Mapping of the locus associated with tolerance to high manganese in rapeseed (*Brassica napus* L.).

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ABSTRACT

Rapeseed germplasm tolerant to high levels of manganese (Mn^{2+}) is becoming increasingly important in areas where oilseed rape is grown on acidic soils. Although rapeseed germplasm tolerant to high Mn^{2+} is available currently there are no molecular marker(s) associated with tolerance to high Mn^{2+} which may facilitate the introgression of this trait into sensitive material. This study was conducted to develop a molecular marker associated with tolerance to high Mn^{2+}. We phenotyped 200 individuals from an F_2 population derived from doubled haploid (DH) parental genotypes Mutu-2 (98-6) (Mn-tolerant) and RSO94-67 (Mn-Sensitive) using a full nutrient solution culture with a Mn^{2+} concentration of 125 µM. Genetic variation for tolerance to high Mn^{2+} in this intercross population was determined on 8-11 day old seedlings by visual and digital analysis of the percentage of chlorosis occurring on the margins of the cotyledons. The visual scores and digital image analysis were strongly positively correlated. The scoring of the F_2 population showed a 3:1 tolerant to sensitive segregating ratio which confirmed that tolerance to high Mn^{2+} may be controlled by a single locus. We have genotyped a subset of this population comprising 92 individuals, using Diversity Array Technology (DArT™) markers and mapped a major locus associated with tolerance to high Mn^{2+} in the Mutu-2 (98-6)/RSO94-67 population. A suite of DArT markers were identified that map approximately 13.7 centimorgans from the Mn^{2+} tolerance locus. We are further saturating and validating this locus with molecular markers, enabling marker assisted selection for breeding rapeseed cultivars for tolerance to high Mn^{2+}.

Keywords: Rapeseed – Manganese – Tolerance – Evaluation – Linkage mapping

INTRODUCTION

Rapeseed (*Brassica napus* L. 2n=4X=38, genomes: AACC) is sensitive to elevated levels of manganese (Mn^{2+}) in the soil (Scott and Wratten, 1997). Mn^{2+} toxicity usually occurs in rapeseed due to acidic soils ($pH_{Ca} \leq 5.5$) although it can also occur in waterlogged or other soils where conditions can reduce Mn oxides to the available Mn^{2+} form (Sparrow and Uren, 1987). Symptoms of Mn^{2+} toxicity include chlorosis and necrosis on the margins of the leaf, 'cupping' or distortion of the leaf and potential reduced growth of the root and shoot of the plant (El-Jaoual and Cox, 1998). Genetic variation for tolerance to high Mn^{2+} has been characterised in rapeseed germplasm (Bjarnason *et al.*, 1972; El-Jaoual and Cox, 1998; Moroni *et al.*, 2003; Scott and Wratten, 1997; Siman *et al.*, 1974; Sparrow and Uren, 1987). Moroni *et al.* (2003) has shown that tolerance to high Mn^{2+} is most likely controlled by a single dominate gene. If this is the case, determining the molecular markers linked with the tolerance to high Mn^{2+} locus will assist breeding programs to incorporate the locus into existing *B. napus* germplasm. Developing a marker linked with Mn^{2+} tolerance is desirable due to the risks associated with visual scoring. This includes the erroneous selection of sensitive plants which can lower the rate of inheritance and slow progress of a breeding program. To determine the markers linked with the hypothesised locus conferring tolerance to high Mn^{2+} a population that segregate for this trait was required.

MATERIALS AND METHODS

An F_2 population comprising 200 individuals was raised from a single F_1 developed from Doubled Haploid (DH) parental lines of Mutu-2 (Mn tolerant) and RSO94-67 (Mn sensitive) developed by Moroni *et al.* (unpublished). The Mn^{2+} tolerance was assessed in a nutrient solution culture system as described previously (Moroni *et al.* 2003). All equipment was disinfected (washed with a 1% sodium hypochlorite solution for 10 minutes then rinsed thoroughly with deionised water). Two unconnected 45 L polyethylene containers were filled with 44 L of a complete nutrient solution prepared with deionised water with continuous aeration and nutrient circulation. The 200 seedlings were distributed evenly across the two containers. The Mn treatment of 125 µM Mn was added to each container over the basal nutrient solution Mn concentration (9 µM Mn). The treatment was added to the containers at the start of the experiment with the nutrient solutions. The pH of the solution in each of the containers was monitored daily with a pH meter (TPS WP-80, Springwood, Australia) and adjusted as necessary with 1 N HCl. Deionised water was added to each container when necessary to compensate for water loss due to evaporation and transpiration. The seedlings were germinated and grown in a temperature controlled laboratory with a day/night temperature of 20.0^{o}C ± 0.5. Light was provided by six fluorescent tubes (30 W) situated 36 cm above the plants on a day/night cycle of 16/8 hr. These lights produced an average photon flux density of 130 µmol m^{-2} s^{-1}. After 5 days of growth on the solution, the chlorosis symptoms were monitored by estimating the visual percentage chlorosis on a cotyledon for each seedling and the score recorded for 3 more days. A 2-component mixture of Gaussians with unequal means and variances was fitted to the F_2 visual score data to determine the cut off point between tolerant and sensitive genotypes (McLachlan and Peel, 2000). Digital images of the seedlings were taken on the same days as the visual estimation of chlorosis and each cotyledon image was processed individually in Scion Image software (Scion Corporation, 2001) following the procedure described by Murakami *et al.* (2005) but modified to omit step 14, and the RGB image was used to measure the colour difference instead of the HSV image as it was easier to separate green and yellow based on greyscale. Statistical analysis for the results of the nutrient culture screening was carried out by GenStat Eleventh Edition, version 11.1.0.1575 (VSN International Ltd, UK) and R statistical computing, version 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria). A regression analysis was used to determine the correlation between the visual and digital scores for the F_2 population. DNA was isolated from each F_2 seedling with a subsample of 92 lines of the F_2 population was genotyped using DArT (Diversity Arrays Technology P/L) (Diversity Arrays Technology P/L, Yarralumla, ACT, Australia) for DArT marker analysis. Linkage analysis was carried out using the software package Map Manager QTXb20 version 0.30 (Manly *et al.*, 2001).

RESULTS

The F_2 show a range of tolerance scores (Fig. 1) but there appears to be a large group of highly Mn^{2+} tolerant seedlings (score 0-5 % chlorosis) and a small group of highly Mn^{2+} sensitive seedlings (score 50-80 % chlorosis). Statistical analysis revealed that the most suitable cut-off point between tolerant and sensitive genotypes for this study was at the 15 % chlorosis score (Fig. 1). Calculating the posterior probability from the component mixture analysis for several cut off points over the four days of scoring (Table 1) showed a seedling with an observed score of ≤ 15 % has > 60% chance of belonging to the first ('tolerant') component of the mix. A seedling with an observed score of ≥ 20% however has > 70 % chance of belonging to the second ('sensitive') component. At a 15 % cut off between genotypes the F_2 population segregated into a ratio of 2.84:1.16 tolerance/sensitivity and was consistent with the expected 3:1 ration. This suggests that a single locus condition tolerance to high Mn^{2+}.

Table.1. Posterior probabilities for several potential cut off points between tolerant and sensitive genotype chlorosis % scores over the four days of scoring.

	5 % Chlorosis	10% Chlorosis	15% Chlorosis	20% Chlorosis	25% Chlorosis	30% Chlorosis
Day 1	0.979192	0.967227	0.852281	0.260131	0.006634	3.95426×10^{-05}
Day 2	0.963027	0.936139	0.624994	0.036867	0.000177575	1.66475×10^{-07}
Day 3	0.960169	0.94714	0.797598	0.204094548	0.004913	2.81261×10^{-05}
Day 4	0.940837	0.916758	0.701933	0.134558999	0.003159	1.99491×10^{-05}

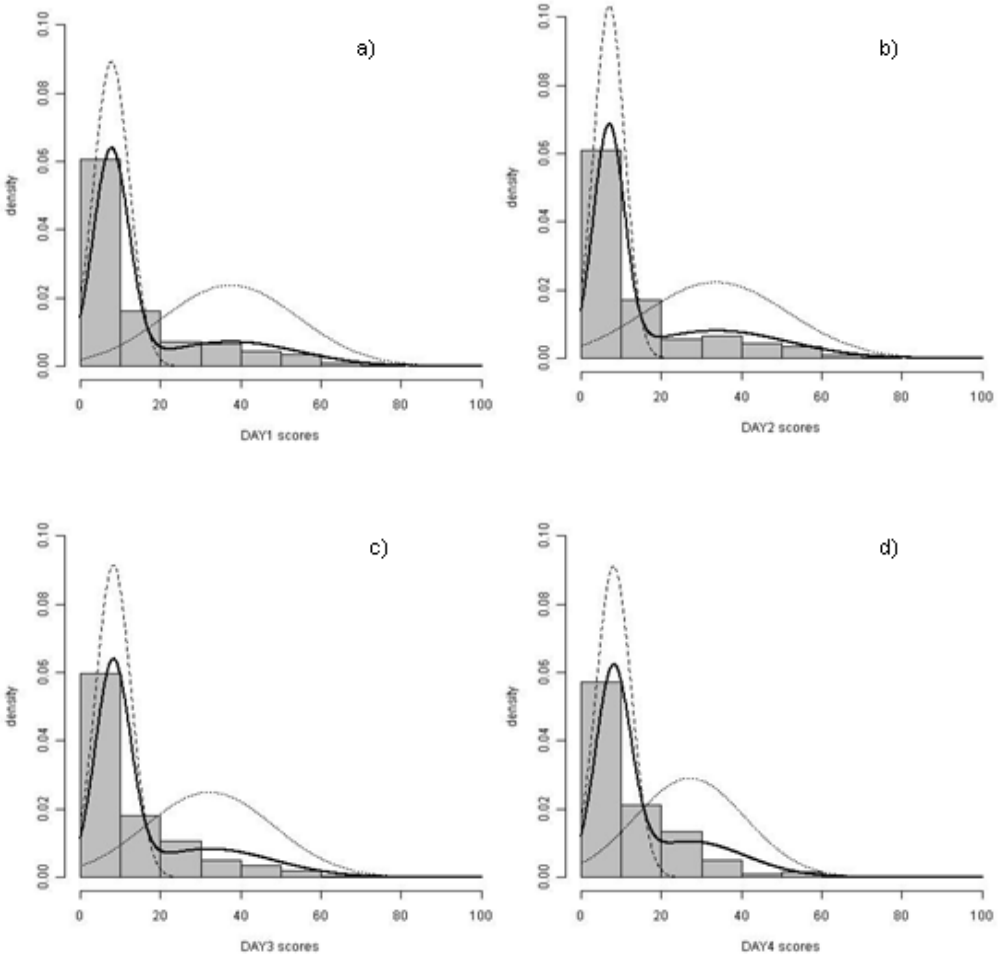

Fig.1. The component densities for the finite mixture modelling analysis for the F_2 visual chlorosis scores on a) Day 1, b) Day 2, c) Day 3 and d) Day 4 of scoring. The dashed line shows the first (or 'tolerant') component density, the dotted line shows the second (or 'sensitive') component density while the solid line shows the average of the two components. A histogram of the F_2 scores is displayed on the graphs for comparison to the component densities.

Digital scoring provided confidence as it enabled removing some of the potential bias associated with visual scoring. The results of the regression (not shown) indicated that the scores were significantly correlated.

DArT marker analysis identified 413 polymorphic DArT markers that exhibited segregation among 91 of the 92 F_2 lines. All markers had a high call rate (up to 96.9 %). Linkage analysis revealed that three DArT markers were linked with the Mn^{2+} tolerance locus. These markers showed co segregation among themselves and were mapped approximately 13.7 centimorgans from the Mn^{2+} locus (Fig. 2).

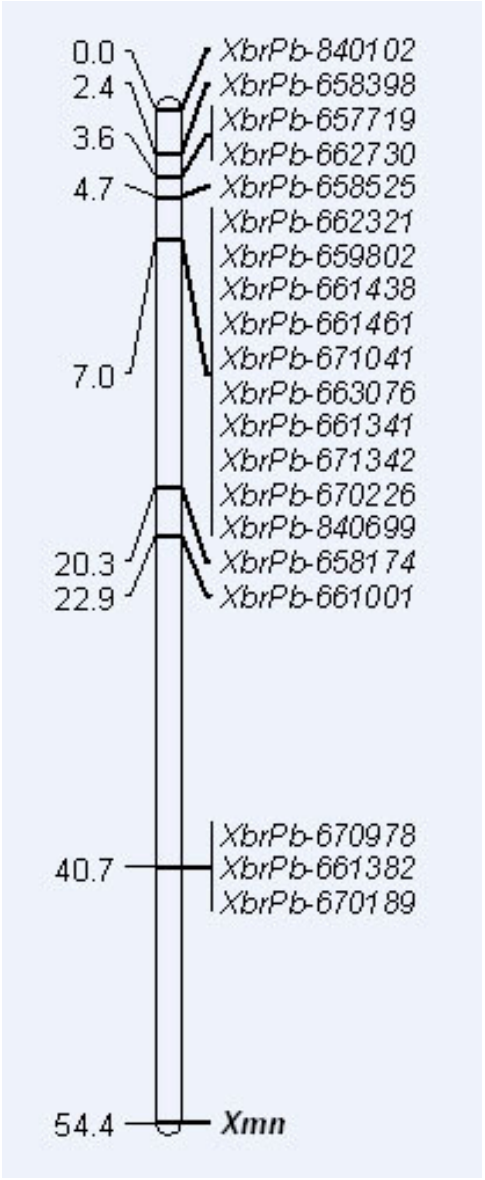

Fig.2. Linkage map for 20 DArT markers of Canola chromosome. Map distances are shown in centiMorgan (cM) are calculated in Map Manager QTX and graphed by MapChart.

DISCUSSION

In selecting a cut off point between tolerant and sensitive genotypes it was important to ensure that the choice was suitable so that there was minimal error in selecting tolerant seedlings as true tolerant and vice versa with sensitive seedlings. Visual scoring of the seedlings was preferable because it was quick and the correlation with the digital analysis showed that it was also accurate. The statistical analysis of the visual and digital data has shown that the rapeseed seedlings could be scored visually with a high degree of accuracy and be separated into genotypes based on their tolerance or sensitivity to high Mn^{2+}. The analysis revealed a 3:1

tolerant/sensitive ratio segregation of the genotypes indicating that inheritance of tolerance to high Mn^{2+} is controlled by a major locus as hypothesised by Moroni *et al.* (2003) and it has been validated in this independent population. With an accurate method of identifying tolerant and sensitive seedlings, the F_2 population was then genotyped to identify the molecular marker(s) associated with the tolerance to high Mn^{2+} locus. Three of those DArT molecular makers map to 13.7 centimorgans away from the tolerance to high Mn^{2+} locus. While these three markers are not closely linked with the tolerance to high Mn^{2+} locus, work is continuing to identify markers that are tightly linked with Mn tolerance. These markers will facilitate an efficient, accurate and cost effective selection of breeding lines for tolerance to high Mn^{2+}.

CONCLUSION

This study has shown that a major locus controls tolerance to high Mn^{2+} in an F_2 population derived from Mutu-2/RSO94-67. We have identified molecular markers linked with tolerance to high Mn^{2+} locus. Currently we are saturating this locus with markers for use in rapeseed breeding programs.

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