Theor Appl Genet (2000) 101:1100–1108

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RFLP mapping of manganese efficiency in barley

Received: 20 October 1999 / Accepted: 18 February 2000

Abstract In many cropping regions of the world, yield is limited by the availability of micronutrients, and micronutrient-efficient cultivars provide a yield advantage. Traditional methods of testing cultivars for micronutrient efficiency are time-consuming and laborious. Molecular markers linked to loci controlling micronutrient efficiency will allow more rapid and efficient selection and introgression of these traits than is currently possible. Using a pot-based bioassay and bulked segregant analysis of an F_2 population, we have identified several RFLPs (grouped distally on chromosome 4HS) linked to a locus for manganese efficiency in barley. This manganese efficiency locus has been designated *Mel1*. Pot bioassay analysis of intercrosses suggests that three useful sources of manganese efficiency are likely to be allelic at the *Mel1* locus. Field evaluation of marker selected F_4 progeny supports the major role of *Mel1* in the genetic control of manganese efficiency. Adoption of marker assisted selection for this trait in the Southern Australian barley breeding program has occurred. This has been facilitated by the demonstration that the *Mel1* allele of Amagi Nijo can be distinguished from 95 other locally useful varieties and breeder's lines on the basis of RFLPs identified by just two molecular markers.

Key words Barley · Manganese efficiency · RFLP mapping · Marker-assisted Selection · Plant nutrition · 4HS

Communicated by J. Dvorak

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Introduction

Manganese (Mn) deficiency in cereals grown on alkaline calcareous soils is a problem affecting a large portion of the cropping area of southern Australia and elsewhere in the world. On these soils manganese, although present, is usually in a complex plant-unavailable form (Marschner 1988). Plants that are deficient in manganese appear chlorotic as manganese has a major role in photosynthetic oxygen evolution. Manganese-deficient plants grow poorly and consequently yield poorly. Additionally, these plants are more susceptible to damage by diseases, such as take-all caused by the root fungal pathogen *Gaeumannomyces graminis* var. *tritici* (Wilhelm et al. 1988). Manganese deficiency is difficult to overcome by the addition of manganese-supplemented fertilizers at sowing as the added manganese is quickly converted to an unavailable form (Reuter et al. 1973**)**. Foliar applications of manganous sulphate are more effective, especially if applied several times during the growing season, but this comes at a significant cost.

Manganese-efficient cultivars are able to reach their yield potential on manganese deficient soils without manganese supplementation (Graham 1988). The exact physiological basis of manganese efficiency in cereals (or any other crop) grown on calcareous soils has not yet been elucidated, although several possibilities have been tested (Huang et al. 1994; Webb 1994; Marschner 1988). It appears that efficient cultivars, compared to inefficient ones, are able to take up more manganese from these soils, rather than being able to make more efficient use of a low amount of manganese within the plant (Graham 1984; Graham et al. 1985). Expression of the efficiency trait can vary depending on a number of factors, such as temperature and soil batch in pot tests and time of sowing and rainfall in the case of field trials. Manganese efficiency is not expressed in solution culture (Huang et al. 1994).

Genotypic variation exists within barley for manganese efficiency (Graham 1988), with some cultivars, such as Amagi Nijo and Weeah, being very manganese-efficient. Earlier genetic studies of the manganese effi-

ciency trait in barley indicated that it may be simply controlled (McCarthy et al. 1988) and thus may be easily transferred to elite breeding lines. However, identifying efficient genotypes in segregating populations is time- and resource-consuming and somewhat imprecise. This applies to both field trials and soil-based pot bioassays in growth chambers because of the significant environmental influence. Furthermore, it can be difficult to distinguish lines heterozygous for the efficiency allele(s), thereby imposing a limitation on backcrossing of the trait into locally adapted varieties. The development of molecular markers for manganese efficiency will greatly enhance the accuracy and speed of selection for this trait, rendering it a practical breeding objective.

Materials and methods

Genetic material

The lines used in this study were the barley cultivars Amagi Nijo, Haruna Nijo, Weeah, Skiff and Galleon and the breeder's lines WI 2585 (Dr. D.H.B. Sparrow, University of Adelaide), WA73S276 (Dr. R. Boyd, University of Western Australia) and VIC 9307, a selection from the cross Haruna Nijo x Schooner (Mr. D. Moody, Victorian Institute for Dryland Agriculture, Horsham, Victoria). Manganese efficiency in the field has been demonstrated for Weeah (Graham et al. 1983), WA73S276 (Longnecker et al. 1990) and Amagi Nijo (unpublished data) over a minimum of 3 years. Manganese inefficiency in the field has been demonstrated for Galleon (Graham et al. 1983) and WI2585 and Skiff (unpublished data) over a minimum of 4 years. The lines Haruna Nijo and VIC 9307 appear to have an intermediate level of manganese efficiency based on field trials over 3 years (unpublished data). Seed of Amagi Nijo, Haruna Nijo, Weeah, WA73S276 and WI 2585 was obtained from Dr. R.C.M. Lance (currently at Agriculture Western Australia, Perth) and seed of Skiff, Galleon and VIC 9307 was obtained from Mr. J. Lewis (University of Adelaide, Adelaide). All seed used in this study came from collections at the University of Adelaide. A doubled haploid (DH) population was generated from F_1 plants of the cross Amagi Nijo×WI 2585 (Davies and Morton 1997). A subset of 55 individuals from this DH population was used in this study.

Pot bioassay for Mn efficiency

The method and soil used for the bioassay was essentially as described by Huang et al. (1994) with the following modifications. All plants used in any particular bioassay were derived from plants grown in the same batch of soil to provide seed with similar and adequate nutrient profiles. This is necessary as seed nutrient deficiency can markedly retard early seedling growth (Uren et al. 1988), and dissimilar seed levels of manganese can give artefactual results for seedling performance. Each bioassay was performed at one level of added Mn, selected to result in Mn deficiency in Mn-inefficient genotypes. This level was estimated for each bioassay, depending on time and conditions of soil storage as both affect the level of plant-available manganese in the soil (Webb et al. 1993) and ranged from 0 ppm to 10 ppm. Nutrients were added at the following rates: (mg/kg dry soil) $Ca(NO_3)_2.4H_2O$, 918; KH_2PO_4, 179.5; $MgSO_4.7H_2O$, 140; H_3BO_3, 5.6; $ZnSO_4.7H_2O$, 17.16; NaCl, 4.16; $CuSO_4.5H_2O$, 3.9; $CoSO_4.7H_2O$, 0.59; $H_2MoO_4.H_2O$, 0.13; K_2SO_4, 28.4; $FeSO_4.7H_2O$, 17.2. Pot sizes were either 16-cm diameter×15 cm, holding 2 kg of dry soil and 12 seedlings, or 20-cm diameter× 15 cm, holding 3 kg of dry soil and 16 seedlings. Seeds were surface-sterilized by treatment with 1% NaOCl for 15 min, washed three times with sterilized nano-pure water and germinated on Whatman No. 1 filter paper prior to sowing. Pots were watered daily to a set weight, being the combined weight of the pot, plastic lining, dry soil and water equivalent to 20% of the dry soil weight. Plant material for analysis consisted of all leaves except the oldest two, which were omitted as most of the manganese contributed by the seed is in these older two leaves. Samples were digested with nitric acid and concentrations of the elements Mn, Zn, Cu, B, S, Na, K, P, Ca, Mg and Fe were determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES). After the bioassay all plants were repotted into a standard soil mix and allowed to re-grow. Young leaf tissue was collected from these plants for DNA isolation. Seed was harvested from mature plants.

DNA clones

Restriction fragment length polymorphism (RFLP) clones were obtained through the Australian Triticeae Mapping Initiative (Dr. P. Sharp, University of Sydney) and from the probe repository of the International Triticeae Mapping Initiative.

RFLPs

DNA extraction

DNA extraction was achieved using a DNA mini-prep method adapted from Rogowsky et al. (1991). Variations to the method were as described below. For the initial extraction, 750 µl of extraction buffer and phenol-chloroform-isoamyl alcohol (25:24:1) were used. The extraction buffer was 0.1 *M* TRIS-HCl pH 8, 10 m*M* EDTA, 0.1 *M* NaCl, 1% sarkosyl. After the second phenol-chloroform-isoamyl alcohol extraction the aqueous phase was extracted once with an equal volume of chloroform. DNA was precipitated by the addition of 0.1 vol of 3 *M* sodium acetate (pH 4.8) and 1 vol of propan-2-ol.

Restriction endonuclease digestion and Southern hybridization followed standard methods.

Bulked segregant analysis (BSA) and mapping methodology

F_2 plants of the cross Amagi Nijo (Mn efficient)×WI 2585 (Mn inefficient) were bioassayed in two experiments, and 17 plants were identified as likely homozygotes, based on their leaf Mn concentrations and plant vigor. Previous experiments have shown a positive correlation between Mn uptake of plants grown under controlled conditions in calcareous soil and their manganese efficiency ranking as determined by field trials (Huang et al. 1994). DNA isolated separately from the selected plants was pooled to create two bulks (Michelmore et al. 1991); an "inefficient bulk" consisting of DNA from ten manganese-inefficient plants and an "efficient bulk" consisting of DNA from seven manganese-efficient plants. Parents and bulks were digested with the restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII and screened with RFLP markers selected from a consensus linkage map for barley (Langridge et al. 1995). Markers were chosen to cover the genome at a maximum of 30-cM intervals, as this genetic distance should give a high probability of detecting all regions of linkage to the trait (Darvasi et al. 1994). Additional candidate linked markers were identified from published genetic maps of barley and wheat (Liu and Tsunewaki 1991; Heun et al. 1991; Graner et al. 1991; Gale et al. 1995; Langridge et al. 1995; Qi et al. 1996).

Linkage analysis

Markers showing association with the efficient and inefficient bulks were further analyzed for linkage to the trait in four populations, one consisting of 84 F_2 plants of the cross Amagi Nijo×WI 2585, the second 45 doubled haploid plants of the cross Amagi Nijo×WI 2585, the third consisting of 46 F_2 plants of the cross WA73S276×WI 2585 and the fourth consisting of 85 F_2 plants of the cross Haruna

Nijo×WI 2585. Single-point linkage analysis ($P<0.001$) was performed using MAP MANAGER QT version b16 software (Manly and Cudmore 1997) for both leaf manganese concentration and leaf manganese content. Results were similar for both parameters so data for leaf manganese concentration only are presented. Estimates of map distance were calculated directly from recombination fractions without the use of mapping functions. Interval analysis was performed with the computer program qGene (Nelson 1997).

Field trial and biostatistical analysis

A field trial was conducted at a Mn-deficient site at Marion Bay on Southern Yorke Peninsula in South Australia during the winter of 1997. The trial consisted of 13 F_4 lines derived from individual F_2 plants (Amagi Nijo×WI 2585) and seven control lines (two entries of each of the parental lines, and one entry each of the cultivars Skiff, Galleon and VIC 9307). Seed of the F_4 lines came from manganese-supplemented F_3 double rows grown at Marion Bay in 1996. Seed of the control lines came from either manganese supplemented plots grown at Marion Bay in 1996 or from plots grown at a site which was not manganese-deficient. Each entry was replicated four times except for 4 of the F_4 lines where seed availability was limited (minimum of two replications). Each replicate consisted of a paired plot. One plot of the pair received no manganese supplementation (nil Mn treatment), and the other received three supplementary manganese treatments (+Mn treatment). These were: one at sowing (20 kg Mn/ha as manganese oxysulphate); and two foliar spray treatments, each 1 kg/ha of manganese as manganese sulphate (Mangasol 47, Top Australia Ltd) during the growing season, at approximately the maximum tillering to early elongation stage (71 days after sowing) and then the early grain filling stage (110 days after sowing).

Prior to sowing, the area was treated with a mixture of Paraquat (112.5 g/ha), Diquat (22.5 g/ha) and Trifluralin (480 g/ha). At sowing, the basal fertilizer 15:18 plus trace elements (1.3% S, 1.16% Cu, 0.014% Co, 1.18% Zn and 0.125% Mo) was applied at the rate of 160 kg/ha to all plots. Seeding rate was 170–180 seed/m^2, and plots were 6 rows (15-cm spacing) by 5 m. The harvested plot area was 3.35 m^2. At 55 days after sowing the herbicides Tralkoxydin (200 g/ha), Bromoxynil (280 g/ha) and MCPA (280 g/ha) were applied for weed control.

Plots were assessed visually approximately 3 weeks prior to harvest at the early ripening stage and grain yield of plots measured post-harvest. Spatial analysis of the trial data was undertaken to remove some of the natural and extraneous variation (Gilmour et al. 1997). The lines were grouped according to allele type at the *Mel1* locus, and a Wald test for the difference between the two groups was performed (Cox and Hinkley 1971).

Production of the subclone abg714.1.2.

Restriction digestion of the clone abg714 with *Pst*I releases two non-vector fragments (0.3 kb, 1.5 kb). The 1.5-kb fragment was mapped in the NABGMP population Harrington×TR306 (Olin Anderson, personal communication). Standard methods (Sambrook et al. 1989) were used to produce a clone containing the 1.5-kb *Pst*I fragment of abg714 in pZErO™-2 (Invitrogen Corp). The resulting clone was named abg714.1. Subsequently, a 572-base *Bam*HI fragment of abg714.1 was cloned into pZErO™-2. The resulting subclone was named abg714.1.2.

Results

Genetic studies

Prior to the RFLP screen, preliminary pot bioassay studies were undertaken to establish the consistency of the Mn efficiency trait between cultivars. A pot bioassay of parents and reciprocal F_1 plants of the crosses WA73S276×WI 2585, Amagi Nijo×Haruna Nijo and Amagi Nijo×Weeah showed no evidence of a maternal effect. The F_1 plants showed an intermediate phenotype relative to their respective parents in all crosses. A similar observation was made in the pot bioassay experiments involving F_2 plants of the crosses Amagi Nijo×WI 2585 and Haruna Nijo×WI 2585 in which F_1 and parental plants were included as control lines.

Identification of F_2 lines for use in bulked segregant analysis (BSA)

The cross Amagi Nijo×WI 2585 was chosen for genetic analysis as, of the efficient varieties, Amagi Nijo (a malting-quality variety) is the preferred source of manganese efficiency for the breeding program. Two bioassays involving a total of 173 F_2 plants allowed the selection of 17 plants that exhibited extreme phenotypes. Ten lines from the ”inefficient” end and 7 lines from the ”efficient” end of the distribution were selected. Figure 1 shows the phenotype distributions of the two pot bioassays and also the data points at which plants were selected for the two bulks. Subsequently, 14 selfed progeny of each selection were tested by pot bioassay. The progeny of 1 selection belonging to the ‘efficient’ pool showed a range of phenotypes from ‘inefficient’ (equivalent to the inefficient parent) to ‘efficient’ (equivalent to the efficient parent), indicating the selection was heterozygous for manganese efficiency. All other selections were confirmed to have been homozygous for the trait as tested by the pot bioassay.

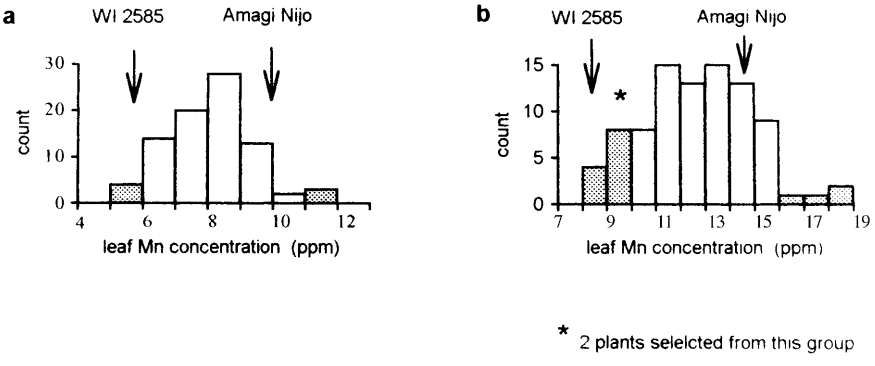

Fig. 1a, b Frequency distributions of manganese concentrations in the leaf tissue of 4-week-old F_2 plants of the cross Amagi Nijo×WI 2585. Results are from 84 plants tested in bioassay 1 (**a**) and 89 plants tested in bioassay 2 (**b**). Plants selected for use in the bulked segregant analysis are from areas of the distribution marked by *shading*. Two plants only were selected from the group marked with the *asterisk*

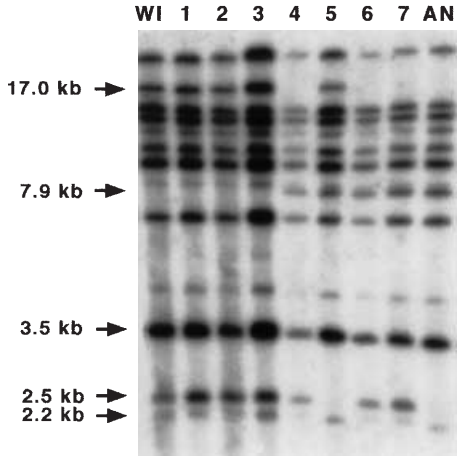

Fig. 2 Autoradiograph of genomic DNA of F_2 plants digested with *Eco*RI and hybridized to the probe cdo583. The 7.9-kb fragment from Amagi Nijo is associated with manganese efficiency, the 17-kb fragment from WI 2585 is associated with manganese inefficiency. *Lanes*: *WI* and *AN* WI 2585 and Amagi Nijo, respectively, *1–3* homozygous Mn-inefficient, *4*, *6* and *7* homozygous Mn-efficient, *5* heterozygote

RFLPs linked to manganese efficiency in the cross Amagi Nijo×WI 2585

A total of 250 RFLP markers were tested on the parental lines (Amagi Nijo and WI 2585) and the selected F_2 bulks. Of these, 143 (57.2%) detected a polymorphism between the parents with at least one of the five enzymes tested. The 4HS RFLP loci *Xwg622*, *Xabg714*, *Xmwg77*, *Xcdo669*, *Xwg876*, *Xcdo795* and *Xwg232* (Graner et al. 1991; Langridge et al. 1995; Qi et al. 1996) all showed association with the trait. The probe cdo583, previously mapped to 5HL (Heun et al. 1991), detected 16 bands when hybridized at 'normal' stringency to a gel blot of genomic DNA digested with *Eco*RI. One *Xcdo583* RFLP locus, shown in Fig. 2, showed association with the trait. MAP MANAGER QT version b16 analysis of 84 individual F_2 plants (Fig. 1a) (see also below) placed the polymorphism of *Xcdo583* linked to manganese efficiency in the distal region of 4HS, within the interval WG622 - CDO669. This RFLP locus was designated *Xcdo583B* to distinguish it from the previously mapped locus. The 4HS locus for manganese efficiency detected in this study was named *Mel1*.

No other regions showing an association to the trait were detected in this study. However, there were several segments of the genome where a lack of polymorphism between the parents for the available RFLP markers meant that BSA-screened markers were more than 50 cM apart. Specifically, the interval *Xwg241* to *Xabg494* on 1H, the interval *Xbcd402* to *Xcdo650* on 4H and the interval *Xmwg820* to *Xpsr167* on 6H remain unscreened.

To confirm the linkage of markers detected by BSA to a locus for manganese efficiency, we tested all candidates on two Amagi Nijo×WI 2585 populations. In the first population of 84 F_2 plants (Fig. 1a), linkage was determined by simple regression analysis and interval analysis. Results for the regression analysis of loci *Xwg622*, *Xcdo583B*, *Xabg714*, *Xmwg77*, *Xcdo669* and *Xwg876*, which all showed significant (P<0.001) likelihood of linkage, are given in Table 1. Figure 3 shows the results of the interval analysis using the above loci and *Xcdo795*. Loci *Xcdo583B* and *Xabg714* appeared to be most closely associated with the trait, with *Xcdo583B* explaining 67% of the total trait variation (Table 1). This equates to approximately 77% of the genetic variation, assuming that error (based on the variance observed for the parents and F_1 plants in the bioassay) contributed 12.9% of the total F_2 variance. Broad sense heritability is 0.87, calculated from the formula $H^2=V_G/(V_G+V_E)$ where V_G is the genetic component and V_E is the error component of the total variation.

Table 1 Likelihood of marker linkage to shoot Mn concentration for a population of 84 F_2 plants from the cross Amagi Nijo×WI 2585 grown in a manganese deficient soil. Based on results from MAP MANAGER QT version b16 (P<0.001)

Locus	LOD	Probability	Percentage of total variance
Xwg622	8.8	$1.7\ e^{-9}$	46
Xcdo583B	17.9	$1.4\ e^{-18}$	67
Xabg714	17.6	$2.3\ e^{-18}$	65
Xmwg77	10.4	$3.8\ e^{-11}$	45
Xcdo669	8.3	$4.7\ e^{-9}$	38
Xwg876	7.2	$6.1\ e^{-8}$	37

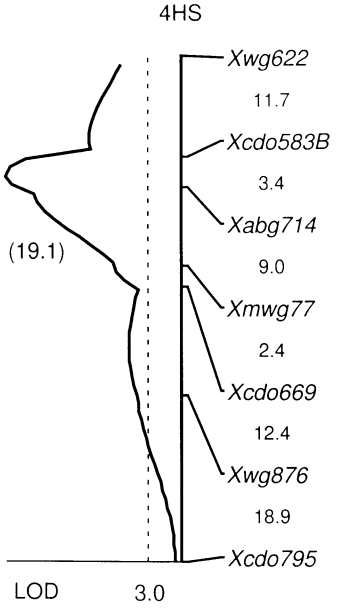

Fig. 3 Location on chromosome 4HS of a major locus for manganese efficiency based on an interval analysis without adjustment for missing marker values. The centromere is towards the bottom of the map. Maximum LOD score is given in *parenthesis*. The *fine dashed line* shows the LOD 3.0 threshold. The map distances, shown between marker loci, are derived from an analysis of 84 F_2 lines from the cross Amagi Nijo×WI 2585 using MAP MANAGER QT version b16

Table 2 Spatially adjusted mean yields and standard errors of F_4 lines and control lines grown in 1997 in a replicated field trial at the manganese-deficient site Marion Bay (A. Nijo Amagi Nijo)

Line	Treatment	Yield (g/plot)	Standard error (g)	Line	Treatment	Yield (g/plot)	Standard error (g)
WI 2585	+Mn	802	33.8	F_4-77	+Mn	1135	45.3
WI 2585	–Mn	173	33.8	F_4-77	–Mn	683	45.3
A. Nijo	+Mn	727	33.8	F_4-101	+Mn	710	45.1
A. Nijo	–Mn	654	33.8	F_4-101	–Mn	489	45.1
Skiff	+Mn	1103	45.0	F_4-104	+Mn	748	51.2
Skiff	–Mn	160	45.0	F_4-104	–Mn	298	51.2
Galleon	+Mn	802	45.0	F_4-105	+Mn	788	45.1
Galleon	–Mn	212	45.0	F_4-105	–Mn	300	45.1
Vic 9307	+Mn	1067	45.0	F_4-106	+Mn	738	51.3
Vic 9307	–Mn	664	45.0	F_4-106	–Mn	531	51.3
F_4-31	+Mn	747	45.3	F_4-113	+Mn	752	51.2
F_4-31	–Mn	282	45.3	F_4-113	–Mn	163	51.2
F_4-36	+Mn	813	45.1	F_4-138	+Mn	725	61.4
F_4-36	–Mn	281	45.1	F_4-138	–Mn	179	61.4
F_4-44	+Mn	883	45.0	F_4-140	+Mn	882	45.1
F_4-44	–Mn	372	45.0	F_4-140	–Mn	303	45.1
F_4-54	+Mn	1010	45.1	F_4-157	+Mn	1035	45.1
F_4-54	–Mn	719	45.1	F_4-157	–Mn	814	45.1

The second population tested to confirm marker linkage to *Mel1* originally consisted of 55 DH lines. Bioassay results enabled classification of most lines as either "inefficient" or "efficient". However, 10 lines had inconsistent results, and only the 45 lines that could be unambiguously assigned to a phenotype class were used for the marker linkage analysis. Close association of the tested markers and *Mel1* was again observed, with *Xcdo583B* and *Xabg714* showing the highest likelihood of linkage. The small population size prevented an accurate estimate of marker order. However, the "best fit" order of markers obtained using MAP MANAGER QT version b16 was the same in both populations.

Estimates of map distance and the most likely order of markers in the region *Xwg622* to *Xcdo795* using data from 84 F_2 plants are shown in Fig. 3.

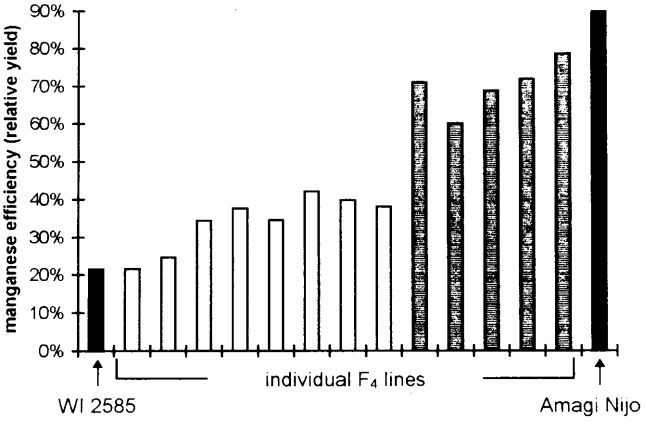

Fig. 4 Manganese efficiency (percentage of relative grain yield) of F_4 lines and control lines grown in a replicated field trial at the manganese deficient site Marion Bay in 1997. *White columns* are individual F_4 lines that have WI 2585 alleles in the interval *Xwg622* to *Xmwg77*. *Dark-grey columns* are individual F_4 lines that have Amagi Nijo alleles in the interval *Xwg622* to *Xmwg77*

Assessing the effect of the *Mel1* locus on field performance under manganese-deficient conditions

To validate the role of *Mel1* in the field expression of manganese efficiency, we grew F_4 progeny lines derived from individual F_2 plants with parental lines Amagi Nijo and WI 2585 and control lines Skiff, Galleon and VIC 9307 in a trial with four replicates at Marion Bay in South Australia, where manganese deficiency in barley is reliably obtained. Of the 13 F_2-derived lines in the trial, 5 were homozygous for Amagi Nijo alleles in the region *Xwg622* to *Xmwg77*, and 8 were homozygous for the corresponding WI 2585 alleles. Mean yield data (spatially adjusted) for each genotype and treatment is presented in Table 2. Field manganese efficiency scores were derived from relative yield performance of plots without added manganese compared to plots with added manganese by the formula

manganese efficiency (%)=
100×(yield of –Mn plot / yield of +Mn plot).

Manganese efficiency scores for the individual F_4 lines and parental lines are shown in Fig. 4. Spatial analysis of the trial showed a significant (P=0.01) treatment effect (nil versus + manganese) for all F_4 lines and the inefficient parent WI 2585, but no significant treatment effect (P<0.01) for the efficient parent Amagi Nijo. This suggested that the locus *Mel1* alone did not account for the trait manganese efficiency in the field. However, a Wald test between the two marker-selected groups of F_4 lines gave a statistic of 219.6 on 1 *df*. This is very significant (P=0), indicating that although the *Mel1* locus may not account for all of the trait's genetic variation, it is associated with a large increase in manganese efficiency in the field, at least in this particular genetic background.

Table 3 Likelihood of linkage to shoot Mn concentration for a population of 85 F_2 plants from the cross Haruna Nijo×WI 2585 grown in manganese deficient soil. Based on results from MAP MANAGER QT version b16 ($P<0.001$)

Locus	LOD	Probability	Percentage of total variance
Xmwg634	8.4	$3.8\ e^{-9}$	38
Xabg714	20.1	$3.7\ e^{-21}$	69
Xmwg77	14.2	$6.1\ e^{-15}$	55

The same chromosome region as *Mel1* of Amagi Nijo conditions the manganese efficiency of the lines WA73S276 and Weeah

The breeder's line WA73S276 showed a level of manganese efficiency similar to Amagi Nijo in both field trials and our pot bioassay (unpublished data) and has some potential as a source of efficiency for barley improvement. Of the markers linked to *Mel1*, only *Xcdo669* was polymorphic in the cross WA73S276×WI 2585. It was tested on a population consisting of 46 F_2 plants. Manganese efficiency was assessed in a pot bioassay and linkage of *Xcdo669* tested using single-point linkage of the marker to the quantitative trait locus (QTL) leaf manganese concentration. Significant linkage to the trait was detected, which was of a similar order to that observed in the Amagi Nijo×WI 2585 populations, with a LOD value of 7.3 ($P<0.001$). This result suggested that the *Mel1* locus is responsible (at least in part) for manganese efficiency in both Amagi Nijo and WA73S276. A pot bioassay of 73 F_2 plants and 11 F_1 plants of the cross Amagi Nijo×WA73S276, and 22 plants of each parent detected no transgressive F_2 segregants (data not shown), supporting this conclusion.

The cultivar Weeah also is highly manganese efficient and is of use as a source of efficiency. Data from yield trials over several years have suggested that Weeah may be less efficient than Amagi Nijo in the field, however in our pot bioassay the two performed similarly (unpublished data). A pot bioassay of 68 F_2 plants and 11 F_1 plants of the cross Amagi Nijo×Weeah, and 22 plants of each parent showed no transgressive F_2 segregants (data not shown), indicating Weeah, like WA73S276, to be allelic to Amagi Nijo at the *Mel1* locus.

A *Mel1* allele is present in the moderately manganese efficient cultivar Haruna Nijo

Haruna Nijo (Aida 1979) and Amagi Nijo (Kihara et al. 1998) are related in their pedigrees, and RFLP analysis puts their genetic similarity at approximately 85% based on 1325 polymorphisms (S Jefferies, personal communication). Field trials indicated that Haruna Nijo carries a moderate level of manganese efficiency. This means that in years where the manganese availability is very low, Haruna Nijo would perform more poorly than Amagi Nijo but better than WI 2585. In our pot bioassay Haruna Nijo performed significantly better than WI 2585 and marginally poorer than Amagi Nijo (data not shown).

Mel1-linked RFLP loci *Xabg714*, *Xmwg77* and the distal 4HS marker *Xmwg634* were tested for likelihood of linkage on a population of 85 F_2 plants of the cross Haruna Nijo×WI 2585. Manganese efficiency was assessed in a pot bioassay and marker linkage was tested by single-point linkage ($P<0.001$) to the leaf manganese concentration. The loci *Xcdo583* and *Xwg622* did not detect a polymorphism between Haruna Nijo and WI 2585 with any of the three enzymes tested (*Bam*HI, *Dra*I and *Eco*RI). As in the Amagi Nijo×WI 2585 population, both *Xabg714* and *Xmwg77* showed a high likelihood of linkage to the trait (Table 3). At an estimated 5.5 cM from the *Mel1* locus, *Xabg714* explained 69% of the total variation (MAP MANAGER version b16) or an estimated 82% of the genetic variation in the bioassay, based on a calculation of error contributing 16.1% to the total variation (data not shown). These values suggested that in the Haruna Nijo cross there was little genetic variation in the pot bioassay left unexplained by the locus *Mel1*.

Further support for this conclusion came from the observation that the phenotype distribution for the parameter manganese concentration in the pot bioassay of the Haruna Nijo×WI 2585 population statistically fit a single-gene model more closely than a two-gene model, as shown in Table 4. The statistical analysis method used compared the observed variance of the 85 F_2 plants with an expected F_2 variance derived assuming either single-gene segregation or two-gene segregation (Mather and Jinks 1977). The analysis assumes normality, no linkage and no epistasis and equal additive effects of the genes in the two-gene model. The expected genetic component of the variance (V_G) for each of the two models was estimated using the means of the 18 plants of each parent and the means of the 8 F_1 plants included in the bioassay. The estimate of variance due to experimental error (V_E)

Table 4 Test of fit to a one-gene or two-gene model for the distribution of bioassay phenotypes of a population of 85 F_2 plants of the cross Haruna Nijo X WI 2585. Reject the null hypothesis that the data fit a model if the expected (total) F_2 variance lies outside the 99.9% confidence interval (CI) of the observed (total) F_2 variance

	Expected (total) F_2 variance	Observed (total) F_2 variance	99.9% CI for observed F_2 variance
Single-gene model	1.47	1.27	0.82–2.15
Two-gene model	0.6	1.27	0.82–2.15

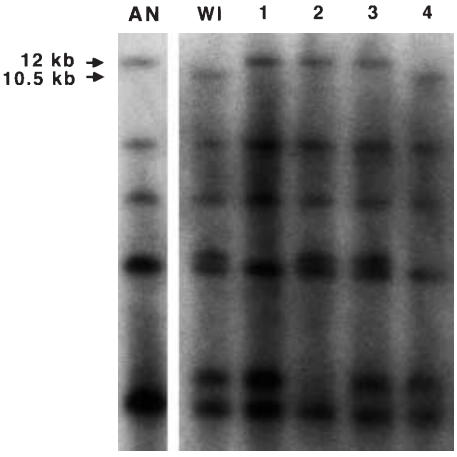

Fig. 5 Autoradiograph of genomic DNA of plants digested with *Dra*I and hybridized to the probe abg714.1.2. The 12-kb fragment from Amagi Nijo is associated with manganese efficiency, the 10.5-kb fragment from WI 2585 is associated with manganese inefficiency. *Lanes*: *WI* and *AN* WI 2585 and Amagi Nijo, respectively, *1–4* DH lines of the cross Amagi Nijo X WI 2585

was calculated from the variance components of the parents and F_1 hybrids by the formula $V_E=0.25V_{P1}+0.5 V_{F1}+0.25V_{P2}$, where V_{P1} was the observed variance for WI 2585 plants, V_{F1} was the observed variance for F_1 plants and V_{P2} was the observed variance for Haruna Nijo plants. For each model an estimate of the expected total variance was derived from the sum of V_G and V_E. Confidence intervals for the observed variances were determined from chi-square tables.

Potential usefulness of the identified markers in the local breeding program

The usefulness of RFLP markers linked to *Mel1* was assessed using the probe/enzyme combinations wg622/*Eco*RI, cdo583/*Eco*RI, abg714/*Dra*I and mwg77/*Dra*I, which were tested on a moderately diverse collection consisting of 96 barley cultivars or breeders' lines that have contributed significantly to breeding programs in southern Australia. At least four alleles were detected for the locus Xabg714 and for Xcdo583 and Xmwg77, three and four alleles, respectively. Only two alleles were evident for Xwg622. The locus *Xabg714* may be particularly useful for marker-assisted breeding in our local breeding program where Amagi Nijo is a prime source of manganese efficiency as only 2 of the other 95 plants tested have the same allele as Amagi Nijo. By scoring this plus one other locus, *Xcdo583*, all 95 lines can be distinguished from Amagi Nijo at this locus (data not shown). However the 1.5-kb *Pst*I fragment of the clone abg714 (obtained from the probe repository of the International Triticeae Mapping Initiative) which was used in these studies contains our sequence that is highly repeated in the barley genome, and this creates some difficulties when using it as a marker. Therefore, we produced a sub-clone (abg714.1.2) of 572 bp in which some of the repeated sequence has been removed (Fig. 5). This sub-clone is now being tested in the Waite barley breeding program (S. Jefferies, personal communication).

Discussion

Previous research has indicated that manganese efficiency in the barley cultivar Weeah, as assessed in a pot bioassay, is under simple genetic control (McCarthy et al. 1988). The detection of close linkage of the trait in bioassays of populations derived from several manganese-efficient parents to only one group of RFLP markers on chromosome 4HS confirms and extends those results. Bulked segregant analysis was performed using markers spaced at 30-cM intervals across the seven barley chromosomes to examine this trait in the cross Amagi Nijo (efficient)×WI 2585 (inefficient). Due to a lack of polymorphisms between the chosen parents in some regions of the genome, complete genome coverage was not achieved. Three chromosome segments, one on each of chromosomes 1H, 4H and 6H, have intervals between screened markers covering in total about 200 cM (18%) of the genome. Nevertheless, the region accounting for a large part of the trait variation in this cross was identified. Furthermore, our analyses of two other cultivars, Weeah and WA73S276, have shown that the excellent manganese efficiency of each is primarily controlled by the same locus, named *M*anganese *e*fficiency *l*ocus *1* or *Mel1*.

The available pedigrees for the cultivars used in this study were examined to determine whether the origin of the manganese efficiency trait might be deduced. Whilst further bioassay and marker analyses of additional lines is essential to clarify this issue, a common progenitor of each of the efficient cultivars used in this study (Amagi Nijo,Weeah and WA73S276) is Plumage-Archer, a variety bred in England and first released in 1914 (Beaven 1947; Hunter 1952). However, pedigree data is insufficient to determine which of the six releases of Plumage-Archer contributed to each of the studied cultivars. Haruna Nijo, which is moderately efficient, does not have Plumage-Archer in its pedigree. Identification of alternative loci for manganese efficiency would be of interest in determining whether 'pyramiding' might further enhance agronomic performance under manganese-deficient conditions.

Examination of the performance of 'marker-selected' F_4 lines in the field (Fig. 4) provided genetic confirmation that the pot-based bioassay for manganese efficiency adequately predicts the manganese efficiency of plants in the field environment and that the *Mel1* locus is a major determinant that distinguishes efficient from inefficient genotypes in this genetic background. However, two lines of evidence suggest that additional 'minor' genes contribute to the outstanding manganese efficiency of Amagi Nijo under field conditions. Firstly, the moder-

ately efficient cultivar Haruna Nijo also has an efficiency locus that maps to the *Mel1* region. Although Amagi Nijo consistently outperformed Haruna Nijo in the pot bioassay, the difference in mean leaf manganese concentration between the two is not statistically significant. In the field bioassay the two could be distinguished statistically in a year of particularly low manganese availability (data not shown). Therefore, it is possible that Amagi Nijo and Haruna Nijo carry the same *Mel1* allele but differ at some other locus (loci), not detected in our 4-week pot bioassay, which affects the level of expression of manganese efficiency in the field. It is also possible that Amagi Nijo and Haruna Nijo carry different alleles at *Mel1*. However, this seems less likely given the genetic similarity of the two cultivars.

The second line of evidence for an additional minor genetic component of manganese efficiency in the field is that the variation observed for the population of F_2 plants of Amagi Nijo×WI 2585 was significantly less than that expected for segregation at a single locus (P=0.05), but was significantly more than that expected for segregation at two loci of equal additive effect (P=0.05) (data not shown). This is in contrast to the good fit of data to the single locus model for the Haruna Nijo×WI 2585 population (Table 4). Additionally, the locus *Xabg714* explains 82% of the genetic variation of the trait (pot bioassay) in the Haruna Nijo population but only 75% in the Amagi Nijo×WI 2585 F_2 population. These data may be explained by two loci of unequal additive effects in Amagi Nijo. However, the bioassays of the two populations were performed separately, and the contribution to the variation of genetic×environmental components was not taken into account. An example of the differences in absolute performance between bioassays can be seen in Fig. 1. This underscores the importance of developing a robust alternative to the bioassay for selection in the cereal breeding programs.

The difficulties associated with traditional selection methods for this trait (field or pot bioassay) meant that in the past relatively few lines within the local breeding program were screened for manganese efficiency, despite its economic importance in the region. Identification of the co-dominant RFLP loci *Xcdo583B* and *Xabg714* has greatly simplified selection. Whilst our research was in progress, the population of DH lines of the cross Amagi Nijo×WI 2585 reached 440 lines (Davies et al. 1997). Further field trials involving a large number of DH lines are underway to assess the effect of the *Mel1* locus and to identify other loci affecting the trait. Incorporation of the trait into elite breeding lines by marker-assisted backcrossing has also become feasible, particularly as we have marker loci which flank the *Mel1* locus. At present, 2 years of early generation material have been screened for the *Mel1* locus using the identified markers, and marker-assisted backcrossing is being used to transfer the trait into manganese-inefficient, high-yielding breeding lines (Jefferies et al. 1997). Field trials of this material will determine whether genetic backgrounds differentially influence expression of the trait.

Acknowledgments We gratefully acknowledge Anna Athanasopoulos for assistance in making the initial crosses, Jodie Kretschmer and Sue Manning for preparation and hybridization of some probes, Jim Lewis for managing the field trial, and Teresa Fowles and Nick Robinson for ICPAES analysis of samples. We thank Mr. John McEvoy for accommodating our field trials on his property each year. Spatial analysis of the field trial was performed by Dr. Ari Verbyla of BiometricsSA. We are indebted to Dr. Jeff Paull for advice on experimental design and to Dr. Chunyuan Huang for help in applying and developing the manganese efficiency bioassay. This project was funded initially by a grant from the University of Adelaide Australian Research Council Small Grants Scheme, with further financial support from the ARC Special Research Centre for Basic and Applied Plant Molecular Biology. All of the experiments performed comply with the current laws of Australia.

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