High-throughput root phenotyping screens identify genetic loci associated with root architectural traits in *Brassica napus* under contrasting phosphate availabilities

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**Background and Aims** Phosphate (Pi) deficiency in soils is a major limiting factor for crop growth worldwide. Plant growth under low Pi conditions correlates with root architectural traits and it may therefore be possible to select these traits for crop improvement. The aim of this study was to characterize root architectural traits, and to test quantitative trait loci (QTL) associated with these traits, under low Pi (LP) and high Pi (HP) availability in *Brassica napus*.

**Methods** Root architectural traits were characterized in seedlings of a double haploid (DH) mapping population (*n* = 190) of *B. napus* ['Tapidor' × 'Ningyou 7' (TNDH)] using high-throughput phenotyping methods. Primary root length (PRL), lateral root length (LRL), lateral root number (LRN), lateral root density (LRD) and biomass traits were measured 12 d post-germination in agar at LP and HP.

**Key Results** In general, root and biomass traits were highly correlated under LP and HP conditions. 'Ningyou 7' had greater LRL, LRN and LRD than 'Tapidor', at both LP and HP availability, but smaller PRL. A cluster of highly significant QTL for LRN, LRD and biomass traits at LP availability were identified on chromosome A03; QTL for PRL were identified on chromosomes A07 and C06.

**Conclusions** High-throughput phenotyping of *Brassica* can be used to identify root architectural traits which correlate with shoot biomass. It is feasible that these traits could be used in crop improvement strategies. The identification of QTL linked to root traits under LP and HP conditions provides further insights on the genetic basis of plant tolerance to P deficiency, and these QTL warrant further dissection.

**Key words:** Phosphate, phosphorus, root, *Brassica napus*, oilseed rape, QTL, biomass, genetic, heritability.

**INTRODUCTION**

Phosphorus (P) is essential to plants. Their roots acquire P from the rhizosphere solution as phosphate (Pi), primarily in the form of H₂PO₄⁻ (Vance et al., 2003; Hammond et al., 2004; White and Hammond, 2008). The concentration of Pi in the soil solution is often low (2–10 μM) and, consequently, the supply of Pi to the root surface by diffusion and mass flow is slow (Biesleski, 1973; Barber, 1995). Hence, P is one of the least-available mineral elements in the soil and frequently limits plant growth (Vance et al., 2003; Tiessen, 2008).

Over 85% of mined P is used in food production (Heffer et al., 2006) and consumption of this non-renewable resource could lead to a peak P scenario (akin to peak oil; Raven, 2008; Cordell et al., 2009). It is therefore likely that there will be increasing pressures on Pi fertilizer availability and, consequently, cost in the future. These pressures will be exacerbated by increasing demand on food production systems as the human population increases and by fluctuation in oil prices (Cordell et al., 2009). Inappropriate use of inorganic Pi fertilizers can also perturb the nutrient balance of natural ecosystems and reduce biodiversity (White and Hammond, 2008, 2009).

Breeding crops that acquire Pi and/or use Pi more efficiently is one strategy to reduce the use of Pi fertilizers (White et al., 2005; Veneklass et al., 2012). Root traits represent a potential source of genetic variation to improve P acquisition for breeding such crops (Lynch, 2007; Römheld and Kirkby, 2010; Powison et al., 2011; De Smet et al., 2012; Lynch and Brown, 2012). Assessment of root traits in crop breeding material can be slow and expensive, involving a combination of field-, glasshouse- and laboratory-based screens (Clark et al., 2011). The latter of these is amenable to high-throughput screens to identify germplasm with altered root growth and morphology. Genetic loci associated with these traits have the potential for use in breeding new crop varieties with improved root phenotypes.

In arabidopsis and other plant species, root responses to low Pi availability have been well characterized (White et al., 2005). Typically, a reduction in the development of the...
primary root (Ticconi et al., 2004; Sánchez-Calderón et al., 2005; Svistoonoff et al., 2007; Jain et al., 2007; Fang et al., 2009; Hammond et al., 2009) and increases in the number and length of lateral roots are observed under low Pi availability (Williamson et al., 2001; Linkohr et al., 2002; López-Bucio et al., 2002, 2003, 2005; Al-Ghazi et al., 2003; Nacy et al., 2005; Reymond et al., 2006). In some species, root agravitropism or topsoil foraging, is observed (Zhu et al., 2005). Both density and length of root hairs are also increased when plants are grown on a low Pi supply (Bates and Lynch, 1996; Gahoonia and Nielsen, 1997; Brown et al., 2012), thus increasing the capacity for Pi acquisition. Biochemical adaptations, including the release of organic anions to release Pi from organic compounds are also observed (Li et al., 2011). Root architectural traits were scored using an open-top growth chamber (OSR), to characterize the component traits of root architecture (Williamson et al., 2011).}

**Materials and methods**

**Plant material and growth conditions**

Plant material consisted initially of 190 double haploid (DH) lines representing the ‘Tapidor’ × ‘Ningyou 7’ (TNDH) mapping population (Qiu et al., 2006), plus the parents. The TNDH mapping population was generated through anther culture of the F₁ generation of a cross between Brassica napus cultivar ‘Tapidor’ (a European winter OSR) and B. napus cultivar ‘Ningyou 7’ (a Chinese semi-winter OSR). A new genetic linkage map was developed combining 53 gene-based markers (Ding et al., 2011) with an existing well-defined genetic map (Long et al., 2011). The new genetic linkage map had a total of 798 molecular markers and an average distance between two adjacent markers of 2.6 cM. The linkage map was constructed using JoinMap 4.0 (Van Ooijen, 2006) and the mapping procedure followed the method of Long et al. (2011) using RFLP, SSR and STS with default parameters and linkage groups distinguished at LOD (logarithm of the ratio of likelihoods) values between 8 and 19. The order of the markers on the new linkage map agreed well with our published maps (Shi et al., 2009; Long et al., 2011).

Seeds were first surface sterilized in 70 % (v/v) ethanol, rinsed in deionized water and then surface sterilized for 1 min using NaOCl (2.5 % active chlorine). Seeds were then rinsed three times in sterile deionized water, before being placed in sterile deionized water at 4 °C for 24 h to imbibe. Imbibed seeds were then sown into vented polystyrene trays (QTray; 240 × 240 × 20 mm; Molecular Devices, Hampshire, UK) containing 300 mL 0.8 % agar and a modified basal salt mix (Murashige and Skoog, 1962) with either 0.625 mM KCl added to provide K. The mean (± s.e.m., n = 3) total Pi concentration was 0.652 ± 0.011 mM P for HP agar and 0.082 ± 0.002 mM P for LP agar. Root responses of parental genotypes to external Pi concentrations ([P]ext) were also assessed at 0, 0.006, 0.312, 0.625 and 1.25 mM P, with KCl added to balance K. Seeds were then sown 3 cm from the top edge of the tray, with four seeds per line and two lines per tray. Trays were sealed with Nesofilms and placed 10 °C from vertical in a growth room under a 16-h photoperiod at a constant temperature of 24 °C. Illumination was provided by a bank of 84 100-W cool fluorescent tubes (Philips, Eindhoven, Netherlands), giving a photon flux density between 400 and 700 nm of 80–100 μmol photons m⁻² s⁻¹ at plant height.

For each line, 16 seeds were sown across four independent replicates, at both LP and HP. Trays were then placed randomly within the growth room. However, due to variation in germination rate, the total number of observations for each line varied between 4 and 16, with the average number of observations per line per treatment being 11.

Images of the root systems were captured using a flatbed scanner (Scanjet 3670; Hewlett-Packard, Palo Alto, CA, USA) 12 d after sowing. At harvest, shoot and root fresh weight (SFW and RFW, respectively) were determined. Tissue samples were dried at 80 °C and dry weights (shoot, SDW; root, RDW) determined. Tissue samples were digested by the addition of 2 mL nitric acid to 0.3 g dried ground material and processed in a closed vessel acid digestion microwave (MARSXpress; CEM Corporation, Matthews, NC, USA). Digested samples were diluted with 23 mL of deionized water and analysed using inductively coupled plasma emission spectrometry (JY Ultima 2; Jobin Yvon Ltd, Stanmore, Middlesex, UK) to determine tissue Pi concentrations.

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Image analysis

Images were loaded into ImageJ (Abramoff et al., 2004). Primary root length (PRL, cm) and lateral root length (LRL, cm) were measured. Lateral root numbers were counted and used to calculate lateral root density (LRD, cm\(^{-1}\)). Total root length (TRL, cm) was calculated as the sum of PRL and LRL.

Data analysis

Raw data were entered into GenStat (Release 13.1.0-4.470; VSN International, Oxford, UK). Data for parent lines grown at different \([P]_{ext}\) were analysed using a two-way ANOVA. Due to variation in germination of lines within the mapping population, data for the mapping population grown at two \([P]_{ext}\) were analysed using REML (residual maximum likelihood) procedures to allocate sources of variation and estimate individual line means (Patterson and Thompson, 1971; Robinson, 1987). Prior to analysis SDW and RDW were ln transformed and LRN, LRD and TRL were square root transformed to improve the normality and variance of the data. PRL and LRL did not require transformation. A random term [(Replicate/Run/Plate/Position) \(\times\) Line] and no defined fixed factors was used to allocate sources of variation for individual traits. Subsequently, line means were estimated using the [(\([P]_{ext}\) \(\times\) Line)] term as a fixed factor, retaining [(Replicate/Run/Plate/Position)] as a random factor.

QTL positions were estimated using the zmapQTL model (Churchill and Doerge, 1994). Thresholds for the LOD ranged between 3.04 and 3.43.

RESULTS AND DISCUSSION

Parents of the TNDH mapping population show differences in root architecture

To determine optimal assay conditions for screening the large TNDH mapping population, root responses to different \([P]_{ext}\) were quantified in the parents of the mapping population (Fig. 1). The effect of \([P]_{ext}\) was significant \((P < 0.01)\) for both RDW and SDW. Root and shoot DW was greatest in both parents at 0-625 mM \([P]_{ext}\), with SDW declining with declining \([P]_{ext}\) and also being lower at 1-25 mM \([P]_{ext}\) compared with 0-625 mM \([P]_{ext}\) and RDW declining with declining \([P]_{ext}\) and remaining similar at 1-25 mM \([P]_{ext}\) compared with 0-625 mM \([P]_{ext}\) (Fig. 1A, B). There was a significant difference between lines for both RDW \((P = 0.012)\) and SDW \((P < 0.005)\), with ‘Ningyou 7’ accumulating a greater biomass than ‘Tapidor’ at all \([P]_{ext}\) (Fig. 1A, B).

Root traits differed significantly between the parents of the TNDH mapping population and showed characteristic changes in root architectural traits with declining \([P]_{ext}\) (Fig. 1). There was a significant decrease in PRL with declining \([P]_{ext}\), with the PRL of ‘Tapidor’ seedlings declining from 6-27 cm at 1-25 mM \([P]_{ext}\) to 3-68 cm at 0 mM \([P]_{ext}\) (Fig. 1C). This is consistent with primary root responses of other B. napus (Akhtar et al., 2008; Yang et al., 2010) and B. oleracea (Hammond et al., 2009) cultivars observed previously. At all \([P]_{ext}\), the PRL of ‘Tapidor’ was greater than the PRL of ‘Ningyou 7’ seedlings. In contrast, the LRL of ‘Ningyou 7’ seedlings was consistently greater than the LRL of ‘Tapidor’ seedlings at all \([P]_{ext}\) (Fig. 1D). Whilst the LRL of ‘Ningyou 7’ seedlings increased up to 0-625 mM \([P]_{ext}\) before declining again at 1-25 mM \([P]_{ext}\), the LRL of ‘Tapidor’ seedlings did not change significantly with \([P]_{ext}\) (Fig. 1D). The increase in LRL with \([P]_{ext}\) for ‘Ningyou 7’ seedlings is consistent with the root responses of other B. napus (Salaman et al., 2007; Hu et al., 2010; Yang et al., 2010) and B. oleracea (Hammond et al., 2009) cultivars observed previously, but the LRL response of ‘Tapidor’ seedlings is atypical. The reduction in LRL of ‘Ningyou 7’ seedlings when \([P]_{ext}\) is reduced contrasts with the increase in LRL observed for arabidopsis when \([P]_{ext}\) is reduced (Williamson et al., 2001; Linkohr et al., 2002; López-Bucio et al., 2002, 2003, 2005; Al-Ghazi et al., 2003; Nacry et al., 2005; Reynold et al., 2006). Also, in a comparison between two B. napus cultivars with either high or low physiological PUE, Akhtar et al. (2008) showed a significant increase in LRL in both cultivars when supplied with low \([P]_{ext}\) or rock phosphate. Consequently, this phenotype may be specific to the parental lines used in this study, or may represent a more general phenotype within B. napus species. The number of lateral roots was greatest in both parents at 0-312 and 0-625 mM \([P]_{ext}\), but decreased significantly with both increasing and decreasing \([P]_{ext}\) (Fig. 1E). LRD increased with \([P]_{ext}\) for ‘Tapidor’, and increased for ‘Ningyou 7’ up to 0-312 mM \([P]_{ext}\) before declining slightly at lower \([P]_{ext}\) (Fig. 1F).

Given the significant differences observed between the two parental lines for key root traits under low \([P]_{ext}\), the TNDH mapping population was screened for root architectural traits at low (0 mM added P, LP) and high (0-625 mM added P, HP) \([P]_{ext}\) to identify genomic regions (QTL) associated with these traits.

Root traits show transgressive segregation in the TNDH mapping population

Seeds from 190 lines of the TNDH mapping population were sown on plates containing either LP or HP agar, to facilitate high-throughput phenotyping of root traits. Root architectural traits were scored for all lines at 12 d (Fig. 2 and Table 1). Transgressive segregation was observed for all traits. At the population level, variance attributed to line, \([P]_{ext}\), and their interaction was highly significant \((P < 0.001)\) for all traits (Table 1), and correlations between LP and HP treatments for individual traits were all significant \((P < 0.001);\) Fig. 2). Mean SDW and RDW for the population did not change significantly when plants were grown on HP agar compared with LP agar, although the individuals within the population showed large responses to \([P]_{ext}\) with the difference in SDW between LP and HP varying from –2-2 to 4-4 mg, and the difference in RDW between LP and HP varying from –0.6 to 0.9 mg (Fig. 2A, B).
Across the TNDH population, mean PRL and LRL were both significantly lower at LP compared with HP; however, there were a range of responses within the population with some individuals increasing their LRL or PRL by 1.0 and 0.4 cm, respectively, at LP compared with HP. ‘Tapidor’ had a longer primary root, and shorter lateral roots, compared with ‘Ningyou 7’ (Fig. 2C, D). The mean LRN and LRD were both higher at LP compared with HP, with a greater range in values for LRD when plants were grown at LP compared with plants grown at HP (Fig. 2E, F). Overall, TRL (data not shown) was greater at HP (mean 17.60 cm, n = 4536) compared with LP (mean 13.76 cm), with a greater range in values observed at HP (5.13–34.22 cm) than at LP (5.35–25.12 cm).

**Fig. 1.** Changes in shoot dry weight (A), root dry weight (B), primary root length (C), lateral root length (D), lateral root number (E) and lateral root density (F) in ‘Tapidor’ and ‘Ningyou 7’ at different external Pi concentrations. Seedlings were grown on trays containing 300 mL 0.8% (w/v) agar and a modified basal salt mix with 0, 0.006, 0.312, 0.625 or 1.25 mM P for 8 d and images of root systems analysed for root traits. Symbols represent means ± s.e.m. (n = 4).
FIG. 2. Variation in shoot dry weight (A), root dry weight (B) primary root length (C), lateral root length (D), lateral root number (E) and lateral root density (F) in the ‘Tapidor’ × ‘Ningyou 7’ double haploid (TNDH) mapping population. Seedlings were grown on trays containing 300 mL 0.8 % (w/v) agar and a modified basal salt mix with 0 (LP) or 0.625 (HP) mM P for 12 d. Data are REML-estimated means (n varies between 4 and 16, average 11 observations per line per treatment). Symbols ‘T’ and ‘N’ represent the mapping population parental values for ‘Tapidor’ and ‘Ningyou 7’, respectively. The continuous line represents the 1 : 1 line.
**Root architectural traits are heritable in Brassica napus**

Breeding new cultivars with improved acquisition and/or utilization of Pi through selection for root architectural traits requires them to be heritable. Within the variance components in the REML analyses, the Line component represents variance attributed to genetic factors. This variance component approximates the population wide additive genetic variation, or narrow-sense heritability. Shoot dry weight and RDW had the highest heritabilities, of 30.70 and 28.01, respectively (Table 1). The root architectural traits had heritabilities ranging from 13.08 to 16.06, suggesting the underlying genetic control of these traits can be selected for, and used in breeding new cultivars with improved root traits. Interestingly, the variance attributed to the $[P]_{ext}$ treatment varied between the root traits. For LRL and LRN, the trait variance attributed to $[P]_{ext}$ was relatively small, compared with the trait variance attributed to $[P]_{ext}$ for PRL, LRD and TRL (Table 1). This implies that PRL, LRD and TRL traits are more responsive to $[P]_{ext}$ in this mapping population, and in *B. napus* may represent key adaptive root architectural traits to low Pi availability.

**QTL associated with biomass and root architectural traits are conserved within the Brassicaceae**

A total of 38 QTL, associated with root architectural and biomass traits, were identified across nine of the 19 chromosomes (Table 2). Significant QTL associated with SDW at both LP and HP co-localized to 44.2 cM on chromosome A03, explaining 27.9% of the genetic variation for PRL at LP (Table 2). QTL associated with PRL at LP and HP, and also TRL at HP, were located between 10.8 and 18.8 cM on A03, and were associated with the functional markers BnWRKY-A3 and BnPHT3-A3 (Ding et al., 2011; Table 2). The remaining three loci associated with PRL at LP and HP co-located to chromosome C06 at 16 cM (support interval from 17.6 to 33.6 cM), together with a QTL associated with LRD at LP. No QTL were identified for LRL traits at either LP or HP within this population, despite the large variation between the parents in the initial experiment (Fig. 1D).

The ancestral genome segments of Brassicaceae species have been relatively well defined in terms of rearrangements and duplication events (Parkin et al., 2005; Mun et al., 2009; Wang et al., 2011b). These segments facilitate comparative genomics between Brassicaceae species, including other *Brassica* species and the model plant arabidopsis. QTL associated with plant responses to low Pi availability have been identified in both *Brassica* species and arabidopsis (Bentsink et al., 2003; Vreugdenhil et al., 2004; Reymond et al., 2006; Svinstoooff et al., 2007; Zhao et al., 2007, 2008; Hammond et al., 2009, 2011; Ding et al., 2010, 2012; Yang et al., 2010, 2011) and share common genomic regions to those identified in this study. For example, QTL identified here, associated with SDW at LP and HP, and RDW and LRN at LP, and located on chromosome A03 between 36.8 and 46 cM, co-locate with QTL associated with SDW, RDW, root volume and root surface area at LP, and plant height at LP and HP, determined in a cross between P-efficient and P-inefficient *B. napus* cultivars (Ding et al., 2010, 2012; Yang et al., 2010, 2011). This corresponds to a pleiotropic QTL associated with multiple measures of biomass and flowering time and seed weight (Shi et al., 2009) and overlaps with a region of chromosome C03 in *B. oleracea* which has previously been associated with shoot biomass and PUE traits (Hammond et al., 2009). This region is syntenous with ancestral block J on arabidopsis chromosome 2, where QTL for SDW (Loudet et al., 2003) and rosette and root weight (Prinzenberg et al., 2010) have previously been identified, further supporting the presence of a pleiotropic gene in this region.

The unique QTL associated with PRL at LP between 28.8 and 54.6 cM on chromosome A07, corresponds to two...
ancestral blocks, E and N, on the bottom of arabidopsis chromosomes 1 and 3, respectively. The region containing QTL associated with PRL and LRD on chromosome C06 (Table 2), is also syntenous with the ancestral block E, suggesting these regions may contain paralogues of genes involved in the regulation of PRL. Arabidopsis genes implicated in primary root development. One for PRL at LP on A07, which co-locates with several QTL associated with root architectural traits were also identified, including one for PRL at LP on A07, which co-locates with several arabidopsis genes implicated in primary root development.

Identification of the genetic elements associated with these traits will provide targets for the future development of crops adapted to growth in low-Pi soils. The use of high-throughput root phenotyping assays has the potential to advance the breeding and selection of these cultivars, but requires cross validation with root characteristics and yield determined under field conditions.

Conclusions

The development of crops that can acquire and/or utilize P more efficiently is essential for the sustainability of future crop production. Plant adaptations to low Pi availability include alterations in the allocation of resources to roots and changes in the distribution of those roots in the soil. Since the assessment of root traits in plants can be slow and expensive, we employed an agar based high-throughput root phenotyping screen to characterize the root traits of a large B. napus mapping population and identify genetic loci controlling these traits under low Pi availability (Fig. 2 and Table 2). Significant QTL associated with biomass and root architectural traits were identified on A03, and co-locate with QTL for biomass traits in B. napus, B. rapa and arabidopsis. Significant QTL associated with root architectural traits were also identified, including one for PRL at LP on A07, which co-locates with several arabidopsis genes implicated in primary root development. Identification of the genetic elements associated with these traits will provide targets for the future development of crops adapted to growth in low Pi soils. The use of high-throughput root phenotyping assays has the potential to advance the breeding and selection of these cultivars, but requires cross validation with root characteristics and yield determined under field conditions.

Table 2. Significant QTL associated with biomass and root architectural traits in the Brassica napus TNDH mapping population

<table>
<thead>
<tr>
<th>Trait</th>
<th>QTL name</th>
<th>Chromosome</th>
<th>Marker</th>
<th>Position (cM)</th>
<th>LOD score</th>
<th>2 LOD support interval (cM)</th>
<th>Additive effect</th>
<th>R² (%)</th>
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<tr>
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<td>SDW_LP_A02a</td>
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<td>5-17.2</td>
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<td>8.5–15</td>
<td>0.36</td>
<td>7.2</td>
</tr>
<tr>
<td>TRD at LP</td>
<td>TRD_LP_A03a</td>
<td>A03</td>
<td>BRMS-043</td>
<td>44-2</td>
<td>3.89</td>
<td>43.2–46</td>
<td>-0.07</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>TRD_LP_C04a</td>
<td>C04</td>
<td>sN3761b</td>
<td>11-9</td>
<td>3.33</td>
<td>8.5–15</td>
<td>0.36</td>
<td>7.2</td>
</tr>
</tbody>
</table>

REML estimated means for biomass and root architectural traits for 176 lines in the TNDH mapping population were used to estimate QTL positions associated with these traits using the zmapQTL model 6 composite interval mapping function (Wang et al., 2011). Significant (P < 0.05) LOD thresholds for individual traits were determined using 1000 permutations. A positive additive effect indicates a positive contribution of the ‘Tapidor’ allele to the trait value.

Conclusions

The development of crops that can acquire and/or utilize P more efficiently is essential for the sustainability of future crop production. Plant adaptations to low Pi availability include alterations in the allocation of resources to roots and changes in the distribution of those roots in the soil. Since the assessment of root traits in plants can be slow and expensive, we employed an agar based high-throughput root phenotyping screen to characterize the root traits of a large B. napus mapping population and identify genetic loci controlling these traits under low Pi availability (Fig. 2 and Table 2). Significant QTL associated with biomass and root architectural traits were identified on A03, and co-locate with QTL for biomass traits in B. napus, B. rapa and arabidopsis. Significant QTL associated with root architectural traits were also identified, including one for PRL at LP on A07, which co-locates with several arabidopsis genes implicated in primary root development. Identification of the genetic elements associated with these traits will provide targets for the future development of crops adapted to growth in low Pi soils. The use of high-throughput root phenotyping assays has the potential to advance the breeding and selection of these cultivars, but requires cross validation with root characteristics and yield determined under field conditions.

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Svistoonoff, 2007). An AINTEGUMENTA-like gene, named PRD (At1g79700), the mutant of which was identified as having reduced primary and lateral root development under low Pi availability compared with the wild-type, also co-locates to this region (Camacho-Cristóbal et al., 2008). The latter of these ancestral blocks also co-locates with a QTL for PRL in arabidopsis (Loudet et al., 2005).
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LITERATURE CITED


