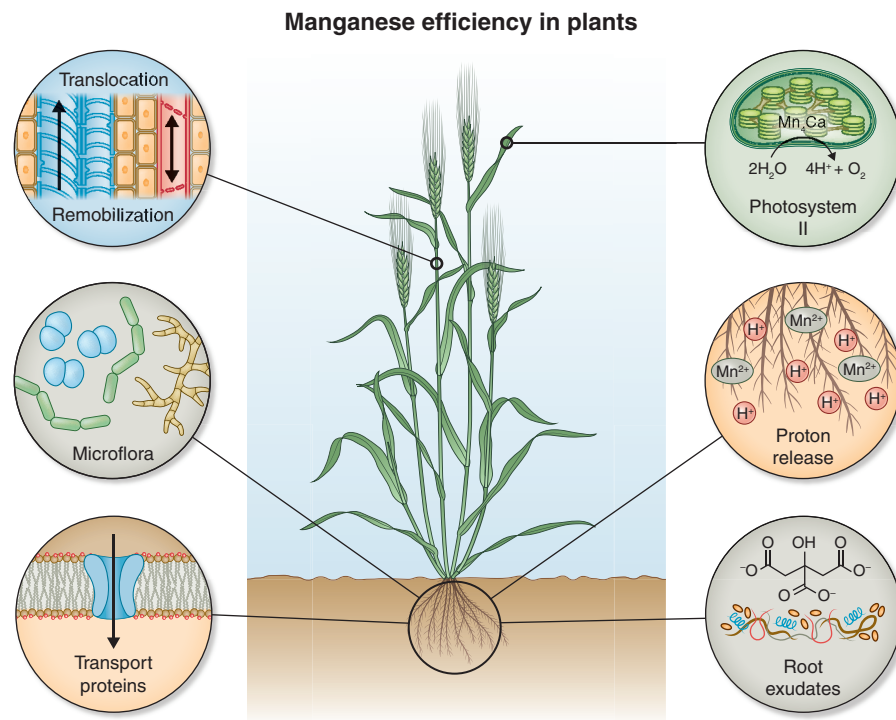


Fig. 6. Conceptual model summarizing selected key processes controlling manganese (Mn) efficiency in plants. Proton release: increased proton-pumping activity releases H^+ into the rhizosphere and promotes lowering of pH, which dissolves Mn(IV) oxides into plant-available Mn^{2+} (Mizuno *et al.*, 2006). Root exudates: exudation of organic acids such as citrate, malate, oxalate and oxaloacetate to the rhizosphere changes soil Mn solubility through their ability to reduce Mn(IV) oxides by ligand exchange and complexing the released Mn^{2+} to increase plant availability (Gherardi and Rengel, 2004; Rengel and Marschner, 2005; Chen *et al.*, 2015; Rengel, 2015; Liu *et al.*, 2017). Under Mn deficiency, root phytase exudation can be upregulated in plants, to solubilize Mn^{2+} complexed with inositol phosphates (George *et al.*, 2014). Microflora: rhizosphere micro-organisms play important roles in plant tolerance towards Mn deficiency. The rhizosphere is rich in bacteria and fungi, with the ability to change the redox state of Mn and thereby influence the plant availability of Mn (Kothari *et al.*, 1991; Posta *et al.*, 1994; Marschner *et al.*, 2003). Transport proteins: a range of plasma membrane-bound proteins localized at the root surface (IRT1 and NRAMP1;5) (Pedas *et al.*, 2008; Cailliatte *et al.*, 2010; Sasaki *et al.*, 2012; Wu *et al.*, 2016) and endogenous transport proteins (VIT1, CAX2, ECA1;3, ZIP1, NRAMP2;3;4 and MTP8, 11) (Pedas *et al.*, 2014; Alejandro *et al.*, 2017; Shao *et al.*, 2017) are regulated by the Mn status of plants and are involved in primary uptake of Mn^{2+} . Translocation and remobilization: upon Mn uptake by the roots, Mn loading and unloading of xylem and phloem is mediated by Mn transport proteins (Yamaji *et al.*, 2013). Furthermore, the chemical environment of the vascular tissue fluid (Álvarez-Fernández *et al.*, 2014), and leaf transpiration (Hebborn *et al.*, 2009) influence Mn mobility within plants. Photosystem II: at the leaf level, the chloroplast Mn transporters, CMT1 (Eisenhut *et al.*, 2018) and PAM71 (Schneider *et al.*, 2016), are involved in Mn^{2+} homeostasis and required for Mn delivery to PSII super- and subcomplexes to fulfil the catalytic function of Mn in water splitting in photosynthesis. The peripheral proteins PsbP and PsbQ of PSII protect the Mn cluster of PSII and are important for PSII stability and functionality (Schmidt *et al.*, 2016a, b).

element concentrations of an elite genotype and Bere barley landraces grown in hydroponics. Table S4: leaf element concentration for plants grown in pot and field experiments. Table S5: phenotypic scores recorded for Orkney and mainland Dundee field trials. Figure S1: enlarged version of Fig. 1A.

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