High throughput phenotyping of root and shoot traits in Brassica to identify novel genetic loci for improved crop nutrition

Catherine Louise Thomas, BSc, MSc

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Abstract

Despite the success of breeding for high-yielding varieties during and since the 'Green Revolution', there are still an ever increasing number of people who suffer from mal-nutrition, due to both inadequate calorie intake and 'hidden hunger' from insufficient essential nutrients. There are also adverse impacts of such high-input, intensive agriculture on the wider environment. It is necessary therefore to focus breeding efforts on improving nutrient uptake and composition of crops, as well as improved yield. Roots have been an under-utilised focus of crop breeding, because of difficulty in observation and accurate measurement. Furthermore, genetic diversity in crop roots may have been lost in commercial varieties because of the focus on above-ground traits and the use of fertilisers. Techniques which can accurately measure phenotypic variation in roots, of a diverse range of germplasm at a high throughput, would increase the potential for identifying novel genetic loci related to improved nutrient uptake and composition.

The aim of this PhD was to screen at high throughput in a controlled-environment, the roots of an array of *Brassica napus* germplasm. The validity of the system to predict field performance, in traits including early vigour, nutrient composition and yield was assessed. Genetic loci underlying variation for the root traits were also investigated. A high throughput screen of the mineral composition of a mutagenised *B. rapa* population was also conducted, with the aim of identifying mutants with enhanced mineral composition of human essential elements, particularly magnesium.

It has been demonstrated that root traits in the high throughput system can predict field performance, particularly primary root length which has the greatest 'broad-sense heritability' and relates to early vigour and yield. Lateral root density on the other hand was found across the studies to relate to mineral composition, particularly of micro-nutrients. Genetic loci underlying root traits, and enhanced magnesium accumulation have been identified, and have potential for use in breeding Brassica with improved mineral nutrition.

Publications arising from the PhD programme:

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Contents

Chapter 1

Introduction

The ‘Green Revolution’ from the 1960s helped to feed an expanding population in the developing world through the breeding of modern high-yielding crop varieties, particularly of rice (*Oryza sativa*), maize (*Zea mays*) and wheat (*Triticum* spp.). These varieties have a typical ideotype: shorter, stiffer stems which devote more resources to producing grain rather than vegetative biomass. This ideotype was achieved through incorporating genes for short stature *sd1* in rice, and *Rht1* and *Rht2* in wheat (Khush, 2001). Furthermore, these varieties respond better to fertilised conditions (Evenson and Gollin, 2003). Since 1960 the worldwide rate of application of nitrogen fertilisers has increased by seven times, and exceeds 72k tonnes of nitrogen per year (Tilman, 1998). These crops had an increased yield of 15-20% compared to conventional varieties (Khush, 2001). However, in most regions the number of undernourished people is still growing (Tilman *et al*., 2002). The human population is predicted to increase 40% by 2030, which will outpace the current rate of crop yield increases and the availability of arable land (Ingram *et al*., 2013), and the majority- 83% of this population increase is in the developing countries of Asia, Africa and Latin America (Khush, 2001). Low-fertility soils and drought are often the major problem for crop production in many of these countries (Lynch, 2007). However, due to cost, fertilisers and elite varieties are often not available to developing nations. Furthermore, increases in fertiliser applications are unlikely to be effective at increasing yields and nutrient uptake, because of diminishing returns over time, whereby poor fertiliser management creates soil degradation including a loss of soil organic matter and soil acidification (Tilman *et al*., 2002). As exemplified by the 150 year Broadbalk experiment at Rothamsted, which shows that average wheat yields have been higher on manured plots compared with plots receiving complete NPK fertilisation (Johnston *et al*., 1994). Fertiliser also adds environmentally detrimental levels of nutrients through leaching to

off-site ecosystems causing eutrophication, therefore nitrogen inputs are being managed by legislation which limits their use in agriculture (Tester and Langridge, 2010). Phosphate rock reserves are a finite resource (Ristova *et al*., 2013). Therefore, modern crop breeding generally selects for yield, however for socio-economic and environmental reasons it is now necessary to breed for crops which also have improved NAE (nutrient acquisition efficiency), such as through selecting for desired root traits. Root traits related to NAE have very rarely been used as selection criteria (Lynch, 2007), and the focus on breeding for high yield under sufficient fertilisation has led to the loss of genetic diversity for root traits in commercial varieties (Crowley and Rengel, 1999).

More than half of the world's population suffer from malnutrition of iron, zinc, and selenium. Calcium, magnesium and copper can also be deficient in some diets (White and Broadley, 2009; Broadley and White, 2010). Nutrients in soil are often limiting for plant growth, particularly the plant macro-nutrients nitrogen (N), phosphorus (P) and potassium (K) which are required in the greatest quantity. Acidic soils with low pH are a worldwide problem affecting 30% of the total of the world (Von Uexkull and Mutert, 1995), caused by the leaching of more readily soluble soil minerals particularly base cations e.g. Ca (calcium), Mg (magnesium) and K, and leading to potentially toxic levels of Fe (iron), Al (aluminium) and other trace metals e.g. Mn (manganese) (Bohn *et al*., 1979). Conversely, in high input agricultural systems supplied with NPK, a lack of secondary elements Ca, Mg and S (sulphur) and micro-nutrients- Fe, Cu (copper) and Zn (zinc) can impact production and nutritional quality of harvests- the 'soil dilution effect' (Edmeades, 2004; Davis 2009). Likewise, modern high-yielding varieties which grow larger and faster may not acquire secondary nutrients at a sufficient rate- the 'genetic dilution effect' (Hermans *et al*., 2013). For example, strong inverse relationships between wheat (*Triticum* spp.) grain yield and grain micro nutrient concentration (Peterson *et al*, 1983; Oury *et al*., 2006) have been observed. The efficacy of improving nutrient composition through fertiliser application is affected by the bioavailability of the applied mineral, influenced by complex soil and plant physiological interactions (Stroud *et al*., 2010). Furthermore, for edible crops the phloem-mobility and translocation to the edible portions of the crop can be a limitation to the use of fertiliser for biofortification (White and

Broadley, 2009). Therefore, biofortification of crops for improved nutritional composition is essential to counter 'hidden hunger'. Genetic improvement of crops is a sustainable and economic solution to mineral and vitamin deficiencies (White and Broadley, 2005; Potrykus, 2008; Mithen *et al*., 2003).

Root system architecture (RSA) is the morphology and topology of the root system, morphology being the variety of components constituting the system and their relationship to one another and topology referring to the location of the root in space (Fitter *et al*., 1987; Hodge *et al*., 2009). The spatial configuration of the root system is plastic and varies greatly depending on soil composition and water and mineral nutrient availability (Macmillan *et al*., 2006). Different root types, their age and branching order, influence the function performed by the root, such as a decrease in nutrient uptake in older roots (Hodge *et al*., 2009). Yet, mechanisms do exist which constrain developmental plasticity, whereby the environmental response pathway may target an intrinsic pathway (Malamy, 2005). The fact that species within a genus often show high similarities in architecture suggests that there may be important historical factors determining root architecture (Fitter *et al*., 1991). Thus root architectural traits although complex and with environmental plasticity do depend on detectable inherent properties.

Root traits have been shown to correlate with improved field performance and nutrient uptake (Steele *et al*., 2013; Hochholdinger *et al*., 2008; Tuberosa *et al*., 2002; Zhao *et al*., 2004; Bonser *et al*., 1996; Ho *et al*., 2005; Hammond *et al*., 2009; Yang *et al*., 2010; Miguel *et al*., 2013). A modelling scenario has shown that historical increases in maize yield were better explained by increasing root depth rather than shoot traits (Hammer *et al*., 2009). However, under drought, Bruce *et al*., (2002) observed that an increased root biomass actually decreased yield. Richards (1991) suggests that for many crops and environments, there is too much carbon invested in the roots. Root traits have rarely been used directly in crop breeding, the few examples are in rice (Steele *et al*., 2007, 2013; Uga *et al*., 2013; Arai-Sanoh *et al*., 2014), wheat (Placido *et al*., 2013) and chickpea (*Cicer arietinum*, Varshney *et al*., 2013). Genes regulating root traits have been identified in *Arabidopsis* (Rosas *et al*., 2013; Gifford *et al*., 2013; Meijon *et al*., 2014; Slovak *et al*., 2014) and *Brassica* (Yang 2010; Shi *et al*. 2013; Fletcher *et al*., 2014, 2016; Basnet *et al*., 2015; ArifUzZaman *et al*., 2016). However,

in *B. napus* root traits have yet to be employed directly for crop improvement (Zhao, 2015).

If seedling emergence in crops is inadequate there is a non-reversable effect on crop yield (Finch-Savage *et al*., 2010). Seedlings have restricted root placement and poor root/soil contact, and can be exposed to stress just after planting (Grossnickle, 2005). During establishment seedlings are highly prone to attack by pests (Evans and Bhatt, 1977). A fast growing and deep rooting system will improve competitiveness with weeds during early establishment (Khan *et al*., 2012). An increase in the germination and vigour of seed lots of sugar beet has been an important factor in the improvement of crop yield in the UK (Ellis, 1992). Therefore, early seedling root growth is an important characteristic for crop improvement. A link between seed size and seedling development has also been observed (Evans and Bhatt, 1977, Nieuwhof *et al*., 1989, Bretagnolle *et al*., 1995, Walters and Reich, 2000, Bettey *et al*., 2000, Rodo and Filho, 2003, Kennedy, 2004; Finch-Savage *et al*., 2010, Khan *et al*., 2012, Koscielny and Gulden, 2012). Stored nutrients in the seed must be the major source of nutrient reserves for initial seedling development because newly planted seedlings cannot exploit the surrounding soil until they develop roots (Grossnickle, 2005). In tomato, (*Solanum lycopersicum*) a correlation between seed weight and root weight and total root size and lateral root number, but not main root length or lateral root density per branching zone, was observed (Khan *et al*., 2012). In *B. oleracea* there was a clear link between seed size and the resulting seedling size, but not between seed size and germination time and germination success (Finch-Savage *et al*., 2010). In maize, roots began to take up significant amounts of exogenous P 5 days after sowing and continued to import seed P reserves for up to 2 weeks, also the amount of P contained in the seed did not influence the onset or rate of uptake of exogenous P (Nadeem *et al*., 2011). Therefore, although seed size and reserves may influence initial seedling growth rates, a vigorous root system for nutrient uptake may still be necessary for early seedling vigour.

Roots from in-situ field settings are difficult to extract and measure without destruction. Therefore, alternative soil-based growth systems/proxy measures have been developed, such as minirhizotrons (Nagel *et al*., 2012), ground penetrating radar (GPR), 3D scanning- CT (Tracy *et al*., 2010) and MRI, but these methods are limited

to thick roots and are not high throughput. Therefore methods have been developed for high throughput phenotyping (HTP) in which the plants are not grown in soil, such as using agar plates (Yazdanbakhsh and Fisahn, 2009; Shi *et al*., 2013), gel columns (Lobet and Draye, 2013; Ingram *et al*., 2013), hydroponics (Chen *et al*., 2011), and 'pouch and wick' filter paper-based systems (Bonser *et al*., 1996; Hund *et al*., 2009; Miguel *et al*., 2013; Watt *et al*., 2013; Adu *et al*., 2014; Le Marie *et al*., 2014; Atkinson *et al*., 2015; Hammond *et al*., 2009). Digital imagery and automated analysis also provide opportunities for greater speed and efficiency (Clark *et al*., 2013). Clark *et al*. (2011) found that traits measured in 2D and 3D were highly correlated, and reliable estimations of the root spatial distribution in 3D could be obtained from 2D. However, the costs of acquiring reliable phenotypic data on large numbers of lines (de Dorlodot *et al*., 2010), and the "tedious nature of phenotyping hundreds of recombinants" (Coudert *et al*., 2010) hinders progress. There is a need for robust, high throughput laboratory screens which may be a proxy for field performance (Bai *et al*., 2013), otherwise phenotyping remains a bottleneck for genetic analysis of roots.

Various techniques exist for identifying genomic loci of interest using forward genetic approaches, whereby the phenotype of interest is linked to the underlying genetic/physical loci, including; linkage mapping, GWAS (genome wide association studies), BSA (bulked segregant analysis) and genomic selection (Zhao, 2015). Reverse genetics on the other hand produces mutations in genes for which the function is already known in a species, and then establishes the phenotype controlled by the gene in the species of interest. This latter approach depends upon the sequence for the genes of interest being annotated, this is most often in model plant species, thus these genes must also have homologous relevance for more distantly related species. However, in most crops,a full genome sequence is yet to be attained and few genes are sequenced.

Most agronomic traits are quantitative and are controlled by multiple genes of varying effect which also interact with the environment and improving such traits through conventional breeding is complex. Marker-assisted backcrossing (MAB) uses genotype rather than phenotype markers to screen for the desired trait, this addresses the issue of the linkage drag of unwanted alleles and accelerates the time to recovery of the recurrent parent genome. Therefore, identification of the genetic loci underlying

desirable traits is a necessary prerequisite for MAB. In Brassica, EMS (ethyl methanesulfonate) mutagenesis was used to identify mutants with low linolenic acid in *B. rapa* and *B. napus* (Auld *et al*., 1991) and *B. oleraceae* (Singer *et al*., 2014). In the *B. napus* mutant the *FAD2* and *FAD3* genes responsible for the phenotype have since been cloned (Yang *et al*., 2012), and the associated SNP markers patented for use in marker-assisted introgression (Hu *et al*., 2015).

The Brassicaceae family includes *B. napus* (allotetraploid AA and CC genomes) which contains the root vegetable swede as well as the major oil crop oilseed rape (OSR)/canola. Yields of OSR in the UK have remained constant since 1990, at around 3-2– 3-4t ha^{-1}, and it is thought there is scope for improvement based on improving water and nitrogen (N) acquisition (Berry *et al*., 2010; White *et al*., 2013). *B. napus* is a complex allotetraploid hybrid of its diploid progenitor species, *B. rapa* and *B. oleracea* (Chalhoub *et al*., 2014). *B. rapa* (A genome) vegetable crops include Chinese cabbage, pak choi, mizuna, turnip roots and greens, it is also used for vegetable oils and fodder crops. *B. oleracea* (C genome) vegetable crops include cabbage, broccoli, cauliflower, brussel sprouts, kohlrabi, collards and kale. The genome of *B. napus* (Chalhoub *et al*., 2014) and of its progenitor species have been sequenced: *B. rapa* (The *Brassica rapa* Genome Sequencing Project Consortium: Wang *et al*., 2011) and *B. oleracea* (Liu *et al*., 2104). *Brassica napus* has undergone limited chromosomal rearrangements since the fusion of the constituent genomes, although there has been significant homoeologous recombination between the genomes (Chalhoub *et al*., 2014). Brassica and the model plant *Arabidopsis thaliana* share a common ancestor. There is 85% genic synteny between the *Arabidopsis* and *B. napus* genomes (Parkin *et al*., 2005), and *B. napus* has two to eight orthologous genes corresponding to each single-copy gene in *Arabidopsis* (Cavell *et al*., 1998; Parkin *et al*. 2005; Zhao *et al*., 2012). Comparative genetics between Brassica crops and the annotated genome of *Arabidopsis* is therefore very effective for identifying conserved gene sequences. For example, there are homologous genes in Brassica species to those in Arabidopsis controlling for flowering time (Osborn *et al*., 1997), disease resistance (Kuwabe *et al*., 2006) and root traits (Shi *et al*., 2013).

Chapter 2. High-throughput phenotyping (HTP) identifies seedling root traits linked to variation in seed yield and nutrient capture in field-grown oilseed rape (*Brassica napus* L.)

In *B. napus*, breeding for the double-low character (low erucic acid low glucosinolate) has led to a significant genetic bottleneck in modern cultivars (Snowdon and Luy, 2012). In order to identify traits for crop improvement it is necessary to screen the genetic diversity within wider pools of germplasm such as wild genotypes and landraces. Non-field adapted germplasm will need to be grown under suitable controlled conditions.

Roots grown under controlled conditions have been observed to correlate with field performance. Young seedling root growth in wheat corresponded to mature plant height in the field (Bai *et al*., 2013). In *B. napus*, root traits in 7 day old seedlings explained final seed yield in the field. A better relationship between root length and yield was observed when sampling at the 1-2 leaf stage rather than at the 3-4 leaf stage, because of increased root loss and root growth plasticity which occurred by the later stage (Koscielny and Gulden, 2012). Research on common bean has indicated that the effects of P on basal roots are consistent in paper, sand, and soil culture experiments (Liao *et al*. 2001). Therefore, because root traits are under significant genetic control, the controlled environment has been demonstrated as a suitable screening system for field performance.

The aim of this study was to screen the root architecture of elite *B. napus* cultivars under a controlled condition in a 'pouch and wick' system, and in the field. In order to establish the reliability of different root traits as suitable proxies for field performance, including measures of emergence, early vigour, root growth, yield and nutrient uptake.

Chapter 3. QTL meta-analysis of root traits in *Brassica napus* under contrasting phosphorus supply in two growth systems

The concentration of the plant available form of P- Pi (phosphate) in the soil solution is often low, because it is extremely insoluble and is readily converted into unavailable organic forms with high adsorption capacity (Lopez-Bucio *et al*., 2003).

QTL detected in independent experiments and located in a given region of a chromosome may represent several estimations of the position of a single QTL, therefore meta-analyses of QTLs from numerous studies/populations helps to validate the likely significance of a QTL (Zhou *et al*., 2014). The aim of this study was to make a QTL meta-analysis of the root traits of a *B. napus* doubled-haploid population grown under high and low Pi supply in two different HTP growth systems. QTL meta-analyses in Brassica have been performed for yield-related traits (Zhou *et al*., 2014; Shi J *et al*., 2009), and for root traits in different experiments in the same population (Yang *et al*., 2010), but not previously for root traits under different growth systems.

Chapter 4. Root morphology and seed and leaf ionomic traits in a *Brassica napus* L. diversity panel show wide phenotypic variation and are characteristic of crop habit

Phenotypic diversity in leaf and seed mineral composition has been studied in diverse accessions (Conn *et al*., 2012; Buescher *et al*., 2010; Baxter *et al*., 2012) and mutagenized populations (Lahner *et al*., 2003) of *Arabidopsis*, and natural populations of Brassica. In a *B. rapa* doubled-haploid population leaf mineral concentration ranged from 2-fold for P to 6.5-fold for Fe (Wu *et al*., 2008). In a large *B. oleracea* diversity panel shoot Ca and Mg varied 2 - 2.3-fold, respectively (Broadley *et al*., 2008). The concentration of elements within different plant tissues is relatively independent (Buescher *et al*., 2010; Baxter *et al*., 2012). In *Arabidopsis*, no macro nutrient concentrations except for P were found to correlate between roots, leaves and seeds, (Baxter *et al*., 2012). Although it varies with the mineral, for example shoot and seed P were of similar proportions whereas K was 4-fold lower in the seed compared to the leaf (Buescher *et al*., 2010). Translocation between tissues varies between minerals due to differences in phloem-mobility (White and Broadley, 2009), compartmentalisation in the central vacuole, root plasma-membrane transport kinetics, root architecture and transpiration (Conn *et al*., 2012). Leaf/seed translocation ratios

between chemical analogues e.g. K/Rb (rubidium) indicate the potential for mineral translocation from leaf to seed of plant essential elements, which is important for improvement of grain mineral composition. They have been considered in *Arabidopsis* (Baxter *et al*., 2012) but have not been looked at comprehensively in Brassica.

There is much potential in Brassica to identify genotypes with enhanced mineral concentrations and the underlying ion transporters. The ERANET-ASSYST (Associative expression and systems analysis of complex traits in oilseed rape) is the largest *B. napus* diversity panel (Bus *et al*., 2011; Körber *et al*., 2012). The phenotypic diversity of leaf mineral composition and seedling development traits such as leaf area (Bus *et al*., 2014), as well as agronomic traits such as emergence and seed quality (Korber *et al*., 2016), have been studied previously in this panel. This study has built on this work by also measuring leaf and seed mineral composition, analogue mineral leaf/seed translocation ratios, seed size, as well as root traits which have not been studied previously in a *B. napus* diversity panel. It also measured the effect of seed size on root growth in the HTP system, which may be of relevance for improved crop seedling establishment and vigour.

Chapter 5. Root, leaf mineral composition and seed size traits in *B. napus* L.; Meta-correlation and QTL/GWAS (Genome Wide Association scan) analyses

Various root traits have been shown to increase nutrient capture including lateral root elongation (Zhang and Forde, 1998; Sanchez-Calderon *et al*., 2006; Linkohr *et al*., 2005; Williamson *et al*., 2001), lateral root number (Gruber *et al*., 2013; Sanchez-Calderon *et al*., 2006; Perez-Torres *et al*., 2008), root hairs (Zhu *et al*, 2005; Yan *et al*., 2004; Giehl *et al*., 2012), a shallower root angle (Zhao *et al*., 2004; Bonser *et al*., 1996; Ho *et al*., 2005; Miguel *et al*., 2013), and smaller root diameter (Fitter *et al*., 1991). Zhu and Lynch (2004) suggest that the arrangement of long laterals under deficient supply is an ‘extensive’ foraging strategy suitable when resources are patchy, whereas a greater density of laterals is an ‘intensive’ strategy suitable in agro-ecosystems under non-patchy conditions, and where there is more inter-plant competition. However, there is uncertainty and contradiction as to the role of different

root traits in nutrient uptake. Furthermore, although N and P have been considered in detail, most other nutrients have not.

The aim of this study was to correlate root traits with leaf mineral concentrations of macro and micro nutrients in three *B. napus* populations, and to identify QTL for these traits. A meta-analysis of both root and leaf mineral concentration traits has not been conducted previously in *B. napus*.

Chapter 6. Genetic loci conferring a three-fold increase in magnesium concentration in *Brassica rapa* identified using a high-throughput forward genetic screen and bulked-segregant analysis

The importance of Mg in crop production has been underestimated (Cakmak and Yazici, 2010). There are substantial indications that concentrations of Mg are deficient in Western diets (Grzebisz, 2011; Rosanoff *et al*., 2013; White and Broadley, 2009; Broadley and White, 2010; Fulgoni *et al*., 2011). Magnesium supply can be low in light, sandy soils with a low CEC (cation exchange capacity), and in acid soils (Craighead, 2001; Joy *et al*., 2015), also under drought as it is predominantly taken up via mass flow (Gransee and Fuhrs, 2013), and under high light intensity (Cakmak and Kirkby, 2008). Competitive inhibition of Mg uptake is caused by Ca and K and to a lesser extent Na and Mn (Craighead, 2001; Romheld and Kirkby, 2007). Mg is highly prone to leaching because it sorbs less strongly to soil colloids than other cations (Craighead, 2001; Gransee and Fuhr, 2013). Therefore, soil resources/conditions have a large influence on crop Mg supply, for example it was observed that the Mg content of sorghum was 16-fold greater in West as compared with East Africa (Joy *et al*., 2013) and Mg concentration of maize (*Zea mays*) grain from calcareous soils was greater than that from non-calcareous soils in Malawi (Joy *et al*., 2015).

Variation in leaf Mg concentration has been observed in various Brassica species (Farnham *et al*., 2000; Kopsell *et al*., 2005; Broadley *et al*., 2008; Wu *et al*., 2008), ranging from 0.2% in a *B. rapa* doubled-haploid population (Wu *et al*., 2008) to 0.8% in a diverse collection of 355 *B. oleracea* accessions (Broadley *et al*., 2008). Much of the genetic variation in shoot Mg concentration occurs at the ordinal level or above in

angiosperms (Broadley *et al*., 2003, 2004; Thompson *et al*., 1997; Watanabe *et al*., 2007) and leaf Mg is under strong genetic control in Brassicaceae species (Wu *et al*., 2008; Farnham *et al*., 2000; Broadley *et al*., 2008; Thomas *et al*. 2016), *Arabidopsis thaliana* (Baxter *et al*., 2012), as well as in wheat grain (Peterson *et al*., 1983; Oury *et al*., 2006). Numerous genes controlling Mg transport in *Arabidopsis* have been identified (Li *et al*., 2001; Gerbert *et al*., 2009; Shaul *et al*., 1999; Pineros *et al*., 1995; Guo *et al*., 2010; Mao *et al*., 2014; Oda *et al*., 2016). However, despite the considerably higher concentrations of Mg in dicots generally and in Brassica particularly, and its considerable genetic control, no Mg accumulators have been identified.

The aim of this study was to make a high throughput screen of a *B. rapa* mutagenised population in order to identify Mg accumulator mutants, and the underlying genetic loci. *B. rapa* is an important vegetable crop worldwide and the loci could be introgressed into vegetable varieties such as pak choi, chinese cabbage or turnip, or through intraspecific crosses into *B. napus* and *B. oleracea*, for improved human nutrition, as well as for improving crop productivity on Mg deficient soils.

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Chapter 2

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High-throughput phenotyping (HTP) identifies seedling root traits linked to variation in seed yield and nutrient capture in field-grown oilseed rape (*Brassica napus* L.)

C. L. Thomas[1,2], N. S. Graham[1], R. Hayden[1], M. C. Meacham[1], K. Neugebauer[1,2], M. Nightingale[3], L. X. Dupuy[2], J. P. Hammond[4], P. J. White[2,5] and M. R. Broadley[1]*

[1]Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK, [2]Ecological Sciences, The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK, [3]Elsoms Seeds Ltd, Pinchbeck Rd, Spalding PE11 1QG, UK, [4]School of Agriculture, Policy and Development, University of Reading, Whiteknights, PO Box 237, Reading RG6 6AR, UK and [5]Distinguished Scientist Fellowship Program, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia
**For correspondence. E-mail martin.broadley@nottingham.ac.uk*

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• **Background and Aims** Root traits can be selected for crop improvement. Techniques such as soil excavations can be used to screen root traits in the field, but are limited to genotypes that are well-adapted to field conditions. The aim of this study was to compare a low-cost, high-throughput root phenotyping (HTP) technique in a controlled environment with field performance, using oilseed rape (OSR; *Brassica napus*) varieties.
• **Methods** Primary root length (PRL), lateral root length and lateral root density (LRD) were measured on 14-d-old seedlings of elite OSR varieties ($n = 32$) using a 'pouch and wick' HTP system (~40 replicates). Six field experiments were conducted using the same varieties at two UK sites each year for 3 years. Plants were excavated at the 6- to 8-leaf stage for general vigour assessments of roots and shoots in all six experiments, and final seed yield was determined. Leaves were sampled for mineral composition from one of the field experiments.
• **Key Results** Seedling PRL in the HTP system correlated with seed yield in four out of six ($r = 0{\cdot}50$, 0·50, 0·33, 0·49; $P < 0{\cdot}05$) and with emergence in three out of five ($r = 0{\cdot}59$, 0·22, 0·49; $P < 0{\cdot}05$) field experiments. Seedling LRD correlated positively with leaf concentrations of some minerals, e.g. calcium ($r = 0{\cdot}46$; $P < 0{\cdot}01$) and zinc ($r = 0{\cdot}58$; $P < 0{\cdot}001$), but did not correlate with emergence, general early vigour or yield in the field.
• **Conclusions** Associations between PRL and field performance are generally related to early vigour. These root traits might therefore be of limited additional selection value, given that vigour can be measured easily on shoots/canopies. In contrast, LRD cannot be assessed easily in the field and, if LRD can improve nutrient uptake, then it may be possible to use HTP systems to screen this trait in both elite and more genetically diverse, non-field-adapted OSR.

Key words: *Brassica napus* (OSR, canola), lateral root density, mineral concentration, primary root length, seed yield.

INTRODUCTION

Direct phenotypic selection for yield, appearance and quality is routinely conducted on the roots of domesticated crops such as carrot (*Daucus carota*; Stein and Nothnagel, 1995) and cassava (*Manihot esculenta*; Nassar and Ortiz, 2007). For most non-root crops, including cereals and legumes, direct phenotypic selection of root traits has not been widely attempted. However, indirect selection of root traits has undoubtedly supported historical yield increases (Lynch, 2007, 2011). Examples of root traits shown to correlate with improved field performance include thicker, longer roots in rice (*Oryza sativa*; Steele *et al*., 2013), increased root hair elongation in maize (*Zea mays*; Hochholdinger *et al*., 2008) and shallower roots under low phosphorus (P) conditions in soybean (*Glycine max*; Zhao *et al*., 2004) and common bean (*Phaseolus vulgaris*; Bonser *et al*., 1996; Ho *et al*., 2005; Miguel *et al*., 2013).

In rice, beneficial root traits have recently been introgressed successfully into elite crop varieties. For example, multiple quantitative trait loci (QTL) associated with thicker and longer roots were introgressed from a *japonica* into an *indica* line via marker-assisted selection (Steele *et al*., 2007, 2013). This led to improved field performance under favourable field conditions and a new rice variety (Birsa Vikas Dhan 111) has subsequently been released. The *Phosphorus Uptake 1* (*PUP1*) QTL has been introgressed into commercial rice varieties, increasing root growth and yield in P-deficient soils (Chin *et al*., 2010). The *Deeper Rooting 1* (*DRO1*) QTL has been back-crossed into a shallow-rooting rice variety, increasing yield under drought conditions by increasing root depth (Uga *et al*., 2013; Arai-Sanoh *et al*., 2014). There are fewer reports on the introgression of root traits in other crops; however, QTL-linked root traits in wheat (*Triticum aestivum*; Placido *et al*., 2013) and chickpea (*Cicer arietinum*; Varshney *et al*., 2013) have been

introduced into elite varieties and shown to improve drought tolerance.

Direct selection of root traits under field conditions is possible, albeit challenging in many environments. For example, low-cost root excavations can be made using hand-held tools ('shovelomics'; Trachsel *et al*., 2011; Wishart *et al*., 2013; Bucksch *et al*., 2014). More involved methods of observing root traits include soil-coring/washing/breaking (Gregory and Eastham, 1996; Ostonen *et al*., 2007; Watt *et al*., 2013; Wasson *et al*., 2014) and the use of interfaces such as 'windows', trenches and minirhizotrons (Jose *et al*., 2001; Dupuy *et al*., 2010; Maeght *et al*., 2013; Vansteenkiste *et al*., 2014). *In situ* proxy measurements for studying root traits include: (1) using buried herbicides to monitor root depth (Maeght *et al*., 2013; Al-Shugeairy *et al*., 2014); (2) electromagnetic induction to estimate root biomass from water depletion (Srayeddin and Doussan, 2009; Shanahan *et al*., 2015); (3) measuring root capacitance (Dietrich *et al*., 2012, 2013); and (4) quantifying plant root DNA concentrations (Huang *et al*., 2013). *Ex situ* methods for studying the roots of soil-grown plants include X-ray microcomputed tomography (μCT; Tracy *et al*., 2010; Mooney *et al*., 2012) and magnetic resonance imaging (MRI; Rogers and Bottomley, 1987; Metzner *et al*., 2015), which may have potential for field-grown crops in the future. Despite these methodological advances, measuring root traits in the field is still a major constraint to direct phenotypic selection for crop improvement due to cost, time and logistical implications (e.g. soil hardness).

A further constraint to selecting root traits directly is the paucity of phenotypic diversity in many crops arising from domestication/genetic bottlenecks (Bus *et al*., 2011). However, more diverse genetic material cannot easily be studied in the field when it is not adapted to local environmental conditions. The use of high-throughput phenotyping (HTP) systems *in vitro* can potentially overcome this constraint (Hammond *et al*., 2009; Chen *et al*., 2011; Koscielny and Gulden, 2012; Downie *et al*., 2015). Numerous HTP techniques have been developed, including rhizotrons for soil-grown plants (Nagel *et al*., 2012; Lobet and Draye, 2013) and transparent media, which enable 3D root imaging *in vitro* (Iyer-Pascuzzi *et al*., 2010; Clark *et al*., 2011; Downie *et al*., 2012). Simpler, 2D HTP systems include agar plate-based systems (Armengaud *et al*., 2009; French *et al*., 2009; Shi *et al*., 2013) and 'pouch and wick' systems, in which roots are grown on filter paper, suspended in nutrient solution, which is surrounded by a pouch that conceals roots from light (Bonser *et al*., 1996; Hund *et al*., 2009; Miguel *et al*., 2013; Watt *et al*., 2013; Adu *et al*., 2014; Le Marie *et al*., 2014; Atkinson *et al*., 2015). An important observation from these HTP studies is that root traits are often highly variable, and so require large numbers of replicates to assess genotypic versus non-genotypic sources of variation (Adu *et al*., 2014). Whilst such HTP systems cannot allow the assessment of roots in soil, they can enable promising germplasm to be identified which could then be crossed into field-adapted material for direct testing in the field. However, few HTP studies have been designed to test the associations between root traits and field performance in elite germplasm explicitly (White *et al*., 2013*b*).

The aim of this study was to test a low-cost HTP technique for screening seedling root traits in oilseed rape (OSR; *Brassica napus*). *Brassica napus* is a complex allotetraploid hybrid of its diploid progenitor species, *Brassica rapa* and *Brassica oleracea* (Chalhoub *et al*., 2014). We selected OSR for study as it is an important crop, providing 18 % of global vegetable oil for human consumption, plus industrial oils, biodiesel, lubricants and animal feeds. Over 60 million tonnes of seed are produced annually worldwide (FAOSTAT, 2015). In the UK, it is grown on >700 000 ha, primarily in rotation with wheat. Yields of OSR in the UK have remained constant since 1990, at ~3·2–3·4 t ha^{-1}, and it is thought there is scope for improvement based on improving water and nitrogen (N) acquisition, especially post-anthesis, through improved rooting traits (Berry *et al*., 2010; White *et al*., 2013*b*). There is limited published information on OSR seedling root traits in relation to yield, although Koscielny and Gulden (2012) reported a relationship among eight varieties of *B. napus* between root length measured on 7-d-old seedlings and their seed yield in the field in Canada. Previous HTP studies on *Brassica oleracea*, *B. napus and B. rapa* show that QTL for seedling root traits correlate with plant shoot biomass (Hammond *et al*., 2009; Shi *et al*., 2013; Adu *et al*., 2014). The work presented here extends these observations to test whether root architectural traits measured on seedlings of *B. napus* relate to key crop characteristics in the field.

MATERIALS AND METHODS

Plant material

Thirty-two elite winter OSR varieties were grown in an HTP pouch and wick system in a controlled environment and at two UK field sites each year, for three years. Root traits measured in the HTP system included primary root length (PRL), lateral root length (LRL) and lateral root density (LRD). Traits measured in the field experiments included final seed yield in all experiments and assessments of early crop establishment and nutrient acquisition in a subset of experiments. The OSR varieties were chosen primarily based on varieties in the Recommended List (RL) of the UK Agriculture and Horticulture Development Board (AHDB), to enable root traits measured in the HTP system to be tested in the field on UK-adapted material. Varieties included conventional types ($n = 20$), recombinant hybrid types ($n = 11$) and fodder types ($n = 1$).

HTP experiments

A pouch and wick hydroponic-based HTP system (Atkinson *et al*., 2015) was deployed in this study (Fig. 1). This system comprised growth pouches assembled from blue germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA), re-cut to 24 × 30 cm and overlaid with black polythene (Cransford Polythene Ltd, Woodbridge, UK). Along their shorter edges, the paper and polythene were clipped together to each side of an acrylic bar (Acrylic Online, Hull, UK) using bulldog-type fold-back clips. The growth pouches were suspended above plastic drip trays, supported within lightweight aluminium/polycarbonate frames, as described in Atkinson *et al*. (2015). Each drip tray contained 2 L of 25 % strength Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture,

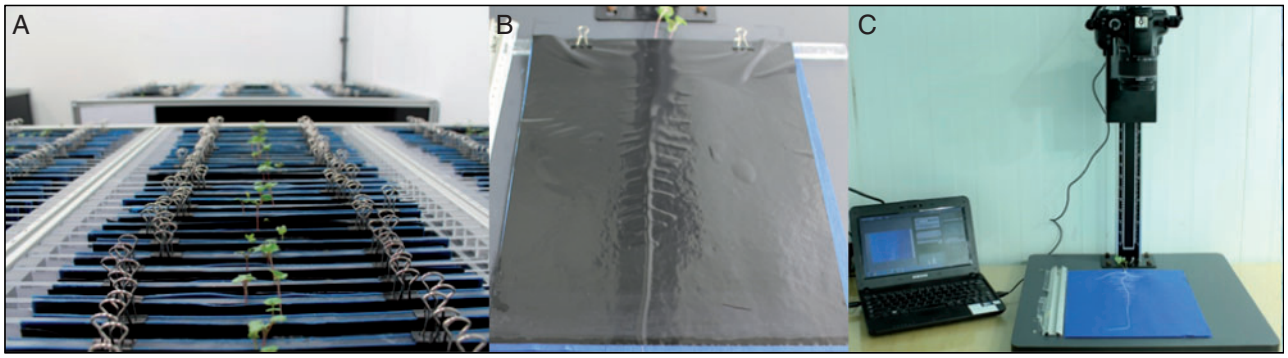

FIG. 1. (A) *Brassica napus* seedlings growing in the hydroponic pouch and wick system. (B) Growth pouch 14 d after sowing (DAS). (C) Stand-mounted camera at fixed height above germination paper with root 14 DAS.

Sigma Aldrich, Dorset, UK) made with deionized water. Drip trays were replenished with 500 mL of deionized water every 3 d. Prior to sowing, the pouches were suspended above the nutrient solution for a minimum of 4 h to become fully saturated. A single seed was sown in the middle of the upper edge of each germination paper by pressing the seed into the paper. Within each aluminium frame, nine drip trays were used, arranged in three columns and three rows. Pouches were allocated randomly to drip trays, 10 or 11 pouches per drip tray, thus giving 96 pouches and 192 plants per frame. A total of four frames were used in each experimental run, giving a potential sample size of 768 plants per run within a single controlled-environment room. The controlled-environment room was 2·2 m wide, 3·3 m long and 3·0 m high, set to a 12-h photoperiod with 18/15 °C day/night temperatures and relative humidity of 60–80 %. Photosynthetically active radiation (PAR; measured at plant height with a 190 SB quantum sensor; LI-COR Inc., Lincoln, NE, USA) was 207 $\mu mol\ m^{-2}\ s^{-1}$, generated by 400-W white fluorescent lamps (HIT 400w/u/Euro/4K, Venture Lighting, Rickmansworth, UK). Fourteen days after sowing (DAS), the polythene sheets were removed from all pouches and images were taken of the germination paper and root system for downstream image analysis. Images were taken using a digital single-lens reflex (DSLR) camera (Canon EOS 1100D, Canon Inc., Tokyo, Japan) with a focal length of 35 mm at a fixed height of 75 cm, using Canon software.

Root image processing

The root images from the HTP system were renamed with each sample's unique experimental design information using Bulk Rename Utility (Version 2.7.1.3, TGRMN Software, www.bulkrenameutility.co.uk). Images were cropped by reducing extraneous pixels on bulked images, using XnConvert (Version 1.66, www.xnconvert.com). Cropped images were analysed using RootReader2D (RR2D; Clark *et al.*, 2013). First, a batch process was carried out which automatically 'thresholds', 'skeletonizes' and 'builds segments' of all images in bulk. The root system was then measured on individual images by placing a marker at the base and tip of the primary root. From these markers, RR2D automatically calculates PRL, LRL of all laterals, and lateral root number (LRN). Further traits calculated from these data included total root length (TRL = PRL + LRL), mean lateral root length (MLRL = LRL/LRN) and LRD (LRN/PRL). A database was developed which integrated the experimental design information from the image name with the RR2D measurements for each sample, using a programming script (2.7.10; Python Software Foundation, www.python.org).

Field experiment locations

Experiments were conducted in 2012–13, 2013–14 and 2014–15 at two field sites each year: Bingham (Nottinghamshire) and Deeping St Nicholas (Lincolnshire) in 2012–13 and 2013–14; the Bingham site was replaced by a site at Harlaxton (Lincolnshire) in 2014–15 (Table 1). Experiments were managed by Elsoms Seeds Ltd (Spalding, UK) according to standard National List protocols for winter oilseed rape. Untreated seeds of each variety were sown at 60 seeds m^{-2} in plots of 12 m length × 1·85 m width, allocated to a randomized-block design of three replicates. Standard field assessments included final seed yields for all experiments, and emergence (scored 5 weeks after drilling by an experienced oilseed breeder on a subjective scale of 1–8, where 8 represents best possible emergence) for most experiments. Additional trait assessments are described in the following sections.

General early vigour assessments of shoots and roots in the field

Plants were sampled at an early growth stage (~6- to 8-leaf stage) from every plot, before roots became too large to excavate easily, with six to eight plants sampled per plot. From the mid-sections of each plot, roots and shoots were removed using a garden fork and stored in plastic bags at <5 °C. Subsequently, roots were washed with detergent (1 % v/v; Teepol, Kent, UK). For the 2012–13 and 2013–14 experiments, roots were photographed as described for the HTP experiments using the DSLR camera. Images of field-sampled roots were analysed using ImageJ (Rasband, 1997–2014, http://rsbweb.nih.gov/ij/docs/faqs.html). The length of the longest root axis (i.e. PRL) and the base diameter of the primary root (PRBD), were traced using the 'segmented line' tool. The mean diameter of the primary root (PRMD) was calculated by dividing the root length into quarters and obtaining the mean root diameter at these points. Dry weights of roots and shoots were obtained for all field experiments; samples were oven-dried at 50 °C for ~48 h and weighed.

TABLE 1. *Locations, timings, soil types, soil pH and cation exchange capacity (CEC) of sites used for the field experiments conducted in 2012–13, 2013–14 and 2014–15*

Site/grid reference	Sowing date	Harvest date	Soil type[a]	pH	CEC(meq 100 g^{-1})
Bingham	30/8/2012	17/8/2013	Slightly acid, loamy and clayey soils with impeded drainage.	7·0	14·9
SK 6 955 738 274	30/8/2013	17/8/2014	Fertility: moderate to high		
Deeping	6/9/2012	29/8/2013	Loamy and clayey soils of coastal flats with naturally high groundwater.	7·6	32·1
TF 2 170 017 100	6/9/2013	29/8/2014	Fertility: lime-rich to moderate		
	3/9/2014	28/7/2015			
Harlaxton	27/8/2014	3/8/2015	Slowly permeable seasonally wet slightly acid but base-rich loamy and clayey soils.	7·2	13·1
SK 8 891 134 066			Fertility: moderate		

[a]Data from Landis: http://www.landis.org.uk/soilscapes/#.

Leaf sampling in the field and preparation for elemental analysis

Leaves were sampled at flowering, prior to seed-set (June 2014) from every plot at the Bingham site from the 2013–14 experiment. For each of the 96 plots, a composite sample was taken comprising one fully expanded leaf removed from the tops of the stems of four randomly selected plants. Leaf samples were dried in paper bags at 50 °C for 48 h and hand-crushed to a powder within the bag, and 0·1 g was subsampled for elemental analysis. Samples were digested using a solution of 1 mL of 30 % H_2O_2, 2 mL of 50 % trace analysis grade HNO_3 and 1 mL of Milli-Q water (18·2 MΩ·cm; Fisher Scientific UK Ltd, Loughborough, UK). Solutions were placed in a Multiwave 3000 microwave with a 48-vessel 48MF50 rotor (Anton Paar GmbH, Graz, Austria) and heated for 45 min at a controlled pressure of 2 MPa, within vessels comprising perfluoroalkoxy liner material and polyethylethylketone pressure jackets (Anton Paar GmbH). Digested samples were diluted to 15 mL with Milli-Q water and stored at room temperature. Immediately prior to analysis, digested samples were diluted 1-in-10 with Milli-Q water. The concentrations of 28 elements were obtained using inductively coupled plasma mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany): Ag, Al, As, B, Ba, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, U, V and Zn. For each data point, element-specific operational blank concentrations (ICP-run means) were subtracted. An external Certified Reference Material was included (IAEA-359 Cabbage; LGC, Teddington, UK).

Statistical analyses

Pearson's correlation coefficients were calculated for root traits of plants grown in the HTP system and emergence, root and shoot traits, and yield in field experiments. One variety (ID 29) was excluded from data analysis because it is a forage type with low seed yield. Variance components were calculated for all HTP traits using a residual maximum likelihood (REML) procedure, with all factors classed as random factors so that the proportional contribution of genotype to overall variation in traits could be determined. The experimental power to compare root traits of different genotypes was determined for both the HTP system and the field. For this, a *t*-test sample-size calculation was used, whereby the measure of variance was the residual mean square from the REML procedure and the response to be detected was set as a percentage of the grand mean of all varieties; data were plotted as contour plots. All statistical analyses were conducted using GenStat 15th Edition (VSN International Ltd, Hemel Hempstead, UK).

RESULTS

A total of 1200 images were obtained from the HTP system and 2428 images from the field experiments. In the HTP system, recombinant hybrid varieties had a longer PRL than conventional varieties (*t*-test, $F = 2{\cdot}07$; $P < 0{\cdot}001$; Table 2) and had greater emergence (*t*-test, $F = 1{\cdot}63$; $P < 0{\cdot}05$; Supplementary Data Table S1) and seed yield (*t*-test, $F = 1{\cdot}87$; $P < 0{\cdot}05$; Table 2) under field conditions.

There were general overall positive relationships between root traits measured in the HTP system and field traits for most sites and years. For example, there were positive relationships between PRL and emergence in three of the field experiments ($r = 0{\cdot}59$, $r = 0{\cdot}22$, $r = 0{\cdot}49$; Supplementary Data Table S1). Thus, whilst plants established much more rapidly at Bingham than at Deeping in 2012–13, varieties with the longest PRL showed better emergence and crop establishment at both sites (Fig. 2). There were also significant positive relationships between PRL and final seed yield in four of the field experiments ($r = 0{\cdot}50$, $r = 0{\cdot}50$, $r = 0{\cdot}33$, $r = 0{\cdot}49$) (Fig. 3; Supplementary Data Table S1).

The relationships among root traits measured within and between the HTP and all field experiments are shown in Table 3. Within the HTP system, there were significant positive relationships between most root length-related traits. For example, there were positive relationships between PRL and TRL ($r = 0{\cdot}84$). However, there was no significant correlation between PRL and LRD. The only significant negative correlation among the root traits measured in the HTP system was between LRD and MLRL ($r = -0{\cdot}38$), which suggests a potential trade-off for these traits. Consistent with these observations, LRD measured in the HTP system did not correlate with yield or any other traits measured in the field, such as emergence or root and shoot dry weights at the 6- to 8-leaf stage. Field experiments indicated that early growth correlated positively with final seed yields. Thus, root and shoot dry weights were correlated at the 6- to 8-leaf stage ($r = 0{\cdot}90$), and both these traits were correlated with final seed yield ($r = 0{\cdot}49$, $r = 0{\cdot}46$, respectively).

TABLE 2. Brassica napus *varieties grown in the HTP system; variety type; recommended area for cultivation in the UK; year of first inclusion the recommended list (RL), primary root length (PRL; mean ± s.d.); and final seed yield in the field. HTP data are from a pouch and wick system with seedlings imaged at 14 DAS (n = ~40). Field data are means of six field experiments (mean ± s.d.). Varieties are ranked by PRL*

Variety	Variety type	Year on RL	Recommended area	PRL (cm)	Yield (t ha^{-1})
16	Recombinant hybrid	2013–14	Club root-infected land	18·5 ± 6·7	5·3 ± 0·9
8	Recombinant hybrid	2011–12	All UK	17·4 ± 6·0	5·5 ± 0·8
18	Recombinant hybrid	2004–05	Club root-infected land	17·3 ± 8·2	5·2 ± 1·8
29	Fodder	Not on RL	–	17·3 ± 5·7	–
11	Conventional	2009–10	E/W	17·2 ± 6·7	5·3 ± 0·8
1	Recombinant hybrid	2013–14	E/W	17·2 ± 6·4	4·9 ± 0·5
9	Recombinant hybrid	2012–13	E/W	17·2 ± 5·3	6·0 ± 0·6
17	Conventional	2005–06	All UK	16·6 ± 5·3	4·6 ± 0·5
25	Recombinant hybrid	2009–10	N	16·3 ± 6·7	5·2 ± 0·7
14	Conventional	2005–06	All UK	16·2 ± 7·0	5·3 ± 0·3
10	Conventional	2010/11	E/W	16·1 ± 5·2	5·1 ± 1·0
2	Conventional	Not on RL	–	15·9 ± 5·3	5·2 ± 0·7
12	Recombinant hybrid (semi-dwarf)	2012/13	E/W	15·6 ± 8·3	4·9 ± 0·7
15	Conventional	2005/06	All UK	15·5 ± 5·0	4·9 ± 0·6
24	Conventional	Not on RL	–	15·4 ± 4·7	5.1 ± 0·8
21	Recombinant hybrid	2013/14	E/W	15·3 ± 6·6	5·5 ± 0·5
13	Recombinant hybrid	2008/09	All UK	15·2 ± 5·3	5·7 ± 0·7
5	Conventional	2011/12	E/W	14·9 ± 6·5	5·2 ± 0·5
3	Conventional	Not reported	E/W	14·8 ± 4·8	5·4 ± 0·5
26	Conventional	2008/09	N	14·7 ± 5·7	5·1 ± 0·7
4	Conventional	2012/13	E/W	14·7 ± 5·8	5·1 ± 0·9
28	Conventional	Not reported	–	14·1 ± 6·1	5·1 ± 0·7
27	Conventional	Not reported	E/W	14·1 ± 3·8	5·1 ± 0·7
6	Conventional	2009/10	E/W	14·1 ± 4·4	5·1 ± 0·4
32	Conventional	2004/05	All UK	13·7 ± 3·9	4·6 ± 0·8
23	Conventional	Not on RL	–	13·2 ± 6·5	4·7 ± 1·0
19	Conventional	Not on RL	–	12·9 ± 5·0	4·3 ± 0·8
20	Conventional	2010/11	N	12·5 ± 5·1	4·9 ± 1·1
7	Conventional	2010/11	E/W	12·4 ± 5·7	5·2 ± 0·8
30	Conventional	2005/06	All UK	12·3 ± 4·7	4·6 ± 0·5
31	Conventional	Not on RL	–	11·0 ± 4·0	4·9 ± 0·5
22	Recombinant hybrid	2011/12	E/W	–	5·5 ± 0·5

–, not applicable.
N, North Region, comprising Scotland, Northern Ireland and Northern England; E/W, East/West Region, comprising England (south of Teesside) and Wales.
Variety 18 did not grow in the field in 2012–13 (i.e. $n = 4$). Variety 22 did not grow in the HTP system.

The LRD measured in the HTP system was significantly positively related to leaf mineral concentrations, e.g. calcium ($r = 0{\cdot}46$) and zinc ($r = 0{\cdot}58$), measured in the field (Fig. 4; Supplementary Data Table S2). Given that no yield or emergence penalties were observed among varieties with greater LRD (Table 3), it is plausible that varieties with greater LRD were able to take up these nutrients more efficiently.

The experimental power to detect differences in trait means between varieties was determined as a function of the number of replicates for the HTP system and for one of the field sites (Bingham 2012–13; Fig. 5). When measured in the HTP system, fewer replicates are needed to detect differences in PRL than in LRD and MLRL. For example, to have an 80 % power to detect 30 % differences in PRL, LRD and MLRL would require 15, 25 and 36 replicates, respectively. In the field experiments, fewer replicates are needed to detect differences in PRBD than in PRL or root dry weight (RDW). For example, to have an 80 % power to detect 30 % differences in PRBD, PRL and RDW would require 11, 14 and 34 replicates, respectively. Varietal (type + variety) factors accounted for relatively little of the total variation in root traits in the HTP system (≤9 %; Table 4 and Supplementary Data Table 3).

DISCUSSION

The estimated time for obtaining root data (sowing, imaging, data analysis) from the HTP system described in this paper was ~2 min per individual plant. The costs of consumables and growth space were <£1 per plant. This compares to ~20 min per plant to extract, wash, image and analyse root data when grown in the field (excluding sowing, crop management and harvesting).

Hybrid OSR types tended to have greater PRL than conventional OSR types in the HTP system (Table 2). Koscielny and Gulden (2012) also observed greater root growth in hybrids compared with open-pollinated varieties in OSR seedlings. Similarly, Hoecker *et al*. (2006) observed heterosis (hybrid vigour) in PRL and LRD of maize 4 d post-germination. Thus, some of the variation in OSR root length traits could be due to hybrid vigour; this hypothesis could be tested on larger collections of OSR germplasm. Two OSR varieties bred for resistance to club root disease were among the top three varieties ranked for PRL (Table 2), indicating that these genotypes have vigorous roots. The interactions between all soil-borne diseases and root traits is clearly an area that warrants further

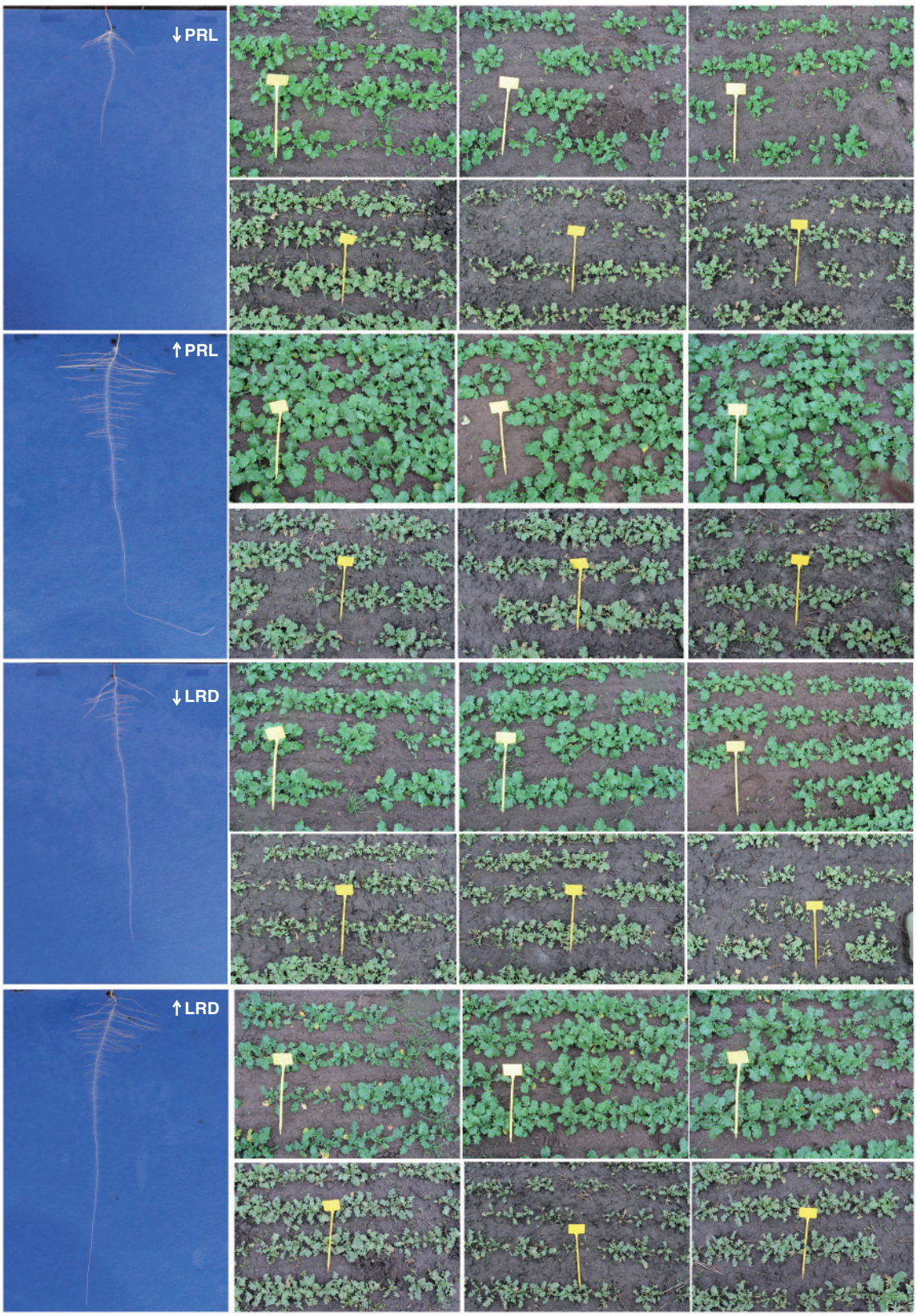

FIG. 2. Varieties ID 31 and ID 16 with the minimum (↓PRL) and maximum (↑PRL) primary root length and varieties ID 15 and ID 17 with the minimum (↓LRD) and maximum (↑LRD) lateral root density, when grown for 14 d in the pouch and wick system (illustrative examples; vertical axis = 32 cm). Field images are triplicate plots of the same varieties at two UK field sites (2012–13): Nottinghamshire (greener, more established plots, upper panels); Lincolnshire (paler, less-established plots, lower panels; markers = 20 cm).

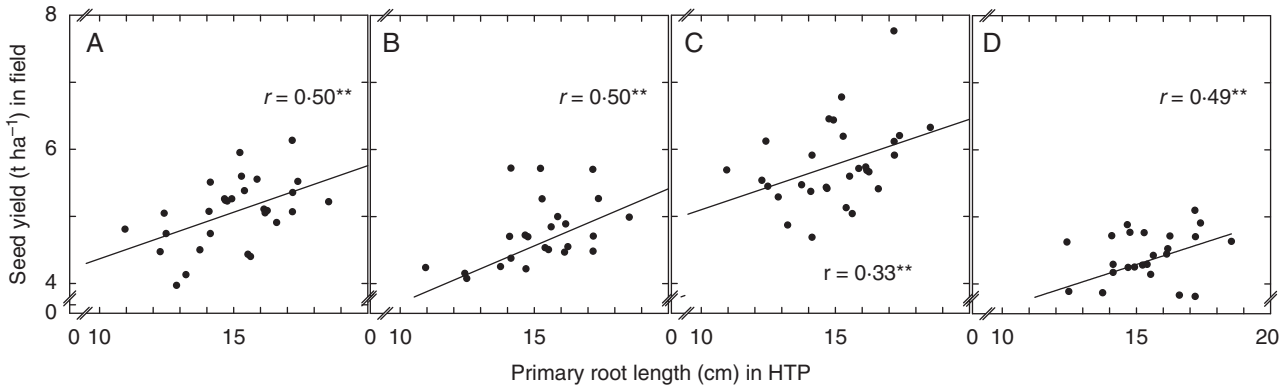

FIG. 3. Seed yield of *Brassica napus* varieties grown in the field as a function of primary root length in the HTP system. Field data are means of triplicate plots at Bingham and Deeping in 2012–13 (A, B), at Bingham in 2013–14 (C) and at Deeping in 2014–15 (D). HTP data are means of ~40 replicates imaged 14 d after sowing. $^{**}P < 0{\cdot}01$.

TABLE 3. *Pearson correlation coefficients (*r*) between root traits of* Brassica napus *varieties grown in the HTP system, and root and shoot traits of varieties grown in all six field experiments.*

		PRL	LRD	MLRL	TRL						
HTP	PRL	–									
	LRD	0·20	–								
	MLRL	0·14	–0·38*	–							
	TRL	0·84***	0·45**	0·32	–						
Field	PRL	0·32	0·31	−0·22	0·26	–					
	PRBD	0·42*	0·08	0·06	0·33	0·68***	–				
	RDW	0·51***	0·02	0·01	0·42**	0·34	0·58***	–			
	SDW	0·49***	−0·07	0·08	0·37**	0·09	0·41*	0·90***	–		
	EM	0·55***	0·16	0·12	0·50***	0·70***	0·81***	0·64***	0·50**	–	
	YIELD	0·35	−0·05	0·06	0·26	0·26	0·39*	0·49**	0·46**	0·66***	–
		PRL	LRD	MLRL	TRL	PRL	PRBD	RDW	SDW	EM	YIELD

***, ** and * represent $P < 0{\cdot}001$, $P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively.

investigation; for example, diseases such as *Rhizoctonia solani* can cause profound damage to primary roots of OSR (Sturrock *et al*., 2015). Variety ID 29 had one of the longest PRLs in the HTP system and also had the largest shoot dry weight (SDW) and one of the largest RDWs in most field experiments at the 6- to 8-leaf stage. However, ID 29 was not considered in subsequent analyses because it is a forage variety type and therefore much lower-yielding in terms of seed.

Length-related root traits measured in the HTP system were associated with traits related to general vigour in the field (Table 3). Thus, correlations were routinely seen between seedling root length traits and growth-related traits in the field, including emergence (2–3 weeks post-sowing), SDW and RDW at the 6- to 8-leaf stage, and final seed yield in most of the six field site × year combinations. This observation is again consistent with Koscielny and Gulden (2012), who compared four hybrid and four open-pollinated OSR varieties and found that PRL at the 1- to 2- and 3- to 4-leaf stages was a predictor of seed yield. In diploid *B. oleracea*, total root length correlated strongly with shoot biomass in pot-grown plants under both low and high external P supply (Hammond *et al*., 2009). In the variance component analysis (Table 4), variety type (i.e. hybrid or conventional) had the largest effect on PRL, which is consistent with PRL having the strongest relationship to general field vigour of all traits in the HTP system. This current study is based on a relatively small number of genotypes and further field experiments and yield stability assessments, on more variety × location combinations, are required to understand how factors, e.g. weather and disease, influence these undoubtedly complex relationships. For example, seed yield at Deeping in 2013–14 correlated negatively (albeit non-significantly) with seed yield in all of the other site × year combinations. Ultimately, the direct test of the value of HTP will be when root traits from non-field-adapted material have been crossed into elite varieties to improve field performance at multiple locations.

Roots of young plants in the field must acquire water and nutrients quickly, especially in small-seeded species with limited nutrient reserves (Wright *et al*., 1996; White and Veneklaas, 2012). So it is not surprising that seedling root length traits explain general early vigour under field conditions in OSR, which then explains some of the variation in final yield. Correlations between seedling development under glasshouse and field conditions, including final yield and quality traits, has also been

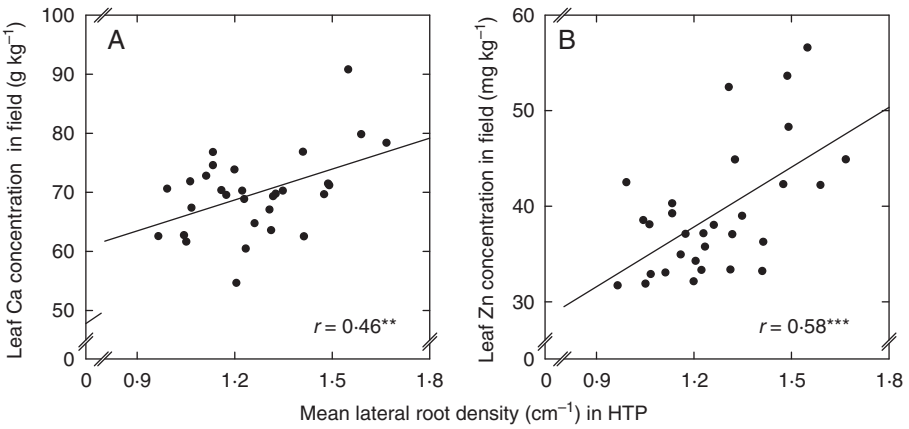

FIG. 4. Leaf dry weight concentrations of calcium (A) and zinc (B) in the leaves of *Brassica napus* varieties grown in the field as a function of lateral root density in the HTP system. Field data are means of triplicate plots at Bingham in 2013–14. HTP data are means of ~40 replicates imaged 14 d after sowing. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

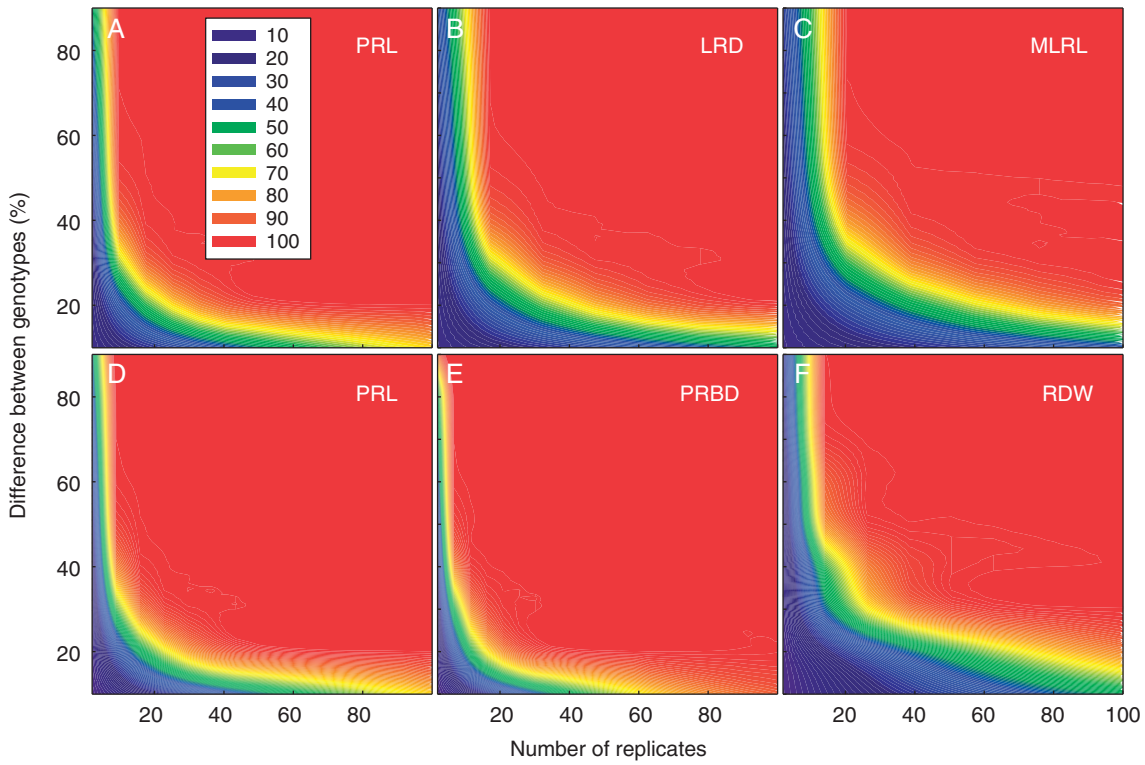

FIG. 5. The experimental power (*z*-axis, legend inset in percentage units) to detect percentage differences in trait means between varieties (*y*-axis) as a function of the number of replicates (*x*-axis). (A, B, C) HTP system. (D, E, F) Field data from Bingham 2012–13.

reported in a large diversity panel of *B. napus* (Körber *et al.*, 2012). Thus, given that establishment and early vigour traits can be measured easily on shoots/canopies, root length traits in OSR might have limited additional selection value for field-adapted germplasm. However, seedling root length can still be measured rapidly and cheaply in an HTP system alongside other traits (e.g. LRD), especially when large numbers of varieties are being compared. Furthermore, an HTP system is advantageous for studying genotypes that are not well adapted to field conditions and therefore require a homogeneous/controlled environment. However, there are, of course, substantial year and site effects that can limit the scope for detecting genotype effects even in field adapted material (Table 4).

In contrast to length-related traits, LRD measured in the HTP system was not significantly correlated with establishment, SDW or RDW at the 6- to 8-leaf stage, or seed yield in the field. However, LRD measured in the HTP system was influenced significantly by variety (Table 4). Furthermore, significant positive correlations between LRD and leaf concentrations of three mineral nutrients (Ca, Zn and Fe) were observed in the field (Table 3, Fig. 3). Given that LRD and field growth traits

TABLE 4. *Variance component analysis of* Brassica napus *root traits in the HTP system, and root and shoot traits in the field from all six field experiments. Variety type component represents recombinant hybrid or conventional. Environment component represent variation allocated to all experimental design factors*

	HTP system				Field			
Variance component (%)	PRL	LRD	MLRL	TRL	PRL	PRBD	RDW	SDW
Variety type	6	1	0	3	0	3	2	1
Variety	3	6	1	5	2	5	1	n.f.
Year	–	–	–	–	n.f.	n.f.	22	16
Site	–	–	–	–	3	30	31	43
Environment	17	10	5	18	15	9	11	13
Residual	74	83	94	73	80	54	33	27

n.f., not fitted during residual maximum likelihood procedure.

were not negatively associated, it is likely that varieties with greater LRD were able to acquire these nutrients more efficiently. This hypothesis could now be tested directly.

Roots of nutrient-limited plants growing through soils with heterogeneous nutrient availability often show increased LRD in places with greater nutrient supply (Drew, 1975; Robinson, 1994; Lopez-Bucio *et al*., 2003; Hodge, 2004; Ostonen *et al*., 2007). Whilst varieties of OSR in the current study will undoubtedly respond to soil nutrient heterogeneity, it seems unlikely that plants were nutrient-deficient on these well-fertilized soils, where the mineral composition of leaves was in a range defined as sufficient for plant growth (White and Brown, 2010). Thus, given that it is not possible to measure fine lateral roots of OSR in the field, the LRD trait warrants further study in HTP systems, with the following foci: (1) determining the effects of LRD on nutrient uptake efficiencies under resource-limited and resource-adequate conditions; and (2) quantifying wider sources of genetic variation in LRD in adapted and non-adapted OSR material to test for selective value in breeding. The inverse relationship between LRD and MLRL in the HTP system is also worth further exploration in terms of trade-offs between resource allocation and acquisition. Adaptive responses to low nutrient availability could also include increased root hair formation, secretion of lytic enzymes, and the release of organic acids and other metabolites into the rhizosphere (Osmont *et al*., 2007; Gruber *et al*., 2013; White *et al*., 2013*a*).

Most variation in traits measured in the HTP system was due to residual factors (plant-to-plant variation) highlighting the variability of these root traits, the need to control environmental factors and the need for high replication. This is consistent with the observations of Adu *et al*. (2014), who reported seedling root trait phenotypes of 16 *B*. *rapa* genotypes grown on germination paper and imaged using high-resolution scanners. The data presented here indicate that between 11 and 36 replicates of OSR would be needed to detect a 30 % difference in root traits in both HTP systems and field environments (Fig. 4). Adu *et al*. (2014) reported that between 6 and 43 replicates would be needed to detect a 50 % difference in root traits grown in their HTP system. In both these studies, the number of replicates required increased in the order PRL < LRD < TRL < MLRL, with varietal factors explaining most variation in PRL and least variation in MLRL. Thus, PRL is the most reliable trait to detect genotypic differences in the HTP system and MLRL the least reliable. It seems likely that residual variation will decrease when wider panels of varieties are used, whilst some of the residual variation will be due to measurable factors such as variation in seed size at the individual plant-to-plant scale (Evans and Bhatt, 1977; Khan *et al*., 2012). Both of these hypotheses are currently being tested in ongoing work.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: all root traits of *Brassica napus* grown in the HTP system, and yield, emergence and root and shoot traits in field experiments. Table S2: leaf mineral concentration of *Brassica napus* grown in the field at Bingham (2013–14).

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Chapter 3

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QTL meta-analysis of root traits in *Brassica napus* under contrasting phosphorus supply in two growth systems

Ying Zhang[1,2,*], Catherine L. Thomas[3,*], Jinxia Xiang[1,2,*], Yan Long[1,4], Xiaohua Wang[1,2], Jun Zou[1], Ziliang Luo[1], Guangda Ding[1,2], Hongmei Cai[2], Neil S. Graham[3], John P. Hammond[5,6], Graham J. King[1,3,6], Philip J. White[7,8], Fangsen Xu[1,2], Martin R. Broadley[3,†], Lei Shi[1,2,†] & Jinling Meng[1,†]

A high-density SNP-based genetic linkage map was constructed and integrated with a previous map in the Tapidor x Ningyou7 (TNDH) *Brassica napus* population, giving a new map with a total of 2041 molecular markers and an average marker density which increased from 0.39 to 0.97 (0.82 SNP bin) per cM. Root and shoot traits were screened under low and 'normal' phosphate (Pi) supply using a 'pouch and wick' system, and had been screened previously in an agar based system. The P-efficient parent Ningyou7 had a shorter primary root length (PRL), greater lateral root density (LRD) and a greater shoot biomass than the P-inefficient parent Tapidor under both treatments and growth systems. Quantitative trait loci (QTL) analysis identified a total of 131 QTL, and QTL meta-analysis found four integrated QTL across the growth systems. Integration reduced the confidence interval by ~41%. QTL for root and shoot biomass were co-located on chromosome A3 and for lateral root emergence were co-located on chromosomes A4/C4 and C8/C9. There was a major QTL for LRD on chromosome C9 explaining ~18% of the phenotypic variation. QTL underlying an increased LRD may be a useful breeding target for P uptake efficiency in *Brassica*.

Phosphorus (P) is an essential macronutrient for plant growth involved in many key metabolic pathways. However, 80% of P content in soil can be fixed in forms unavailable to plants[1]. Hence, P is one of the major limitations for crop productivity worldwide. Enhancing P uptake and utilization efficiency (plant biomass per unit input of P) through breeding and genetic technology is an important strategy to reduce the application of phosphate (Pi) fertilizers to crops. Resolving P efficiency to sub-traits important for P uptake provides a more targeted strategy than selecting for varieties with improved yield under P deficiency, because field settings are complex and the results from one may not be reproducible in another[2–5].

Phosphorus deficiency in plants leads to changes in root morphology and architecture, including the formation of longer, thinner roots, increased lateral root production and growth, cluster root formation, and greater root hair length and density[3,6–9]. Phosphorus is an immobile resource which remains largely in the top soil[1]. Consequently, shallow root angles conferring better top-soil foraging have been shown to increase growth under P deficiency[3,8,10]. To adapt to P stress, it is observed that plants alter their allocation of biomass to roots rather than shoots, thus the root/shoot ratio increases[11,12].

[1]National Key Laboratory of Crop Genetic Improvement and National Centre of Plant Gene Research, Huazhong Agricultural University, Wuhan 430070, China. [2]Key Laboratory of Arable Land Conservation (Middle and Lower Reaches of Yangtze River), Ministry of Agriculture, Huazhong Agricultural University, Wuhan 430070, China. [3]Plant and Crop Sciences Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, United Kingdom. [4]Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China. [5]School of Agriculture, Policy and Development, University of Reading, Reading RG6 6AR, United Kingdom. [6]Southern Cross Plant Science, Southern Cross University, Lismore NSW 2480, Australia. [7]The James Hutton Institute, Invergowrie, Dundee DD2 5DA, United Kingdom. [8]King Saud University, Riyadh 11451, Kingdom of Saudi Arabia. *These authors contributed equally to this work. †These authors jointly supervised this work. Correspondence and requests for materials should be addressed to L.S. (email: leish@mail.hzau.edu.cn)

P efficiency is controlled by complex quantitative traits. Many root traits and QTL associated with P uptake efficiency have been identified. For example, QTL have been found for root hair growth in common bean (*Phaseolus vulgaris*)[13] and maize[14], decreased basal root gravitropism in common bean[15] and adventitious rooting which has greater horizontal growth and specific root length compared to basal rooting[16]. *Pup1*/*PSTOL1* was the first P efficiency QTL/gene to be introgressed into a P intolerant variety of rice (*Oryza sativa*) and enhance grain yield in P deficient soils, which functioned to keep normal development of the root under P deficiency[17,18]. Greater basal root whorl number affords a greater vertical range of soil exploration of common bean[10]. In *B. oleracea*, accessions with greater yield under low P conditions had greater lateral root number, length and growth rate[19]. In *B. napus*, the P-efficient genotype Eyou Changjia developed a larger root system than the P-inefficient genotype B104-2[20]. A *B. napus* RIL population derived from a cross between Eyou Changjia and B104-2 showed that low P specific yield-related QTLs were clustered on chromosomes A1, A6 and A8[21]. In the *B. napus* TNDH population, low-P specific yield-related QTL were observed in field trials clustered on A2, A3 and A5[22]. Yet, to date no root trait QTL have been introgressed into commercial varieties of *Brassica,* in part due to limited validation of conserved QTL across populations and environments[23,24], and poorly resolved chromosomal regions[4].

High throughput phenotyping (HTP) has become a priority for crop breeding research in order to obtain relevant root trait data. However, since large scale field screening remains costly, time demanding and inefficient, obtaining accurate phenotype data is a bottleneck for crop root research. Therefore, various 2D root HTP systems have been deployed, including agar plate-based systems[25,26,27] and 'pouch and wick' systems, in which roots are grown on filter paper, suspended in solution, which is surrounded by a pouch which conceals roots from light[5,10,28–31].

High-density genetic linkage maps are prerequisites for map-based cloning and marker-assisted plant breeding. In recent years, with the development of the next-generation sequencing and SNP genotyping arrays, SNPs have become the marker of choice in most species for construction of high density genetic linkage maps such as rice and maize[32,33]. SNP discovery is challenging in allopolyploid species such as *B. napus*, as they may arise both between allelic (homologous) sequences within subgenomes and between homoeologous sequences among subgenomes, as well as from polymorphisms between paralogous duplicated sequences[34]. In 2011 an international *Brassica* SNP consortium produced an Infinium™ genotyping array, with over 50,000 (60 k) SNPs, for *B. napus*, in cooperation with Illumina Inc. (San Diego, CA, USA)[35]. This introduced a very efficient method for high-density, sequence-based, genome-wide polymorphism screening of *B. napus* populations. For example, Liu *et al.* (2013) constructed the first SNP genetic map with the 60 k SNP chip of *B. napus*, which contains 9164 SNP markers covering 1832.9 cM with an average distance of 0.66 cM between adjacent markers[36].

Oilseed rape (OSR, Canola, *Brassica napus* L.) is a globally important source of oil and provides 18% of global vegetable oil for human consumption, industrial oils, biodiesel, lubricant, and fodder for animal feeds. Over 70 million tonnes of rapeseed are produced annually worldwide[37] (FAOSTAT). In this study, a high-density TNDH SNP-based genetic linkage map of *B. napus* was constructed. QTLs controlling root architecture and shoot traits under low Pi (LP) and 'normal' Pi (NP) conditions were identified using a hydroponic 'pouch and wick' system[5,31], and were integrated with those from an agar based screening system[27] in a meta-analysis. This analysis will investigate conserved QTL for root traits, and reduce the corresponding confidence interval, thus refining the chromosomal region of useful root loci.

Results

Construction of a high-density SNP based genetic linkage map. A total of 30,717 SNP markers scored from the Brassica 60 k array showed polymorphisms between Tapidor and Ningyou7. Of these, the 13,753 SNP markers with $\leq$5% missing data were used for clustering the 'genetic bins', conducting genetic linkage analysis and linkage map construction. SNP bin markers specific to the *B. napus* A and C genomes were clearly separated into different linkage groups at LOD 4–19, respectively. A set of 1,826 SNP bins, consisting of 13,612 SNP markers, were successfully assigned to 19 linkage groups representing the A1-A10 and C1-C9 linkage groups of *B. napus*, with total genetic distances of 913.7 cM and 1004.2 cM for the A and C genomes, respectively. The marker number and density varied considerably across different chromosomes. The map was named the '1826-map' (Table S1).

An integrated genetic linkage map named the '2109-map' was constructed (Table S1), which had 19 linkage groups and a length of 2210.2 cM, with a total of 2109 molecular markers, comprising 1734 SNP bins from the 1826-map and 375 original markers (SSR, RFLP, SNP and STS) derived from the previous TNDH genetic linkage map with 798 markers (798-map)[22,27]. Among the 2109 markers, 916 (43.4%) showed genetic distortion ($P < 0.05$). Thus, of the total segregation distortion (SD) markers, 433 and 483 markers were skewed towards the female parent Tapidor and male parent Ningyou7, respectively. The SD markers were detected on all chromosomes (Fig. 1). The extreme SD markers which significantly deviated from the fit-line in the 2109-map were removed. At the same time, some markers associated with these extreme SD markers were also absent in the newest 2041-map (Fig. 1; Table 1). 60.1% and 39.9% of the 2041 markers were mapped on the A genome and C genome, respectively. The marker density was 1.18 per cM on the A genome, 0.76 per cM on C genome and 0.97 per cM over the whole genome. The number and density of SNP bins on the A genome were significantly greater than those on the C genome in all four maps (Table S1; Table 1). Average density increased from 0.39 markers per cM on the 798-map to 0.95 markers (0.95 SNP bin) per cM on the 1826-map and 0.97 marker (0.82 SNP bin) per cM on 2041-map (Table S1; Table 1). Moreover, perfect collinearity was observed in the alignment of these markers on the three genetic linkage maps, except for a small number of inversions.

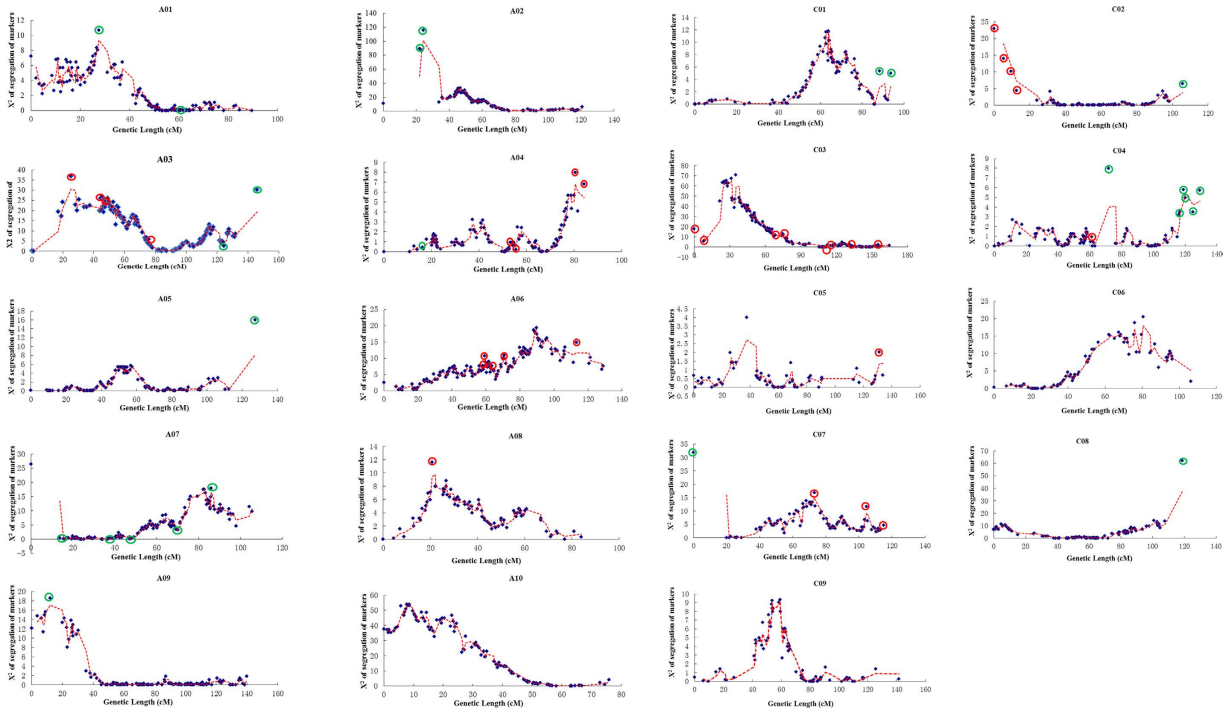

Figure 1. The segregation of markers on each linkage group of the *Brassica napus* TNDH 2109-map. Chi-squared test: n = 2, df (degrees of freedom) = 1, P = 0.05, $\chi^2 = 3.84$, SD* = segregation distortion. When the segregation of markers was 3.84 the segregation ratio of Tapidor and Ningyou7 was 1:1. Red and green circles indicate the extreme markers skewed towards Tapidor and Ningyou7, respectively, which were deleted to construct the new 2041 map.

Linkage Group	Number of molecular Markers	Genetic Distance (cM)	Marker density (markers/cM)	SNP bin	SNP bin density (markers/cM)	Original markers
A1	142	89.4	1.63	132	1.48	10
A2	107	120.6	0.91	91	0.75	16
A3	158	131.8	1.44	121	0.92	37
A4	105	82.0	1.31	91	1.11	14
A5	105	112.7	0.83	94	0.83	11
A6	141	128.8	1.15	120	0.93	21
A7	131	104.9	1.30	108	1.03	23
A8	81	83.8	0.98	67	0.80	14
A9	153	139.7	1.10	102	0.73	51
A10	104	75.9	1.37	85	1.12	19
C1	97	92.6	1.06	90	0.97	7
C2	85	74.4	0.85	78	1.05	7
C3	127	142.4	0.84	104	0.73	23
C4	95	116.2	0.80	79	0.68	16
C5	62	133.4	0.47	52	0.39	10
C6	78	106.2	0.73	59	0.56	19
C7	83	93.5	0.76	67	0.72	16
C8	107	108.1	0.91	98	0.91	9
C9	80	141.4	0.57	60	0.42	20
A genome	1227	1069.6	1.18	1011	0.95	216
C genome	814	1008.3	0.76	687	0.68	127
Total(A + C)	2041	2077.9	0.97	1698	0.82	343

Table 1. Number of molecular markers, genetic distance, marker density in each linkage group of the *Brassica napus* TNDH 2041-map. SNP bin, the smallest unit where chromosome recombination events happen. Original markers, SSR, RFLP and AFLP markers.

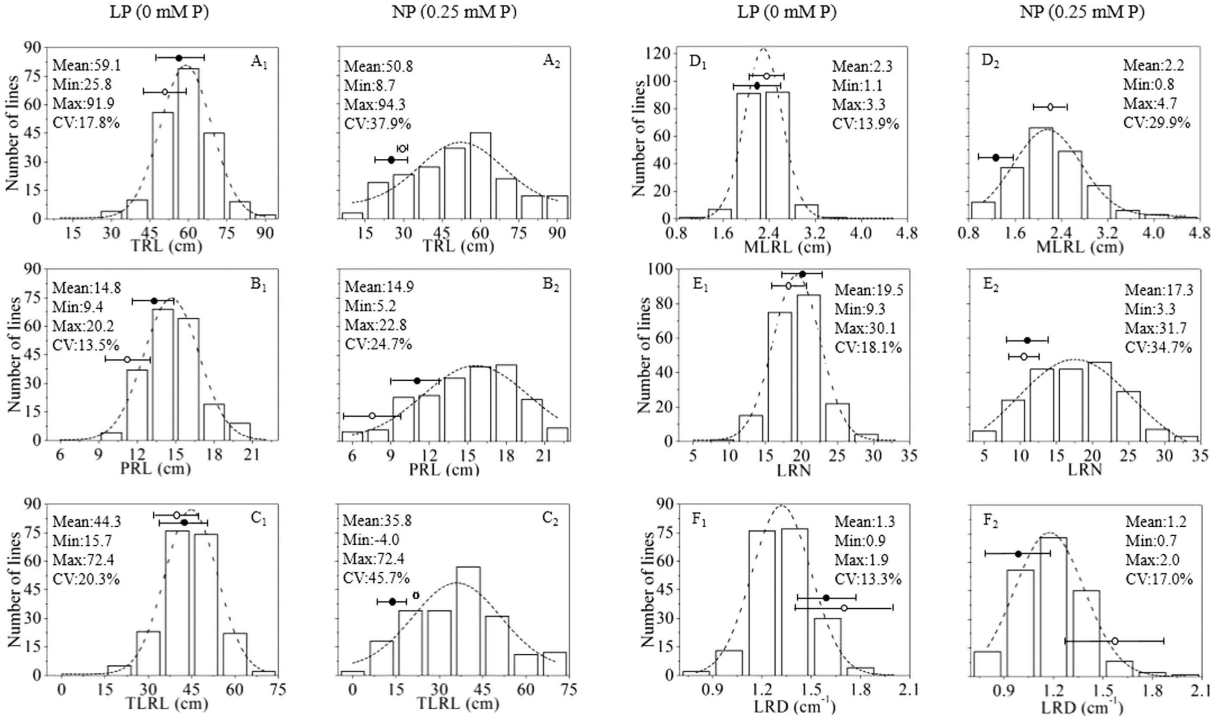

Figure 2. Frequency distribution of root architectural traits of the TNDH population grown for 14 days in a 'pouch and wick' system under low Pi (LP, 0 mM P) and 'normal' Pi (NP, 0.25 mM P). A = TRL (total root length); B = PRL (primary root length); C = TLRL (total lateral root length); D = MLRL (mean lateral toot length); E = LRN (lateral root number); F = LRD (lateral root density). Open circles = parent Ningyou7; filled circles = parent Tapidor.

Root and shoot traits of seedlings grown in the 'pouch and wick' system. All root traits showed approximately normal distribution and transgressive segregation at both LP and NP, with means under LP ranging from: PRL 9.4–20.2 cm; TRL 25.8–91.9 cm; TLRL 15.7–72.4 cm; MLRL 1.1–3.3 cm; LRN 9.3–31.0; LRD 0.9–1.9 cm^{-1}, at LP (Data S1; Fig. 2).

All root traits, except PRL, showed significantly greater growth under the LP as compared with the NP condition ($p < 0.001$) (Fig. 2; Data S2). However, REML variance components analysis showed a greater effect of genotype than treatment, with the genotype effect (a measure of broad-sense heritability) ranging from 2% in MLRL to 11% in PRL, the treatment effect ranging from 0% in MLRL to 5% in LRD (Data S3). Thus, relationships across the LP and NP treatments showed significant positive correlations in PRL, LRN and LRD ($p < 0.05$) (Data S2). Within both LP and NP treatments, all root traits were significantly positively correlated ($p < 0.001$- $p < 0.05$), except for significant negative correlations between PRL and LRD ($p < 0.001$), MLRL and LRD ($p < 0.001$) (LP only), MLRL and LRN ($p < 0.001$) and PRL and MLRL ($p < 0.001$) (NP only) (Data S2).

The parent Tapidor as compared with Ningyou7 had significantly greater TRL, PRL, TLRL and LRN ($p < 0.001$), but a shorter MLRL and a significantly lower LRD ($p < 0.05$) under LP. Likewise, under NP Tapidor had a longer PRL and a significantly lower LRD compared to Ningyou7 ($p < 0.05$). Under LP, Ningyou7 also had a greater shoot dry weight than Tapidor ($p < 0.01$) and a greater shoot/root ratio ($p < 0.01$) (Data S2; Figs 2 and 3).

QTL and meta-analysis of root and shoot traits grown under LP and NP in the 'pouch and wick' system. Using the 2041 map, a total of 60 QTL were identified from the 'pouch and wick' system for root and shoot traits; 46 under LP and 14 under NP, which were located across 13 of the 19 chromosomes (excluding A2, A8, C1, C3, C6 and C7). At LP most QTL were on A9 and C2- 35% and at NP most QTL were on A1- 29%. Considering both Pi treatments together, most QTL for LRD were on C2 and C9- 75%, for LRN most QTL were on A4, C4 and C2- 60%, for MLRL most QTL were on A9 and C2- 67%, for TLRL and TRL most QTL were on A1- 40% and for dry weight most QTL were on A4, A5 and A7- 80%. There were eleven QTL which explained > 10% of the phenotypic variance, most of which were on A9/C9 for LRD/LRN, a QTL for LRD on C9 under LP explaining the greatest variance- 17.6%. The positive allelic effect of the majority of QTL came from Tapidor, except all those for PRL and the majority for MLRL from Ningyou7 (Fig. 4; Table S2).

The QTL within a treatment which had over-lapping confidence intervals (CI) were integrated into combined QTL. Fourteen combined QTL were identified (28 of the original 60 QTL were integrated); at LP there were two combined QTL on A4, one each on A5, A7, and A9, three on C2, one on C4 and two on C9. At NP there was one combined QTL on A3 and two on A1. Most combined QTL were on C2 for LRN/LRD. Confidence intervals were reduced by between 0.9–6.6 cM, a ~33% reduction in combined QTL compared to component QTL (Fig. 5; Table S2).

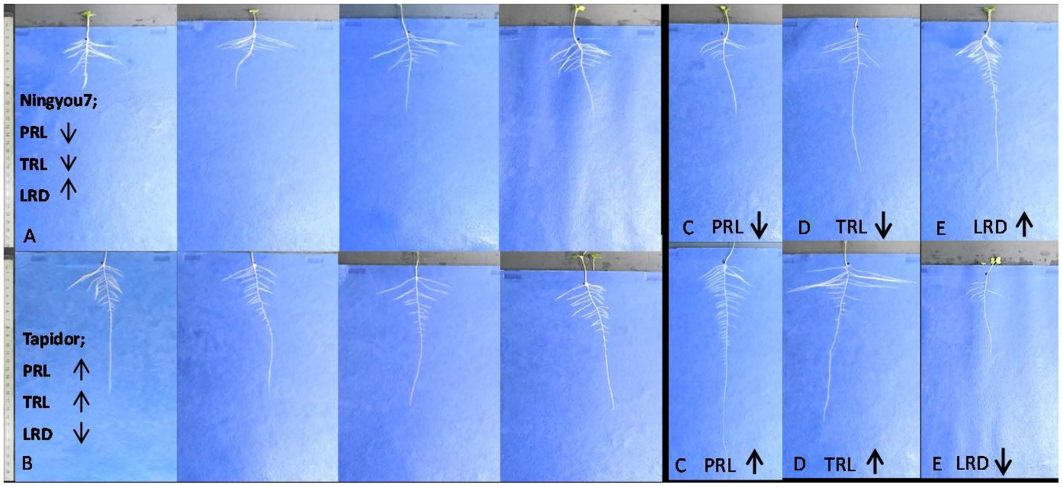

Figure 3. Illustrative examples of parents (**A**) Ningyou7 (**B**) Tapidor and the extreme genotypes with the mean minimum and maximum (**C**) PRL (TN52, TN15, respectively), (**D**) TRL (TN58 and TN38, respectively) and (**E**) LRD (TN40 and TN53), at 14 DAS having grown in a 'pouch and wick' system under a low Pi treatment (LP, 0 mM Pi). Primary root length (PRL), total root length (TRL), lateral root density (LRD), scale = 1 cm.

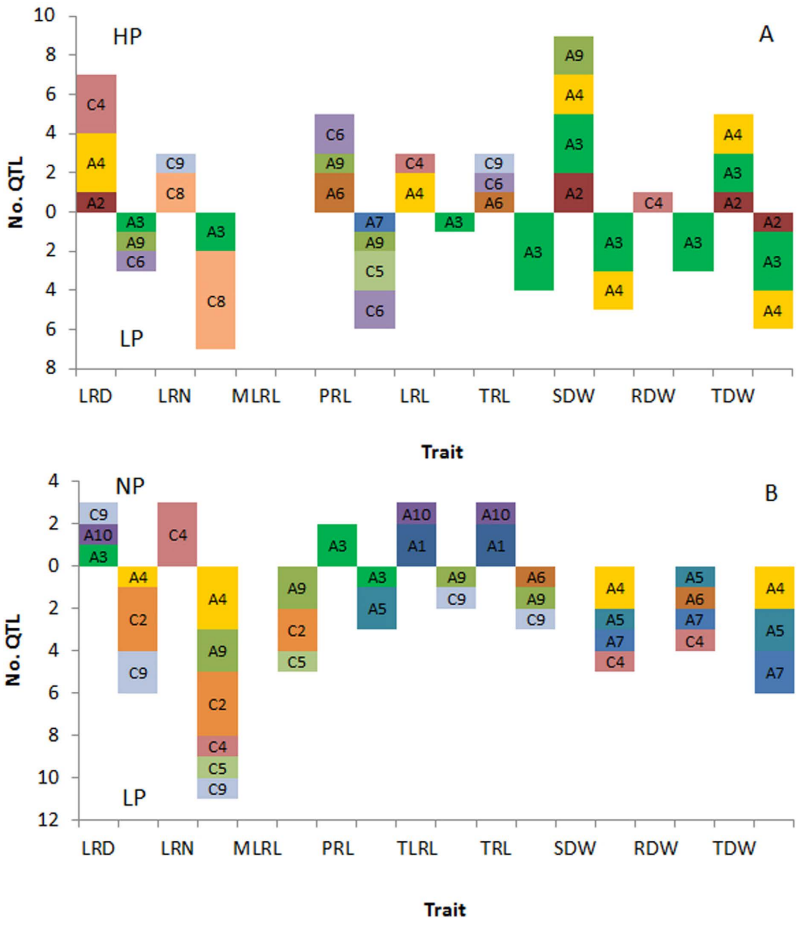

Figure 4. QTL above the LOD threshold of 2.5 for root/shoot traits in (**A**) seedlings grown for 12 d in the agar system, under HP (0.625 mM Pi, above horizontal) and LP (0 mM Pi, below horizontal) and (**B**) seedlings grown for 14 d in the pouch and wick system, under NP (0.25 mM Pi, above horizontal) and LP (0 mM Pi, below horizontal), on chromosomes A1-10 and C1-9. LRD = lateral root density, LRN = lateral root number, MLRL = mean lateral root length, PRL = primary root length, LRL = total lateral root length, TRL = total root length, SDW = shoot dry weight, RDW = root dry weight, TDW = total dry weight.

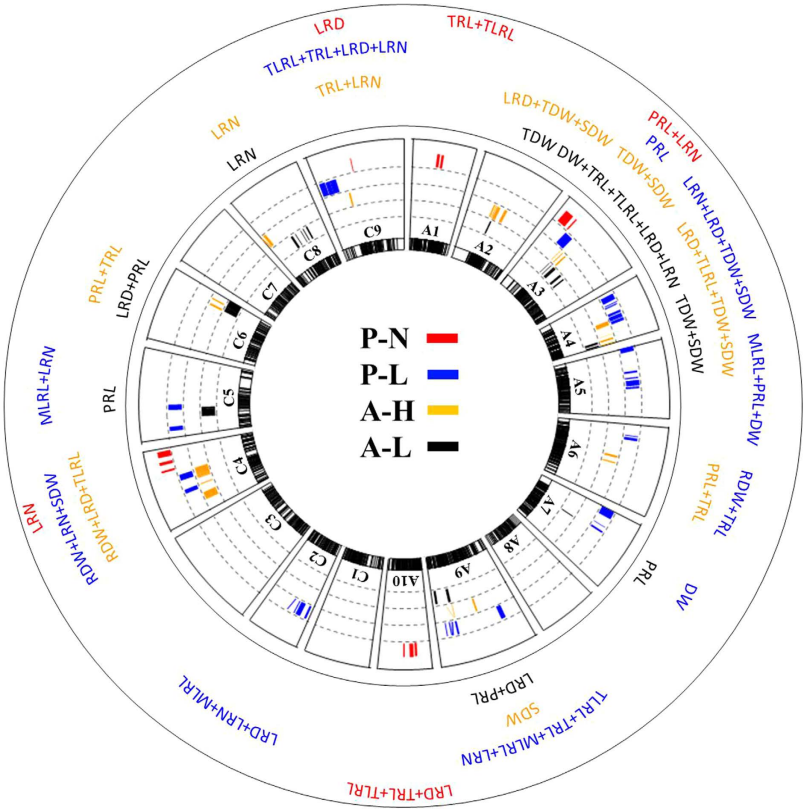

Figure 5. Individual and combined QTL for root and shoot traits within a treatment, shown as colorful bars, with thickness indicating confidence interval, and position indicating location along the chromosome (A1-A10, C1-C9). Red = 'normal' Pi (NP, 0.25 mM Pi) and blue = low Pi (LP, 0 mM Pi) in the hydroponic 'pouch and wick' system (P), yellow = high Pi (HP, 0.625 mM Pi) and black = low P (LP, 0 mM Pi) in the agar screening system (**A**). LRD = lateral root density, LRN = lateral root number, MLRL = mean lateral root length, PRL = primary root length, TLRL = total lateral root length, TRL = total root length, SDW = shoot dry weight, RDW = root dry weight, TDW = total dry weight. DW means QTL for SDW, RDW and TDW. QTLs were detected using the new TNDH 2041-map.

QTL and meta-analysis of root and shoot traits grown under LP and HP in the agar system. Using the 2041 map, a total of 71 QTL for root and shoot traits were detected using the data previously collected in an agar screen; 35 under LP and 36 under HP, which were located across 11 of 19 chromosomes (excluding A1, A5, A8, A10, C1, C2, C3 and C7). At LP most QTL were on A3- 49% and under HP most were on A4- 25%. Considering both Pi treatments together, most QTL for LRD were on A4 and C4- 60%, for LRN most QTL were on C8- 70%, for PRL most QTL were on C6- 40%, for TLRL most QTL were on A4- 50%, for TRL most QTL were on A3- 63% and for dry weight most QTL were on A3- 48%. There were 22 major QTLs which explained >10% of the phenotypic variance, most were on A3 and were for shoot dry weight, a QTL on C6 for PRL under HP explaining the greatest variance- 17.5%. The positive allelic effect of the majority of QTLs came from Ningyou7, this was consistent across all traits, except PRL where the most positive effect was from Tapidor (Fig. 4; Table S3).

The QTL within a treatment which had over-lapping CI were integrated into combined QTL. Fourteen combined QTL were identified (35 of the original 71); at LP there were five combined QTL on A3 and two on A4, at HP there were two combined QTL on A3, two on A4, and one each on A6, C6 and C9. Confidence intervals were reduced by between 0.0 – 4.8 cM, a ~56% reduction in combined QTL compared to the component QTL (Fig. 5; Table S3).

QTL meta-analysis of root and shoot traits across Pi treatments and growth systems. Combined from across the LP and NP treatments under the 'pouch and wick' system there was one QTL with over-lapping confidence intervals (although it was not a combined QTL) for PRL on A3. Under the agar system there were seven combined QTL from across the P treatments; TDW under HP and LP on A2, TDW + SDW at HP and RDW + TDW + SDW + TRL at LP on A3, TDW + SDW at HP and LRL + RDW + TDW + SDW + TRL at LP on A3; and RDW + TRL at LP and SDW at HP also on A3, TDW + SDW at LP and LRD + LRL + TDW + SDW at HP on A4, SDW + TDW at LP and LRL at HP also on A4, LRD at LP and PRL at HP and LP on C6 and PRL at LP and TRL + PRL at HP also on C6. Thus, most combined QTL across treatments were on A3 and integrated QTL for both root traits and dry weight (Fig. 5; Table S4).

Across the pouch and wick and agar based systems there were four combined QTL; for LRN at LP and LRD at HP on A4, MLRL at LP and LRD at LP on A9, SDW at LP and LRD at HP on C4, and LRN at LP and PRL at LP on C5, in the pouch and agar based systems, respectively. The confidence interval reduced from a mean of 7 cM before integration to 4.4 cM after- a reduction of ~35% (Fig. 5; Table S4).

Discussion

Genetic linkage maps are highly valuable tools for studying genome structure and evolution, comparative genome analyses and localizing genes of interest. In this study, a high density SNP-based genetic linkage map of *B. napus* was constructed (Table 1). The number of markers mapped onto the A genome was greater than that onto the C genome (Table 1; Table S1), as in previous reports[36,38,39]. This could be attributed to genetic differences between the two parental lines. Asiatic *B. napus* cultivars such as Ningyou7 include more introgressed alleles in the A genome from the genetic background of Chinese *B. rapa*, which could contribute to increased genetic diversity and hence SNP polymorphisms compared to European oilseed varieties[40,41]. For instance, the *B. napus* genetic map of the AMDH population, derived from a cross between two French winter OSR varieties shows no significant difference in the number of markers mapped on the A and C genomes[42]. This would explain why the majority of QTL were on the A genome and the positive allelic effect on the A genome was predominantly from Ningyou7, whereas on the C genome Tapidor contributed the majority of the positive allelic effect (Tables S2, S3).

Using the new SNP map, there were 71 QTLs detected from the agar screen which compares to 38 detected using the previous map, and 22 as compared to 8 QTLs explained >10% of the phenotypic variation (Table S3). There was ~50% QTL integration across traits, treatments and systems, and integrating QTLs reduced the confidence interval by ~40%- after integration the mean interval was 3.7 cM, compared to 6 cM before (Tables S2, S3, S4). Therefore, a greater density SNP map and QTL integration has considerably improved QTL detection.

Under LP as compared to NP in the pouch system, root growth was greater in all traits, except primary root length (Data S1, S2). Similarly, in the agar system, lateral root number and density were greater and mean primary root length was shorter under LP compared to HP[27], although in contrast to the pouch system lateral root length was also shorter at LP. Previous studies of *B. oleracea*[19] and *B. napus*[20] have also observed greater root length in plants with reduced P supply. A shorter primary root length and greater lateral root density has also been observed previously in response to P deficiency in *Arabidopisis*[43,44]. It could be argued that a reduced PRL in response to a low P supply is simply due to impaired growth, rather than being an adaptive response. For example, in *Arabidopsis* only half of the accessions tested showed a reduced primary root length in response to low Pi[45]. Furthermore, a shorter primary root length inevitably leads to a greater lateral root density because the latter is an inverse function of the former (e.g. LRD = LRN/PRL)[45]. Although, in *Arabidopsis* it was observed that an increase in lateral root density occurred along the branching zone of the primary root in response to low Pi availability, the branching zone is independent of the length of the primary root, thus lateral root density did increase independently of a reduced primary root length[44]. Therefore, although it is possible that a shorter primary root length is a general stress response, a greater lateral number and length suggests an adaptive/plastic response to nutrient deficiency.

As compared with Ningyou7, which is the P-efficient genotype, Tapidor had a longer primary root length but lower lateral root density at LP in both the pouch and wick system (Fig. 3; Data S2) and agar screen. The biomass of shoot and the shoot/root ratio of Ningyou7 were also significantly greater than Tapidor under LP under both culture systems (Data S2). Thus it appears that Ningyou7 trades a greater total root length for a greater lateral root density, and consequently is able to allocate more biomass to shoot rather than root, without any resource acquisition penalty. Likewise, in the field Ningyou7 has greater seed yield, seed weight and number of primary branches compared to Tapidor under low Pi[22]. The *Pup1* P efficiency locus is associated with a greater shoot/root ratio and it was suggested that maintaining a greater shoot biomass with less root is characteristic of P efficiency[46]. It has been suggested that for many crops and environments there is too much carbon invested in the roots[2]. Thus the QTLs associated with a shorter primary root length and greater lateral root density may promote greater shallow rooting and improve P use efficiency.

QTL integration found pleiotropic loci which controlled numerous root traits- including both lateral root length and number. In the agar system there were combined QTL on A3 for LRD + TLRL and on C9 for TRL + LRN, and in the pouch system on A3 for PRL + LRN (Tables S2, S3, S4). This pleiotropism is unsurprising given that these traits were positively correlated (Data S2). Although, interestingly, across the systems there was a combined QTL for LRD and MLRL on A9, this is surprising given that it was observed here and elsewhere that there is a negative relationship between MLRL and LRD[5], suggesting that the same QTL is independently controlling both lateral root length and number.

It seems that there was no predominant effect of P treatment on the QTL detected, there were only two combined QTL from within a P treatment across the culture systems; PRL + LRN at on C5 and LRD and MLRL on A9, both at low P (Table S4). This is most likely explained by there being only a minimal P treatment effect compared to genotype effect, as indicated by the variance components analysis (Data S3). Although, under low P but not under high P, in both growth systems, the greatest number of QTL were for LRN, perhaps indicating the responsiveness of this trait to low P availability.

Most QTL for shoot and root biomass were on chromosomes A3 and A4, and most root traits also had QTL on these chromosomes (Figs 4 and 5). Likewise, most QTL for yield-related traits in the TNDH population in the field were on A3, and many of these co-localise with the QTL observed here[22]. Similarly, in a meta-analysis of QTL studies for yield related traits in *B. napus* it was found, post integration, that A3 had the most unique QTL[24]. Most QTL for root traits in *B. napus* grown in a paper culture system were on A3 and C3[20]. QTL for leaf P were identified in *B. rapa* on A3[47]. In *B. oleracea* it was observed that shoot P and P use efficiency (PUE) QTLs were mostly on C3 and C7[19] and QTL for germination rate and percentage germination were found on C3[48,49]. It seems

that chromosomes A3/C3 consistently influence vigour and yield-related traits, therefore the QTL observed for root length traits in the present study may be linked with general vigour.

Lateral root number and density had fewer QTL on A3 compared to other traits. When both culture systems are considered together, 78% of QTL for lateral root density and 84% of QTL for lateral root number were on chromosomes A4/C4, C2, C8, A9/C9 (Fig. 4). There was also an integrated QTL for LRD and LRN across the growth systems on chromosome A4 (Table S4). Furthermore, there are numerous examples of co-localised QTL on A4/C4 and C8/C9 for LRD and LRN, and it may be that homoeologous exchange has occurred in these chromosomes. For example, A4/C4 had co-localising QTL for LRN/LRD at LP in the pouch and HP in the agar systems, respectively. On C8/C9 there was co-localisation of QTL for LRN under HP and LP in agar, and for LRD/LRN under NP in the pouch and LP in the agar system, respectively (Tables S2, S3). Chromosome C8 has syntenic segments with A8 and C9, and C9 is largely collinear with A9 and C8[50]. Homoeologous exchange has previously been observed throughout the genome in *B. napus*[51] but most extensively on chromosomes A1/C1, A3/C3, and A9/C9[52–54]. QTL related to leaf mineral concentration have also previously been identified on chromosomes A9/C9. In *B. oleracea*, a regulatory hotspot and a QTL were located on chromosome A9 for shoot P at low P, the regulatory hotspot on A9 had GO terms related to the cytoskeleton[55]- a structure intimately related to cell division and therefore perhaps lateral root initiation. In *B. oleracea* the most significant QTL for shoot concentrations of Ca, Mg, Zn and Na occurred on C9[56]. In a *B. napus* diversity panel, significantly associated SNP markers were observed on C9 and A9 for shoot concentrations of Ca, Mg, Zn and Na[57], and in the same panel significantly associated GEMs (gene expression markers) for LRD as well as for shoot mineral concentrations were observed on A9 (Thomas *et al.*, 2016, unpublished).

In the present study QTL were found on chromosome A1 for root length under NP in the pouch system (Fig. 4). Likewise, a meta-analysis, pre-integration, observed that most QTL for yield-related traits in *B. napus* were on chromosome A1[24]. In *B. oleracea*, QTL for percentage germination and rate[48] and hypocotyl upward growth[49] were observed on chromosome C1. Also in *B. oleracea*, a QTL for physiological PUE (Phosphorus Use Efficiency) at optimal P and a regulatory hotspot responding to P availability, were located on chromosome A1, the hotspot had GO terms related to photosynthesis[55]. QTL for leaf P have also been identified on chromosome A1 in *B. rapa*[47]. Thus chromosome A1/C1, as well as A3, appear to be linked to vigorous growth under optimal P conditions.

Using the confidence interval found for the combined QTLs, a BLAST to the Darmor *B. napus* reference sequence[54] identified 19 candidate genes related to root growth and plant genetic responses to low P availability, most of which were on chromosomes A3 and C9, including *LJRHL1-like* (*LRL2*) which controls root hair cell development, *AUXIN-INDUCED IN ROOT CULTURES 12* (*AIR12*) related to lateral root morphogenesis, and *Phosphate deficiency response 2* (*PDR2*) which mediates the developmental response of root meristems to phosphate availability. These candidates lay the foundation for a deeper dissection of the P starvation response mechanisms in *Brassica napus*.

Methods

Plant materials. Tapidor x Ningyou7 doubled haploid population (TNDH, N = 202)[58]. The DH population was derived from a cross between Tapidor and Ningyou7 by microspore culture. Ningyou7 was characterized as a P-efficient cultivar with better growth and higher P acquisition than Tapidor under low P (LP) and optimal P (OP or HP) conditions in pot culture and field trials[22,59].

SNP marker analysis and linkage map construction. Total genomic DNA of 202 genotypes of the TNDH population as well as the two parental lines was extracted from the young leaves by a modified CTAB (cetyl trimethylammonium bromide) method[60]. Five leaves from different individuals of each line were used to construct DNA bulks. DNA concentration in tubes was measured by electrophoresis through a conventional 2% agarose gel. 30 ng/μl, 50 ng/μl and 100 ng/μl λDNA (48,502 bp) was used as reference. The final DNA samples were diluted to 50 ng μL^{-1}. The population and the parents were genotyped using the *Brassica* 60 k SNP BeadChip Array (Illumina Inc., USA). This array, which successfully assays 52,157 Infinium Type II SNP loci in *B. napus*, was developed using preferentially single-locus SNPs contributed from genomic and transcriptomic sequencing in genetically diverse *Brassica* germplasm[61]. DNA sample preparation, hybridization to the BeadChip, washing, primer extension and staining were performed according to the work flow described in the Infinium HD Assay Ultra manual. Imaging of the arrays was performed using an Illumina HiSCAN scanner after BeadChip washing and coating. Allele calling for each locus was performed using the GenomeStudio genotyping software (v2011, Illumina, Inc.). Positions of A-genome SNPs were provided by the array manufacturer, while C genome SNP source sequences were subjected to a BLAST search against the *B. oleracea* genome database (BRAD, http://brassicadb.org/) to locate chromosome positions (E value $\leq$ 1e-50).

The genotype data was visualized by the software Genomestudio (Illumina inc.). The genotype with missing data >5% were removed to reduce the mapping errors and avoid artificial exaggeration of map distances. From the genotypic data, marker pairs with zero recombination were assigned to the same bin. The representative SNP marker of a bin was selected to construct the genetic linkage map using the software packages of JoinMap 4.0[62]. The threshold for goodness-of-fit was set to $\leq$5.0 with a recombination frequency of <0.4 and a minimum logarithm of odds score of 1.0. Markers with a $\chi 2$ value of >3.0 were excluded in all genetic groups. Recombination frequencies were converted to centimorgans (cM) using the Kosambi method[63] for map distance calculation according to the method described by Long *et al.*[64]. A reference genetic map was constructed with SNP bins being designated according to the best SNP marker in each bin. The last SNP-based map was integrated with the previous 798-map composed of SSR (single sequence repeat) and RFLP (restriction fragment length polymorphism) markers[22,27,64]. Then, Chi-square (χ^2) tests were used for testing segregation distortion[65]. The markers with

$\chi^2 > 3.84$ ($p = 0.05$) were designated segregation distorted markers (SD markers). The extreme SD markers which significantly deviated from the fit-line were abandoned[66,67] (Fig. 1).

High throughput root and shoot phenotyping. A 'pouch and wick' hydroponic-based HTP (high throughput phenotyping) system[31] and image analysis procedure[5] was deployed in this study. This system comprised growth pouches assembled from blue germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA), re-cut to 24 × 30 cm and overlain with black polythene (Cransford Polythene Ltd, Woodbridge, UK). Along their shorter edges, the paper and polythene were clipped together using 'bulldog'-type fold-back clips to each side of an acrylic bar (Acrylic Online, Hull, UK) giving 2 germination papers per pouch. The growth pouches were suspended above plastic drip trays, supported within lightweight aluminium/polycarbonate frames. Prior to sowing, the pouches were suspended above the nutrient solution for a minimum of 4 h to become fully saturated. A single seed was sown in the middle of the upper edge of each germination paper, by pressing the seed into the paper. Within each aluminium frame there were nine drip trays with 10 or 11 pouches per drip tray, arranged in three columns. A total of 4 frames were used in each experimental run, thus 96 pouches and 192 seedlings per frame, giving a potential sample size of 768 seedlings per run, within a single CE room. The CE room was 2.2 m wide, 3.3 m long, 3.0 m high, set to a 12 h photoperiod with 18/15 °C day/night temperatures and relative humidity of 60–80%. Photosynthetically Active Radiation (PAR; measured at plant height with a 190 SB quantum sensor; LI-COR Inc., Lincoln, NE, USA) was 207 μmol m^{-2} s^{-1}, generated by 400 W white fluorescent lamps (HIT 400w/u/Euro/4K, Venture Lighting, Rickmansworth, UK). Drip trays were replenished with 500 mL of deionised water every 3 d. Fourteen days after sowing (DAS), the polythene sheets were removed from all pouches and images were taken of the germination paper and root system for downstream image analysis. Images were taken using a Digital Single Lens Reflex (DSLR) camera (Canon EOS 1100D, Canon Inc., Tokyo, Japan) with a focal length of 35 mm at a fixed height of 75 cm, using Canon software.

Seedlings of 199 of the genotypes and the parents (n = 4872) were screened at a 'normal' Pi concentration of 0.25 mM (NP treatment) using a ¼ strength Hoagland's solution (No. 2 Basal Salt Mixture, Sigma Aldrich, Dorset, UK). Seedlings of all 202 genotypes and the parents (n = 5376) were also screened at a low Pi concentration of 0 mM (LP treatment) using a zero Pi Hoagland's solution recipe, with K_2SO_4 added for balanced K. The pH of the nutrient solution was adjusted to be ~5.7 by HCl/NaOH, consistent with a pH of 5.8 in the NP treatment. Each genotype was grown in one experimental run, pouches were randomly allocated to a position within each column of each tank, giving ~24 replicates per run. Two additional oilseed rape lines were grown per run to serve as a reference for normalisation of the data, to account for run effects. Roots and shoots grown under the LP treatment were dried at 50 °C for 48 hours, and dry weights were taken (SDW, RDW) and total dry weight calculated (TDW, root + shoot). This data was not collected for the NP treatment due to unforeseen issues with sample storage.

Root image analysis from the 'pouch and wick' system. The root images from the HTP system were renamed with each sample's unique experimental design information using Bulk Rename Utility (Version 2.7.1.3, TGRMN Software, www.bulkrenameutility.co.uk). Images were cropped by reducing extraneous pixels on bulked images, using XnConvert (Version 1.66, www.xnconvert.com). Cropped images were analysed using RootReader2D[68] (RR2D). First, a 'batch process' was carried out which automatically 'thresholds', 'skeletonises' and 'builds segments' of all images in bulk. The root system was then measured on individual images by placing a marker at the base and tip of the primary root. From these markers, RR2D automatically calculates primary root length (PRL), total lateral root length (TLRL) of all lateral roots and lateral root number (LRN). Total root length (TRL) = PRL + LRL, mean lateral root length (MLRL) = LRL/LRN and lateral root density (LRD) = LRN/PRL. A database was developed which integrated the experimental design information from the image name, with the RR2D measurements for each sample, using a programming script (2.7.10; Python Software Foundation, www.python.org).

The root traits of the TNDH population had been screened previously using an agar plate based system, at a Pi concentration of 0.625 mM (HP treatment) and 0 mM (LP treatment)[27].

Data analysis. The genotypic and non-genotypic variance components of root traits were calculated using a REML (REsidual Maximum Likelihood) procedure. In the REML model all factors were classed as random factors with no defined fixed factor[27]; [(Run/Frame/Column/Position/Paper side) + ([P]ext × Line)]. To acquire adjusted means the same model was used, however genotype was classed as a fixed factor and all other factors were random. Adjusted means were used for all statistical and QTL analyses. Pearson's correlation coefficients calculated the relationship between traits within a P treatment and across P treatments. T-tests were performed to calculate differences in root growth between the P treatments and between the parents. All statistical analyses were conducted using GenStat (15th Edition, VSN International Ltd, Hemel Hempstead, UK).

QTL analyses and meta-analyses in the 'pouch and wick' and agar systems. QTL detection for root and shoot traits was carried out using the composite interval method (CIM), WinQTLCart (version 2.5)[69]. Walk speed was set as 1 cM. The estimated additive effect and the percentage of phenotypic variation explained by each putative QTL were obtained using the CIM model. A QTL was regarded as significant with manual input threshold value more than 11.5 (LOD threshold of 2.5). QTL support intervals were determined by 2-LOD intervals around the QTL peak.

QTLs for root and shoot traits across both treatments and culture systems were integrated in a meta-analysis using BioMercator[70] (version 4.2). The Gerber & Goffinet meta-analysis model with the smallest AIC value was chosen for QTL integration. The principle of integration is that the peak position of component QTLs should be located within the confidence interval of integrated QTL.

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Author Contributions

Conceptual and experiment designs by L.S., J.M., F.X. and M.R.B.; Experiments were conducted by Y.Z., C.L.T., J.X. and Y.L.; Data analysis performed by Y.Z., C.L.T., X.W., J.Z., Z.L., G.D., H.C. and N.S.G.; Reagents/materials/analysis tools were contributed by J.M., J.P.H., G.K., M.R.B. and P.J.W. and the report was written by Y.Z. and C.L.T. All the authors have commented, read and approved the final manuscript.

Additional Information

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Supplementary information

Supplementary Table 1. Number of molecular markers, genetic distance and marker density on each linkage group of the *Brassica napus* TNDH 798-map, 1826-map and 2109-maps. Original markers means the traditional molecular markers; SSR, RFLP, AFLP.

	798 map			1826 map				2109 map				
Linkage Group	No. of markers	Genetic Length (cM)	Marker density (markers/cM)	Bin	No. of markers	Genetic Length (cM)	Marker density (markers/cM)	No. of markers	SNP Bin	Original markers	Genetic Length (cM)	Marker density (markers/cM)
A1	48	98.3	0.49	139	754	76.5	1.82	146	136	10	89.5	1.63
A2	39	96.0	0.41	97	507	111.7	0.87	110	92	18	120.6	0.91
A3	81	130.5	0.62	156	1100	124.8	1.25	164	126	38	145.9	1.12
A4	34	86.9	0.39	113	701	70.5	1.60	110	92	18	84.2	1.31
A5	45	115.4	0.39	96	616	96.0	1.00	106	94	12	127.4	0.83
A6	55	134.5	0.41	126	599	110.1	1.14	148	125	23	128.9	1.15
A7	52	113.0	0.46	113	919	73.8	1.53	137	111	26	105.5	1.30
A8	31	92.3	0.34	68	283	65.3	1.04	82	67	15	84.0	0.98
A9	81	143.1	0.57	102	605	120.9	0.84	154	101	53	139.8	1.10
A10	35	88.5	0.40	85	359	64.2	1.32	104	85	19	75.9	1.37
C1	36	72.9	0.49	94	828	91.7	1.03	99	91	8	93.8	1.06
C2	31	80.7	0.38	80	604	87.8	0.91	90	79	11	105.7	0.85
C3	57	180.0	0.32	110	998	150.3	0.73	139	110	29	164.8	0.84
C4	40	141.6	0.28	103	2298	113.3	0.91	104	86	18	129.3	0.80

C5	20	88.4	0.23	52	377	125.6	0.41	63	52	11	133.7	0.47
C6	38	99.0	0.38	60	569	90.7	0.66	78	59	19	106.2	0.73
C7	26	104.0	0.25	70	469	109.2	0.64	87	70	17	114.9	0.76
C8	23	81.4	0.28	102	721	116.5	0.88	108	98	10	118.8	0.91
C9	26	106.6	0.24	60	305	119.0	0.50	80	60	20	141.4	0.57
A genome	501	1098.4	0.46	1095	6443	913.7	1.20	1261	1029	232	1101.6	1.14
C genome	297	954.6	0.31	731	7169	1004.2	0.73	848	705	143	1108.6	0.76
Total (A+C)	798	2053.1	0.39	1826	13612	1917.9	0.95	2109	1734	375	2210.2	0.95

Supplementary Table 2. Component and integrated QTL (in bold) for root and shoot traits in the TNDH population grown for 14 days at low Pi (LP, 0 mM Pi) and normal Pi (NP, 0.25 mM Pi) in a 'pouch and wick' system. The QTL analysis was based on the new high-density 2041 map constructed in this study. TRL = total root length, PRL = primary root length, TLRL = total lateral root length, LRN = lateral root number, LRD = lateral root density, SDW = shoot dry weight, RDW = root dry weight, TDW = total dry weight, Chr. = chromosome, R^2 = the explained phenotypic variation, CI = confidence interval at P=0.05, Add. Effect = additive effect; + allele from the parent Tapidor, - allele from the parent Ningyou7.

P condition	Chr.	Component QTL	Combined QTL	LOD	R^2 (%)	Peak position (cM)	CI (cM)	Add. Effect
NP	A1		***uq.P_NP_A1_1***	**4.0**	**8.1**	**45.5**	**43.6 - 47.3**	
		qTLRL_NP_A1a			**7.6**	**45.2**	**41.7 - 46.9**	+
		qTRL_NP_A1a			**8.7**	**45.7**	**41.7 - 46.9**	+
			uq.P_NP_A1_2	**5.0**	**10.4**	**52.1**	**50.0 - 54.2**	
		qTRL_NP_A1b			**10.2**	**52.1**	**46.9 - 53.3**	+
		qTLRL_NP_A1b			**10.6**	**52.1**	**47.6 - 53.3**	+
	A3		***uq.P_NP_A3_1***	**4.5**	**10.1**	**15.0**	**8.1 - 21.8**	
		qPRL_NP_A3a			**12.4**	**13.1**	**1.1 - 17.8**	-
		qLRN_NP_A3a			**7.9**	**18.8**	**2.5 - 26.3**	-
		qPRL_NP_A3b		3.4	6.8	28.8	27.8 - 29.9	-
	A10	*qLRD_NP_A10a*		3.6	7.3	37.1	35.4 - 37.7	-
		qTRL_NP_A10a		2.5	5.6	50.6	45.4 - 51.4	-
		qTLRL_NP_A10a		3.5	6.9	53.0	52.2 - 55.6	-
	C4	*qLRN_NP_C4a*		2.7	5.3	81.3	80.4 - 83.4	+
		qLRN_NP_C4b		4.5	8.8	91.4	91.0 - 95.7	+
		qLRN_NP_C4c		3.1	6.0	106.7	102.5 - 111.5	-
	C9	*qLRD_NP_C9a*		3.6	7.1	61.4	60.6 - 61.9	+
LP	A3	*qPRL_LP_A3a*		3.2	6.3	33.7	28.8 - 43.5	-

	A4	*qLRN_LP_A4a*		2.9	4.8	31.7	31.0 - 35.8	-
		qLRN_LP_A4b		4.2	6.8	39.2	35.8 - 42.4	-
		qLRN_LP_A4c		3.3	5.5	45.0	44.1 - 48.1	-
		qLRD_LP_A4a		3.2	5.2	59.0	58.1 - 60.0	-
			uq.P_LP_A4_5	**3.2**	**6.0**	**61.7**	**60.9 - 62.4**	
		qTDW_LP_A4a			**5.8**	**61.2**	**60.7-64.0**	**-**
		qSDW_LP_A4a			**6.2**	**61.8**	**60.9-62.6**	**-**
			uq.P_LP_A4_6	**4.0**	**7.3**	**71.0**	**66.2 - 75.8**	
		qTDW_LP_A4b			**6.4**	**71.0**	**64.0-80.0**	**-**
		qSDW_LP_A4b			**8.2**	**71.0**	**67.9-80.0**	**-**
	A5	*qMLRL_LP_A5a*		4.2	8.2	0.0	0.0 - 8.0	-
			uq.P_LP_A5_2	**4.9**	**9.2**	**40.0**	**38.4 - 41.6**	
		qPRL_LP_A5a			**8.8**	**40.0**	**37.8-42.5**	**-**
		qSDW_LP_A5a			**9.7**	**40.0**	**37.8-42.2**	**-**
		qPRL_LP_A5b		3.7	7.3	49.5	48.1 - 50.3	-
		qTDW_LP_A5a		4.7	8.9	53.8	53.5 - 54.5	-
		qRDW_LP_A5a		3.8	7.3	56.4	54.5 - 63.0	-
		qTDW_LP_A5b		3.3	6.3	65.2	63.8 - 66.9	-
	A6	*qRDW_LP_A6a*		3.5	6.9	18.1	16.8 - 18.2	-
		qTRL_LP_A6a		2.7	5.2	21.5	20.9 - 25.4	-
	A7		***uq.P_LP_A7_1***	**4.9**	**9.1**	**67.3**	**65.8 - 68.8**	
		qSDW_LP_A7a			**10.8**	**67.1**	**65.7-73.2**	**-**
		qTDW_LP_A7a			**7.5**	**67.3**	**65.7-69.0**	**-**
		qTDW_LP_A7b		3.4	6.4	77.2	76.3 - 77.3	-
		qRDW_LP_A7a		2.6	5.9	101.3	90.6 - 103.3	-
	A9		***uq.P_LP_A9_1***	**2.8**	**6.0**	**30.4**	**26.5 - 34.2**	
		qTLRL_LP_A9a			**6.0**	**30.1**	**26.1 – 35.2**	**+**
		qTRL_LP_A9a			**5.9**	**31.1**	**26.1-40.6**	**+**
		qMLRL_LP_A9a		5.8	11.7	104.8	104.3 - 107.5	-

		qMLRL_LP_A9b		6.3	12.5	112.8	111.3 - 113.6	-
		qLRN_LP_A9a		4.0	6.8	115.3	115.0 - 117.0	+
		qLRN_LP_A9b		6.3	10.6	123.9	122.9 - 124.5	+
	C2		***uq.P_LP_C2_1***	**3.7**	**6.6**	**5.9**	**4.1 - 7.6**	
		qLRD_LP_C2a			**4.7**	**5.3**	**3.1 - 7.5**	**+**
		qLRN_LP_C2a			**8.6**	**6.9**	**3.5 - 9.4**	**+**
			uq.P_LP_C2_2	**5.0**	**8.3**	**15.9**	**13.9 - 17.9**	
		qLRD_LP_C2b			**6.4**	**15.9**	**15.3 - 21.6**	**+**
		qLRN_LP_C2b			**10.2**	**15.9**	**15.3 - 20.6**	**+**
		qMLRL_LP_C2a		5.8	12.4	19.2	16.2 - 22.9	-
			uq.P_LP_C2_4	**4.0**	**6.8**	**24.7**	**23.9 - 25.5**	
		qLRD_LP_C2c			**4.7**	**24.7**	**22.5 - 25.7**	**+**
		qLRN_LP_C2c			**8.6**	**24.7**	**23.7 - 25.6**	**+**
		qMLRL_LP_C2b		3.7	7.1	34.7	34.3 - 35.7	+
	C4		***uq.P_LP_C4_1***	**2.7**	**4.6**	**42.5**	**39.1 - 45.9**	
		qRDW_LP_C4a			**5.1**	**42.5**	**38.3-53.8**	**+**
		qLRN_LP_C4a			**4.1**	**42.5**	**40.3 - 47.8**	**+**
		qSDW_LP_C4a		3.1	5.5	63.4	60.6 - 70.3	+
	C5	*qMLRL_LP_C5a*		3.3	6.3	16.2	10.9 - 17.8	+
		qLRN_LP_C5a		3.4	6.0	48.7	44.2 - 53.0	-
	C9		***uq.P_LP_C9_1***	**3.6**	**7.3**	**9.4**	**4.6 - 14.2**	
		qTLRL_LP_C9a			**7.3**	**9.4**	**0.0 - 17.6**	**+**
		qTRL_LP_C9a			**7.3**	**9.4**	**6.0-17.6**	**+**
		qLRD_LP_C9a		6.7	12.8	18.6	14.7 - 22.1	+
			uq.P_LP_C9_3	**6.5**	**11.8**	**27.6**	**22.5 - 32.7**	
		qLRN_LP_C9a			**6.0**	**26.1**	**22.1 - 40.5**	**+**
		qLRD_LP_C9b			**17.5**	**28.3**	**24.0 - 36.2**	**+**

Supplementary Table 3. Integrated QTL (in bold) from component QTL for root and shoot traits in the TNDH population grown for 12 days at low Pi (LP, 0 mM Pi) and high Pi (HP, 0.625 mM Pi) in an agar system (Shi *et al*., 2013). The QTL analysis was based on the new high-density 2041 map constructed in this study. TRL = total root length, PRL = primary root length, TLRL = total lateral root length, LRN = lateral root number, LRD = lateral root density, SDW = shoot dry weight, RDW = root dry weight, TDW = total dry weight, Chr. = chromosome, R^2 = the explained phenotypic variation, CI = confidence interval at P=0.05.

P condition	Chr.	Component QTL	Combined QTL	LOD	R2	Peak position (cM)	CI (cM)	Add. effect
HP	A2	*qLRD_HP_A2a*		4.7	9.4	57.0	55.2 - 60.3	-
		qTDW_HP_A2a		3.0	6.6	73.6	67.6 - 73.9	-
		qSDW_HP_A2a		4.4	8.8	74.8	73.9 - 75.4	-
		qSDW_HP_A2b		3.9	7.9	79.9	77.5 - 80.1	-
	A3		***uq.A_HP_A3_1***	**4.3**	**8.8**	**44.8**	**44.3 - 45.3**	
		qTDW_HP_A3a			**7.5**	**44.8**	**43.8-45.3**	-
		qSDW_HP_A3a			**10.2**	**44.8**	**43.9 - 45.3**	-
			uq.A_HP_A3_2	**5.2**	**10.6**	**50.8**	**50.0 - 51.6**	
		qSDW_HP_A3b			**12.4**	**50.8**	**48.9 - 51.2**	-
		qTDW_HP_A3b			**8.8**	**50.8**	**48.9-51.2**	-
		qSDW_HP_A3c		5.3	11.0	56.5	56.0 - 58.9	-
	A4		***uq.A_HP_A4_1***	**4.5**	**9.4**	**2.7**	**1.8 - 3.5**	
		qLRD_HP_A4a			**11.9**	**2.5**	**0.0 - 4.0**	-
		qLRL_HP_A4a			**10.1**	**2.6**	**1.5 - 4.0**	-
		qTDW_HP_A4a			**6.3**	**2.8**	**0.0-4.5**	-
		qSDW_HP_A4a			**9.3**	**2.8**	**0.4 - 4.0**	-
		qLRL_HP_A4b		4.8	9.9	7.7	6.9 - 8.4	-
			uq.A_HP_A4_3	**6.0**	**12.6**	**9.3**	**8.0 - 10.7**	
		qLRD_HP_A4b			**17.2**	**8.9**	**7.3 - 10.8**	-
		qSDW_HP_A4b			**7.9**	**10.0**	**8.4 - 12.8**	-
		qTDW_HP_A4b		2.7	5.4	15.1	14.8 - 15.4	-
		qLRD_HP_A4c		2.6	5.0	37.0	31.0 - 38.6	+

	A6		***uq.A_HP_A6_1***	**3.7**	**7.3**	**55.7**	**53.7 - 57.7**	
		qPRL_HP_A6a			**6.2**	**55.7**	**52.3 - 59.2**	**-**
		qTRL_HP_A6a			**8.3**	**55.7**	**54.0 - 58.8**	**-**
		qPRL_HP_A6b		2.6	4.5	65.7	64.7 - 67.1	-
	A9	*qPRL_HP_A9a*		3.3	5.6	68.0	65.5 - 68.8	+
		qSDW_HP_A9a		3.0	5.7	104.3	104.2 - 105.4	-
		qSDW_HP_A9b		3.0	5.5	110.9	110.5 - 111.3	-
	C4	*qRDW_HP_C4a*		3.0	6.7	23.2	17.4 -30.6	-
		qLRD_HP_C4a		3.0	6.0	44.9	44.6 - 46.4	-
		qLRD_HP_C4b		3.6	7.0	55.4	54.7 - 56.6	-
		qLRL_HP_C4a		2.6	5.4	58.5	58.3 - 62.0	-
		qLRD_HP_C4c		2.5	6.0	67.4	62.0 - 75.2	-
	C6	*qPRL_HP_C6a*		5.9	11.3	12.0	11.3 - 13.9	+
			uq.A_HP_C6_2	**6.4**	**11.9**	**23.0**	**21.2-24.8**	
		qTRL_HP_C6a			**6.3**	**21.4**	**10.8 - 23.7**	**+**
		qPRL_HP_C6b			**17.5**	**23.1**	**19.9 - 23.6**	**+**
	C8	*qLRN_HP_C8a*		2.6	5.9	1.1	0.0 - 1.9	-
		qLRN_HP_C8b		5.0	11.0	8.1	3.9 - 9.0	-
	C9		***uq.A_HP_C9_1***	**2.8**	**5.9**	**47.1**	**45.5 - 48.6**	
		qTRL_HP_C9a			**5.3**	**47.0**	**46.5 - 49.8**	**+**
		qLRN_HP_C9a			**6.5**	**47.7**	**41.8 - 51.3**	**+**
LP	A2	*qTDW_LP_A2a*		2.9	5.9	72.7	71.2 - 73.9	-
	A3		***uq.A_LP_A3_1***	**4.5**	**9.5**	**45.7**	**45.2 - 46.2**	
		qRDW_LP_A3a			**6.1**	**45.3**	**36.9 - 45.8**	**-**
		qTDW_LP_A3a			**11.0**	**45.3**	**43.9-46.4**	**-**
		qSDW_LP_A3a			**12.0**	**45.3**	**44.7 - 46.4**	**-**
		qTRL_LP_A3a			**8.7**	**46.1**	**45.4 - 46.9**	**-**
			uq.A_LP_A3_2	**5.0**	**10.5**	**51.6**	**51.0 - 52.2**	
		qLRL_LP_A3a			**6.4**	**51.6**	**50.8 - 54.5**	**-**
		qRDW_LP_A3b			**10.1**	**51.6**	**50.8 - 52.4**	**-**
		qSDW_LP_A3b			**13.7**	**51.6**	**50.8 - 54.1**	**-**
		qTDW_LP_A3b			**13.1**	**51.6**	**50.8-54.1**	**-**

		qTRL_LP_A3b			**8.9**	**51.6**	**50.8 - 54.1**	**-**
			uq.A_LP_A3_3	**3.2**	**7.1**	**57.0**	**56.0 - 58.8**	
		qRDW_LP_A3c			**7.5**	**57.0**	**56.0 - 58.8**	**-**
		qTRL_LP_A3c			**6.6**	**58.6**	**56.0 - 58.9**	**-**
			uq.A_LP_A3_4	**6.3**	**12.7**	**59.4**	**59.1 - 59.7**	
		qSDW_LP_A3c			**13.2**	**59.4**	**58.0 - 62.3**	**-**
		qTDW_LP_A3c			**12.2**	**59.4**	**58.9-59.5**	**-**
		qTRL_LP_A3d		3.0	6.7	61.0	59.5 - 62.6	-
			uq.A_LP_A3_6	**3.5**	**7.2**	**76.9**	**74.8 - 79.0**	
		qLRD_LP_A3a			**5.9**	**76.9**	**74.9 - 80.9**	**-**
		qLRN_LP_A3a			**8.4**	**76.9**	**74.9 - 81.0**	**-**
		qLRN_LP_A3b		3.1	6.6	84.7	84.0 - 86.7	-
	A4		***uq.A_LP_A4_1***	**5.5**	**11.1**	**2.6**	**1.3 - 3.9**	
		qTDW_LP_A4a			**9.4**	**2.6**	**0.1-4.0**	**-**
		qSDW_LP_A4a			**12.7**	**2.6**	**0.4 - 4.0**	**-**
			uq.A_LP_A4_2	**6.1**	**12.1**	**8.9**	**6.8 - 11.0**	
		qSDW_LP_A4b			**13.3**	**8.9**	**6.8 - 12.7**	**-**
		qTDW_LP_A4b			**10.9**	**8.9**	**6.8-12.7**	**-**
	A7	*qPRL_LP_A7a*		4.7	8.1	63.4	62.8 - 64.6	+
	A9	*qLRD_LP_A9a*		3.4	7.0	105.4	104.8 - 109.8	-
		qPRL_LP_A9a		3.0	5.3	131.9	130.4 - 134.8	+
	C5	*qPRL_LP_C5a*		4.2	7.7	40.5	31.1 - 44.8	-
		qPRL_LP_C5b		4.2	7.3	47.2	44.8 - 49.1	-
	C6	*qLRD_LP_C6a*		2.8	5.8	6.7	0.0 - 13.9	-
		qPRL_LP_C6a		3.0	5.3	14.6	13.2 - 18.6	+
		qPRL_LP_C6b		4.2	7.2	22.6	18.6 - 23.7	+
	C8	*qLRN_LP_C8a*		4.7	9.9	45.7	41.6 - 46.0	+
		qLRN_LP_C8b		3.6	7.7	51.0	50.6 - 52.0	+
		qLRN_LP_C8c		5.8	13.0	62.8	61.7 - 63.0	-
		qLRN_LP_C8d		7.7	17.1	68.7	68.2- 69.8	-
		qLRN_LP_C8e		5.8	12.9	74.5	74.4 - 77.4	-

Supplementary Table 4. Integrated QTL (in bold) from component QTL for root traits in the TNDH population grown for 12 and 14 days, in 'pouch and wick' systems and agar screening under contrasting Pi concentrations: low Pi (LP, 0 mM Pi) and normal Pi (NP, 0.25 mM Pi) and low Pi (LP, 0 mM Pi) and high Pi (HP, 0.625 mM Pi), respectively. The QTL analysis was based on the new high-density 2041 map constructed in this study. TRL = total root length, PRL = primary root length, TLRL = total lateral root length, LRN = lateral root number, LRD = lateral root density, SDW = shoot dry weight, RDW = root dry weight, TDW = total dry weight, Chr. = chromosome, R^2 = the explained phenotypic variation, CI = confidence interval at P=0.05.

Chr.	Combined QTL	Component QTL	LOD	R^2 (%)	Peak position (cM)	CI (cM)
A1	***uq.A1_1***	*qP_TLRL_NP_A1a*	**4.0**	**8.1**	**45.5**	**43.6 - 47.3**
		qP_TRL_NP_A1a				
	uq.A1_2	*qP_TRL_NP_A1b*	**5.0**	**10.4**	**52.1**	**50.0 - 54.2**
		qP_TLRL_NP_A1b				
A2	*uq.A2_1*	*qA_LRD_HP_A2a*	4.7	9.4	57.0	55.2 - 60.3
	uq.A2_2	*qA_TDW_LP_A2a*	**2.9**	**6.2**	**72.8**	**71.6 - 74.1**
		qA_TDW_HP_A2a				
	uq.A2_3	*qA_SDW_HP_A2a*	4.4	8.8	74.8	73.9 - 75.4
	uq.A2_4	*qA_SDW_HP_A2b*	3.9	7.9	79.9	77.5 - 80.1
A3	***uq.A3_1***	*qP_PRL_NP_A3a*	**4.5**	**10.1**	**15.0**	**8.1 - 21.8**
		qP_LRN_NP_A3a				
	uq.A3_2	*qP_PRL_NP_A3b*	3.4	6.8	28.8	27.8 - 29.9
	uq.A3_3	*qP_PRL_LP_A3a*	3.2	6.3	33.7	28.8 - 43.5
	uq.A3_4	*qA_TDW_HP_A3a*	**4.6**	**9.4**	**45.0**	**44.6 - 45.4**
		qA_SDW_HP_A3a				
		qA_RDW_LP_A3a				
		qA_TDW_LP_A3a				
		qA_SDW_LP_A3a				
	uq.A3_5	*qA_TRL_LP_A3a*	3.9	8.7	46.1	45.4 - 46.9
	uq.A3_6	*qA_SDW_HP_A3b*	**5.1**	**10.5**	**51.3**	**50.9 - 51.8**

		qA_TDW_HP_A3b				
		qA_LRL_LP_A3a				
		qA_RDW_LP_A3b				
		qA_SDW_LP_A3b				
		qA_TDW_LP_A3b				
		qA_TRL_LP_A3b				
	uq.A3_7	*qA_SDW_HP_A3c*	**4.4**	**9.3**	**56.8**	**55.8 - 57.8**
		qA_RDW_LP_A3c				
	uq.A3_8	*qA_TRL_LP_A3c*	3.0	6.6	58.6	56.0 - 58.9
	uq.A3_9	*qA_SDW_LP_A3c*	**6.3**	**12.7**	**59.4**	**59.1 - 59.7**
		qA_TDW_LP_A3c				
	uq.A3_10	*qA_TRL_LP_A3d*	3.0	6.7	61.0	59.5 - 62.6
	uq.A3_11	*qA_LRD_LP_A3a*	**3.5**	**7.2**	**76.9**	**74.8 - 79.0**
		qA_LRN_LP_A3a				
	uq.A3_12	*qA_LRN_LP_A3b*	3.1	6.6	84.7	84.0 - 86.7
A4	***uq.A4_1***	*qA_LRD_HP_A4a*	**4.9**	**10.0**	**2.6**	**1.9 - 3.4**
		qA_TDW_LP_A4a				
		qA_SDW_LP_A4a				
		qA_LRL_HP_A4a				
		qA_TDW_HP_A4a				
		qA_SDW_HP_A4a				
	uq.A4_2	*qA_LRL_HP_A4b*	4.8	9.9	7.7	6.9 - 8.4
	uq.A4_3	*qA_SDW_LP_A4b*	**6.1**	**12.3**	**9.2**	**8.1 - 10.3**
		qA_TDW_LP_A4b				
		qA_LRD_HP_A4b				
		qA_SDW_HP_A4b				
	uq.A4_4	*qA_TDW_HP_A4b*	2.7	5.4	15.1	14.8 - 15.4
	uq.A4_5	*qP_LRN_LP_A4a*	2.9	4.8	31.7	31.0 - 35.8
	uq.A4_6	*qA_LRD_HP_A4c*	**3.4**	**5.9**	**38.3**	**35.8 - 40.7**
		qP_LRN_LP_A4b				
	uq.A4_7	*qP_LRN_LP_A4c*	3.3	5.5	45.0	44.1 - 48.1
	uq.A4_8	*qP_LRD_LP_A4a*	3.2	5.2	59.0	58.1 - 60.0

	uq.A4_9	*qP_TDW_LP_A4a*	**3.2**	**6.0**	**61.7**	**60.9 - 62.4**
		qP_SDW_LP_A4a				
	uq.A4_10	*qP_TDW_LP_A4a*	**4.0**	**7.3**	**71.0**	**66.2 - 75.8**
		qP_SDW_LP_A4a				
A5	*uq.A5_1*	*qP_MLRL_LP_A5a*	4.2	8.2	0.0	0.0 - 8.0
	uq.A5_2	*qP_PRL_LP_A5a*	**4.9**	**9.2**	**40.0**	**38.4 - 41.6**
		qP_SDW_LP_A5a				
	uq.A5_3	*qP_PRL_LP_A5b*	3.7	7.3	49.5	48.1 - 50.3
	uq.A5_4	*qP_TDW_LP_A5a*	4.7	8.9	53.8	53.5 - 54.5
	uq.A5_5	*qP_RDW_LP_A5a*	3.8	7.3	56.4	54.5 - 63.0
	uq.A5_6	*qP_TDW_LP_A5b*	3.3	6.3	65.2	63.8 - 66.9
A6	*uq.A6_1*	*qP_RDW_LP_A6a*	3.5	6.9	18.1	16.8 - 18.2
	uq.A6_2	*qP_TRL_LP_A6a*	2.7	5.2	21.5	20.9 - 25.4
	uq.A6_3	*qA_PRL_HP_A6a*	**3.7**	**7.3**	**55.7**	**53.7 - 57.7**
		qA_TRL_HP_A6a				
	uq.A6_4	*qA_PRL_HP_A6b*	2.6	4.5	65.7	64.7 - 67.1
A7	*uq.A7_1*	*qA_PRL_LP_A7a*	4.7	8.1	63.4	62.8 - 64.6
	uq.A7_2	*qP_SDW_LP_A7a*	**4.9**	**9.1**	**67.3**	**65.8 - 68.8**
		qP_TDW_LP_A7a				
	uq.A7_3	*qP_TDW_LP_A7b*	3.4	6.4	77.2	76.3 - 77.3
	uq.A7_4	*qP_RDW_LP_A7a*	2.6	5.9	101.3	90.6 - 103.3
A9	***uq.A9_1***	*qP_TLRL_LP_A9a*	**2.8**	**6.0**	**30.4**	**26.5 - 34.2**
		qP_TRL_LP_A9a				
	uq.A9_2	*qA_PRL_HP_A9a*	3.3	5.6	68.0	65.5 - 68.8
	uq.A9_3	*qA_SDW_HP_A9a*	3.0	5.7	104.3	104.2 - 105.4
	uq.A9_4	*qP_MLRL_LP_A9a*	**4.6**	**9.3**	**105.0**	**103.6 - 106.3**
		qA_LRD_LP_A9a				
	uq.A9_5	*qA_SDW_HP_A9b*	3.0	5.5	110.9	110.5 - 111.3
	uq.A9_6	*qP_MLRL_LP_A9b*	6.3	12.5	112.8	111.3 - 113.6
	uq.A9_7	*qP_LRN_LP_A9a*	4.0	6.8	115.3	115.0 - 117.0
	uq.A9_8	*qP_LRN_LP_A9b*	6.3	10.6	123.9	122.9 - 124.5
	uq.A9_9	*qA_PRL_LP_A9a*	3.0	5.3	131.9	130.4 - 134.8

A10	*uq.A10_1*	*qP_LRD_NP_A10a*	3.6	7.3	37.1	35.4 - 37.7
	uq.A10_2	*qP_TRL_NP_A10a*	2.5	5.6	50.6	45.4 - 51.4
	uq.A10_3	*qP_TLRL_NP_A10a*	3.5	6.9	53.0	52.2 - 55.6
C2	***uq.C2_1***	*qP_LRD_LP_C2a*	**3.7**	**6.6**	**5.9**	**4.1 - 7.6**
		qP_LRN_LP_C2a				
	uq.C2_2	*qP_LRD_LP_C2b*	**5.0**	**8.3**	**15.9**	**13.9 - 17.9**
		qP_LRN_LP_C2b				
	uq.C2_3	*qP_MLRL_LP_C2a*	5.8	12.4	19.2	16.2 - 22.9
	uq.C2_4	*qP_LRD_LP_C2c*	**4.0**	**6.7**	**24.7**	**23.9 - 25.5**
		qP_LRN_LP_C2c				
	uq.C2_5	*qP_MLRL_LP_C2b*	3.7	7.1	34.7	34.3 - 35.7
C4	*uq.C4_1*	*qA_RDW_HP_C4a*	3.0	6.7	23.2	17.4 -30.6
	uq.C4_2	*qP_RDW_LP_C4a*	**2.7**	**4.6**	**42.5**	**39.1 - 45.9**
		qP_LRN_LP_C4a				
	uq.C4_2	*qA_LRD_HP_C4a*	3.0	6.0	44.9	44.6 - 46.4
	uq.C4_3	*qA_LRD_HP_C4b*	3.6	7.0	55.4	54.7 - 56.6
	uq.C4_4	*qA_LRL_HP_C4a*	2.6	5.4	58.5	58.3 - 62.0
	uq.C4_5	*qP_SDW_LP_C4a*	**2.8**	**5.8**	**64.8**	**60.9 - 68.7**
		qA_LRD_HP_C4c				
	uq.C4_6	*qP_LRN_NP_C4a*	2.7	5.3	81.3	80.4 - 83.4
	uq.C4_7	*qP_LRN_NP_C4b*	4.5	8.8	91.4	91.0 - 95.7
	uq.C4_8	*qP_LRN_NP_C4c*	3.1	6.0	106.7	102.5 - 111.5
C5	*uq.C5_1*	*qP_MLRL_LP_C5a*	3.3	6.3	16.2	10.9 - 17.8
	uq.C5_2	*qA_PRL_LP_C5a*	4.2	7.7	40.5	31.1 - 44.8
	uq.C5_3	*qA_PRL_LP_C5b*	**3.8**	**6.7**	**47.5**	**45.6 - 49.4**
		qP_LRN_LP_C5a				
C6	*uq.C6_1*	*qA_LRD_LP_C6a*	2.8	5.8	6.7	0.0 - 13.9
	uq.C6_2	*qA_PRL_HP_C6a*	5.9	11.3	12.0	11.3 - 13.9
	uq.C6_3	*qA_PRL_LP_C6a*	3.0	5.3	14.6	13.2 - 18.6
	uq.C6_4	*qA_TRL_HP_C6a*	**5.6**	**10.3**	**22.8**	**21.4 - 24.3**
		qA_PRL_LP_C6b				
		qA_PRL_HP_C6b				

C8	*uq.C8_1*	*qA_LRN_HP_C8a*	2.6	5.9	1.1	0.0 - 1.9
	uq.C8_2	*qA_LRN_HP_C8b*	5.0	11.0	8.1	3.9 - 9.0
	uq.C8_3	*qA_LRN_LP_C8a*	4.7	9.9	45.7	41.6 - 46.0
	uq.C8_4	*qA_LRN_LP_C8b*	3.6	7.7	51.0	50.6 - 52.0
	uq.C8_5	*qA_LRN_LP_C8c*	5.8	13.0	62.8	61.7 - 63.0
	uq.C8_6	*qA_LRN_LP_C8d*	7.7	17.1	68.7	68.2- 69.8
	uq.C8_7	*qA_LRN_LP_C8e*	5.8	12.9	74.5	74.4 - 77.4
C9	***uq.C9_1***	*qP_TLRL_LP_C9a*	**3.6**	**7.3**	**9.4**	**4.6 - 14.2**
		qP_TRL_LP_C9a				
	uq.C9_2	*qP_LRD_LP_C9a*	6.7	12.8	18.6	14.7 - 22.1
	uq.C9_3	*qP_LRN_LP_C9a*	**6.5**	**11.8**	**27.6**	**22.5 - 32.7**
		qP_LRD_LP_C9b				
	uq.C9_4	*qA_TRL_HP_C9a*	**2.8**	**5.9**	**47.1**	**45.5 - 48.6**
		qA_LRN_HP_C9a				
	uq.C9_5	*qP_LRD_NP_C9a*	3.6	7.1	61.4	60.6 - 61.9

Chapter 4

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Root morphology and seed and leaf ionomic traits in a *Brassica napus* L. diversity panel show wide phenotypic variation and are characteristic of crop habit

C. L. Thomas[1,2], T. D. Alcock[1], N. S. Graham[1], R. Hayden[1], S. Matterson[1], L. Wilson[1], S. D. Young[1], L. X. Dupuy[2], P. J. White[2,3], J. P. Hammond[4], J. M. C. Danku[5], D. E. Salt[5], A. Sweeney[6], I. Bancroft[6] and M. R. Broadley[1*]

Abstract

Background: Mineral nutrient uptake and utilisation by plants are controlled by many traits relating to root morphology, ion transport, sequestration and translocation. The aims of this study were to determine the phenotypic diversity in root morphology and leaf and seed mineral composition of a polyploid crop species, *Brassica napus* L., and how these traits relate to crop habit. Traits were quantified in a diversity panel of up to 387 genotypes: 163 winter, 127 spring, and seven semiwinter oilseed rape (OSR) habits, 35 swede, 15 winter fodder, and 40 exotic/unspecified habits. Root traits of 14 d old seedlings were measured in a 'pouch and wick' system ($n = \sim 24$ replicates per genotype). The mineral composition of 3–6 rosette-stage leaves, and mature seeds, was determined on compost-grown plants from a designed experiment ($n = 5$) by inductively coupled plasma-mass spectrometry (ICP-MS).

Results: Seed size explained a large proportion of the variation in root length. Winter OSR and fodder habits had longer primary and lateral roots than spring OSR habits, with generally lower mineral concentrations. A comparison of the ratios of elements in leaf and seed parts revealed differences in translocation processes between crop habits, including those likely to be associated with crop-selection for OSR seeds with lower sulphur-containing glucosinolates. Combining root, leaf and seed traits in a discriminant analysis provided the most accurate characterisation of crop habit, illustrating the interdependence of plant tissues.

Conclusions: High-throughput morphological and composition phenotyping reveals complex interrelationships between mineral acquisition and accumulation linked to genetic control within and between crop types (habits) in *B. napus*. Despite its recent genetic ancestry (<10 ky), root morphology, and leaf and seed composition traits could potentially be used in crop improvement, if suitable markers can be identified and if these correspond with suitable agronomy and quality traits.

Keywords: Canola, Ionomics, Mineral concentration, High-throughput phenotyping, Root morphology, Seed size, Leaf/seed elemental ratios

* Correspondence: martin.broadley@nottingham.ac.uk
[1]School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK
Full list of author information is available at the end of the article

Background

Plants require at least 14 essential mineral elements to complete their life-cycles [1]. These include nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S), which are macronutrients required in large amounts (typically 1000– > 10,000 mg kg^{-1} leaf dry weight, DW). The micronutrients chlorine (Cl), boron (B), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), nickel (Ni) and molybdenum (Mo) are required in smaller amounts (typically 0.1–100 mg kg^{-1} leaf DW) [2]. Plants also accumulate non-essential elements, some of which have little or no effect on plant growth and development at the concentrations they occur in nature, and others of which may have beneficial and/or detrimental effects depending upon their concentrations in plant tissues. These include arsenic (As), cadmium (Cd), selenium (Se), silicon (Si) and sodium (Na).

Most mineral elements are taken up in ionic form from the soil solution by plant roots. Traits/phenes affecting root morphology and anatomy play a key role in the acquisition of mineral nutrients by plants and impact on crop yields [3–5]. For example, increased root hairs and shallower basal root growth angles can increase P uptake [6, 7]. Reduced allocation of carbon to root structures via increased aerenchyma and reduced cortical cell file formations [8] and smaller root diameter [9] may allow some plants more efficient access to larger soil volumes, and thereby water and nutrients. The subsequent uptake and utilisation of mineral elements by plants is controlled by traits affecting ion transport, translocation and sequestration [1]. Mineral elements in both chelated and free-ionic forms move across the root via apoplastic (extracellular) and symplastic (intracellular) pathways to the stele. Following xylem loading and subsequent transport to transpiring leaf tissues, elements are taken up from the leaf apoplast by specific cell types. Translocation of mineral elements in the plants to non-transpiring or xylem-deficient tissues occurs via the phloem [10, 11]. Some elements are highly mobile in phloem tissues (K, Na, Mg, Cd, N, P, S, Se and Cl), some are relatively immobile in the phloem (Ca and Mn), and some elements have intermediate mobility (B, Fe, Zn, Cu, Mo and I) [10–12].

The term 'ionome' defines the complement of mineral elements in all of their chemical forms within an organism or tissue, irrespective of whether they are essential or non-essential [13]. The ionome is thus the inorganic subset of the metabolome at a given moment in space and time, which varies at all scales. Within an individual plant, an ionome is specific to tissue type and developmental stage; e.g. seed, fruit and tuber concentrations of Ca are lower than leaf concentrations of Ca due to its limited phloem mobility [14, 15]. Between individuals, the ionome of a specific tissue type varies due to environmental and genetic factors at all scales and this can be observed as differences between populations, species, and plant families [13, 14, 16–18].

Variation in the ionomes of edible crop tissues has enabled identification of quantitative trait loci (QTL) linked to mineral composition and important to human and animal nutrition in several crop species [3, 19, 20]. For example, genetic loci affecting the mineral composition of leaves of *Brassica oleracea* [21], *Brassica rapa* [22], *Brassica napus* [23] and *Lotus japonicus* [24] have been identified. In the study of Bus et al. [23], there were strong pair-wise positive correlations in the shoot concentrations of many of the 11 mineral elements in 30 d old *B. napus* (>500 genotypes). Furthermore, there were many pair-wise negative correlations between the shoot concentrations of several elements, notably Ca and K, and numerous leaf and seedling size related traits. Plant ionomes are also amenable to genetic dissection using natural and induced genetic variation via mutagenesis, using association mapping and reverse genetic approaches. Several genes underlying variation in mineral nutrient acquisition and translocation have recently been identified. For example, in *Arabidopsis thaliana*, a deletion mutant with a reduced leaf Ca concentration led subsequently to the identification of *ESB1* (*Enhanced Suberin Biosynthesis 1*) which affects Casparian Band formation [25, 26]. A mutant with reduced leaf Mg, Ca, Fe, and Mo and increased leaf Na and K concentration was similarly associated with reduced sphingolipid biosynthesis [27]. A variety of other *Arabidopsis* genes are associated with phenotypic variation in leaf As [28], Cd [29], K [30], S and Se [31].

Brassica napus is an important crop in global terms, with crop types including oilseed rape (OSR), vegetable swede, and fodder crops. Currently, oilseed types of OSR are the third largest source of vegetable oil globally after soybean and oil palm. Worldwide production of OSR was 72.8 Mt in 2013 [32]. Other uses for OSR oils include biodiesel and rape meal for animal feeds, and co-products, including vitamin E (tocopherol) and cholesterol lowering compounds (phytosterols) from the oil, and waxes from pod walls with medical/cosmetic properties. Further industrial oils are currently underexploited but could increase economic margins for farmers. There is considerable scope for improvement of yield of seeds and co-products if suitable traits can be identified and introduced into well-adapted varieties, for example, through improvements in yield and resource-use efficiency [33, 34]. Worldwide average yields for OSR have increased from 1.5 to 2 t ha^{-1} from 2000 to 2013. Yields are higher in Western Europe, with 2013 average yields of 3.5 t ha^{-1}. The long term average yield of UK OSR is 3.1 t ha^{-1} [35], which is much less than UK wheat (8.1 t ha^{-1}) and UK barley (6.4 t ha^{-1}) yet it is similarly nutrient-intensive [36].

The yields of UK OSR are also far less than their estimated potential of >6.5 t ha^{-1} [35].

The aim of this study was to determine the phenotypic diversity in root morphology, shoot ionomic (leaf and seed) and seed size/yield traits within a broad genetic diversity panel of *B. napus* (encompassing all crop types) and to identify their relationship to crop habit. Determining the phenotypic diversity in these traits, and their interrelationships, in this population could inform subsequent studies to dissect the genetic bases and identify markers in traits relevant for crop improvement [37]. An increased understanding of these traits could also help in breeding strategies via more conventional means. To our knowledge, no previous studies have simultaneously characterised the phenotypic variation in root morphology, ionomes and seed size from such a large diversity panel, which is likely to capture most of the species-wide variation in these traits in *B. napus*.

Methods

Plant material for all experiments

Inbred lines of *Brassica napus* L. genotypes were used in this study. These were from the ERANET-ASSYST consortium diversity population [23, 38–40]. A core panel of 387 genotypes were selected, comprising 163 winter, 127 spring, and seven semiwinter oilseed rape (OSR), 35 swede, 15 winter fodder, and 40 exotic/unspecified habits (Additional file 1: Table S1). Two cultivation systems were deployed. Seedling root traits were determined in a 'pouch and wick' hydroponic system in a controlled environment (CE) room. Leaf and seed mineral composition traits were measured on compost-grown plants grown in a designed experiment in a polytunnel.

Root phenotyping in a pouch and wick system

The 'pouch and wick' high-throughput phenotyping (HTP) system was reported previously [5, 41]. This system comprised growth pouches assembled from blue germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA), re-cut to 24 × 30 cm and overlain with black polythene (Cransford Polythene Ltd, Woodbridge, UK). Along one of the shorter edges, the paper and polythene were clipped together to an acrylic rod (Acrylic Online, Hull, UK) using 'bulldog'-type fold-back clips. The growth pouches were suspended above plastic drip trays, supported within lightweight aluminium/polycarbonate frames (KJN Aluminium Profiles, Leicester, UK). Each drip tray contained 2 L of 25 % strength Hoagland's solution (No. 2 Basal Salt Mixture, Sigma Aldrich, Dorset, UK) made with deionised water. Drip trays were replenished with 500 mL of deionised water every 3 d. Prior to sowing, the pouches were suspended above the nutrient solution for a minimum of 4 h to become fully saturated. Within each aluminium frame, nine drip trays were used, arranged in three columns and three rows. Pouches were allocated randomly to drip trays, 10 or 11 pouches per drip tray, thus 96 pouches and 192 plants per frame (i.e. a single plant on each side of the paper). A total of four frames were used in each experimental run, giving a potential sample size of 768 plants per run within the CE room. The CE room was 2.2 m width, 3.3 m length, 3.0 m height, set to a 12 h photoperiod 18/15 °C day/night temperatures and relative humidity of 60–80 %. Photosynthetically Active Radiation (PAR; measured at plant height with a 190 SB quantum sensor; LI-COR Inc., Lincoln, NE, USA) was approximately 207 μmol m^{-2} s^{-1}, generated by 400 W white fluorescent lamps (HIT 400w/u/Euro/4 K, Venture Lighting, Rickmansworth, UK).

A single seed was sown on each germination paper, in the middle of the upper edge of the paper, by pressing the seed into the paper. The potential effect of seed size on root traits was controlled for by selecting individual seeds which spanned a range of sizes for each genotype, therefore giving a mean seed diameter of ~1.8 mm for each genotype. Seeds of each genotype were sieved using mesh with a diameter (Ø) of 1.4, 1.7, 2.0 and 2.36 mm (Scientific Laboratory Supplies Ltd, Hessle, UK). Seed retained within the mesh of each faction were selected such that 25 % of seed represented each Ø-category for each genotype. Where insufficient seeds were available for a given Ø-category, the next smallest Ø-category was used instead.

Fourteen days after sowing (DAS), the polythene sheets were removed from all pouches and images were taken of the germination paper and root system for downstream image analysis. Images were taken using a Digital Single Lens Reflex (DSLR) camera (Canon EOS 1100D, Canon Inc., Tokyo, Japan) with a focal length of 35 mm at a fixed height of 75 cm. The root images from the HTP system were renamed with each sample's unique experimental design information using Bulk Rename Utility (Version 2.7.1.3, TGRMN Software, www.bulkrenameutility.co.uk). Images were cropped by reducing extraneous pixels on bulked images, using XnConvert (Version 1.66, www.xnconvert.com). Cropped images were analysed using RootReader2D (RR2D) [42]. First, a 'batch process' was carried out which automatically 'thresholds', 'skeletonises' and 'builds segments' of all images in bulk. The root system was then measured on individual images by placing a marker at the base and tip of the primary root. From these markers, RR2D automatically calculates primary root length (PRL), lateral root length (LRL) of all laterals, and lateral root number (LRN). Further traits calculated from these data included total root length (TRL = PRL + LRL), mean lateral root length (MLRL = LRL/LRN) and lateral root density (LRD = LRN/PRL). A database was developed which integrated the experimental design information from the image name, with the RR2D

measurements for each sample, using a programming script (2.7.10; Python Software Foundation, www.python.org).

Of the core panel of 387 genotypes, 354 genotypes comprising 156 winter, 124 spring and seven semiwinter OSR, 14 winter fodder, 33 swede and 20 exotic/unspecified types were screened. Two additional reference winter OSR lines were screened in each experimental run. Each experimental run comprised 32 genotypes, of 24 individuals per genotype. There were 16 experimental runs in total. This equates to a total of 11,176 potential images. An image was removed from analysis if the seed had failed to germinate, or if the seed had rolled down the paper and therefore the shoot failed to emerge above the pouch, or if the seedling was stunted with a radicle < 3 cm, or the radicle appeared deformed such as being twisted around the seed. Overall, 29 % of samples were removed from analysis; excluded data are noted in Additional file 1: Table S2 and all images are available on request.

The relative contribution of genotypic and non-genotypic variance components underlying variation in root traits were calculated using a REML (REsidual Maximum Likelihood) procedure according to the model [(run/frame/column/tray/paper-side) + habit + seed size + genotype]. Genotype was subsequently added as a fixed factor to estimate genotype-means of root traits.

Leaf and seed mineral composition traits in soil-grown plants

Growth of plant material

Seed of all genotypes were sown directly into fine-grade (<3 mm particle size) compost-based growing media (Levington Seed & Modular + Sand -F2S; Everris Ltd., Ipswich, UK) in modular propagation trays (650 plants m^{-2}; internal Ø 2.5 cm, module volume 55 cm^3; Type '104', Desch Plantpak, Essex, UK). Sowing took place from 22 to 29 October 2013. The compost was covered with perlite and transferred to a glasshouse vented at 15 °C (controlled by TomTech μClimate, Spalding, Lincs). Supplementary, artificial lighting (Philips Master GreenPower SON-T 400 W bulbs controlled by Grasslin Uni 45 timer) was used to maintain day lengths of 12 h light d^{-1}. Watering was once daily by hand as required until transplantation. From 16 to 29 January 2014, five plants of each genotype were transplanted into individual 5 L pots (internal Ø 22.5 cm; height 18 cm) containing Levington C2 compost (Scotts Professional, Ipswich, UK). Pots were arranged within two single-skinned polytunnels (with a Visqueen Luminance Skin, Northern Polytunnels, Colne, UK) with no additional lighting or heating, at the Sutton Bonington Campus of the University of Nottingham (52°49'58.9" N, 1°14'59.2" W).

Pots were arranged in a randomised block design of five replicate blocks using an R script (personal communications, Edmondson RA, superseded [43]). Three replicates were allocated to one polytunnel, two to the other. Each replicate comprised 432 units, including one of each of the 387 core genotypes, plus 16 reference genotypes added to enable more accurate normalisation. A further 29 genotypes were included to fill gaps. Each replicate block was split into 12 sub-blocks of 36 genotypes, which were allocated at random. Where a lack of germination meant that insufficient plants were available at the transplanting stage, empty, compost-filled pots were used in their place.

Automatic irrigation was controlled in each polytunnel by a Hunter Irrigation Controller (Hunter Industries, San Marcos, CA, USA, provided by Hortech Systems Ltd., Holbeach, UK). Water from a header tank was distributed by a pump (DAB Active JI112M; DAB Pumps Ltd. Bishop's Stortford, UK) such that each pot received 133 mL of water at 08:00, 12:00 and 16:00 each day, via a low density polyethylene (LDPE) pipe based irrigation system fitted with compensated, non-leaking (CNL) drippers at 4 L h^{-1} capacity. Each CNL dripper supplied four pots using an attached, four-tipped manifold (Netafim, Tel Aviv, Israel, provided by Hortech systems Ltd.). Each system was also fitted with a Dosatron D3GL-2 feed injector (Tresses, France) used to provide plants with Kristalon Red NPK fertiliser (Yara, Grimsby, UK) between 24 March and 22 May 2014. This was set to mix fertiliser from a stock solution (made up at 100 g fertiliser per litre water) into water at a ratio of one part stock solution to 100 parts water before being sent to the pots. Plants were covered by 380 × 900 mm micro-perforated pollination bags (Focus Packaging & Design Ltd, Brigg, UK) once inflorescences began to show to prevent cross pollination. Any side shoots that emerged after bagging were cut. Watering was reduced to 50 % from 2 June 2014 and switched off completely from 1 July 2014 to encourage senescence. All plants were sprayed with 0.1 % (v/v) azoxystrobin (Amistar, Syngenta, Cambridge, UK) to control first signs of Phoma and some Botrytis on 20 November 2013 and were sprayed again on 29 January 2014 and 17 February 2014. Tebuconazole (Folicur, Bayer, Cambridge, UK) and Amistar were applied at a rate of 0.06 % (v/v) on 28 April 2014. Aphid control was by 0.05 % (w/v) Pirimicarb (Aphox, Syngenta) on 20 May 2014 and 0.07 % (v/v) Deltamethrin (Decis, Bayer) on 2 June 2014.

The total quantity of experimental units was 2160. All plants were harvested from the polytunnels from 14 to 17 July 2014. Stems were cut just above the bottom of the micro-perforated bag containing the top of plants. Each bag was then tied up such that no plant material could escape. Labelled bags were placed into 1 m^3 ventilated crates for storage prior to threshing. Crates containing plant material were transported to Elsoms Seeds

(Spalding, Lincolnshire), where they were threshed for seed with an SRC single plant thresher (Nickerson Brothers Limited, Lincoln) and cleaned using a Selecta seed cleaner (Selecta Machinefabriek B.V., Enkhuizen, Netherlands). Thousand seed weight for each plant was measured using a Contador seed counter (Pfeuffer GmbH, Kitzingen, Germany). Total seed yield per plant are indicative data, since side-stems were removed where these grew outside of the bags.

Sampling, digestion and analysis of leaf samples

Leaves were sampled at the rosette stage (typically 6–8 true leaves showing) from 5 to 11 March 2014. A minimum of three fully expanded leaves were cut from the plant, weighed and photographed while fresh. Leaves from each plant were stored in separate labelled paper bags at -20 °C. All samples were freeze dried (CHRIST Alpha 2-4 LD freeze dryer; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) for 48–60 h, and re-weighed. Leaves were homogenised in liquid N_2 using a pestle and mortar and kept frozen prior to analyses.

Subsamples (~0.20 g DW) of leaf were digested using a microwave system comprising a Multiwave 3000 platform with a 48-vessel MF50 rotor (Anton Paar GmbH, Graz, Austria); digestion vessels were perfluoroalkoxy (PFA) liner material and polyethylethylketone (PEEK) pressure jackets (Anton Paar GmbH). Leaf material was digested in 2 mL 70 % Trace Analysis Grade HNO_3, 1 mL Milli-Q water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, UK), and 1 mL H_2O_2 with microwave settings as follows: power = 1400 W, temp = 140 °C, pressure = 2 MPa, time = 45 min. Two operational blanks were included in each digestion run. Duplicate samples of certified reference material (CRM) of leaf (Tomato SRM 1573a, NIST, Gaithersburg, MD, USA) were included approximately every fourth digestion run; laboratory reference material (LRM) from pooled / freeze-dried *Brassica napus* leaves was also used for later digests. Following digestion, each tube was made up to a final volume of 15 mL by adding 11 mL Milli-Q water and transferred to a 25 mL universal tube (Sarstedt Ltd., Nümbrecht, Germany) and stored at room temperature.

Leaf digestates were diluted 1-in-5 using Milli-Q water prior to elemental analysis. The concentrations of 28 elements were obtained using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany); Ag, Al, As, B, Ba, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, U, V, Zn. Operational modes included: (i) a helium collision-cell (He-cell) with kinetic energy discrimination to remove polyatomic interferences, (ii) standard mode (STD) in which the collision cell was evacuated, and (iii) a hydrogen collision-cell (H_2-cell). Samples were introduced from an autosampler incorporating an ASXpress™ rapid uptake module (Cetac ASX-520, Teledyne Technologies Inc., Omaha, NE, USA) through a PEEK nebulizer (Burgener Mira Mist, Mississauga, Burgener Research Inc., Canada). Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Sc (20 μg L^{-1}), Rh (10 μg L^{-1}), Ge (10 μg L^{-1}) and Ir (5 μg L^{-1}) in 2 % trace analysis grade HNO_3 (Fisher Scientific UK Ltd). External multi-element calibration standards (Claritas-PPT grade CLMS-2; SPEX Certiprep Inc., Metuchen, NJ, USA) included Ag, Al, As, B, Ba, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti (semi-quant), U, V and Zn, in the range 0–100 μg L^{-1} (0, 20, 40, 100 μg L^{-1}). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, Courtaboeuf, France) was used to create Ca, K, Mg and Na standards in the range 0–30 mg L^{-1}. Boron, P and S calibration utilized in-house standard solutions (KH_2PO_4, K_2SO_4 and H_3BO_3). In-sample switching was used to measure B and P in STD mode, Se in H_2-cell mode and all other elements in He-cell mode. Sample processing was undertaken using Qtegra™ software (Thermo Fisher Scientific) with external cross-calibration between pulse-counting and analogue detector modes when required. In total, 2096 samples were analysed in 14 ICP-MS runs.

Digestion and analysis of seed samples

Dry seeds (three seeds per tube and occasionally four for very small seeds) were transferred into Pyrex test tubes (16 × 100 mm). After weighing an appropriate number of samples the masses of the remaining samples were calculated using method of Danku et al. [44]. The Seed samples were left overnight to pre-digest in 1.16 mL trace metal grade HNO_3 (J. T. Baker Instra-Analyzed; Avantor Performance Materials; Scientific & Chemical Supplies Ltd, Aberdeen, UK) spiked with indium internal standard and 1.2 mL H_2O_2 (Primar-Trace analysis grade, 30 %; Fisher Scientific, Loughborough, UK) was also added. They were then digested in dry block heaters (DigiPREP MS, SCP Science; QMX Laboratories, Essex, UK) at 115 °C for 4 h.

Seed digestates were diluted to 11.5 mL with Milli-Q water (18.2 MΩ cm, Merck Millipore, Watford, UK) and aliquots transferred to 96-well deep well plates using adjustable multichannel pipette (Rainin; Anachem Ltd, Luton, UK) for analysis. Elemental analysis was performed with an ICP-MS (PerkinElmer NexION 300D equipped with Elemental Scientific Inc. autosampler and Apex HF sample introduction system; PerkinElmer LAS Ltd, Seer Green, UK and Elemental Scientific Inc., Omaha, NE, USA, respectively) in the standard mode. Twenty elements (Li, B, Na, Mg, P, S, K, Ca, Mn, Fe, Co, Ni, Cu,

Zn, As, Se, Rb, Sr, Mo, and Cd) were monitored. Liquid reference material composed of pooled samples of the digested seed materials was prepared before the first sample run and was used throughout the remaining sample runs. The liquid reference material was included after every ninth sample in all ICP-MS sample sets to correct for variation between and within ICP-MS analysis runs [44]. Sample concentrations were calculated using external calibration method within the instrument software. The calibration standards (with indium internal standard and blanks) were prepared from single element standards (Inorganic Ventures; Essex Scientific Laboratory Supplies Ltd, Essex, UK) solutions. In total, 1986 samples were analysed across four ICP-MS runs.

Data processing of leaf and seed mineral composition traits

For each data-point, an element-specific operational blank concentration (mean of each ICP-MS run) was subtracted. Data were then multiplied by initial sample volume, divided by the initial dry mass of plant material, and converted to mg element kg^{-1} of dry leaf or seed material. Element-specific limits of detection (LODs) were reported as three times the standard deviation (SD) of the operational blank concentrations, assuming a notional starting dry weight of 0.200 g for leaf and 0.015 g for seed data (Additional file 1: Table S3). For leaves, element-specific recoveries from CRMs ranged from 68 to 134 %, for 18 elements with certified CRM values (Additional file 1: Table S4). From leaf mineral concentration data, seven elements (Ag, Co, Cr, Ni, Pb, U, V) were removed from further analysis due to having mean mineral concentrations which were less than or close to the LOD (Additional file 1: Table S5). Likewise, seven elements (As, Co, Cr, Fe, Ni, Pb, Se) were removed from seed mineral concentration data (Additional file 1: Table S6). For those elements retained for analysis, data for individual leaf and seed element concentrations which were below element-specific LODs were replaced with half LOD values. Leaf and seed element concentrations >5 standard deviation (SDs) above the global arithmetic mean for each element were also removed as a precaution against using contaminated samples (125 out of 58,688 values for leaves; 107 out of 42,504 values for seed).

The relative contribution of genotypic and non-genotypic variance components underlying variation in leaf and seed composition traits was calculated using a REML procedure in GenStat. Genotype, habit and experimental sources of variation were classed as random factors according to the model [habit + genotype + polytunnel + polytunnel/replicate + polytunnel/replicate/sub-block]. For leaf composition traits, a further model was used [replicate + (replicate/sub-block) + genotype + (replicate/genotype)] in which genotype was subsequently added as a fixed factor to estimate genotype-means. For seeds, the arithmetic mean data were used for each genotype.

Multivariate analysis of root morphology and mineral composition traits

Correlation analysis was conducted on all 945 possible pairwise combinations of the 44 root, leaf and seed trait variate sets (genotype means). Five stepwise discriminant analyses were conducted in GenStat, one each for the root morphology-, leaf- and seed mineral- and seed weight variate sets, which contained 6, 21,15 and two variates, respectively, and one for the variate set of all traits combined. Genotypes were grouped according to 'crop habit'. The Wilks' Lambda 'forward selection' stepwise algorithm option was selected, which, at each step, adds the trait-variate which explains the most between-group variation from all of the remaining trait-variate sets. Specificity plots were drawn, to view the proportion of genotypes of each 'crop habit' correctly allocated to each group, at each step. Discrimination plots were drawn to represent the separation of variation in the crop habits in two dimensions. All statistical analyses were conducted using GenStat 15th Edition (VSN International Ltd, Hemel Hempstead, UK).

Results and discussion

Root growth was influenced strongly by seed size

Seed diameter accounted for a large proportion of the variation in total root length (TRL; 44 %), primary root length (PRL; 35 %), lateral root length (LRL; 41 %) and lateral root number (LRN; 41 %), but not for mean lateral root length (MLRL; 6 %) or lateral root density (LRD; 3 %) in 14 d old seedlings (Table 1; Fig. 1a). Genotype/habit factors accounted for between 7 % (MLRL) and 17 % (PRL) of the total variation in the six root traits. Residual (plant-to-plant) variation accounted for the largest single source of variation in the study, up to 75 and 81 % for LRD and MLRL, respectively, indicating that lateral roots traits are particularly responsive to the environment. This large residual source of variation is consistent with previous studies of *Brassica* seedling root traits, which show that large numbers of individuals are required to detect subtle differences in root traits between genotypes with confidence [5, 45]. Thousand seed weight (TSW) in the 2013 seed, from which all plants were grown, varied significantly within and between crop habits, from largest to smallest in: semiwinter OSR, winter OSR, spring OSR, winter fodder and swede types ($P < 0.001$, Fig. 1b). However, whilst seed diameter had a significant positive correlation with root length, based on the data for individual seedlings, potential correlations between TSW and root length could not be tested in this study because seeds were selected

Table 1 Variance components analysis of root morphology, seed yield and leaf and seed mineral composition traits in *Brassica napus*, showing the variation (%) in the trait associated with genotype, habit, experimental design and residual factors, (seed size effect was calculated for the root traits only), as determined by Residual Maximum Likelihood (REML) analyses

Variate	Genotype	Habit	Experimental	Seed diameter	Residual
Root traits					
TRL	9	1	4	44	41
PRL	11	6	3	35	45
LRL	9	1	5	41	44
MLRL	6	1	6	5	81
LRN	9	4	5	41	43
LRD	13	2	8	3	75
Seed yield					
TSW	8	3	3	-	87
Leaf mineral composition					
Al	3	0	44	-	53
As	4	2	67	-	27
B	22	3	42	-	34
Ba	23	1	42	-	34
Ca	26	12	27	-	34
Cd	11	1	59	-	29
Cs	2	1	74	-	23
Cu	17	5	29	-	49
Fe	13	6	43	-	38
K	35	4	30	-	32
Mg	36	17	14	-	34
Mn	15	5	48	-	32
Mo	22	10	6	-	61
Na	37	21	6	-	36
P	21	8	17	-	55
Rb	30	7	35	-	28
S	40	15	17	-	28
Se	0	0	85	-	15
Sr	24	7	41	-	28
Ti	9	3	65	-	23
Zn	24	6	30	-	40
Seed mineral composition					
B	13	2	40	-	45
Ca	16	9	26	-	49
Cd	9	8	40	-	42
Cu	32	0	6	-	62
K	21	1	25	-	53
Li	10	5	16	-	69
Mg	6	18	52	-	24
Mn	12	1	55	-	32
Mo	41	21	7	-	31
Na	12	3	26	-	59
P	12	5	50	-	32
Rb	14	3	36	-	46
S	31	13	37	-	19
Sr	9	8	27	-	57
Zn	21	12	14	-	53

TRL total root length, *PRL* primary root length, *LRL* total lateral root length, *MLRL* mean lateral root length, *LRN* lateral root number, *LRD* lateral root density, *TSW* Thousand Seed Weight. See Additional file 1: Table S10 for detailed information

for uniformity between genotypes based on diameter classification and not by individual seed weights.

Positive relationships have been reported previously between seed size and the seminal root length and total root weight of barley (*Hordeum vulgare*) [46], and between seed size and total root size and lateral root number in tomato (*Solanum lycopersicum*) [47]. Larger seeds have also been shown to improve seedling establishment, shoot weight, biomass and final yield in some, but not all field studies of OSR in Canada [48–51]. However, larger-sized seeds typically had more vigorous early growth [51]. Thousand seed weight was also shown to correlate positively with absolute growth rate 21 days after germination [52]. Improved seed size-related root growth of *B. napus* seedlings might also increase tolerance to shoot pests (e.g. flea beetle, *Phyllotreta* spp.) [48] and root diseases such as *Rhizoctonia solani* which can damage the primary roots of *B. napus* [53]. Seed weight has previously been associated with pre-emergence growth in a bi-parental mapping population of *Brassica oleracea*, but under separate genetic control to germination [54, 55]. Additionally, the present study found that the thousand seed weight (TSW) in the winter OSR varieties from different release periods has steadily increased over time, suggesting that larger seeds may have been bred for (Additional file 2: Figure S6). This present study shows there is scope to exploit the genetic control of seed size-related root growth as a potential route to improve early vigour in the small-seeded *B. napus*.

Winter OSR and fodder types had larger root systems than other crop habits

Winter OSR and winter fodder types had a greater mean TRL, PRL, TLRL, and LNR at 14 d than the other crop habits ($P < 0.001$, Fig. 2; Additional file 1: Table S7).

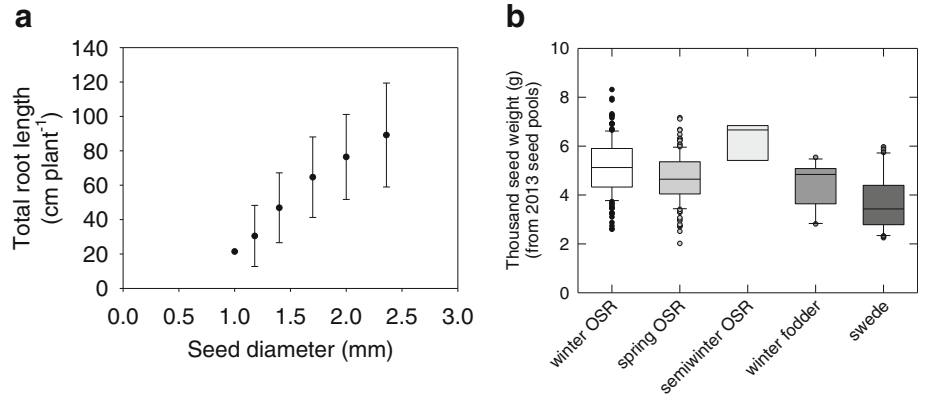

Fig. 1 a Total root length (TRL) as a function of *Brassica napus* seed diameter from a 'pouch and wick' system. Data represent mean ± standard error of the mean for all seedlings grown, from seeds with diameters of 1.18, 1.40, 1.70, 2.0 and 2.36 mm; $n = 44$, 1349, 2055, 2242 and 1059, respectively, averaged across 361 genotypes. **b** Thousand seed weight (TSW) of the *B. napus* seed used in all experiments in this study. Data are means of up to 320 genotypes, including winter OSR ($n = 142$), spring OSR ($n = 124$), semiwinter OSR ($n = 7$), winter fodder ($n = 14$) and swede ($n = 33$) habits. Boxes represent the mid two quartiles with the median drawn; whiskers are the 95 % confidence limits plus extremes

Semiwinter OSR had a shorter mean PRL than all other habits ($P < 0.001$, Fig. 2b), and a greater mean LRD ($P < 0.001$, Fig. 2f). It is important to note that these differences in root system size between OSR crop types were observed when seeds of uniform diameter were sown for each genotype. Increased root length in seedlings is likely to indicate increased early vigour. Velicka et al. [56] observed that early sowing afforded a greater root collar thickness and leaf number, and these earlier sown plants had greater over-winter survival and more rapid accumulation of matter in the apical bud in spring. Furthermore, Scott et al. [57] observed that earlier sowing significantly increased seed yield because of increased leaf and root growth. Seedling root-length traits, measured in this same 'pouch and wick' system, correlated with early plant growth and final seed yield in 30 commercial winter OSR *B. napus* genotypes [5]. Finch-Savage et al. [55] suggested that vigorous early root growth is

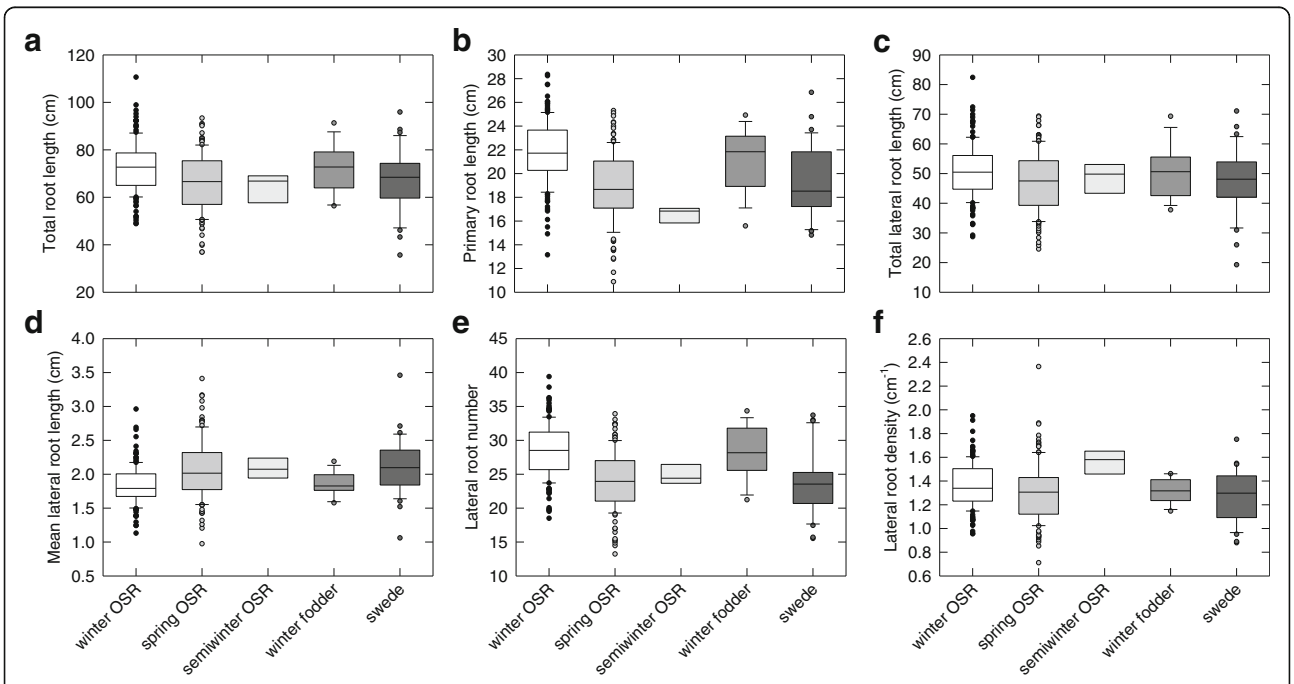

Fig. 2 Root traits of *Brassica napus* grown in a 'pouch and wick' system. Data are means of up to 319 genotypes, including winter OSR ($n = 142$), spring OSR ($n = 124$), semiwinter OSR ($n = 7$), winter fodder ($n = 14$) and swede ($n = 32$) habits. Boxes represent the mid two quartiles with the median drawn; whiskers are the 95 % confidence limits plus extremes. Panels **a**–**f** represent different root traits

essential for small seeded crops such as *Brassica* to acquire resources before desiccation occurs. A fast-growing, thick root collar contains large amounts of soluble carbohydrates which will enable the plant to withstand frost and afford a rapid re-growth in spring [58]. Thus, sufficient early root growth is necessary for winter crop survival and may have been selected for based on yield in previous breeding programs, whereas spring sown crops have less need for a rapid development to ensure hardiness.

Spring varieties typically had higher leaf concentrations of macronutrients and some micronutrients than winter varieties

The mean leaf concentration of 21 elements varied by more than six orders of magnitude across genotypes, from 0.01 mg kg^{-1} (As) to >50,000 mg kg^{-1} (K) (Fig. 3; Additional file 1: Table S7). Genotypic variation in leaf mineral concentration ranged from 1.8-fold (Fe) to >40-fold (Se). Among the macronutrients, leaf mineral concentrations varied 2.0-fold for P, 2.1-fold for K, 3.0-fold for Ca, 2.6-fold for Mg, and 2.5-fold variation in S. In comparison, among a panel of ~450 *B. oleracea,* also grown in compost and sampled during early vegetative growth, shoot concentrations of: Ca and Mg varied 2.0- and 2.3-fold [21], respectively; P and K varied 4.9- [59] and 3.4-fold [60], respectively. Among a panel of soil-grown 509 inbred lines of *B. napus*, the shoot mineral concentrations of 30 d old seedlings varied (approximately) 2.0-fold for Ca, 1.6-fold for Mg, 6.7-fold for P and 2.0-fold for K [23].

Winter OSR, winter fodder and swede had lower mean leaf macronutrient (Ca, Mg, P, K, S) concentrations than spring and semiwinter OSR (Ca, Mg; $P < 0.001$, Fig. 3). Semiwinter OSR also had higher mean leaf Ca and Mg concentrations compared to other habits ($P < 0.001$). Among the micronutrients, leaf Cu was greater in spring OSR than other habits ($P < 0.001$). Leaf Fe concentrations were greater in winter and spring OSR ($P < 0.001$). The mean leaf Mo concentrations were greatest in Spring OSR and swede ($P < 0.001$), although there was substantial variation within crop type. The mean leaf concentrations of beneficial and non-essential elements (As, Cd, Na, Se) were consistently higher in the semiwinter OSR leaves used in this study, typically followed by spring OSR (Fig. 3). Likewise, in the study of Bus et al. [23], winter OSR also had lower mean shoot Ca, K and S concentrations than spring and semiwinter OSR, but similar P concentrations and semiwinter OSR had the highest shoot S and Zn of the crop types. Despite these overall trends in the data, there is wide variation in shoot mineral composition within all crop types of *B. napus*, which will be influenced strongly by the nutritional environment in which the plant is grown as well as genotypic factors.

Variance components analysis (Table 1; Additional file 1: Table S10) shows that genotype had the largest influence on leaf S concentration (40 %) and the smallest influence on leaf Se concentrations (0 %). Habit accounted for the least variation in all traits (generally less than 10 %) but had the greatest effect on leaf Na, Mg and S concentration. The trends of heritabilities (i.e. genotype effect) for leaf composition traits follow a similar pattern to those observed previously in soil grown leaves of *Arabidopsis* [61], whereby leaf Mg was the most heritable macro nutrient in their study, and the second most heritable in this study. Leaf Ca, K and Mo concentration were ranked among the most heritable leaf composition traits in both studies. Leaf Fe, Mn and Cu concentration were among the least heritable traits in both studies. The variance components analysis indicates that the effect of experimental variance is generally higher for micronutrients than macronutrients.

Seed mineral concentrations were consistent across habits for many nutrients, but S concentrations were lower and Mo concentrations were higher in OSR types

The mean seed concentration of 15 elements varied by more than six orders of magnitude across genotypes, from 0.01 mg kg^{-1} (Cd) to >13,000 mg kg^{-1} (K) (Fig. 4; Additional file 1: Table S7). Genotypic variation in seed mineral concentration varied 1.7-fold (P) to 14-fold (Na). Among the macronutrients, seed mineral concentrations varied 3.1-fold for Ca, 1.9-fold for Mg, 2.0-fold for K, and 7.5-fold for S. We are not aware of previous reports of species-wide variation in seed mineral composition traits in a *Brassica* species. White and Broadley [14] reviewed variation in the mineral composition of edible cereal grains and dicot seeds for several species, typically core germplasm collections, which had been grown under comparative conditions. Among the dicots, seed Ca concentration varied 3.7-, 2.0-, 9.1-, 1.5- and 1.9-fold, and seed Mg concentration varied 2.4-, 1.4-, 2.3-, 1.3-, and 1.6-fold, for chickpea, peanut, pea, bean and soybean, respectively. Therefore, the seed macronutrient composition of this *B. napus* panel appears to be a similar range as other dicot species.

Winter and spring OSR had similar seed macronutrient concentrations, except for P, in which spring and semiwinter OSR had higher seed P concentrations than winter types ($P < 0.001$, Fig. 4) and Mg in which the semiwinter had higher concentrations than other types ($P < 0.001$). Winter fodder and swede had higher seed S concentrations than OSR crop types ($P < 0.001$), presumably due to the smaller proportion of "double-low" (low glucosinolate, low erucic acid) compared to "double-high" varieties, because winter fodder and swede have not been selected for low seed glucosinolate concentration. Glucosinolates are sulphur and nitrogen-containing secondary metabolites common in the *Brassicaceae* family [62, 63],

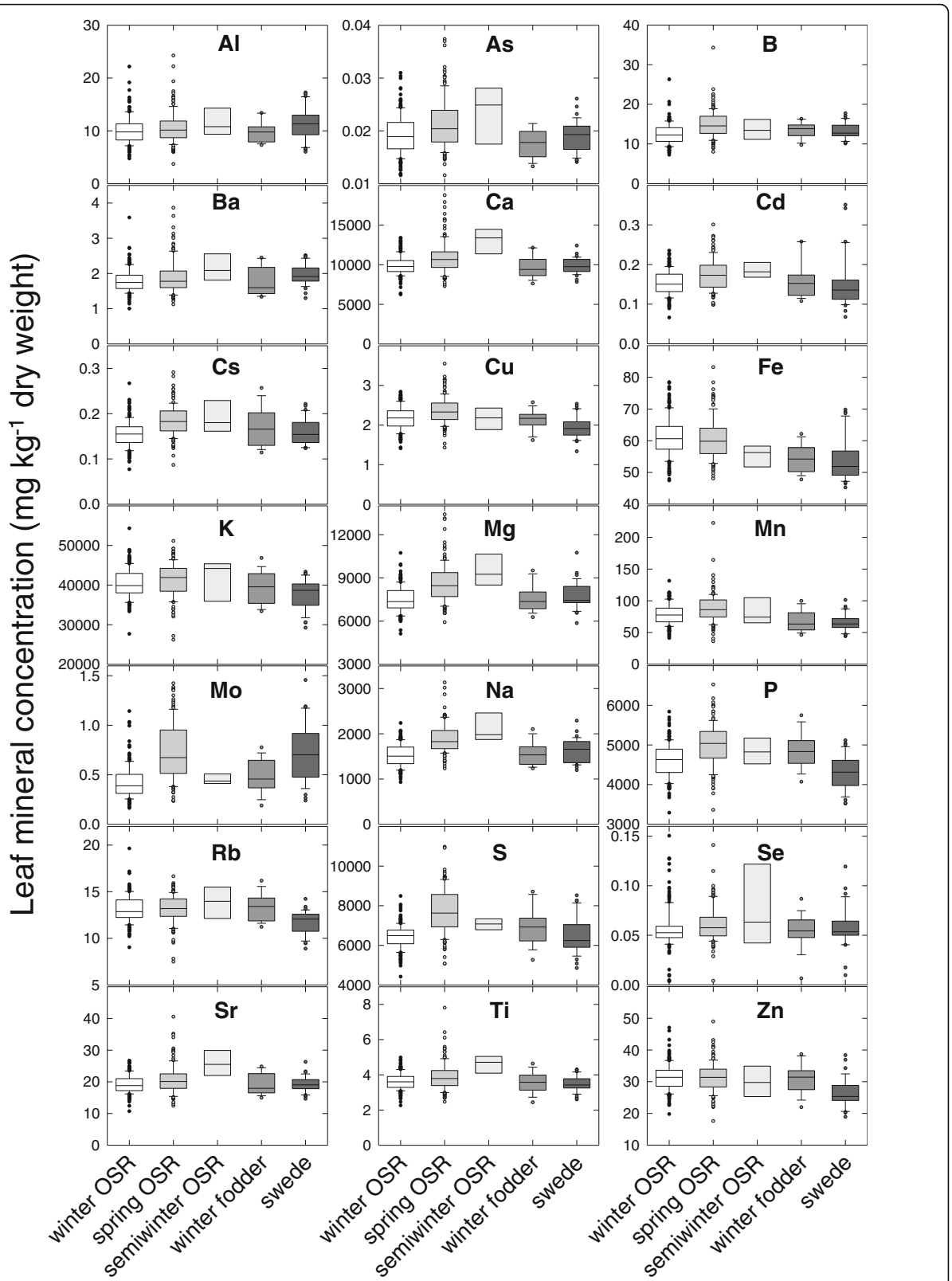

Fig. 3 Leaf mineral concentrations of *Brassica napus* grown in compost. Data are means of up to 385 genotypes, including winter OSR ($n = 163$), spring OSR ($n = 127$), semiwinter OSR ($n = 7$), winter fodder ($n = 15$) and swede ($n = 35$) habits. Boxes represent the mid two quartiles with the median drawn; whiskers are the 95 % confidence limits plus extremes

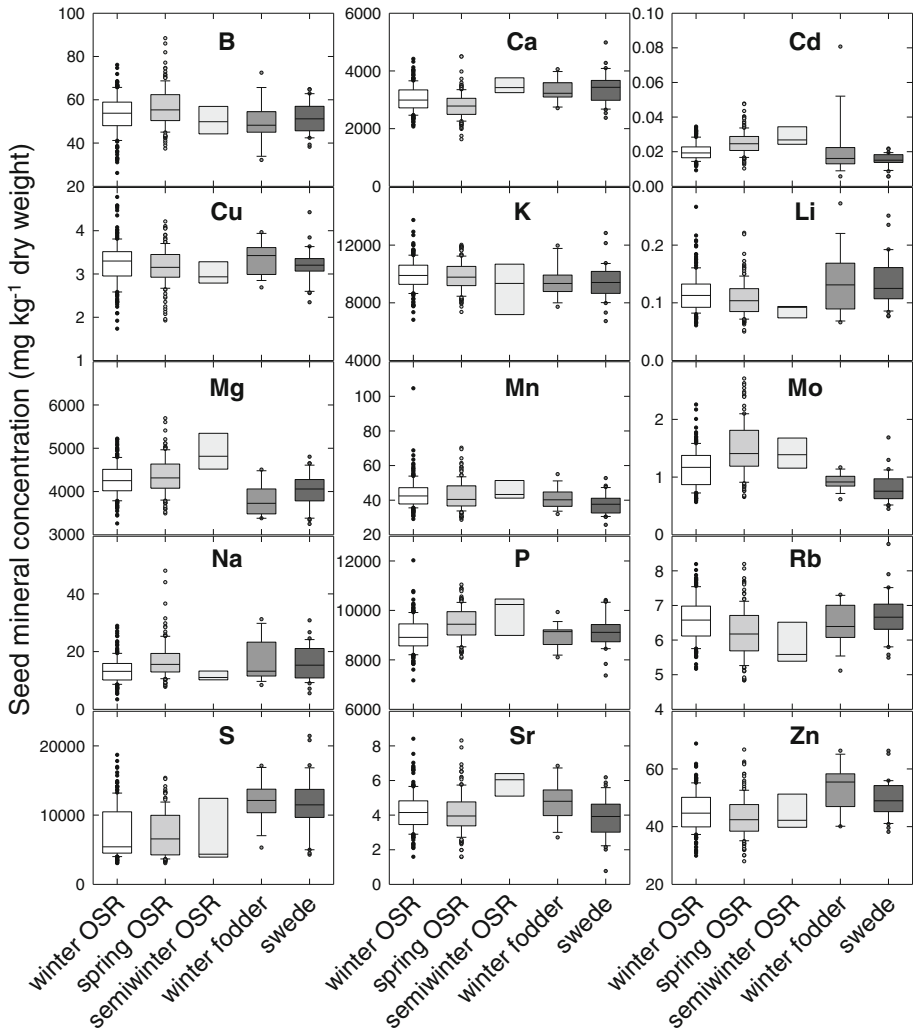

Fig. 4 Seed mineral concentrations of *Brassica napus* grown in compost. Data are means of up to 380 genotypes, including winter OSR ($n = 162$), spring OSR ($n = 127$), semiwinter OSR ($n = 7$), winter fodder ($n = 15$) and swede ($n = 31$) habits. Boxes represent the mid two quartiles with the median drawn; whiskers are the 95 % confidence limits plus extremes

and some genes of the sulphate assimilation pathway are members of the glucosinolate biosynthetic network [64]. Seed Mo concentrations showed the opposite pattern, with higher concentrations in OSR than other types ($P < 0.001$). An antagonistic effect of sulphate on Mo concentration in the shoot, root and seeds of OSR has previously been observed [65, 66]. Given that S and Mo are known to share some assimilation and transport pathways [67, 68], this could imply a relationship between seed glucosinolates, S and Mo. It has previously been suggested that tissue specific demand can regulate the expression of sulphate transporters [69]. It could be surmised that plants with low seed S/glucosinolates display a slightly different sulphate transporter profile leading to differences in Mo accumulation. Nevertheless, how S and Mo are interacting in relation to glucosinolates is yet to be determined. Consistent with S/Mo transport being under strong genetic control, variance components analysis (Table 1; Additional file 1: Table S10) showed that genotype had the largest influence on seed Mo concentration (41 %) out of all the elements analysed in this study. As observed previously in *Arabidopsis* seeds [61], Mo, Cu, S and K concentrations were influenced by genotype to a greater extent than seed Mg and P concentrations. Of the micronutrients, seed B, Cu and Mn concentrations were generally consistent across habits; B was highest in spring OSR ($P < 0.001$), Cu was highest in winter fodder and winter OSR ($P < 0.001$), Mn was highest in semiwinter OSR ($P < 0.001$). Zn concentrations were the highest in winter fodder ($P < 0.001$), Cd concentrations were highest in semiwinter OSR ($P < 0.001$), and seed Na concentrations were highest in spring OSR.

Differences in nutrient translocation between crop habits can be detected by comparing leaf and seed concentration ratios of elements with potentially similar transport or assimilation pathways

Four pairs of elements were selected, for which both leaf and seed concentration data were available, and for which there are reports of shared transport/assimilation pathways. These element pairs are S and Mo [67, 68], Ca and Sr [70, 71], K and Rb [72–74], and Zn and Cd [75]. The second element of each of these pairs is either not a nutrient, or, for Mo, is only required in very small amounts compared to the first element in each pair. The first hypothesis tested was that the ratio of first:second element concentration in leaves is ≫ 1:1, as expected from external nutrient supply and plant requirements. The second hypothesis was that the seed ratio/leaf ratio of elements is >1:1. For example, in the case of S and Mo, this would be expressed as $([S]_{seed}/[Mo]_{seed}) / ([S]_{leaf}/[Mo]_{leaf})$; Fig. 5a). A seed ratio/leaf ratio of elements >1:1 indicates that the net seed accumulation of the essential, or more abundant, nutrient element, is greater than the non-essential, or less abundant, nutrient. This could be due to selective processes in the source (e.g. increased mobilisation from the leaf) and/or sink (e.g. decreased remobilisation from the pod) tissues. As expected, the ratio of first:second element is ≫ 1:1 in leaves. For S:Mo, the mean of 387 genotypes is 15902:1 (range 4808–37661:1); for Ca:Sr 519:1 (433–604:1); for K:Rb 3106:1 (2652–3760:1); for Zn:Cd 200:1 (85–449:1). However, there was considerable variation in the seed/leaf ratio of elements (Fig. 5; Additional file 1: Table S7). For S:Mo, the differences between habits was most apparent. For the OSR habits, most OSR genotypes had seed ratio/leaf ratios <1:1, indicating net accumulation of Mo in seeds is greater than S in relative terms (Fig. 5c). In contrast, most swede genotypes and almost half of the winter fodder genotypes had a greater net accumulation of S than Mo. This relationship is indicative of a potential link between glucosinolates, S and Mo content of seeds but the nature of the interactions between these components has yet to be elucidated and is the focus of current studies on this population. For Ca:Sr, most genotypes showed slight increase in net accumulation of Ca compared to Sr in seeds, with swede habits having the highest mean (Fig. 5b). Interestingly, all genotypes showed increased net accumulation of Rb in seeds compared to K, with swede habits showing the lowest relative net accumulation of K compared to Rb (Fig. 5c). The reasons for this observation are not clear. For Zn:Cd, all genotypes showed increased net Zn accumulation in seeds compared to Cd.

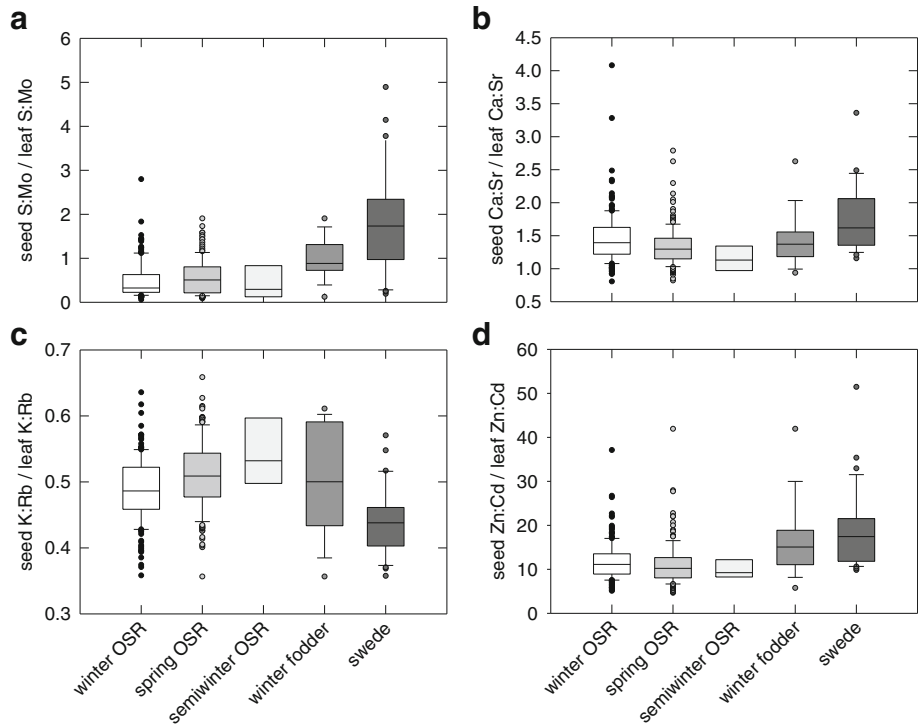

Fig. 5 Leaf and seed element concentration ratios of *Brassica napus* grown in compost. Data are means of up to 385 and 380 genotypes, for leaf and seed concentrations, respectively, including winter OSR ($n = 163$), spring OSR ($n = 127$), semiwinter OSR ($n = 7$), winter fodder ($n = 15$) and swede ($n = 35$) habits. Boxes represent the mid two quartiles with the median drawn; whiskers are the 95 % confidence limits plus extremes. Ratios are calculated, e.g. for Ca:Sr, as $([S]_{seed}/[Mo]_{seed}) / ([S]_{leaf}/[Mo]_{leaf})$. Panels **a**–**d** represent different element pairs

This was again greater in swede habits, and also winter fodder; both had greater net accumulation of Zn in seeds, compared to Cd, than did the OSR habits.

Root traits and leaf and seed mineral composition traits correlate within, but not between tissues

There were strong correlations between root traits. The strongest positive correlations were between traits relating directly to the total length of the root system (i.e. comprising PRL and LRL components, $P < 0.001$) and also between these traits and LRN ($P < 0.001$, Fig. 6; Additional file 1: Table S8). There was a weak negative relationship between PRL and LRD ($r = -0.28$; $P < 0.001$), since the latter is derived from LRN/PRL. In addition, there was also a weak negative relationship between MLRL and LRD ($r = -0.38$; $P < 0.001$), suggesting a trade-off between lateral root length and number. These correlations are consistent with previous observations on a much smaller panel, of 32 UK-field adapted OSR genotypes, grown in the same 'pouch and wick' system [5]. There were many strong positive correlations between the leaf concentrations of pairs of elements (Fig. 6; Additional file 1: Table S8). The

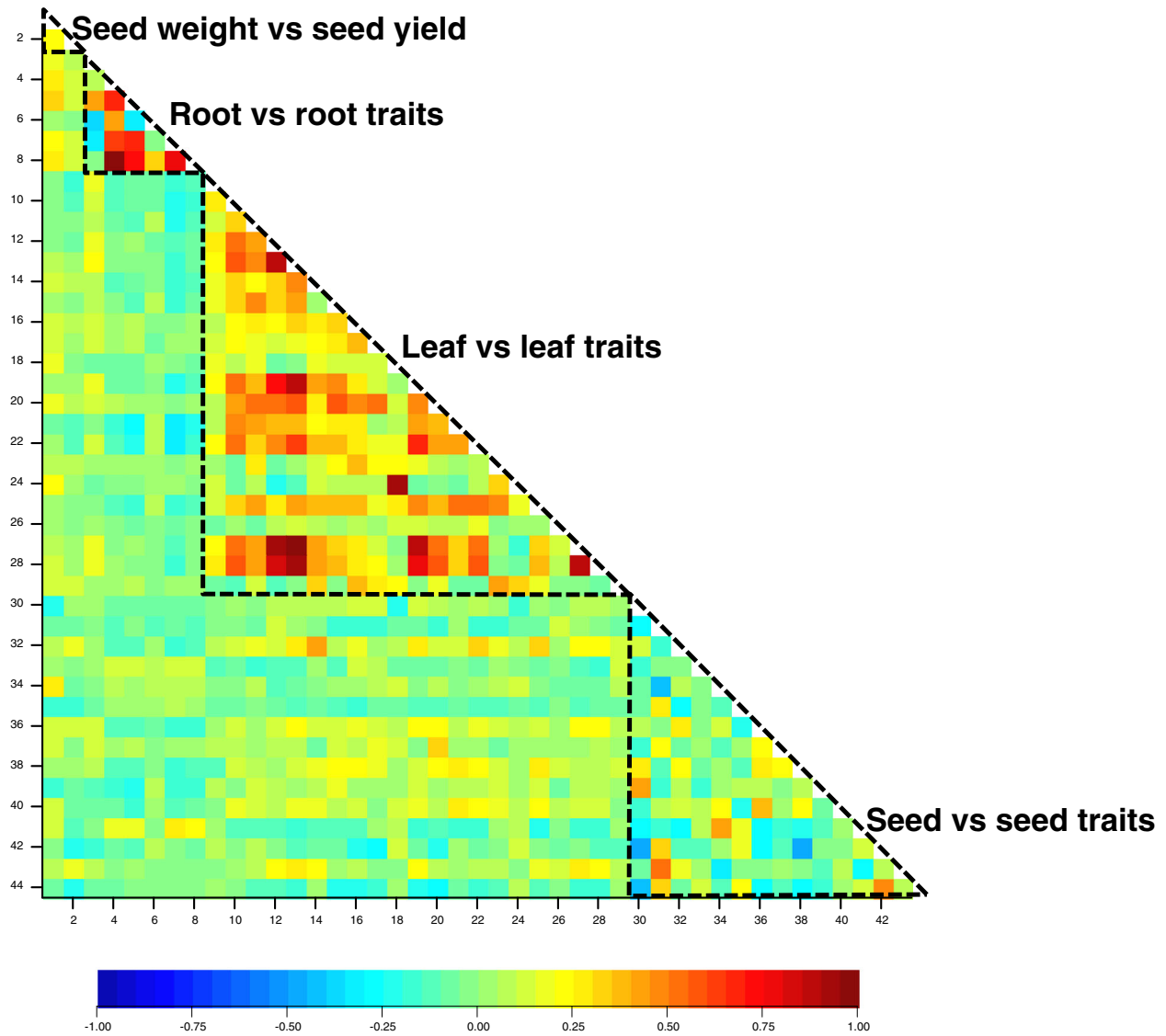

Fig. 6 Pair-wise correlations of all 44 traits; TSW and seed yield, root morphology and leaf and seed mineral composition traits in *Brassica napus* genotypes. Plants were grown in a 'pouch and wick' system (root traits, up to 319 genotypes) or compost (leaf and seed traits; up to 385 and 380 genotypes, respectively). Correlation coefficients are scaled from -1.0 (*dark blue*) to +1.0 (*dark red*) and are from genotype trait means. Traits: (1) TSW; (2) seed yield; (3–8) root traits: (3) LRD; (4) LRL; (5) LRN; (6) MLRL; (7) PRL; (8) TRL; (9–29) leaf concentration traits: (9) Al; (10) As; (11) B; (12) Ba; (13) Ca; (14) Cd; (15) Cs; (16) Cu; (17) Fe; (18) K; (19) Mg; (20) Mn; (21) Mo; (22) Na; (23) P; (24) Rb; (25) S; (26) Se; (27) Sr; (28) Ti; (29) Zn; (30–44) seed concentration traits: (30) B; (31) Ca; (32) Cd; (33) Cu; (34) K; (35) Li; (36) Mg; (37) Mn; (38) Mo; (39) Na; (40) P; (41) Rb; (42) S; (43) Sr; (44) Zn

strongest positive relationships were between leaf concentrations of Ca and Sr ($r = 0.97$), Sr and Ba ($r = 0.93$), K and Rb ($r = 0.92$) and Ca and Mg ($r = 0.87$); all $P < 0.001$. Positive correlations between the leaf concentrations of Group II elements reflect the relative lack of selectivity between these elements during transport within the plant [70]. Such relationships between Ca and Mg have been observed previously within-species, including among panels of diverse *B. oleracea* [21], OSR [23], Arabidopsis [61] and multi-species datasets [17, 73]. The strong positive relationship in the leaf concentration of Group I elements K and Rb is as expected from previous observation across many species [74]. However, the transport of other Group I elements is typically much more selective than Group II elements [71]. There were few negative relationships between the leaf concentrations of pairs of elements (Fig. 6), but weak negative relationships were observed between Rb and the Group II elements, Ba ($r = -0.24$), Sr ($r = -0.18$), and Ca ($r = -0.16$); all $P < 0.001$.

There were few strong positive correlations between the seed concentrations of pairs of elements (Fig. 6; Additional file 1: Table S8). The strongest positive correlation was between Ca and Sr ($r = 0.51$; $P < 0.001$), followed by between K and Rb ($r = 0.40$; $P < 0.001$). Both of these correlations were much weaker than those observed in leaves. The strongest negative correlations between the seed concentrations of pairs of elements were between S and Mo ($r = -0.47$), S and B ($r = -0.46$), and Ca and K ($r = -0.40$); all $P < 0.001$ (Fig. 6).

In general, leaf and seed mineral composition traits correlated very weakly (Fig. 6; Additional file 1: Table S8). The strongest positive correlation in a compositional trait between plant parts was a positive correlation between leaf Cd and seed Cd ($r = 0.41$; $P < 0.001$). All other correlations between leaf and seed mineral composition traits, and between root traits and leaf or seed composition traits were weaker, with correlation coefficients ranging from -0.26 to +0.33. Likewise, with the exception of P, no correlations were observed between elemental concentrations of leaf, root and seed tissues in *Arabidopsis* [61].

The strongest positive correlations between a seedling root trait and a leaf composition trait were between LRD and leaf Ca ($r = 0.16$; $P = 0.006$), Sr ($r = 0.15$; $P = 0.008$) and Ba ($r = 0.14$; $P = 0.01$) concentrations. A similar weak positive correlation between seedling LRD and leaf Ca (and Zn) concentrations was also seen previously in some field experiments [5]. Interestingly, PRL had weak, but significant, negative correlations with most leaf composition traits e.g. Mo ($r = 0.31$, $P = 0.001$) and Na ($r = 0.30$, $P = 0.001$). These data provide some evidence that LRD might be a beneficial trait for nutrient resource and above-ground biomass acquisition in *B. napus* [5]. Some leaf and seed mineral concentrations e.g. Ca and Mg and some beneficial elements (Fig. 3) of semiwinter OSR genotypes were greater than in other habits (Fig. 2f). Semiwinter OSR genotypes used as starting material for this study had the greatest LRD and greatest mean TSW of the five crop habits, which may have led to improved overall root size and function, and mineral acquisition in this study (Additional file 2: Figure S5). Semiwinter types are likely to have a distinct breeding history from winter and spring OSR habits due to having more introgressions from *B. rapa* and a longer period of domestication [76, 77]. These differences in pedigree might also explain some of the variation in root and shoot traits assigned in this present study to crop habit, and warrant further study in a range of environments.

Combining root, leaf and seed traits in a discriminant analysis is characteristic of crop habit

Combining all traits within the discriminant analysis provided the most accurate characterisation of crop habit (Fig. 7). Using the variate set of all traits combined, and after the addition of the 20 most informative trait variate sets, genotypes with the winter (86 %), spring (85 %) and semiwinter (81 %) OSR habits were correctly allocated to the correct group, as were 76 % of swede and 68 % of the winter fodder habits. The trait which contributed the most in terms of allocation to crop habit was leaf S concentration, followed by PRL, and seed Ca and Mo concentration, followed by the TSW of the 2013 seed from which plants were grown. The next most important root trait after PRL was MLRL, which was ranked 12th. The relative contributions of all 44 traits in this variate set to the discriminant analysis are presented in Additional file 1: Table S9. The discriminant analyses for each of the root-, leaf- and seed-trait variate sets was less accurate (Additional file 2: Figures S1–S4).

Conclusions

Brassica napus has been shown to be amenable to rapid marker identification linked to useful agronomic traits in wide diversity populations. For example, using associative transcriptomics (AT) with a panel of 84 *B. napus* genotypes from within the same panel used here, Harper et al. [37] showed that seed contents of both erucic acid and glucosinolates (GS) were associated with specific genes known to be involved in their biosynthetic pathways, whilst identifying additional new target loci of potential use in breeding. Similarly, Koprivova et al. [78] used a subset of this study's population to identify novel loci linked to shoot anion accumulation. The AT technique is based on transcriptome-sequencing, combined with association mapping. It uses transcribed sequences (mRNA-seq) which allows variation of gene sequences to be detected (through single nucleotide polymorphisms; SNPs) whilst reducing the complexity of the analysis compared to typical genome-wide association

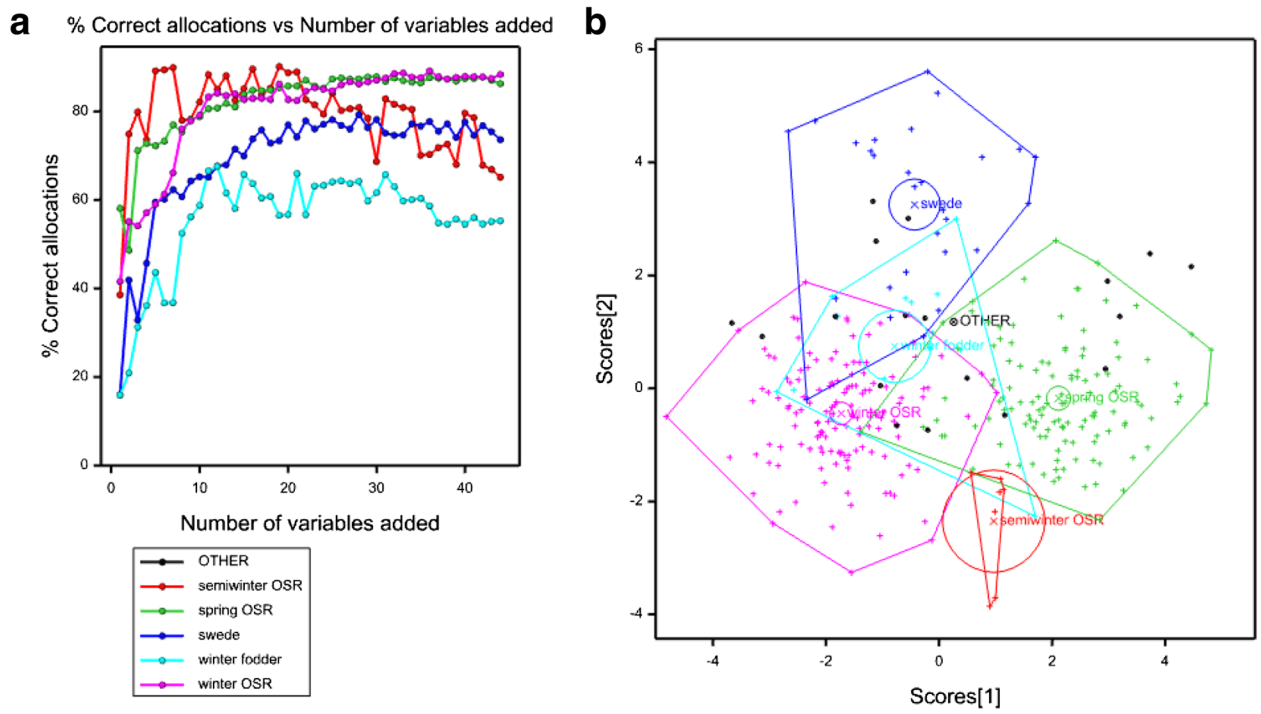

Fig. 7 Step-wise discriminant analysis using the variate set of all 44 traits; root morphology, TSW and seed yield and leaf and seed mineral composition traits combined. **a** Specificity plot showing the proportion of genotypes of each 'crop habit' correctly allocated to each group, at each step of the discriminant analysis; (**b**) discrimination plots drawn to represent the distribution of 'crop habits' in two dimensional variance space. 'Crop habit' means (X), 95 % confidence circles (*circles*) and group polygons enclosing all units for each crop habit are indicated

analysis (GWAS). In addition, transcript abundance (gene expression markers; GEMs) can be quantified simultaneously. Transcript abundance is likely to be of particular relevance in the control of traits in complex polyploid species in which gene duplication may lead to unequal expression of gene paralogues [79].

A substantial proportion of the same inbred population used in this study have also been used to study other traits, including those relating to nutritional composition and seedling growth, in other environments [23, 38–40, 80] and those based on leaf ionome traits [25–31]. The most accurate characterisation of crop habit is when multiple plant part traits are combined in analysis. Thus, combining multiple datasets using this panel has the potential for more accurate candidate trait identification, and to dissect the transport pathways which lead to altered elemental accumulation for crop improvement. The volume and throughput of data obtained from root phenotyping and ionomics platforms is considerable and the challenge now is to combine datasets for a better understanding of the ionome and the key traits involved in elemental accumulation, and to mine for the underlying molecular mechanisms of these useful traits using associative transcriptomics analysis.

Additional files

Additional file 1: Supplementary tables. A collection of tables containing a variety of extra data including raw data gathered in the experiments, limits of detection calculations, data used for analyses of traits, and data used for generation of some of the figures. Also included is a detailed ouput from variance components analysis and a list of the abbreviations used across the tables. (XLSX 1324 kb)

Additional file 2: Supplementary figures. A collection of extra figures which may be of interest to readers but that aren't in the main scope of the submission. Figures S1–S4 show step-wise discriminant analyses plots using different subsets of the traits measured (root morphology traits, leaf mineral composition traits, seed mineral composition traits, & seed yield traits respectively). Plots from analyses using the full set of traits are included as a main figure in the submission; see Fig. 7. Figures S5 and S6 are box and whisker plots of seed yield data by crop habit and thousand seed weight by genotype release date respectively. (PPTX 429 kb)

Abbreviations

Ag: Silver; Al: Aluminium; As: Arsenic; B: Boron; Ba: Barium; Ca: Calcium; Cd: Cadmium; Co: Cobalt; Cr: Chromium; Cs: Caesium; Cu: Copper; Fe: Iron; K: Potassium; LRD: Lateral root density; LRL: Lateral root length; LRN: Lateral root number; Mg: Magnesium; MLRL: Mean lateral root length; Mn: Manganese; Mo: Molybdenum; Na: Sodium; Ni: Nickel; P: Phosphorus; Pb: Lead; PRL: Primary root length; Rb: Rubidium; S: Sulphur; Se: Selenium; Sr: Strontium; Ti: Titanium; TRL: Total root length; TSW: Thousand seed weight; U: Uranium; V: Vanadium; Zn: Zinc

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Availability of data and materials
The data sets supporting the results of this article are included within the article and its additional files. Data are also available at the Brassica Information Portal (BIP; https://bip.earlham.ac.uk/; The Earlham Institute, Norwich, UK), doi:10.5281/zenodo.59927; doi:10.5281/zenodo.59937; doi:10.5281/zenodo.59936.

Authors contributions
CLT collected and analysed root and seed morphology trait data. TDA analysed leaf and seed mineral and discrimination data. RH and SM oversaw growth of plant material in polytunnels. LW and SDY oversaw ICP-MS analysis of leaf tissue. DES and JMCD oversaw ICP-MS analysis of seed tissue. AS advised on effects of S and Mo in plants. MRB and NSG oversaw project management and data analysis. All other authors contributed to project conception and advised on data analysis. All authors have read and approved the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Not applicable.

Author details
[1]School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK. [2]Ecological Sciences, The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK. [3]Distinguished Scientist Fellowship Program, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia. [4]School of Agriculture, Policy and Development and the Centre for Food Security, University of Reading, Whiteknights, PO Box 237, Reading RG6 6AR, UK. [5]University of Aberdeen, Institute of Biological and Environmental Sciences, Cruickshank Building, St Machar Drive, Aberdeen AB24 3UU, UK. [6]Department of Biology, University of York, Heslington, York YO10 5DD, UK.

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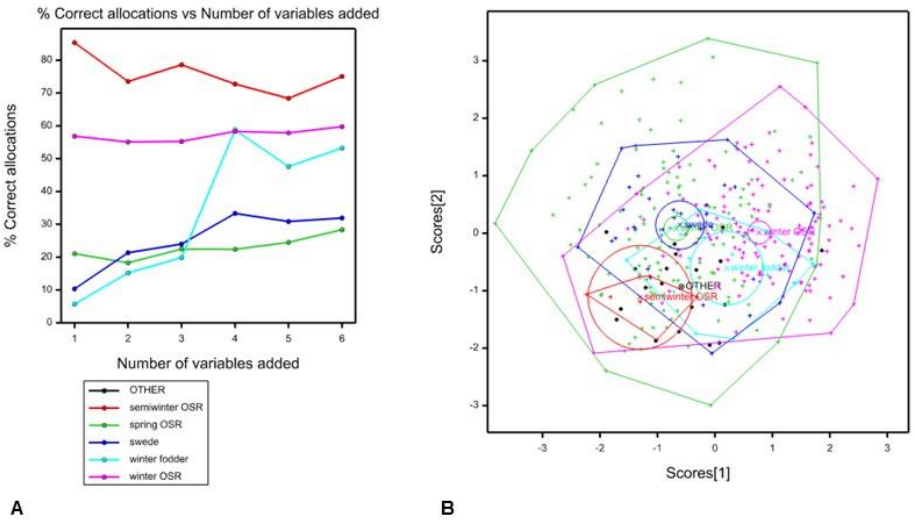
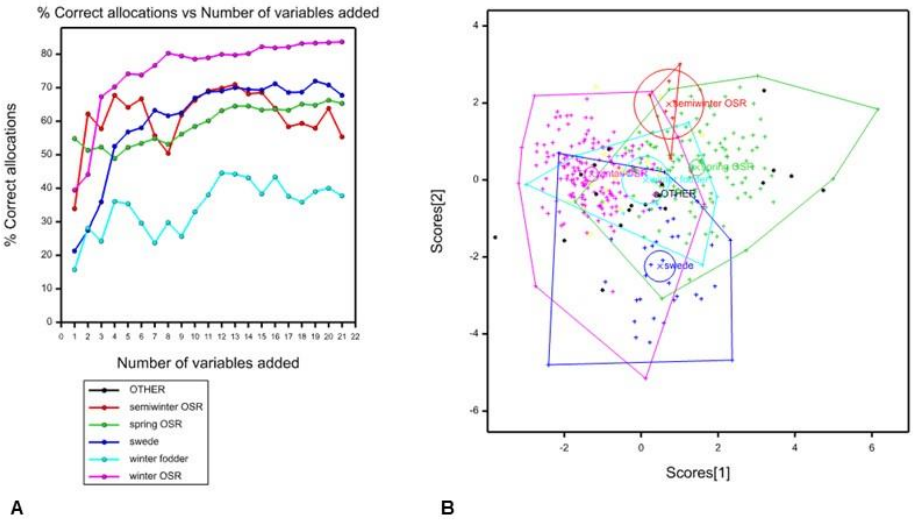
Supplementary Information

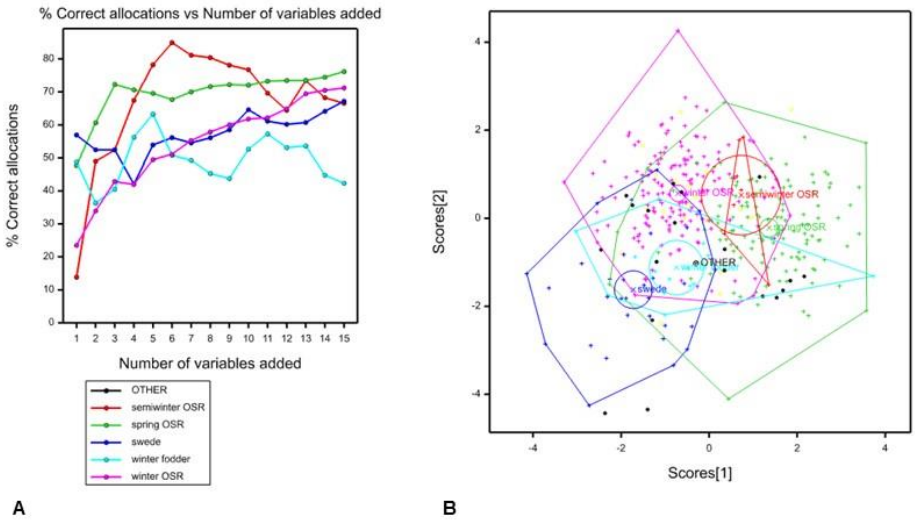
Supplementary Figures



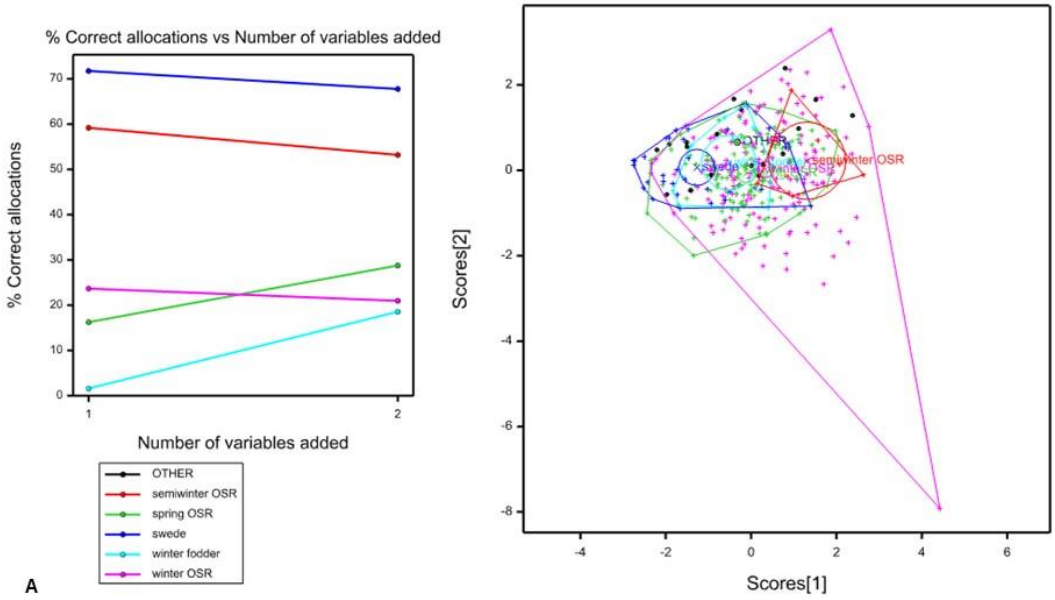
Supplementary Figure 1. Step-wise discriminant analysis using the variate set of 6 root morphology traits. (A) Specificity plot showing the proportion of genotypes of each 'crop habit' correctly allocated to each group, at each step of the discriminant analysis; (B) discrimination plots drawn to represent the distribution of 'crop habits' in two dimensional variance space. 'Crop habit' means (X), 95% confidence circles (circles) and group polygons enclosing all units for each crop habit are indicated.



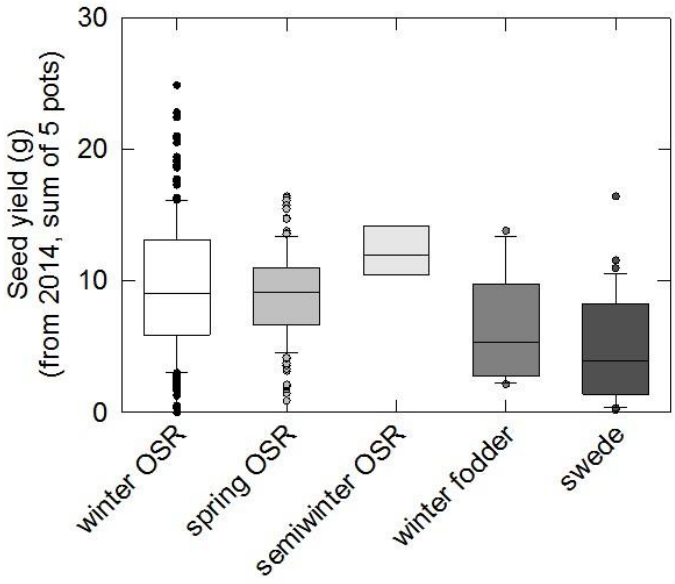
Supplementary Figure 2. Step-wise discriminant analysis using the variate set of 21 leaf mineral composition traits. (A) Specificity plot showing the proportion of genotypes of each 'crop habit' correctly allocated to each group, at each step of the discriminant analysis; (B) discrimination plots drawn to represent the distribution of 'crop habits' in two dimensional variance space. 'Crop habit' means (X), 95% confidence circles (circles) and group polygons enclosing all units for each crop habit are indicated.



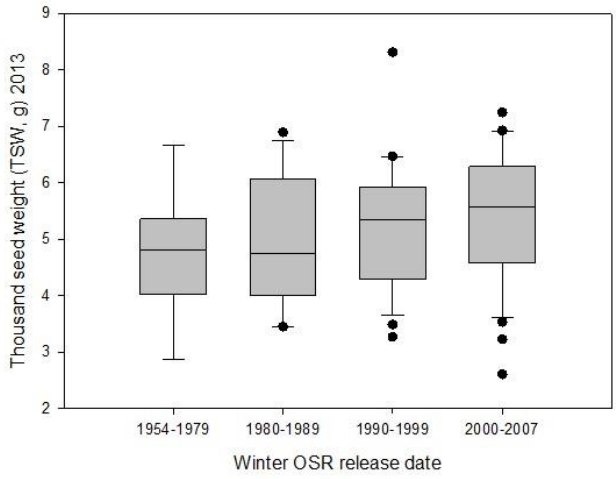
Supplementary Figure 3. Step-wise discriminant analysis using the variate set of 15 seed mineral composition traits. (A) Specificity plot showing the proportion of genotypes of each 'crop habit' correctly allocated to each group, at each step of the discriminant analysis; (B) discrimination plots drawn to represent the distribution of 'crop habits' in two dimensional variance space. 'Crop habit' means (X), 95% confidence circles (circles) and group polygons enclosing all units for each crop habit are indicated.



Supplementary Figure 4. Step-wise discriminant analysis using the variate set of 2013 TSW and 2014 seed yield traits. (A) Specificity plot showing the proportion of genotypes of each 'crop habit' correctly allocated to each group, at each step of the discriminant analysis; (B) discrimination plots drawn to represent the distribution of 'crop habits' in two dimensional variance space. 'Crop habit' means (X), 95% confidence circles (circles) and group polygons enclosing all units for each crop habit are indicated.



Supplementary Figure 5. 2014 seed yield. Data are means of up to 320 genotypes, including winter OSR (n=142), spring OSR (n=124), semiwinter OSR (n=7), winter fodder (n=14) and swede (n=33) habits. Boxes represent the mid two quartiles with the median drawn; whiskers are the 95% confidence limits plus extremes.



Supplementary Figure 6. Thousand seed weight of 2013 seed. Data are means of 84 winter OSR genotypes from different release periods. Boxes represent the mid two quartiles with the median drawn; whiskers are the 95% confidence limits plus extremes.

Chapter 5

Root, leaf mineral concentration and seed size traits in *B. napus;* Meta correlation and QTL/Associative Transcriptomics analyses

Abstract

Background. There is a need to identify novel genetic loci for improved crop nutrition. Screening diverse collections of germplasm will aid this process as there is limited diversity within existing elite varieties. Meta-QTL/GWAS (genome wide association scan) analyses for both root and shoot traits have not been performed previously in *Brassica napus*.

Methods. Root traits including primary root length, lateral root length and lateral root density were screened in a high throughput phenotyping (HTP) 'pouch and wick' system in 734 genotypes from two doubled-haploid populations; TNDH (Tapidor x Ningyou7) and TCDH (Temple x Canard) and in a diversity panel- ASSYST. Leaf concentrations of macro and micro nutrients were screened in glasshouse grown plants, and in the case of the TNDH population in the HTP system. Correlations were made between the root and shoot traits. Quantitative trait loci (QTL) analyses were performed in the TN and TC populations, and associative analyses for SNP and GEM (gene expression markers) in the ASSYST panel. Extreme genotypes with the longest lateral roots and the greatest number of lateral roots, as identified in the HTP system, were screened for more detailed analysis of root and shoot growth and leaf mineral concentration in the glasshouse.

Results and conclusion. Relationships between root traits and leaf mineral concentrations indicate that there may be a 'dilution-effect' whereby larger roots, such as a longer primary root, equate to larger plants, with a lower leaf mineral concentration, particularly of macro nutrients. Whereas, lateral root number and density related positively with some leaf minerals, particularly micro-nutrients. QTL identified for lateral root length on chromosomes A1/C1 and for lateral root density on chromosomes A9/C9 co-located across the populations. These QTL loci have previously been found to have genes controlling flowering time. Candidate genes were also identified for root hair cell differentiation. These could be candidates for

enhancing lateral rooting in Brassica. Furthermore, the up-stream regulation of root traits via the life-history pathway has not been reported previously.

Abbreviations: RSA (root system architecture), LRL (lateral root length), PRL (primary root length), MLRL (mean lateral root length), BZL (branching zone length), LRN (lateral root number), LRD-cmPRL (lateral root density per cm PR), LRD-cmBZL (lateral root density per cm BR), QTL (quantitative trait loci), SNP (single nucleotide polymorphism), GEM (gene expression marker), HTP (high throughput phenotyping), B (boron), Ca (calcium), Cu (copper), Fe (iron), K (potassium), Mg (magnesium), Mn (manganese), Mo (molybdenum), Na (sodium), P (phosphorus), S (sulphur), Zn (zinc), TSW (thousand seed weight), SD (seed diameter).

Introduction

Root system architecture (RSA) is the three-dimensional structure of the root system, including the length of primary/seminal roots, length, number and angle of lateral/adventitious roots, root diameter and root hairs. Roots are highly plastic and have been observed to alter structure in response to heterogeneous nutrient supply, and are therefore highly asymmetric (Lopez-Bucio *et al*., 2003). This is possible because the primary root grows continually through cell division and elongation in the root apical meristem, and lateral roots form post-embryonically in the differentiation zone behind the meristematic zone. Root hairs emerge from the zone of maturation, they are less than 10μm in diameter but can be as long as 1.3 cm, >100 million per day can be produced. Plant hormones including auxin (Evans *et al*., 1994), ABA (abscisic acid, DeSmet *et al*., 2003) and ethylene (Ivanchenko *et al*., 2008) play major roles in the initiation of lateral roots. Hence, under nutrient deficiency, resources are re-distributed to the root through carbon re-partitioning (Cakmak *et al*., 1994; Mollier and Pellerin, 1999), and the root to shoot ratio increases (Drew 1975; Mollier and Pellerin, 1999).

Nutrients in soil are often limiting for plant growth, particularly nitrogen (N), phosphorus (P) and potassium (K). Phosphorus has high immobility it may therefore be enriched in top-soil where it is applied (Lynch and Brown, 2001). Nitrogen is highly soluble and is easily leached, particularly in conventional agricultural settings (Hansen *et al*., 2000). Rapeseed is N inefficient compared to cereals (Bouchet *et al*., 2016). Phosphorus availability is affected by both high and low pH as is K, nitrates decrease at low pH but the ammonium form of N is more available. The available fraction in soil solution of Fe, Zn, Mn and Cu can also be insufficient, these nutrients are often bound to soil colloids and are principally transported by diffusion, thus solubilising these nutrients in the soil rather than transport across the plasma membrane is the more limiting factor (Grusak *et al*., 1999). Generally, micronutrients- B, Mn, Fe, Co, Cu and Zn are less available at high pH, although Mo is more available (Rengel, 1999). Thus, plants have to adapt their root structure differentially depending on the limiting conditions.

It been consistently observed that lateral root elongation increases in homogeneously low P and N, or in patches of high P and N in otherwise deficient conditions e.g. in *Arabidopsis* (Zhang and Forde, 1998; Sanchez-Calderon *et al*., 2006; Linkohr *et al*. 2005; Williamson *et al*., 2001). Sanchez-Calderon *et al*. (2006) observed a 500% increase in lateral root elongation under low P. The same has been observed in Barley (*Hordeum vulgare*, Drew, 1975). It has been suggested that because the concentration of ions in the shoot were not particularly low (Drew, 1975), and there was a response to nitrate limitation in mutants defective in nitrate metabolism, that it is not nitrate metabolism within the plant which is signalling an increase in lateral root growth, but the ion itself at the root interface- a localised stimulatory effect (Zhang and Forde, 1998). However, in Maize (*Zea mays*) grown under low P there was a small non-significant increase in lateral root elongation in the first 7 days of P deficiency, but this response diminished soon after. It was argued that the temporary increase in elongation was because of the smaller leaf and a concomitant lower demand for carbon in the leaf, but later the radiation use efficiency of the leaf was affected and carbon was re-distributed to the shoot, and therefore root changes are dependent on the time-scale of deprivation (Mollier and Pellerin, 1999). The effect of a deficiency in other nutrients on RSA is limited, but in *Arabisopsis* lateral root elongation has also

been observed in response to low Fe (Giehl *et al*., 2012; Gruber *et al*., 2013) and Mn (Gruber *et al*., 2013). In *Brassica*, greater lateral root elongation has been observed in seedlings under P and drought stress (Hammond *et al*., 2009; Akhtar *et al*., 2008, respectively). Similarly, the B-efficient cultivar had greater lateral root elongation than the B-inefficient cultivar (Xu *et al*., 2002), and the Zn efficient genotypes had greater fine root growth compared to inefficient genotypes (Grewal *et al*., 1997).

However, unlike lateral root elongation, the effects of a heterogeneous nutrient supply on primary root length and lateral root initiation are less clear. In *Arabidopsis* often the primary root gets shorter and the lateral root initiation increases (Williamson *et al*., 2001; Linkohr *et al*, 2005), however the increase in lateral root initiation can be seen to be virtually proportional to the decrease in primary root length. This result could be the result of growth defects or iron toxicity (Mollier and pellerin, 1999; Ward *et al*., 2008) rather than an adaptive response. For example, in barley and *Arabidopsis* neither Drew (1975) nor Zhang and Forde (1998), respectively, observed an effect of of N and P supply on root axis length. Conversely in rice (*Oryza sativa*) an elongation rather than reduction in primary root length has been observed (Wissuwa *et al*., 2003; Yi *et al*., 2005). Yet, it was observed in *Arabidopsis* that low N and Fe increased the branching zone length, which is the zone along the primary root from where lateral roots emerge (Gruber *et al*., 2013). Likewise, Sanchez-Calderon *et al.* (2006) observed in *Arabidopsis* that low phosphorus insensitive mutants did not reduce their primary root length and corresponding lateral root branching in response to low P whereas the wild type did, the P content of shoots was similar in the mutant and WT, indicating that the root response of the WT was not simply a growth response. They suggest that this primary root response was the result of root meristem exhaustion, resulting in a dis-continuation of cell division. Lateral root initiation has also been shown to increase under a deficient P supply independently of a decrease in primary root length, in *Arabidopsis* (Sanchez-Calderon *et al*., 2006; Gruber *et al*., 2013; Perez-Torres *et al*., 2008) and barley (Drew, 1975). Furthermore, lateral root primordia increased in response to low Fe (Giehl *et al*., 2012). Although in *Arabidopsis* there was no effect of low N on lateral root initiation (Zhang and Forde, 1998). Moreover, Pariasca-Tanaka *et al*. (2009) observed that most genes up-regulated were stress responses to P rather than tolerance responses.

Root hairs have been observed to increase in length and density independent to reduced radicle elongation under low P, and P uptake per unit root length was increased in *Arabidopsis* (Bates and Lynch, 2000, 2001; Ma *et al*., 2001), in maize (Zhu *et al*, 2005) and common bean, for which heritability was high for root hair traits in solution culture- 53-63% (Yan *et al*., 2004). Increased root hairs have also been observed in response to low Fe (Moog *et al*. 1995; Schmidt 1999; Giehl *et al*., 2012). In *Arabidopsis* Zinc and Mn increased root hair density by 70 - 200%, whereas Ca, Mg, B and Cu had no effect, and N, K and S decreased length (Ma *et al*., 2001). In *B. napus* Hu *et al.* (2010) observed a large increase in root hairs in a P-efficient cultivar after 3 weeks under low P conditions. A shallower lateral root angle has also been demonstrated to increase P uptake under low P (Bonser *et al*., 1996).

Lynch (2011) argues that there are cost/benefits to different root traits. For example, in maize seminal roots play a dominant role in water supply, whereas they acquire less P than nodal roots (Hodge *et al*., 2009). The thickness of the root is strongly correlated with the diameter of the xylem vessels and thick roots contribute to better water flux. A deep root system able to extract water at depth is the most consensual of the traits contributing to drought resistance (Courtois *et al*., 2009). Specific root length is the length to mass ratio of a root fragment (Ostonen *et al*., 2007). Fitter (1991) has proposed that the length/mass ratio is an index of root benefit to root cost because the length is proportional to resource acquisition and the mass to construction and maintenance. Likewise, Gordon and Jackson (2000) observed an inverse relationship with root diameter and nutrient concentrations. Thus, under normal nutrient supply in conventional agriculture the advantage of different root architectures is uncertain. Despite the evidence for root plasticity, genetic loci controlling RSA have been identified, and root plasticity is thought to be under some degree of genetic control (Malamy *et al*., 2005).

There have been far fewer studies on genetic loci controlling the root architecture of Brassica, particularly for seedling root traits (Fletcher *et al*., 2014; Hu *et al*., 2010; Lu *et al*., 2008; Shi L *et al*., 2013; Hammond *et al*., 2009; Yang *et al*., 2010, 2011; Zhang *et al* 2016, Zhang *et al*., 2015) than for yield-related traits e.g (Chen *et al*., 2010; Dechaine *et al*., 2014; Fletcher *et al*., 2014; Schiessl *et al*., 2015; Shi J *et al*., 2009; Shi T *et al*., 2013; Korber *et al*., 2016). Likewise, mineral concentration in Brassica

(Broadley *et al*., 2008; White *et al*., 2010; Wu *et al*., 2008; Liu *et al*., 2009; Bus *et al*., 2014; Hammond *et al*., 2009, 2011; Yang *et al*., 2010, 2011; Zhao *et al*., 2007; Koprivova *et al*., 2014; Ding *et al*., 2010) has been studied less often. Other traits related to life-history such as flowering time, and maternal effects such as seed size (Basnet *et al*., 2015; Dechaine *et al*., 2014; Fletcher *et al*., 2014) are also relevant for root vigour. In Brassica, relationships between root architecture and leaf mineral concentrations have been studied rarely, these have examined the relationships with P uptake (Hammond 2009; Hu 2010; Shi L 2013) and Zn toxicity (Wu 2008). In *B. oleracea*, QTL for measures of PUE were located predominantly on chromosomes C1 and C7, and lateral root number, length and growth rate correlated positively with measures of PUE (Hammond *et al*., 2009). In *B. rapa*, there was a negative relationship between taproot thickness and length (Lu *et al* 2008). In *B. napus* a HTP system identified QTL for root traits on chromosomes A7, C6 and C9 (Shi L *et al*., 2013). QTL co-located for phosphorus uptake and root length particularly on chromosomes A3 and C3 (Yang *et al*., 2010). However, because of a lack of confirmation of QTL across populations and environments (Zhou *et al*., 2014), root trait QTL are yet to be used directly for Brassica crop improvement.

GWAS (genome wide association studies) use panels of genetically diverse genotypes with a greater range of alleles, thus compared to bi-parental populations e.g doubled-haploid (DH) there is greater potential to find variability for traits. Furthermore, compared to bi-parental populations, within a diversity panel there is greater recombination within the genome, thus where there is linkage it is within a smaller region, enabling more refined detection of associated loci. Associative transcriptomics (AT) identifies SNPs (single nucleotide polymorphisms) from transcribed sequences (mRNA-seq), thereby revealing variation in both gene sequence and gene expression (Harper *et al*., 2012). Applying this technique in *B. napus* has been shown to be highly effective in identifying previously identified genes for seed glucosinolates (Harper *et al*., 2012), and has identified candidate genes for increased seedling root length under water stress (Zhang *et al*., 2015).

The aims of this study were to link root architecture traits in seedlings, and seed size, to leaf mineral concentration in three *B. napus* populations; two mapping populations and a diversity panel, as well as to identify QTL/markers for the traits. A BLAST of

candidate gene sequences identified through the GWAS identified a number of orthologous genes related to lateral root initiation in *Arabidopsis*. To our knowledge, a meta-analysis of root traits and leaf mineral concentration in *B. napus* has not previously been performed.

Materials and methods

B. napus plant material

The Temple x Canard doubled-haploid mapping population (TCDH, OREGIN: Oilseed Rape Genetic Improvement Network, 2008-13) consists of 142 F^2 homozygous lines derived from a cross by microspore culture (Elsoms Seeds Ltd, UK). The female parent Temple is a European double-low (low seed erucic acid and glucosinolate content) winter OSR variety with good NUE (Nutrient Use Efficiency), high oil and good autumn vigour. Canard is a European double-high forage variety with low % N, P, K in shoots and average autumn vigour (OREGIN, 2008-13). This study has used 128 of these genotypes and the parents.

The Tapidor x Ningyou7 doubled haploid mapping population consists of 202 F^2 homozygous lines derived from a cross by microspore culture (Qui *et al*., 2006). The female parent Tapidor is a double-low European winter OSR variety (i.e has a high vernalisation requirement). Ningyou7 is a double-high Chinese semi-winter OSR variety (i.e has a low vernalisation requirement). Tapidor was characterized as a P-inefficient cultivar with lower growth and P acquisition than Ningyou7 under low P and optimal P conditions in pot culture and field trials (Shi *et al*., 2010; 2013). This study has used 201 of these genotypes and the parents.

The ERANET-ASSYST (Associative expression and systems analysis of complex traits in oilseed rape) is a *Brassica napus* diversity panel of over 500 genetically diverse inbred or DH lines, originating from across Europe, Asia and North America (Bus *et al*., 2011; Körber *et al*., 2012). This study focused on 362 of the core panel genotypes which had available seed, comprising of 164 winter, 125 spring and 7

semiwinter OSR types, and 14 winter fodder, 33 swede and 19 exotic/unspecified types.

High throughput phenotyping (HTP) of root traits

The populations, as well as two commercial OSR cultivars used as a reference for normalisation (Broadley *et al*., 2010), were screened for root architecture; primary root length (PRL), lateral root length (LRL), mean lateral root length (MLRL: LRL/LRN), branching zone length (BZL: length of PRL with emerging lateral roots, in the TC population only), lateral root number (LRN) and density (LRD^{-cmPRL} = LRN/PRL and LRD^{-cmBZL} = LRN/BZL). Additionally, in the ASSYST population the seed diameter of each seed sown was measured and recorded (according to the methods of Thomas *et al*., 2016[b]). Briefly, seedlings (n = ~24 per genotype) were grown for 14 days in a ¼ strength Hoagland's solution in a 'pouch and wick' hydroponic-based HTP system (Atkinson *et al*., 2015). Each genotype was grown in a single run in a randomised position in each of 3 columns in each of 4 growth tanks. Semi-automatic software *RootReader2D* (Clarke *et al*., 2013), was used to measure root growth. Methods according to Thomas *et al*. (2016 [a,b]).

Growth conditions and sampling for leaf mineral analyses

The TCDH population, including two plants each of 134 lines and ten plants each of the parental lines were grown in Desch Plantpak (Essex, UK) 104 modular propagation trays filled with fine-grade (<3 mm particle size) compost-based growing media (Levington Seed & Modular + Sand -F2S; Everris Ltd., Ipswich, UK). Propagation trays were covered with perlite and transferred to a glasshouse with a daytime temperature of 15°C, venting at 15°C, and a night time temperature of 5°C, venting at 15°C. At the rosette stage (typically 5-6 true leaves showing) the 4th leaf of each plant and the whole shoot of the parent plants were dried in an oven at 50°C for two days for downstream mineral analysis. The whole shoots of the parent plants were weighed once dried.

The TNDH population and the parents were grown as above in the HTP system, under a zero P concentration using a modified Hoagland's solution (according to the methods of Zhang *et al*., 2016). For each genotype all ~24 seedlings were pooled to

acquire an adequate leaf sample quantity. All leaf samples were dried in paper bags at 50°C for 48 h for downstream mineral analysis.

The sample leaves of each genotype were hand-crushed to a powder and 0.05-0.1 g was subsampled for elemental analysis. Samples were digested using a solution of 1 mL 30% H_2O_2, 2 mL 50% trace analysis grade (TAG) HNO_3, and 1 mL milli-Q water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK). Solutions were placed in a Multiwave 3000 microwave with a 48-vessel 48MF50 rotor (Anton Paar GmbH, Graz, Austria) and heated for 45 min at a controlled pressure of 2 MPa, within vessels comprising perfluoroalkoxy (PFA) liner material and polyethylethylketone (PEEK) pressure jackets (Anton Paar GmbH). Digested samples were diluted to 15 mL with milli-Q water and stored at room temperature. Immediately prior to analysis, digested samples were diluted 1-in-10 with milli-Q water. The concentrations of 28 elements were obtained using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany); Ag, Al, As, B, Ba, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, U, V, Zn. An external Certified Reference Material was included (IAEA-359 Cabbage; LGC, Teddington, UK). For each data-point, element-specific operational blank concentrations (from ICP-MS run means) were subtracted. Data were then multiplied by initial sample volume, divided by the initial dry mass of plant material, and converted to mg element g^{-1} of dry leaf material.

The collection of leaf mineral data in the ASSYST panel have been described in Thomas *et al*. (2016 [b]) and correlations with root traits will be outlined, however only results from the associative transcriptomics analyses in root traits and not leaf mineral traits will be discussed in this work.

Seed diameter and TSW analyses (seed size)

In the TCDH and TNDH populations, seeds of 1 seed packet of each genotype were sieved using mesh with a diameter (⌀) of 1.4, 1.7, 2.0 and 2.36 mm (Scientific Laboratory Supplies Ltd, Hessle, UK). Seed retained within the mesh of each sieve were weighed and a weighted-average seed diameter calculated (sum % of each seed size category * seed diameter mm)/100 for each genotype. In the ASSYST panel the

thousand grain weight (TGW) of each genotype was calculated by counting 1000 seeds using an automatic seed counter (Contador seed counter, Hoffman Manufacturing, Oregon, USA), and weighing the total seed. Seed diameter and TGW are equivalent measures of seed size with a strong relationship (Karakoy *et al*., 2012).

Statistical analyses of root, seed diameter and leaf mineral data

In the root and leaf mineral concentration data, outliers with a mean > 2 SDs of the global mean were removed. In the seed data, samples were removed if the total seed weight was less than 1g. To account for the 'run' effect across experiments, REML (Residual Maximum Likelihood) adjusted means were calculated for root traits, whereby genotype was classed as a fixed factor and experimental sources of variation were classed as random factors according to the model: genotype+run/frame/column/reservoir/paper-side. In the ASSYST population the REML model also included seed diameter as a random factor. Pearson's correlation coefficients were calculated for the relationships between root traits, seed diameter/TSW and leaf mineral concentrations. T-tests were used to calculate the trait differences between the DH parents. In the ASSYST panel ANOVAs were used to calculate trait differences between 'types'. All statistical analyses were conducted using GenStat 15th Edition (VSN International Ltd, Hemel Hempstead, UK).

Genetic map construction

Plants of the TCDH population and the parents were grown as above under glasshouse conditions. At the rosette stage (typically 5-6 true leaves showing) the 3rd leaf was removed from each plant, pooled and snap-frozen in liquid nitrogen and stored in foil wrappers at -80°c for downstream RNA extraction. Furthermore, 5 seedlings each of the parents Temple and Canard were grown for 7 days on blue germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA) in petri dishes supplied with a 25% Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture, Sigma Aldrich, Dorset, UK) made with deionised water, in a growth room, and roots were sampled and were immediately frozen in liquid nitrogen and stored at -80°c for downstream RNA extraction. All samples were ground in liquid nitrogen using a mortar and pestle. RNA was extracted from subsamples of ~150mg using the EZNA Plant RNA kit (VWR International Ltd., Lutterworth, UK) as per manufacturer's instructions using

the on-column DNase treatment. RNA quality and quantity was measured using a NanoDrop spectrophotometer (Thermo Scientific). mRNA-seq analysis with 50bp single-end reads was performed on triplicate samples using an Illumina HiSeq 2000 (The Genome Analysis Centre (TGAC), Norwich, UK). A synthetic *B. napus* reference genome sequence was constructed by combining the pseudo-chromosome sequences from *B. oleracea* c.v. TO1000 (Parkin *et al*., 2014) and *B. rapa* c.v. Chiifu (Wang *et al*., 2011). The sequenced reads from the parental lines were aligned to this synthetic reference using TopHat2 version 2.0.11.Linux_x86_64 (Kim *et al*., 2013). SNPs were identified between lines and the reference genome using samtools version 0.1.19-44428cd (Li *et al*., 2009). SNPs were filtered to exclude those with a heterozygous call. SNP calls were designated as 'ambiguous' or 'known' using a phred score threshold of 15. Sequence reads from each individual line were then similarly aligned. SNP calls were sorted by chromosomal position in the synthetic reference, and 'ambiguous' base calls at a SNP locus (due to lack of read coverage in a particular line) were imputed as follows; in each line, each chromosome was partitioned into segments with parental heritage for a segment assigned as either 'ambiguous', or 'known', depending on the 'known' SNP calls found at the loci. For a segment to have known heritage assigned, it was necessary for at least 15 consecutive 'known' SNP calls to indicate one parent, rather than the other. In such segments with 'known' parentage, SNP loci within the segment with ambiguous base calls were assigned to the 'known' parent. After imputation of missing base calls on this basis, SNPs were 'binned' by removing consecutive loci with identical base calls across all lines. Markers were manually selected to represent all recombinations and excess markers removed.

TNDH population map construction was according to the methods of Zhang *et al*. (2016). Briefly, the population and the parents were genotyped using the Brassica 60 k SNP BeadChip Array (Illumina Inc., USA). Positions of A-genome SNPs were provided by the array manufacturer, while C genome SNP source sequences were subjected to a BLAST search against the *B. oleracea* genome database (BRAD, http://brassicadb.org/) to locate chromosome positions (E value ≤1e-50).

ASSYST panel associative transcriptomics was performed according to the methods of Harper *et al*. (2012) and Bancroft (2013). Briefly, transcriptome sequencing (mRNA-

Seq, Illumina, TGAC, Norwich) of the leaves of 387 genotypes of the *B. napus* diversity panel provided data on quantitative expression of genes and sequence variation of transcripts. Marker order was determined via pseudomolecules.

QTL analyses for root, seed diameter and leaf mineral concentration in the DH populations

Quantitative Trait Loci analyses were conducted in the TC (n = 122) and TN (n = 180) populations, with arithmetic means with outliers removed and REML adjusted means. A CIM (Composite Interval Mapping) procedure (Zeng, 1994; Jansen and Stam, 1994) was used, whereby cofactor QTL were sequentially added to the model in order of their significance until the QTLs were no longer significant. This procedure uses a Haldane mapping function, the default settings were selected whereby the genetic predictor step size was set to 1 million cM, the co-factor proximity was set at 50 cM and the threshold of significance was 0.05 (Li and Ji, 2005). The additive effect and estimated percentage of phenotypic variation explained by each putative QTL were obtained. The TCDH and TNDH populations had LOD (Log10likelihood ratio) scores of 3.67 and 3.88 respectively, determined at a 0.05 significance level. QTL confidence intervals (CI) were determined by 2 LOD intervals surrounding the QTL peak. Analyses were conducted using GenStat 15th Edition (VSN International Ltd, Hemel Hempstead, UK).

Associative Transcriptomics analyses for root and TGW traits in the ASSYST panel

In the ASSYST panel (n = 339) association analyses were performed between traits and SNP (single nucleotide polymorphism) markers and GEMs (gene expression markers), using arithmetic means with outliers removed and REML adjusted means. False positive associations between genotype and phenotype are created by population structure and kinship. Therefore, population structure was first analysed using STRUCTURE (Pritchard *et al*., 2000), with a burn-in period of 10,000, MCMC (Bayesian Markov Chain Monte Carlo model) steps at 10,000, an admixture model was selected assuming 1-10 values of K, 3 iterations for each value of K were processed. The Evano (2005) method was used to select the value of K that best fitted the data and assign genotype group membership. CLUMPP (Jakobsson and Rosenberg, 2007) was used to concatenate results into a Q matrix. Relatedness

(kinship) between pairs of individuals was assessed using TASSEL, producing a kinship matrix (K matrix). In TASSEL, markers with minor allele frequencies less than 5% were first removed. A mixed linear model (MLM) which controls for population structure (using the Q matrix) and pairwise relatedness (using the K matrix) performed the SNP marker-trait association analyses. GEM-trait associations were calculated by a fixed effect linear model using R (http://www.R-project.org/), with RPKM (reads per kb per million aligned reads) values and the Q matrix used as the explanatory variables. Marker associations were deemed significant above the FDR (false discovery rate) threshold. The genes were functionally annotated with the best search result after local BLASTX searches in the curated Swissprot database downloaded from NCBI (http://www.ncbi.nlm.nih.gov/).

ASSYST genotypes with the extreme phenotypes maximum LRD and MLRL

HTP had previously indicated a negative relationship between mean lateral root length and lateral root density, therefore a trade-off between having either fewer longer lateral roots or shorter but a greater number of lateral roots. It was hypothesised that these different phenotypes would have implications for nutrient acquisition. Genotypes of the ASSYST panel were selected as candidates from the initial HTP screen, which displayed the maximum LRD; 5, 260, 369 and maximum MLRL; 333, 355, 361, and had an approximately equal PRL and TSW (**suppl. Fig. 1**). The genotypes are all European spring OSR types, except genotype 5 which is a winter type, and genotype 260 which is Australian. Outlier genotypes which had a coefficient of variation (CV) greater than 50% had first been removed from the data. The genotypes were re-screened in the HTP system, alongside genotypes which had been selected for displaying the respective minimum LRD and MLRL, to confirm the phenotype (following the methods above, n = 20). Twelve plants of each genotype were grown in the glasshouse for sampling at the 5-6 leaf stage for 4th leaf mineral analysis, and shoot and root dry weight (after roots had been washed) (n = 6). The remaining 6 plants were grown until maturity for assessment of mature plant shoot and root dry weight and seed yield (following the methods above). A REML procedure calculated the main effects of ‘phenotype’ and shoot dry weight (in order to assess the biomass dilution effect) on the traits, including ‘genotype’ as a random factor.

Results

Root traits, seed size and leaf mineral concentration

In the TC population, 33% of the seedling root data samples were classed as 'stunted' and removed from analysis, and a further ~3% were removed as outliers. The population showed transgressive segregation in all root and leaf mineral concentration traits. Root trait means ranged from; TRL- 55-125 cm, PRL- 14-27cm, LRL- 48–113 cm, MLRL- 2.0-4.3 cm, BZL- 6-11 cm, LRN- 18-37, LRD^{-cmPRL}- 1.0-1.8 and LRD^{-cmBZL}- 2.3 - 4.0. Parent Canard as compared to Temple had greater root length; primary root length, lateral root length ($t = 2.7$, $P < 0.01$), lateral root number ($t = 2.9$, $P < 0.01$) and lateral root density ($t = 2.0$, $P < 0.05$), but a shorter mean lateral root length ($t = 2.9$, $P < 0.01$). Canard had greater leaf mineral concentrations; Mo ($t = 4.7$, $P < 0.001$), K ($t = 3.2$, $P < 0.01$) and Na ($t = 3.2$, $P< 0.01$), however Temple had a greater concentration of B ($t = -3.1$, $P < 0.01$). Canard also had a greater shoot dry weight at the 5-6 leaf stage ($t = 2.4$, $P< 0.05$), (**suppl. Fig. 2**).

In the TN population, 42% of the seedling root data samples were classed as 'stunted' and removed from analysis, and a further ~4% were removed as outliers. The population showed transgressive segregation in all root and leaf mineral concentration traits. Root trait means ranged from; TRL- 19-92cm, PRL- 8-23 cm, LRL- 6-70 cm, MLRL- 0.5-4.0 cm, LRN- 8-30, LRD^{-cmPRL}- 0.8-1.7. Between the parents the differences were minimal, Ningyou7 had a greater lateral root length, number and significantly greater lateral root density ($t = 2.1$, $P< 0.05$), but a shorter primary root length than Tapidor. Tapidor had greater leaf mineral concentrations than Ningyou7; B ($t = -5.7$, $P < 0.01$), Fe ($t = -5.1$, $P < 0.01$), K ($t = -7.4$, $P < 0.01$) and Mg ($t = -2.7$, $P < 0.05$), except that Ningyou7 had a greater concentration of S ($t = 5.6$, $P < 0.01$). Ningyou7 also had a greater shoot dry weight at the 1 true leaf stage ($t = 3.2$, $P<0.01$) (**suppl. Fig. 3**)

In the ASSYST population 29% of the seedling root data samples were classed as 'stunted' and removed from analysis, and a further ~3% were removed as outliers.

Root trait means ranged from; TRL- 24–99cm, PRL- 9–28cm, LRL- 12–76cm, MLRL- 0.8–2.9cm, LRN- 11-37, LRD^{-cmPRL}- 0.7–2.0. (**suppl. Fig. 4**). Winter OSR and fodder types had significantly greater primary root length ($F = 2.8$, $P < 0.01$) and lateral root number than other types ($F = 17.8$, $P < 0.001$). Winter OSR compared to spring OSR generally had lower leaf mineral concentrations ($P < 0.001$) e.g Ca ($F = 10.8$), Mg ($F = 18.3$), S ($F = 25.6$), B ($F = 11.9$), Mo ($F = 27.7$), and Na ($F = 25.0$), except in Zn ($F = 7.0$) where winter OSR had a greater concentration (see further detail in Thomas *et al*, 2016[b]).

Relationships between root traits, seed size and leaf mineral concentration

In the TC population raw data indicate positive relationships between most root traits ($P< 0.001$) e.g. PRL and LRN ($r = +0.74$), but negative relationships between PRL and LRD^{-cmPRL} ($r = -0.29$), MLRL and LRN ($r = -0.36$) and MLRL and LRD^{-cmBZL} ($r = -0.25$). Most significant relationships between root length traits (including lateral root number which is linearly related to PRL) and leaf mineral concentrations were negative e.g. LRL and Cu ($r = -.44$, $P<0.01$) and Se ($r = -0.25$, $P<0.01$), BZL and P ($r = -0.23$, $P < 0.01$), LRN and Na ($r = -.21$, $P< 0.05$), except a positive relationship between BZL and Mn ($r = +0.17$, $P < 0.05$), and LRN and Zn ($r = +0.22$, $P< 0.05$). However, between LRD^{-cmPRL} there were more positive relationships e.g Zn ($r = +0.41$, $P<0.001$), P ($r = +0.19$, $P<0.05$), Mg ($r = +0.19$, $P<0.05$) and Fe ($r = +0.23$, $P<0.05$), and also between LRD^{-cmBZL} and P ($r = +0.19$, $P<0.05$) and Zn ($r = +0.33$, $P<0.001$). Mean seed diameter had positive relationships with all root traits e.g LRL ($r = +0.23$, $P< 0.05$). Conversely it had a number of significant negative relationships with leaf mineral concentration e.g Cu (-0.66, $P<0.001$) and Se (-0.25, $P<0.05$) but a positive relationship with B ($r=+0.22$, $P<0.05$) (**Table 1**).

Table 1. correlations (r) in the TCDH population between root traits, seed diameter and leaf mineral concentration in seedlings grown for 14d in a 'pouch and wick' system in a ¼ strength Hoagland's solution and in the glasshouse sampled at the rosette stage, respectively. Green = positive, orange = negative, pale = P<0.05, dark = P<0.001.

	B	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	P	S	Zn
PRL	0.08	-0.04	-0.40	0.02	-0.01	-0.07	0.05	0.04	-0.12	-0.21	-0.04	-0.12
LRL	0.09	-0.08	-0.44	-0.04	-0.01	-0.07	0.05	0.03	-0.17	-0.18	0.01	-0.06
MLRL	0.10	0.02	-0.34	-0.16	-0.01	-0.13	-0.01	-0.01	0.07	-0.17	-0.01	-0.31
BZL	0.14	0.06	-0.43	0.02	0.07	0.07	0.16	0.15	-0.12	-0.23	0.11	0.00
LRN	-0.02	-0.08	-0.21	0.14	0.01	0.09	0.07	0.01	-0.21	-0.12	-0.01	0.22
LRD/PRL	0.02	-0.01	0.13	0.23	0.14	0.19	0.14	0.07	-0.03	0.19	0.13	0.41
LRD/BZL	-0.13	-0.15	0.10	0.15	-0.03	0.08	-0.05	-0.10	-0.15	0.19	-0.10	0.33
SD	0.22	0.17	-0.66	-0.03	0.12	0.01	-0.05	-0.04	-0.03	-0.19	0.05	-0.18

In the TN population raw data indicate positive relationships between root traits ($P < 0.001$) e.g PRL and LRN ($r = +0.83$), but negative relationships between PRL and MLRL ($r = -0.17$), PRL and LRD^{-cmPRL} ($r = -0.16$) and LRN and MLRL ($r = -0.16$). There were generally weak negative relationships between primary root length and mineral concentration, although there was a significant positive relationship with B ($r = +0.14$, $P<0.05$). Conversely there were positive relationships between lateral root length and leaf mineral concentration particularly between mean lateral root length and Na ($r = +0.32$); Mn ($r = +0.32$); Mo ($r = +0.26$); Fe ($r = +0.26$ and S ($r = +0.25$) ($P <0.01$-0.001). The relationships with lateral root number e.g. Mn ($r = -0.15$, $P <0.05$) and Na ($r = -0.15$, $P <0.05$) and lateral root $density^{-cmPRL}$ e.g Mn ($r = -0.15$, $P< 0.05$), Mo ($r = -0.22$, $P <0.01$) and S ($r = -0.14$, $P <0.05$) were more generally negative, although there was a significant positive relationship with B and lateral root number ($r = +0.15$, $P <0.05$). Between seed diameter and leaf mineral concentrations most relationships were negative ($P <0.001$) particularly in K ($r = -0.42$), Ca ($r = -0.32$), and Mn ($r = -0.27$), except positive relationships with P ($r = +0.33$) and Zn ($r= +0.19$, $p< 0.05$) (**Table 2**).

Table 2. correlations (r) in the TNDH population between root traits, seed diameter and leaf mineral concentration in seedlings grown for 14d in a 'pouch and wick' system in ¼ strength Hoagland's solution and in 0 Pi solution, respectively. Green = positive, orange = negative, pale = P<0.05, dark = P<0.001.

	B	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	P	S	Zn
PRL	0.14	-0.09	0.03	0.04	-0.02	0.01	-0.11	-0.07	-0.13	0.09	0.07	0.12
LRL	0.26	0.04	0.16	0.17	0.11	0.15	0.11	0.08	0.16	0.14	0.17	0.15
MLRL	0.25	0.21	0.15	0.26	0.18	0.20	0.32	0.26	0.32	0.15	0.25	0.16
LRN	0.15	-0.09	0.07	-0.01	-0.01	0.00	-0.14	-0.13	-0.15	0.06	0.00	0.02
LRD/PRL	0.03	-0.05	0.10	-0.05	-0.03	-0.03	-0.15	-0.22	-0.07	-0.03	-0.14	-0.12
SD	-0.16	-0.31	-0.13	-0.12	-0.42	0.07	-0.27	-0.11	-0.25	0.33	-0.03	0.19

In the ASSYST panel raw data indicate positive relationships between most root traits ($P < 0.001$), except between PRL and LRD^{-cmPRL} ($r = -0.28$), PRL and MLRL ($r = -0.12$), MLRL and LRN ($r = -0.20$) and MLRL and LRD^{-cmPRL} (suppl. Table 3). There were weak relationships between root traits and leaf mineral concentrations, with root length the relationships were generally negative e.g. ($P<0.001$) primary root length and B ($r = -0.20$), Mg ($r = -0.25$), Mo ($r = -0.31$) and Na ($r = -0.31$), although there was a weak positive relationship between LRN and Fe ($r = +0.12$, $P< 0.05$) and MLRL and Mo ($r=+0.12$, $P<0.05$). Conversely relationships with LRD^{-cmPRL} were positive e.g Ca ($r = +0.19$) and Mg ($r = +0.16$). Despite attempting to control for the effect of seed size on root growth by sowing an approximately uniform seed size, there were still positive relationships between TSW and all root traits e.g. LRL ($r = +0.45$, $P<0.001$), particularly in data where outliers had been removed. Between TSW and leaf mineral concentrations relationships were generally positive, in the case of macro nutrients relationships were very weak, except with K ($r = +0.15$, $P < 0.01$), whereas with most micro nutrients they were weakly significant; Cu ($r = +0.11$, $P<0.05$), Fe ($r = +0.14$, $P<0.01$), Mn ($r = +0.16$, $P<0.01$) and Zn ($r = +0.17$, $P<0.01$), except that the relationship with Mo was negative ($r = -0.11$, $P< 0.05$) (**Table 3**).

Table 3. correlations (r) in the ASSYST diversity panel between root traits, seed diameter and leaf mineral concentration in seedlings grown for 14d in a 'pouch and wick' system in a ¼ strength Hoagland's solution and in the glasshouse sampled at the rosette stage, respectively. Green = positive, orange = negative, pale = P<0.05, dark = P<0.001.

	B	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	P	S	Zn
PRL	-0.20	-0.16	0.00	0.02	-0.02	-0.25	-0.02	-0.31	-0.31	-0.05	-0.19	0.04
LRL	-0.08	-0.01	0.04	0.03	-0.07	-0.09	0.06	-0.18	-0.12	0.02	-0.10	0.03
MLRL	0.07	-0.01	-0.01	-0.08	0.02	0.00	0.00	0.12	0.09	0.00	0.07	-0.05
LRN	-0.13	-0.01	0.07	0.12	-0.09	-0.10	0.05	-0.29	-0.20	0.03	-0.17	0.10
LRD/PRL	0.06	0.19	0.12	0.13	-0.09	0.16	0.12	-0.01	0.09	0.06	0.01	0.05
TSW	-0.02	0.06	0.12	0.14	0.15	0.02	0.16	-0.11	0.04	0.09	0.02	0.17

Genetic maps

The TCDH map comprised of 135 genotypes. The final map had a total of 1,010 SNP markers, the number of markers ranged from 14 on chromosomes C1 to 116 on chromosome C3, chromosome length ranged from 15.8cM in chromosome A10 to 64.1cM in chromosome C3, and marker density (per cM) ranged from 0.85 on chromosome C2 to 5.38 on chromosome A3 (**genetic map available on request, summary table Suppl. Table 4**).

The TNDH map comprised of 182 genotypes. The map had a total of 2,174 SNP markers, the number of markers ranged from 62 on chromosome C5 to 291 on chromosome A3, chromosome length ranged from 82cM in chromosome A4 to 145.9cM in chromosome A3, and marker density (per cM) ranged from 0.46 on chromosome C5 to 1.99 on chromosome A3.

The ASSYST population was allocated to 4 sub-groups; winter OSR, swede, spring OSR, and semi-winter/kales/winter fodder. There were a total of 355,536 SNP markers, the most being on chromosome C3- 29,817, and the least on chromosome A4- 12,501. There were a total of 53,889 GEM markers, the most being on chromosome C3- 4,524 and the least on chromosome A4.

QTL/GWAS analyses of root traits, seed size and leaf mineral concentration

In the TCDH population, the increasing allele in all root trait and seed diameter QTL came from Canard, except for MLRL and LRD^{-cmPRL} from Temple. QTL were on chromosomes; MLRL- A1, LRL- C1, PRL and LRD^{-cmPRL}- C9, MLRL and LRN- C4, BZL- C6, LRD^{-cmBZL}- C7, and seed diameter- A8. The increasing allele in all leaf mineral concentration QTL was from Temple, except Fe which was from Canard. The QTL were on; Ca- C1, Mg and Zn- C5, P- A10, Fe- A4 and S and Se- C7. The QTL which explained > 20% of phenotypic variation were for LRL on C1- 46%, Ca on C1- 47%, Se on C7- 37%, MLRL on C9- 25%, seed diameter on A8- 21%, S on C7- 20% and Zn on C5- 20%. Between root traits there were QTL with an overlapping CI (confidence interval) for LRL and MLRL on C1 and A1 respectively but the increasing allelewas from different parents; for PRL and PRL^{-cmPRL} on C9 but the increasing allelewas from different parents; and for MLRL and LRN on C4 but the increasing allele was from different parents. Between root and leaf mineral traits there were QTL with an overlapping CI for LRL, Ca, MLRL and P on C1, C1, A1 and A10 respectively; and for LRD^{-cmBZL} and S and Se on C7, but in all instances the increasing allele for the root traits and those for the mineral traits came from different parents. Between the leaf mineral traits there were QTL with an overlapping CI for Mg and Zn on C5; and S and Se on C7 (**Fig. 1**; **Suppl. Table 1**).

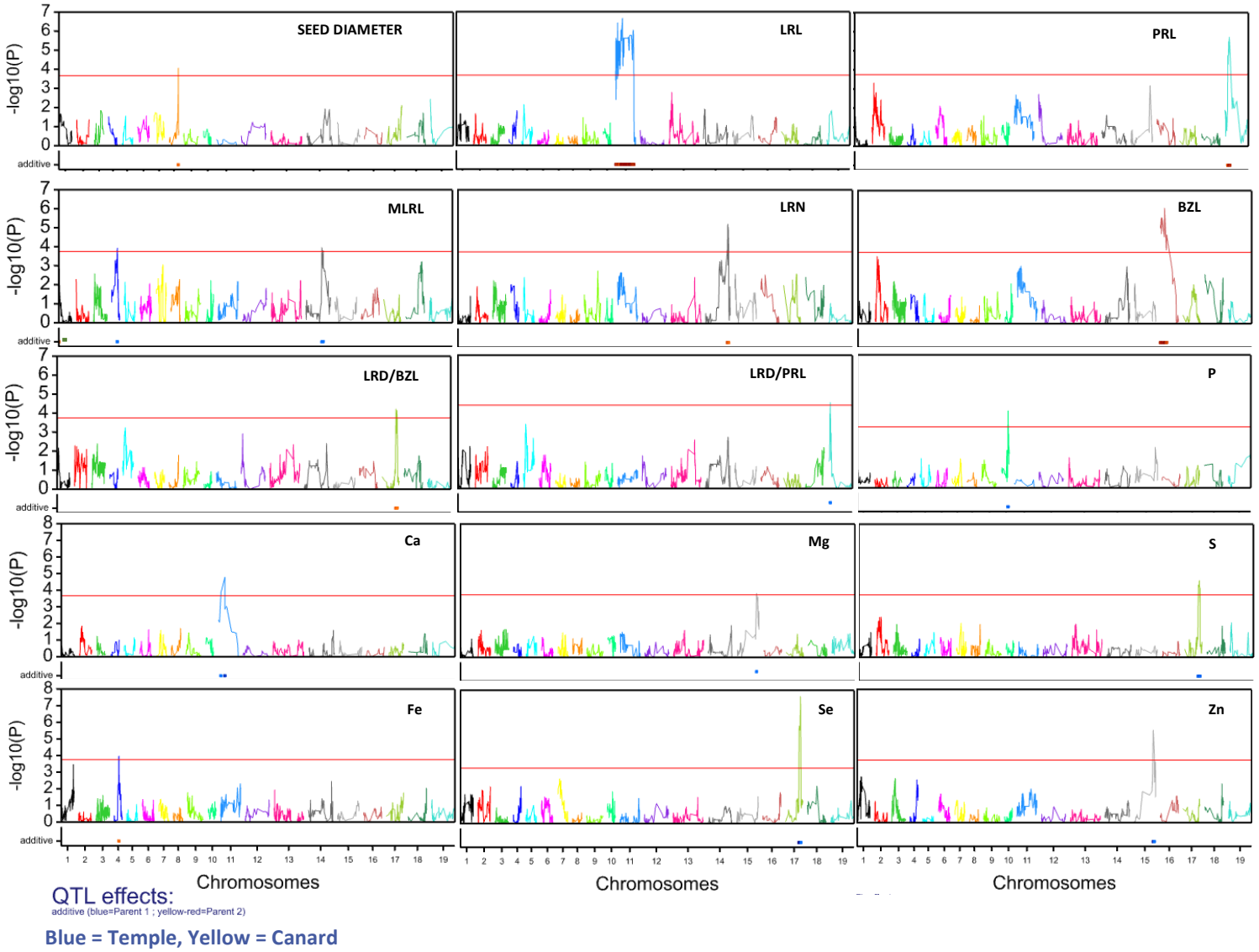


Figure 1. QTL in the TCDH population for seed diameter, root traits and leaf mineral concentration in seedlings grown for 14d in a 'pouch and wick' system in a ¼ strength Hoagland's solution and in the glasshouse sampled at the rosette stage, respectively. Red line = LOD threshold at 0.05 significance.

In the TNDH population, the increasing allele in most root traits came from Tapidor, except for MLRL from Ningyou7, the increasing allele for seed diameter also came from Ningyou7. The QTL were on chromosomes; LRL and LRN- A1; MLRL- C1; LRD^{-cmPRL}- C9; and seed diameter- A2 and A7. The QTL for leaf mineral concentration were on chromosomes; P- C4 the increasing allele came from Ningyou7; Ca- C1 and C7 the increasing allelecame from Ningyou7 and on C2 the increasing allelecame from Tapidor; Mg- A1 the increasing allelecame from Tapidor and on C7 came from Ningyou7; S- A9 and C7 the increasing allelecame from Ningyou7 and on C4 came from Tapidor; K- A9 the increasing allele came from Tapidor and on A10 came from Ningyou7; B- A2 the increasing allelecame from Tapidor; Mo- C7 the increasing allelecame from Ningyou7; Zn- A1 the increasing allelecame from Tapidor and on A4 came from Ningyou7; Cu- A1 and A2 the increasing allelecame from Tapidor. The QTL which explained > 15% of phenotypic variation were for B on A2- 19%, S on C7- 17%, and Mo on C7- 15%. Between root traits there were no QTL with an overlapping CI. Between root traits and leaf mineral traits there were QTL with an overlapping CI for LRL and Mg on A1; for MLRL and Zn on C1/A1 respectively but the increasing allelewas from different parents; for LRN and Mg on A1; for and LRD^{-cmPRL} and S on C9/A9 respectively but the increasing allelewas from different parents. Between the leaf mineral traits there were QTL with an overlapping CI for P and Zn on A4; for Ca, Mg, S and Mo on C7; for B and Cu on A2. The QTL for seed diameter had an overlapping CI with leaf B and Cu concentration on A2 but the increasing allelefor seed diameter and for mineral traits came from different parents. (**Fig. 2**; **Suppl. Table 2**).

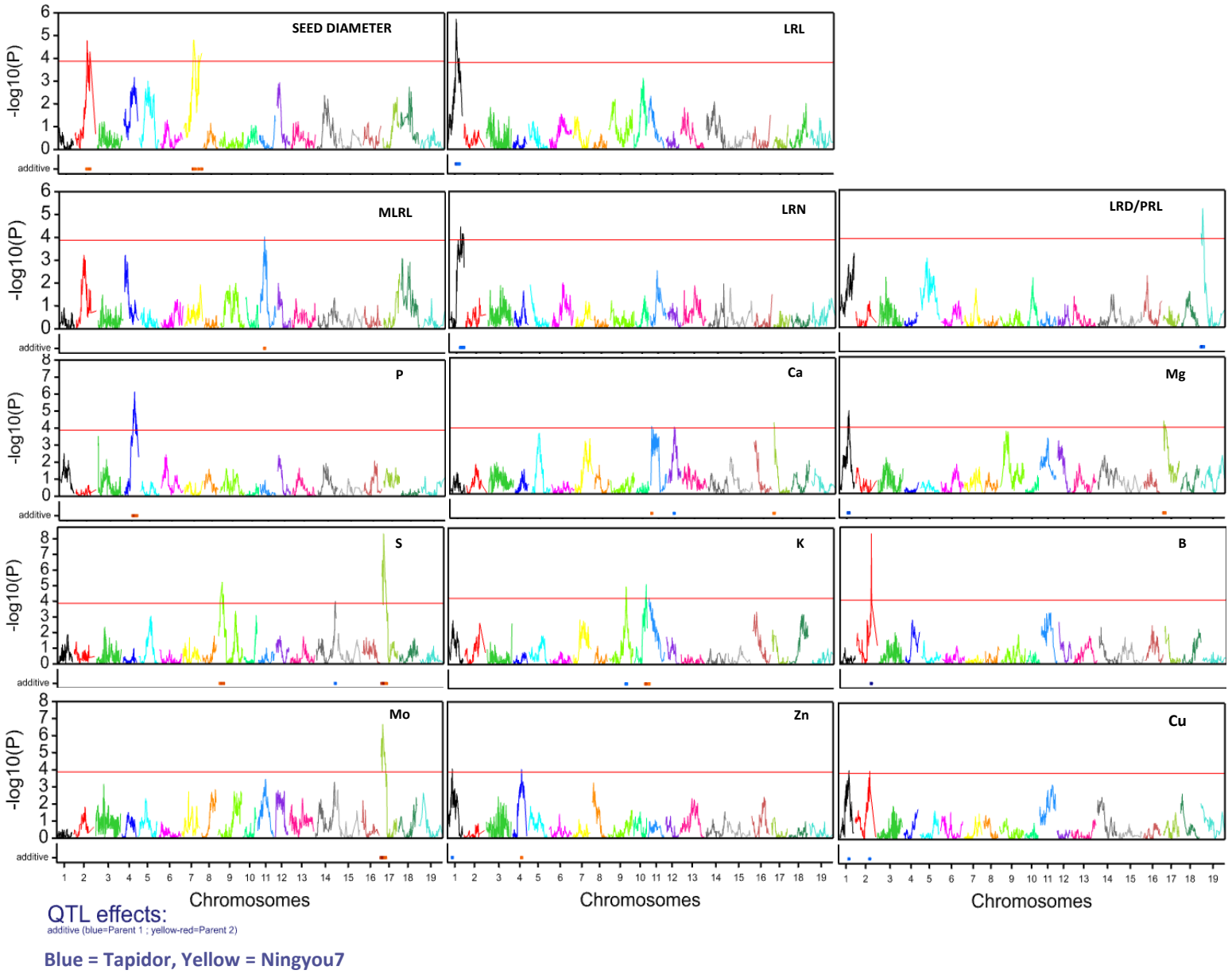
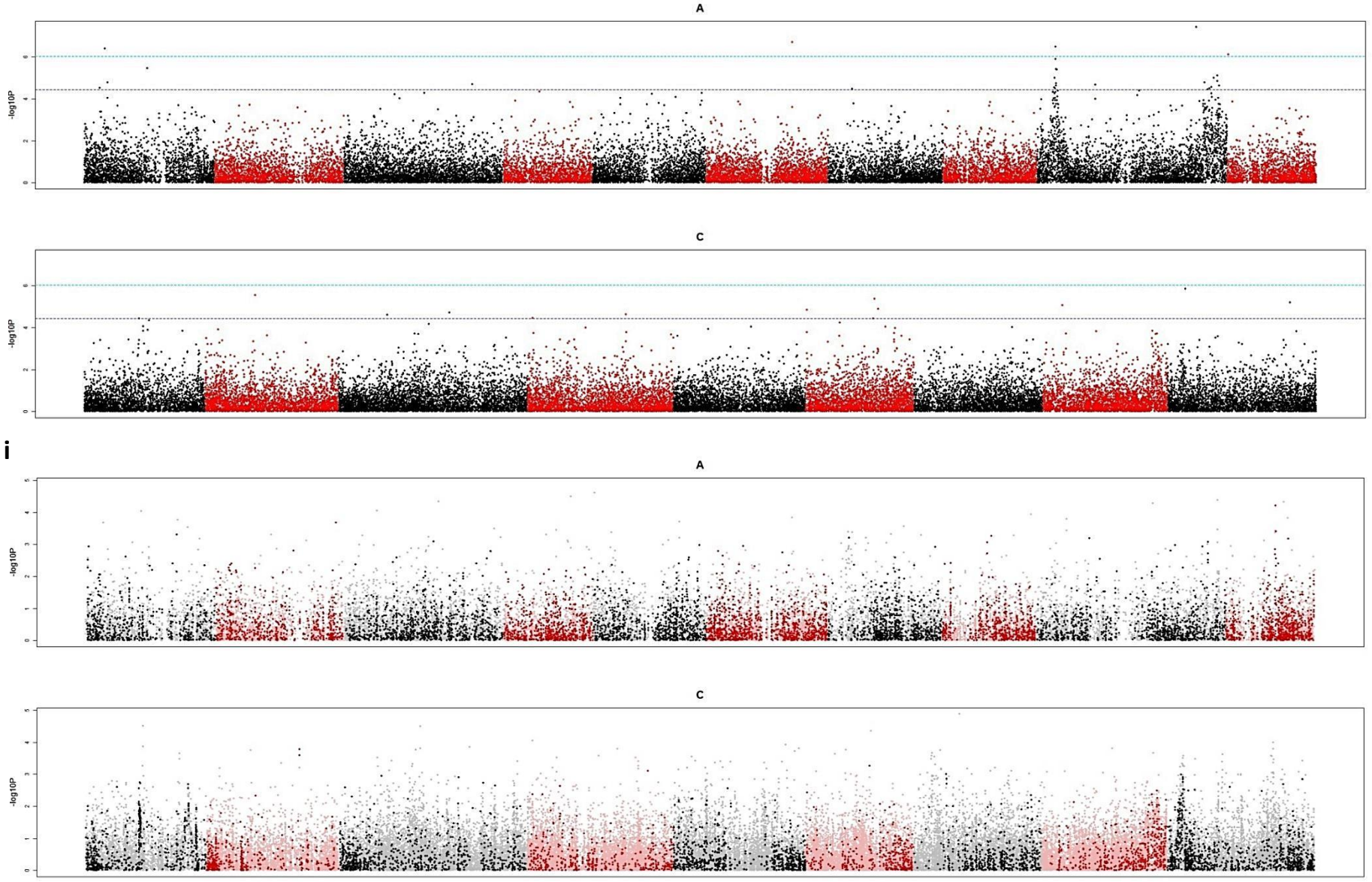


Figure 2. QTL in the TNDH population for seed diameter, root traits and leaf mineral concentration in seedlings grown for 14d in a 'pouch and wick' system in ¼ strength Hoagland's solution and in 0 Pi solution, respectively. Red line = LOD threshold at 0.05 significance.

Across the populations, there were QTL with overlapping CI and showing homeologous exchange for LRL, Ca, MLRL and P on C1, A1 and A10 respectively in the TC population and MLRL and Ca in the TN on C1; for PRL and LRD^{-cmPRL} in the TC and LRD^{-cmPRL} in the TN on C9; for LRN on C4 in the TC and P and Zn on A4 in the TN; for PRL on C9 in the TC and S on A9 in the TN; and for BZL on C6 in the TC and for leaf Ca, Mg, S and Mo on C7 in the TN population (**Suppl. Tables 1 and 2**).

In the ASSYST panel there were 39 GEM markers for LRD^{-cmPRL} significant above the FDR threshold; 20 were on chromosome A9 forming 2 peaks, there were also 5 on A1 and 3 on C6. There were non-significant SNP marker peaks on A8, C1, C3 and C9. A number of the GEM and SNP markers corresponded (**Fig.3A**). For MLRL there were 10 significant GEM markers above the FDR including two each on A1 and A4, and non-significant SNP peaks on A5, C3 and C8. The SNP and GEM markers did correspond to one another (**Fig. 3B**). For TSW there were many significant GEM markers, however there was just a small peak on C9, the most significant marker being on C4. There were a cluster of non-significant SNP markers on C2 and a small non-significant peak on C9 (**Fig 3C**). A number of the GEM and SNP markers corresponded. There were no corresponding markers across traits for either SNPs or GEMs, except that SNPs and GEMs for TSW and leaf micro nutrient concentrations often corresponded (data not shown).

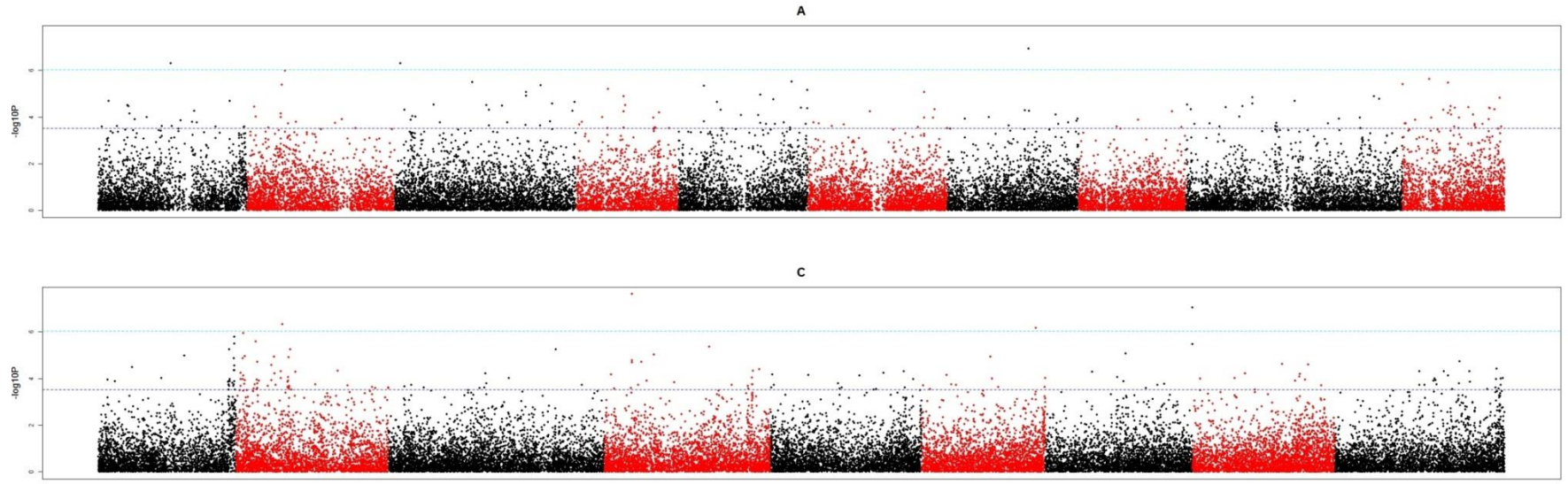
A BLAST of the significant GEM makers for LRD had direct hits to a number of *Arabidopsis* genes related to root cell division and meristematic activity; *GEM/GL2*, *MIA/PDR2*, *COB*/*COBRA*, *ANGUSTIFOLIA* and *ARF1*. There were also direct hits for genes related to ion transport; *CAX11* and *ATECA4/ACA4* and stress signalling; *CRK1*/*CDPK-related kinase 1* and *CNX1*/*calnexin1*, all of the markers were on chromosome A9 except in the case of the latter marker on C4. The most significant SNP marker on A5 for MLRL was an *Arabidopsis* heavy metal transport/homeostasis superfamily protein *HIPP*. The most significant SNP marker on C2 (pseudo-molecule C9) for TSW was *PKP2-BETA1* an *Arabidopsis* gene controlling seed oil biosynthesis and seedling establishment, and another significant SNP marker on C9 was the gene *SUPPRESSOROFMAX2 1/SMAX1* controlling seedling germination (**Suppl. Tables 3 a,b,c**).



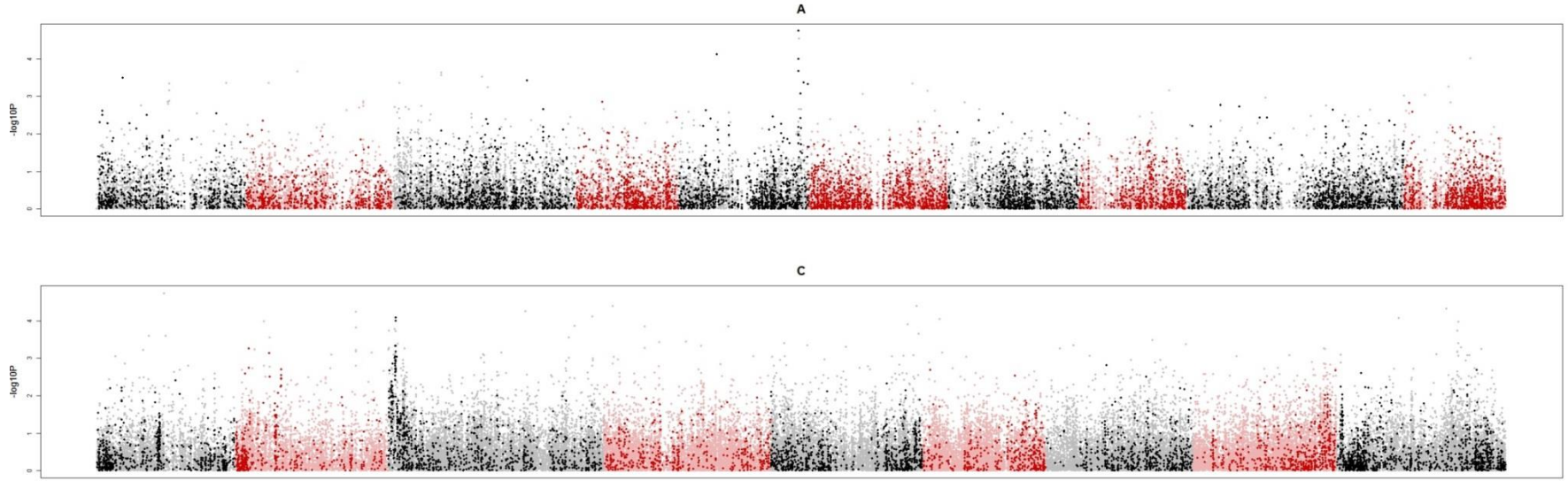
i

ii

A



i



ii

B

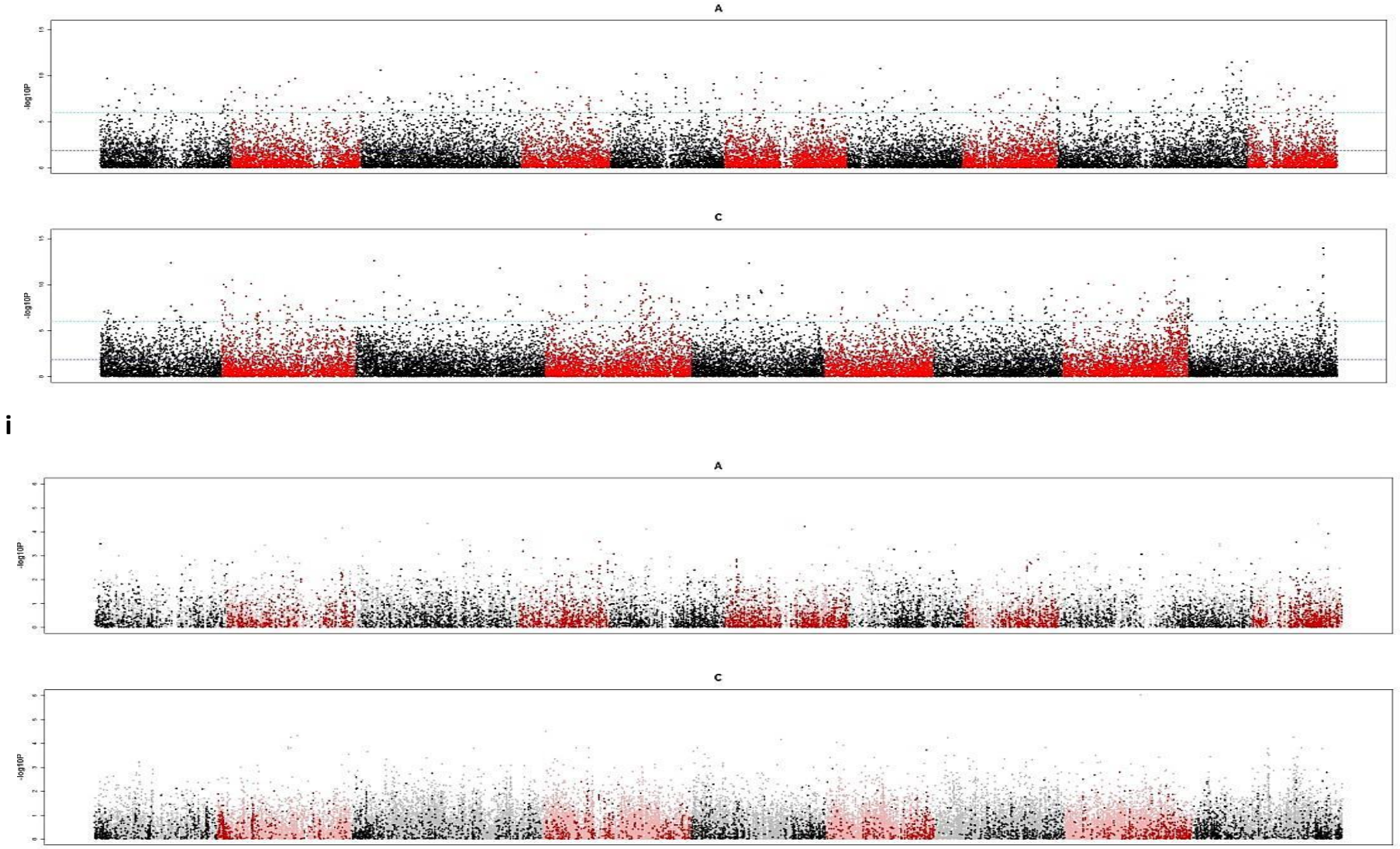


Figure 3. Association analyses in the ASSYST diversity panel between i. GEMs and ii. SNP markers and A. LRD B. MLRL and C. TSW. In seedlings grown for 14d in a 'pouch and wick' system in ¼ strength Hoagland's solution. TSW was calculated from plants grown in the glasshouse. Blue line = FDR significance threshold, black line = 0.05 significance threshold.

Leaf mineral concentration and biomass in the extreme rooting genotypes

Genotypes 5, 260 and 369 were confirmed to have the phenotype of maximum LRD^{-cmPRL} in the HTP re-screen; with means of 1.9 and 1.9, 2.0 and 2.3, and 1.8 and 2.4, in experiments 1 and 2, respectively (**suppl. Fig. 1A**). Whereas only line 355 was confirmed to have the maximum MLRL- 3.3 and 2.0cm in experiments 1 and 2, respectively (**suppl. Fig 1B**), therefore different genotypes were selected as candidates for maximum MLRL; 333 and 361. In the glasshouse experiment, genotypes with the max MLRL compared to those with the max LRD had greater shoot and root (F = 16.7, p< 0.05) dry weight, but a lower shoot/root ratio (F = 19.4, P < 0.05) (**Fig. 4A**). Whereas, genotypes with the max LRD had greater leaf concentrations of Ca (F = 11.2, p < 0.05, **Fig. 4B**) and Mg (F = 8.3, P < 0.05, **Fig. 4C**) but lower concentrations of P (F = 9.6, P<0.05, **Fig. 4D**) and Cu (F = 7.7, P<0.05, **Fig. 4E**). REML analysis shows that there was a greater effect of shoot dry weight than 'phenotype' on all mineral concentrations. Interestingly, the genotypes with the max LRD did not flower **(Fig. 5A** and **B**).

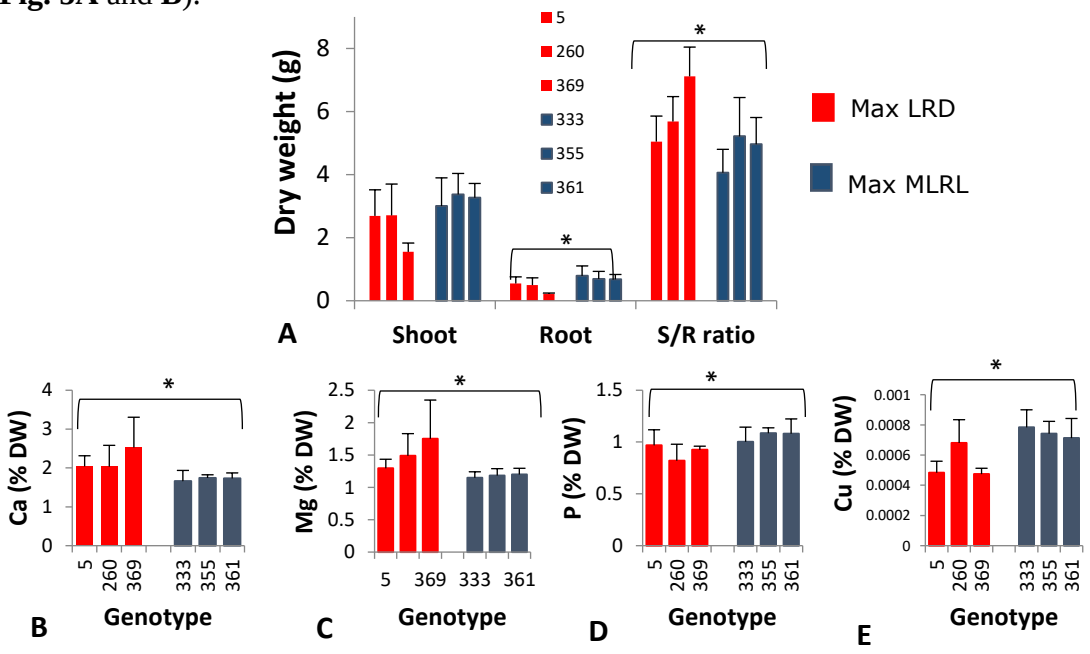


Figure 4. Genotypes with the maximum LRD (black bars) and maximum MLRL (grey bars); A. whole plant dry weight of shoot, root and shoot/root ratio, and 4th leaf mineral concentration of B. Ca C. Mg D. P E. Cu. In plants grown in the glasshouse sampled at the 8th leaf stage. Error bars = 1 SD. * = p<0.05

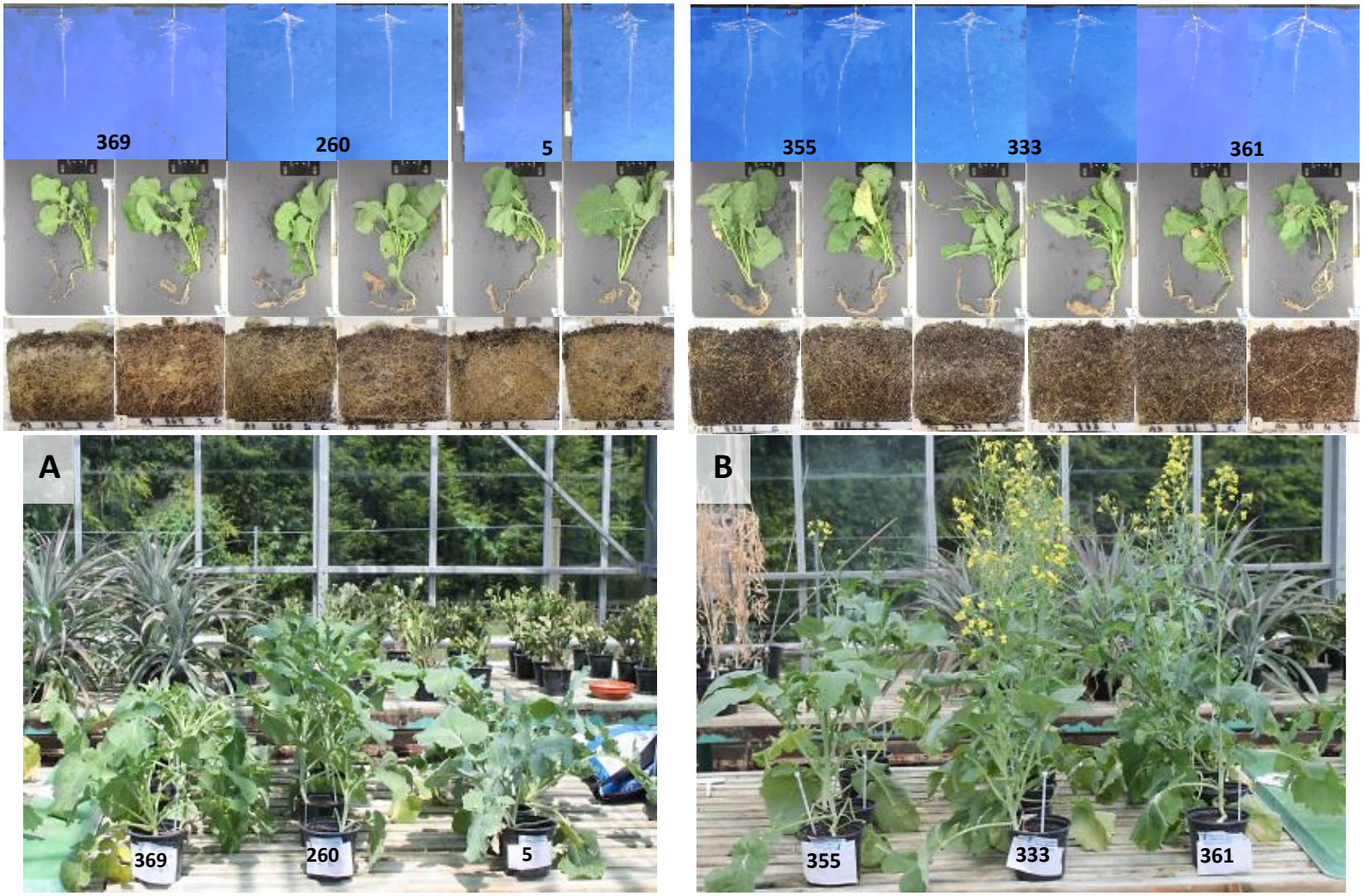


Figure 5. Illustrative images of 2 different plants of each genotype with the max A. LRD; 5, 260, 369 and B. MLRL; 333, 355, 361. Grown for 14d in a 'pouch and wick' system in a ¼ strength Hoagland's solution; in the glasshouse at the rosette stage 14 DAS; and at maturity.

Discussion

There were QTL which co-located across the DH populations for root vigour- total lateral root length and number on chromosomes A1 in the TN, and C1 in the TC (in the TN population, when using a different QTL mapping software, the QTL on A1 were also located on A10, Zhang *et al*., 2016). Chromosomes A1/A10 (Udall *et al*., 2005) and A1/C1 (Cai *et al*., 2012; Udall *et al*., 2006) are homeologous and have a large number of collinear segments. In the TC population these alleles were contributed by canard a forage variety and in the TN population by Tapidor the winter variety. In Brassica, QTLs on chromosomes A1/A10/C1 have often been identified as having pleiotropic effects on multiple traits linked to yield and vigour, including: seed yield (Quijada *et al*., 2006; Korber *et al*., 2016, Fletcher *et al*., 2014; Shi J *et al*., 2009; Dechaine *et al*., 2014; Ding *et al*., 2012; Bouchet *et al*, 2014; Shi T *et al*., 2013); seed number/weight (Shi J *et al*., 2016; Yuhua *et al*., 2016; Dechaine *et al*., 2014); seed oil (Wang *et al*., 2013; Zhao *et al*., 2012); root vigour (Fletcher *et al*., 2014; Lu *et al*., 2008; Yang *et al*., 2010; ArifUzZaman *et al*., 2016); plant height/shoot vigour (Quijada *et al*., 2006; Hammond *et al*., 2009) and PUE (Hammond *et al*., 2009; Yang *et al*., 2011). They have also been linked to germination rate (Basnet *et al*., 2015; Dechaine *et al*., 2014; Finch-savage *et al*., 2010; Bettey *et al*., 2010) and flowering/time to maturity (Quijada *et al*., 2006; Bouchet *et al*., 2014; Fletcher *et al*, 2014, 2016; Hou *et al*., 2012; Raman *et al*., 2016; Schiessl *et al*., 2015; Shi J *et al*., 2009; ArifUzZaman *et al*., 2016; Lou *et al*., 2007). Later flowering has also been observed to correlate negatively with yield (Quijada *et al*., 2006; Fletcher *et al*., 2014; Shi J *et al*., 2009; Shi T *et al*., 2013; Chen *et al*., 2010), due to a greater plant biomass and/or height before flowering (Quijada *et al*., 2006; Fletcher *et al*., 2014; Shi J *et al*., 2009; Shi T *et al*., 2013; Mei *et al*., 2009). For example, in field trials of the TN population, the additive effect for QTL for yield traits was the opposite to those for time to flowering and time to maturity (Shi J *et al*., 2009). A meta-analysis of 15 QTL studies for yield-related traits in *B. napus* found that time to maturity and plant height QTL were mostly on A1 and A9, and for seed yield were on A1 (Zhou *et al*., 2014). It has also been observed previously that winter types have a greater root biomass compared to spring types (Rahman and McClean., 2013; Fletcher *et al*., 2014; ArifUzZaman *et al*., 2016; Yang *et al*., 2010, 2011). A positive relationship between root pulling force and days to flowering was found, whereby the winter lines which

flowered later continued to grow roots for a longer period, the QTL for root vigour and days to flowering co-located on A10. In their follow-up study they found that the flowering gene *FLC1*, which suppresses flowering in *Arabidopsis* flanked thisloci on A10, and in the spring parent this region has an insertion creating a truncated protein (Fletcher *et al*., 2016). Therefore, perhaps yield is affected depending upon the proportion of resources distributed either to vegetative root and shoot growth or to seed production, which is under the control of flowering time genes. Likewise, vigorous root traits also appear to be influenced by the life-history traits, such that winter types develop a more vigorous root system before flowering. Indeed, QTL for flowering time identified on A10 at 17-30 cM was flanked by *BrFLC1* (Dechaine *et al*., 2014), which also co-locates with the QTL found here on chromosome C1 for LRL in the TC at 7.7-22.3 cM and MLRL in the TN at 17.2-32.8 cM.

There were QTL for LRD which co-located on chromosome C9 in the TC and TN populations, which in both cases were from the winter parent, also significant GEM markers were found for LRD on A9 in the ASSYST population. In the TC population there was also a QTL for PRL on C9 which co-located with those for LRD, but was from the forage rather than the winter variety. Chromosome C8 has syntenic segments with A8 and C9, and C9 is largely collinear with A9 and C8 (Cai *et al*., 2012). In an agar-based root screen of the TN population there was a QTL for LRN on C9 in which the additive effect was from the winter parent Tapidor, which co-located with the QTL found here (Shi *et al*., 2013). In *B. rapa* QTL on A9 were found for taproot thickness and taproot length, but in either case the increasing allele came from the opposite parent, this corresponds to the findings here in that LRD has a negative relationship with primary root length (Lu *et al*., 2008). In *B. oleracea*, a regulatory hotspot on A9 was found for shoot P at low P (Hammond *et al*., 2009). In a sub-group of just the winter types from the ASSYST population, most QTL for seed yield were on A9, and QTL overlapped for flowering, yield and plant height, (Schiessl *et al*., 2015). QTL have also consistently been detected for seed number and silique length in Brassica on A9 ([a]Li N *et al*., 2014; [b]Li F *et al*., 2014; Yang *et al*., 2012). In a meta-analysis of 15 QTL studies in *B. napus*, the vast majority of QTL for seed number had been on C9, and the most for maturity time and plant height were on A9 and A1 (Zhou *et al*., 2014). In *B. rapa* there was antagonistic pleiotropy on A9 which increased the number

of seeds but decreased seed mass (Dechaine *et al*., 2014), the same effect on seed number and seed mass was found for QTL on C9 in *B. napus* (Zhang *et al*., 2012). QTL on A9 have also been found to control flowering time in *B. napus* (Ferriera *et al*., 1995; Schiessl *et al*., 2015). Furthermore, it has been suggested that there may be negative linkage/repulsion between QTL detected on A1/A10 and those on C8/C9 (Yang *et al*., 2010), whereby they have the opposite effect on the same trait. Phosphorus uptake and phosphorus efficiency were negatively correlated, the QTL on C8 increased phosphorus uptake whereas the QTL on C1 increased phosphorus efficiency (Yang *et al*., 2011). In the ASSYST panel, one SNP marker association for seed yield was identified, in spring varieties this was on chromosome A1 and explained 13% of the variation, and in the winter varieties was on chromosome A9 and explained 11% of the variation (Korber *et al*., 2016). In *B. napus*, the winter parent contributed additive alleles for seed oil content predominantly on chromosomes A9 and C9, but the spring parent had a negative effect on seed oil on chromosomes A1/A10 (Wang 2013; Shi J (2016). As with the QTL on A1/A10, it seems that QTL on A9/C9 have pleiotropic effects on yield and vigour related traits, and moreover, they also appear to affect flowering time. Furthermore, spring types may attribute the additive effect on A1/A10 and the winter types on A9/C9 for the various traits.

This apparent negative linkage between chromosomes A1/A10 and A9/C9 can perhaps be explained by homoeologous exchange. Linkage group A9 shares homology with linkage group A1 (Ferriera *et al*., 1995), and between linkage groups A10 and C9 there has been an HNRT (homeologous nonreciprocal transposition, Quijada *et al*., 2006; Udall *et al*., 2005), whereby homeologous sections of chromosomes no longer segregate independently. It has been found that the *FLC1* gene which suppresses flowering in *Arabidopsis* (Hou *et al*., 2012) has two homeologous genes in *B. napus*- *Bn.FLC.A10* and *Bn.FLC.C9*, on chromosomes A10 from *B. rapa* and C9 from *B. oleracea*, respectively (Pires *et al*., 2004; Raman *et al*., 2016). In the *B. napus* homologous gene *BnFLC.C9*, the fourth cis-block which corresponds to the crucial 75bp segment for the expression of *AtFLC* in *Arabidopsis* was present in the winter plants but absent in spring varieties, in the TNDH population (Zou *et al*., 2012). Likewise, in the upstream region of *BnFLC.A10* most winter rapeseeds had a 621-bp insertion fragment which delayed flowering but which was absent in the spring types

(Long *et al*., 2007; Quijada *et al*., 2006; Hou *et al*., 2012; Fletcher *et al*., 2016). Therefore, the *FLC1* genes on A10 and C9 which suppress flowering are differentially expressed by spring and winter varieties. This may explain why negative linkage on A1/A10 and A9/C9 has so often been observed for various related traits in *B. napus*. The QTL observed in the present study for LRD on C9 is located between -3.6-17cM, which corresponds to the location of the *FLC1* homologues in *B. napus* at 0 cM on C9 (Zou *et al*., 2012). Thus the *FLC* genes on chromosomes A1/A10 and C9 in *B. napus*, which control various life-history traits including germination, flowering and seed production are also linked to the root traits observed in this study. Alleles for genes underlying this QTL on chromosomes A1/A10 seem to initiate early root length, whereas those on C9 promoted a greater lateral root initiation but shorter primary length. Furthermore, it is interesting that the extreme genotypes selected for the greater LRD in the glasshouse not only developed their root system more slowly than those selected for a greater MLRL, but they also flowered approx. 2 weeks later than the genotypes selected for max MLRL, and had significantly greater fine root growth by this later stage, as can be seen in **fig 5**. This suggests that they may have developed long roots more slowly but developed a larger root system perhaps through a greater number of lateral roots, this was particularly the case in ASSYST 05, which was the only winter variety.

There were numerous QTL detected in this study on C6/C7/A7. There has been significant homeologous exchange between chromosomes C6 and A7 (Quijada *et al*., 2006) and C6 and C7 (Cai *et al*., 2012). There were QTL for BZL on C6, LRD^{-cmBZL} on C7, and leaf S and Se on C7 in the TC population. As well as QTL for leaf Ca, Mg, S and Mo on C7 and seed diameter on A7 in the TN population. Furthermore, across the populations the QTL for BZL on C6 in the TC and the leaf mineral traits in the TN co-located. By contrast to the QTL for root traits on A1 and C9, which appear to be linked to life-history and vigour/yield traits, these chromosomes have been linked previously predominantly with root specific and leaf and seed mineral traits. For example, in the TN population roots were screened in agar plates, the QTL for PRL were predominantly located on chromosomes A7 and C6, and for LRD on C6. The QTL for PRL on A7 co-locates with the QTL here for $LRD^{-cm\ BZL}$ on C7. There was a QTL for root pulling force on C7, which interestingly, they suggest unlike the QTL on

A10 and C2 which affected root biomass downstream of their effects on flowering time, this QTL was specific to the root trait and did not co-localise with those for flowering time and yield (Fletcher *et al*., 2014). In *B. oleracea* QTL were found for various measures of phosphorus use efficiency and shoot P on chromosome C7 (Hammond *et al*., 2009). Co-located QTL for shoot Ca and Mg concentration were found on chromosomes C6 and C7 in *B. oleracea* (Broadley *et al*., 2008). Likewise, in *B. oleracea* a QTL for shoot K was found on C7 (White *et al*., 2010). In *B. rapa* QTL were on A6 for shoot Zn, Mn and P concentration (Wu *et al*., 2008). In *B. napus* QTL for shoot concentrations of B, Mg and P were on A7 and for Ca were on A6 (Liu *et al*., 2009). In seeds of *B. napus*, there was a QTL hotspot on C6 for concentration of P, Ca, Mg, Cu and Zn, as well as QTL on A7 for Mg, on C7 for Cu and Fe, and on A6 for Mn (Ding *et al*., 2010). In *B. napus* QTL for seed oil content was consistent across environments on A7/C6 (Chen *et al*., 2010). In a GWAS study of *B. napus* significant SNP associations for glucosinolate content were found on C7 and for seed weight on A7 (Li F *et al*., 2014)[b]. Therefore, these chromosomes seem to be predominantly linked with mineral composition, perhaps via a link with root traits.

Correlations show a negative relationship between PRL and LRD, this is unsurprising given that there is a linear relationship between PRL and lateral root number. Furthermore, data collected previously in the field (Thomas *et al*., 2016[a]) and in the extreme phenotype experiment in this study, show a very linear relationship between root biomass and shoot biomass, and plants with a greater PRL and LRL in the HTP system have a greater shoot biomass, whereas there was a negative relationship between LRD and shoot biomass. This helps explain the somewhat inconsistent correlations observed between the root and leaf mineral traits between the populations observed here. In both the TC and ASSYST populations, the leaf mineral data were collected from plants grown in nutrient rich compost. In these populations there was a negative relationship between root length and number in the HTP system and most leaf macro nutrient mineral concentrations- particularly Ca and Mg, whereas between lateral root density and leaf mineral concentration there were positive relationships**.** It is likely that the plants with the greater root length- PRL, LRL, LRN were larger, whereas those with the greater LRD were smaller. Therefore, the larger plants had lower mineral concentrations than the smaller plants. It could be that this shows a

biomass dilution effect; whereby the larger and smaller plants acquired an equal amount of nutrient- that which was sufficient for growth, however this was distributed around a greater volume of shoot biomass within the larger plant and therefore at a lower concentration, and vice versa in the smaller plant. In the extreme experiment this was shown to be the case, whereby the effect of shoot dry weight on leaf mineral concentration was greater than the 'phenotype' effect, particularly for Ca and Mg concentration. Whereas, the opposite trends were observed in the TN population- there were positive relationships between mean lateral root length and leaf mineral concentration, but negative relationships between lateral root density and mineral concentration. The leaves for mineral analysis in this population were sampled from young seedlings grown in the HTP system under a zero phosphorus solution. It is likely therefore that these plants were deficient for numerous nutrients because of low P (Ding *et al*., 2010), and the longer lateral root system was necessary to acquire nutrients. A biomass dilution effect has been observed previously in *B. oleracea* in shoot Ca and Mg concentration (Farnham *et al*., 2000), and in leaf Ca, Mg and K concentration in *B. napus* (Bus *et al*., 2014). Confirming this explanation, there were few corresponding QTL for root and leaf mineral traits, and where they did correspond, the additive effect for the root and leaf mineral QTL was in nearly all cases from the opposite parent, indicating the independent control of leaf mineral concentration from those root traits measured here.

On the other hand, Dubrovsky and Forde (2012) have argued that measuring lateral root density per cm of the primary root, rather than per cm of the branching zone under-estimates the lateral root density of plants with a shorter primary root length, because the proportion of the primary root taken by the branching zone increases with development. Thus, in this study the LRD in plants with a shorter primary root length has been underestimated. As mentioned above, the plants with the greater LRD (shorter PRL) when grown in the glasshouse, by the later stage of development, had much greater fine root growth than those genotypes selected for max MLRL, this indicates that lateral root growth was greater in these genotypes, but perhaps initially they are putting resources into lateral root initiation rather than elongation. Indeed, the majority of putative candidate genes identified for LRD were controlling lateral root/root hair growth. For example, the most highly expressed region had a marker

which was homologous for the *Arabidopsis GEM* (GL2/*GLABRA2* expression modulator) gene. *GL2*/*GLABRA 2* is the main gene known to control hair/non hair cell differentiation in the epidermis of the root, it is expressed in atrichoblasts- cells that do not produce hairs and is not expressed in trichoblasts. At*GEM* expression is inversely related to At*GL2* expression, hence it being a modulator. In *gem* mutants root hairs were significantly decreased, and the number of longitudinal cell divisions was increased, therefore At*GEM* increases root hair cell initiation rather than elongation (Caro *et al*., 2007).

Interestingly, there were, infrequently, positive relationships between root length/number and leaf mineral concentration. As well as between seed size (which relates linearly to root growth in the HTP system) (Thomas *et al*., 2016[b]) and leaf mineral concentration. This was the case in the TC population with positive relationships between BZL and Mn and LRN and Zn, and in the ASSYST population in LRN and Fe, and between seed size and mineral concentration of Zn and P in the TN population, and of Cu, Fe, Mn and Zn in the ASSYST population. This suggests that the larger root system was necessary for the uptake of these nutrients in particular. These micro nutrients- Fe, Mn, Zn and Cu, can be at limited supply in high pH soils (El-Fouly, 1983), because they are divalent metals which decrease in solubility by one hundred-fold for every unit increase in pH (Rengel *et al*., 1999). Therefore, perhaps high nutrient compost is at a pH sufficient to limit the supply of micro nutrients and therefore require more proliferate root growth. In *B. napus*, a greater effect of Zn deficiency in pot grown as opposed to field grown plants has been found, and the Zn efficient genotypes had a greater volume of fine roots compared to Zn inefficient genotypes (Grewal *et al.*, 1997). In *Arabidopsis* when seedlings were grown under a deficient Fe supply it was observed that lateral root length increased in a localised supply of Fe (Gruber *et al*., 2013; Giehl *et al*., 2012), and lateral root primordia emerged more quickly (Giehl *et al*., 2012), and in a deficient supply of Fe, Zn and Mn increased root hair density by 70 - 200% (Ma *et al*., 2001). Whereas Ca, Mg, B and Cu had no effect and a deficient supply of N, K and S decreased root hair growth (Ma *et al*., 2001). In contrast to their biomass dilution effect observed for Ca and Mg, a positive relationship was found between shoot biomass and Mn (Bus *et al*, 2014), perhaps indirectly indicating the effect of a greater root biomass on uptake of Mn.

Similarly, it has been observed in lentil that seed size/HSW (hundred seed weight) relates negatively with seed macro nutrient concentrations of P, K, Mg and Ca, whereas with Cu and Mn there were significant positive relationships (Karakoy *et al*., 2012). Balint *et al* (2001) also observed a negative relationship between wheat 1000-kernel weight and grain concentration of Ca and Mg, however relationships were not observed in Fe, Cu and Zn. In *B. napus*, a negative relationship has been observed between TSW and all mineral concentrations except Mn (Ding *et al*., 2010). It was found that Zn concentration in the seed contributed to early root and shoot growth, and plants with a low seed Zn concentration required much greater Zn fertilisation to achieve optimal yield (Rengel and Graham, 1995). Welch (1999) suggests that Cu, Zn, Ni, Mo and Co have a variable phloem-mobility and that which is in the seed has a significant contribution to seedling development. Furthermore, leaf Mg, Ca, K and Mo concentration were ranked as the most heritable in *B. napus* (Thomas *et al* 2016[b]) and in *Arabidopsis* (Baxter *et al*., 2012), and in both studies leaf Fe, Mn and Cu concentration were the least heritable traits and the most effected by the 'environment' factor. This indicates that environmental supplies of micro nutrients are more variable and have a bigger impact on plant uptake compared to macro nutrients. Therefore, vigorous root growth and initial seed reserves may be necessary for the uptake of micro nutrients.

There may be problems with the SNP polymorphisms in the TC population of the present study (Ian Bancroft, pers. comm), therefore the actual number of segregating lines is potentially much smaller than the number tested. The phenotypic variation explained can be overestimated in small populations (Wang *et al*., 2013), thus the results are to be taken with caution. Consensus mapping combines genetic maps which share a sufficient number of common loci, it then makes a projection of the remaining loci, including QTL loci (Goffinet and Gerber, 2000). A combined map with a greater density of markers reduces the QTL confidence interval region, and facilitates the identification of consistent candidate loci. This approach could be used to help refine the QTL region and aid in fine mapping of the QTL regions identified here, particularly on C7/A7 which appear to be more closely linked to mineral concentration.

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Supplementary Table 1. QTL for seed diameter, root traits and 4th leaf mineral concentration in plants grown for 14 days in a 'pouch and wick' system with a ¼ strength Hoagland's solution, and in the glasshouse until 6-7 leaf stage, respectively. Log10(P) threshold of 3.67. Additive effect: 1 = Temple, 2 = Canard.

Trait	Chr.	Position (cM)	CI (cM)	Marker	LOD	R^2 (%)	Add effect
LRL	C1	14.98	7.7-22.3	851	6.16	46	2
PRL	C9	10.13	2.8-17.5	1420	5.27	16	2
BZL	C6	9.85	2.5-17.2	1213	5.56	20	2
MLRL	A1	0.94	-6.4-8.28	3	2.27	7.8	2
	C4	35.06	27.8-42.4	1124	2.11	8.2	1
LRN	C4	48.53	41.2-55.8	1139	4.83	15	2
LRD/BZL	C7	39.76	32.5-47.1	1298	4.00	19	2
LRD/PRL	C9	3.70	-3.6-11.0	1398	3.67	13	1
P	A10	13.75	6.5-21.1	793	4.37	12	1
Ca	C1	7.78	0.5-15.1	840	3.90	47	1
Mg	C5	41.40	34.1-48.7	1195	3.63	14	1
S	C7	42.04	34.7-49.3	1305	4.35	20	1
Fe	A4	11.24	3.9-18.5	328	3.77	19	2
Zn	C5	41.40	34.1-48.7	1195	5.11	20	1
Se	C7	42.58	35.3-50.0	1307	7.62	37	1
Seed diameter	A8	19.41	12.1-26.7	386	3.77	21	2

Supplementary Table 2. QTL for root traits, seed diameter and 4th leaf mineral concentration in plants grown for 14 days in a 'pouch and wick' system with a ¼ strength Hoagland's solution and a glasshouse, respectively. -Log10(P) threshold of 3.88. Additive effect: 1 = Tapidor, 2 = Ningyou7.

Trait	Chr.	Position (cM)	CI (cM)	Marker	R^2 (%)	LOD	Add. effect
LRL	A1	52.38	44.6-60.2	A01-p3059596_A	12	5.48	1
MLRL	C1	24.99	17.2-32.8	s_16055_1-p448968_C	8	3.88	2
LRN	A1	69.17	61.4-77.0	A01-p1951988_A	9	4.26	1
LRD/PRL	C9	9.40	1.6-17.2	KBrH121P05-2_C	10	4.93	1
P	A4	58.08	50.3-65.9	A04-p8348750_A	12	5.76	2
Ca	C1	6.07	-1.70-13.9	A01-p28447868_C	7	3.68	2
	C2	35.6	27.8-43.4	s_16449_1-p251526_C	4	2.28	1
	C7	0.00	-7.8-7.8	s_15762_1-p717223_C	3	1.84	2
Mg	A1	56.81	49.0-64.6	A01-p2574969_A	9	4.32	1
	C7	2.60	-5.2-10.4	s_22310_1-p310994_C	7	3.77	2
S	A9	20.98	13.2-28.8	S001F23-2_A	10	5.35	2
	C4	111.47	103.7-119.3	s_18903_1-p158777_C	6	3.96	1
	C7	9.31	1.5-17.1	s_16450_1-p343032_C	17	8.90	2
K	A9	99.96	92.2-107.8	s_16197_1-p2958011_A	8	4.35	1
	A10	68.92	61.2-76.8	A10-p1406327_A	12	4.44	2
B	A2	85.01	77.2-92.8	BnMGD2-A2b_A	19	8.02	1
Mo	C7	9.31	1.5-17.1	s_16450_1-p343032_C	15	6.89	2
Zn	A1	29.66	21.9-37.5	A01-p19879925_A	8	3.90	1
	A4	51.66	43.9-59.5	s_24979_1-p134759_A	11	5.41	2
Cu	A2	79.86	72.1-87.7	bac-48_A	10	4.14	1
Seed diameter	A2	70.58	62.8-78.4	A02-p6570881_A	10	4.34	2
	A7	104.91	97.1-112.7	IGF2021z_A	9	3.85	2

Supplementary Table 3a. GEMs above the FDR (<0.10) in the ASSYST panel for LRD, in seedlings grown for 14d in a 'pouch and wick' system with a ¼ strength Hoagland's solution.

GEM marker	Chr.	FDR	TAIR ID	GENE
Cab013443.1	A9	0.002	AT2G22475.1	GEM/GL2
Cab047665.1	A9	0.005		
Cab038300.1	A9	0.006	AT5G61400.1	
Cab019786.1	A1	0.006	AT4G29530.1	
Cab029949.1	A10	0.008	AT1G01510.1	ANGUSTIFOLIA
Cab038301.1	A9	0.011	AT5G61390.1	
Bo9g017370.1	C9	0.011	AT5G63040.2	
Bo2g056290.1	A9	0.012	AT1G67850.2	
Cab042468.1	A1	0.020		
Cab038284.1	A9	0.020	AT5G61770.3	
Cab038259.1	A9	0.020	AT5G62580.1	SPR2
Bo6rg091500.1	C6	0.018	AT1G72470.1	EXO70 gene family,
Bo9g163130.1	C9	0.025	AT5G16510.2	RGP5
Cab014244.1	A9	0.030		
GSBRNA2T00140036001	C8	0.030	AT1G21680.1	
GSBRNA2T00094729001	A9	0.033		
Cab014110.1	A9	0.031	AT1G11890.1	ATSEC22
Bo6rg095240.1	C6	0.038	AT1G66580.1	SENESCENCEASSOCIATEDGENE 24
Bo6rg003540.1	C6	0.040	AT1G44790.1	
Cab014241.1	A9	0.039	AT1G08960.1	CAX11
Cab019692.1	A1	0.041	AT4G28470.1	
Cab013766.1	A9	0.041	AT1G17070.1	NTR1
Cab038283.1	A9	0.042	AT5G61790.1	ATCNX1
Bo3g088390.1	C9	0.043	AT2G14740.2	ATVSR3
Cab002089.1	A1	0.043	AT4G22890.2	PGR5-LIKE A
Cab047940.1	A6	0.043		
Cab014309.1	A9	0.045	AT1G07670.1	ATECA4
Bo4g143960.1	C4	0.045	AT5G37890.1	ATSINAL7
Cab038280.1	A9	0.044	AT5G62000.4	ARF1
Bo3g032470.1	C3	0.044	AT2G38430.1	
Cab014030.1	A9	0.048	AT1G13060.2	PBE1
Cab038347.2	A9	0.047	AT5G23630.1	PDR2
Cab019982.1	A1	0.049	AT4G31790.2	
Cab013955.2	A9	0.048		
Cab038319.1	A9	0.048	AT5G60920.1	COBRA
Cab005046.1	A7	0.048	AT3G24350.2	SYP32
Cab013891.1	A9	0.049	AT1G14710.2	
Bo4g012970.1	C4	0.048	AT2G41140.1	CDPK-RELATED KINASE 1
Bo1g055420.1	C1	0.049	AT4G15780.1	ATVAMP724

Supplementary Table 3b. Top ten most significant SNPs in the ASSYST panel for MLRL, in seedlings grown for 14d in a 'pouch and wick' system with a ¼ strength Hoagland's solution.

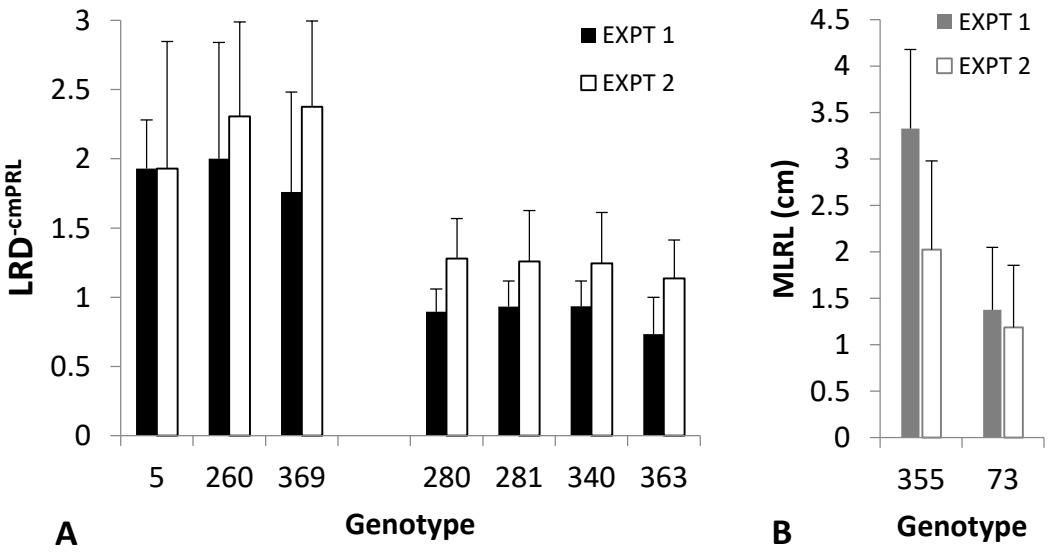
SNP marker	Position	FDR	TAIR ID	Gene
Bo7g097550.1:1662:A	C07	0.3		
Cab023288.1:765:A	A05	0.4		
Bo5g147000.1:775:A	C05	0.4		
Bo1g010670.1:597:T	C01	0.4		
Bo2g097550.1:461:A	C02	0.4		
Bo2g097550.1:762:G	C02	0.4		
Bo4g039080.1:1431:C	C04	0.4		
Cab023288.1:380:G	A05	0.4	AT3G06130.1	HIPP
Bo1g059160.1:1705:G	C01	0.4		
Cab015538.1:610:A	A03	0.4		

Supplementary Table 3c. Top 16 most significant SNPs in the ASSYST panel for TSW, in plants grown in a glasshouse.

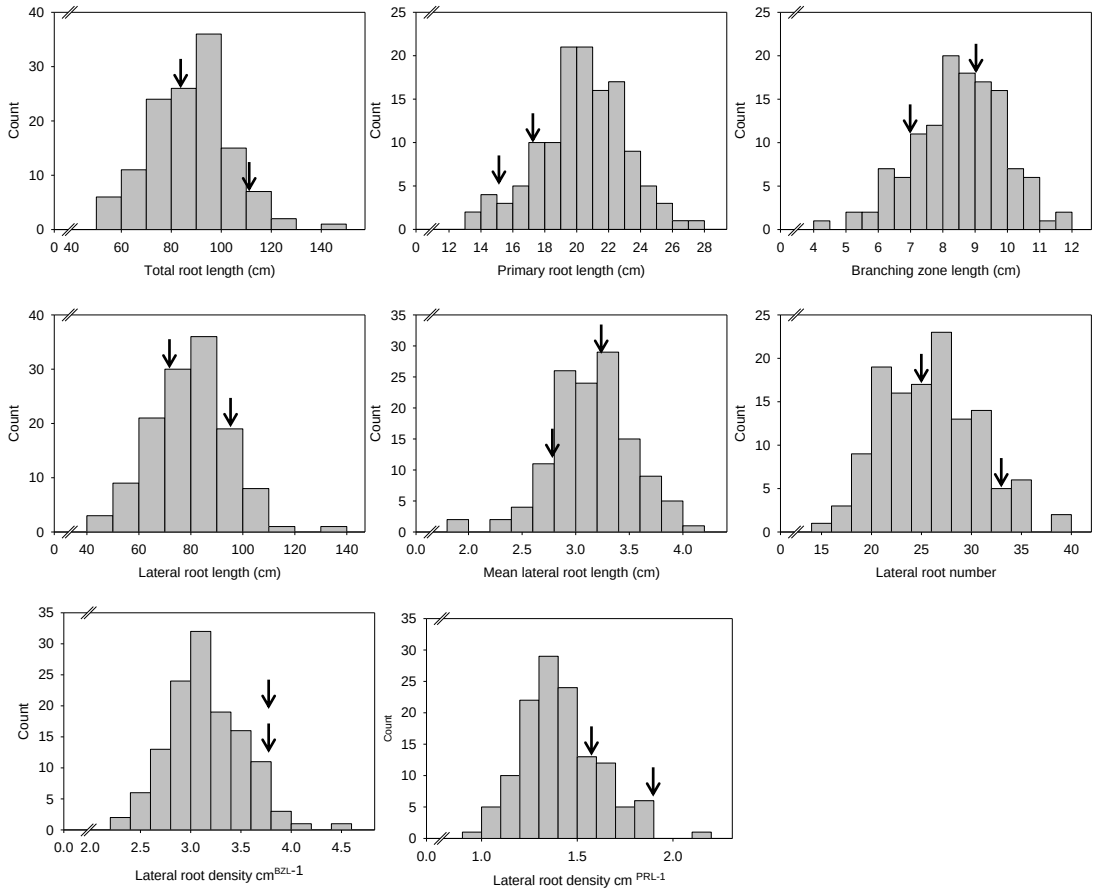
SNP marker	Chr.	FDR	TAIR ID	GENE
Bo2g047320.1:1662:G	C02/C09	0.1	AT5G52920.1	PKP2
Bo8g082880.1:507:C	C08	0.1		
Bo2g051140.1:519:T	C02	0.4		
Bo2g052760.1:183:C	C02	0.6		
Bo2g051140.1:648:G	C02	0.6		
Bo9g149880.1:446:C	C09	0.6		
Bo4g006860.1:902:C	C04	0.6		
Cab000531.1:563:C	A09	0.6		
Bo2g051140.1:564:A	C02	0.6		
Cab003626.1:767:G	A03	0.6		
Cab008008.1:735:T	A10	0.6		
Bo2g068140.1:450:C	C02	0.6		
Bo4g142620.1:3246:A	C04	0.6		
Bo2g100590.1:484:A	C02	0.6		
Bo8g058780.1:465:T	C08	0.6		
Bo9g133450.1:603:G	C09	0.6	AT5G57710.1	SMAX1

Supplementary Table 4. TCDH final genetic map summary.

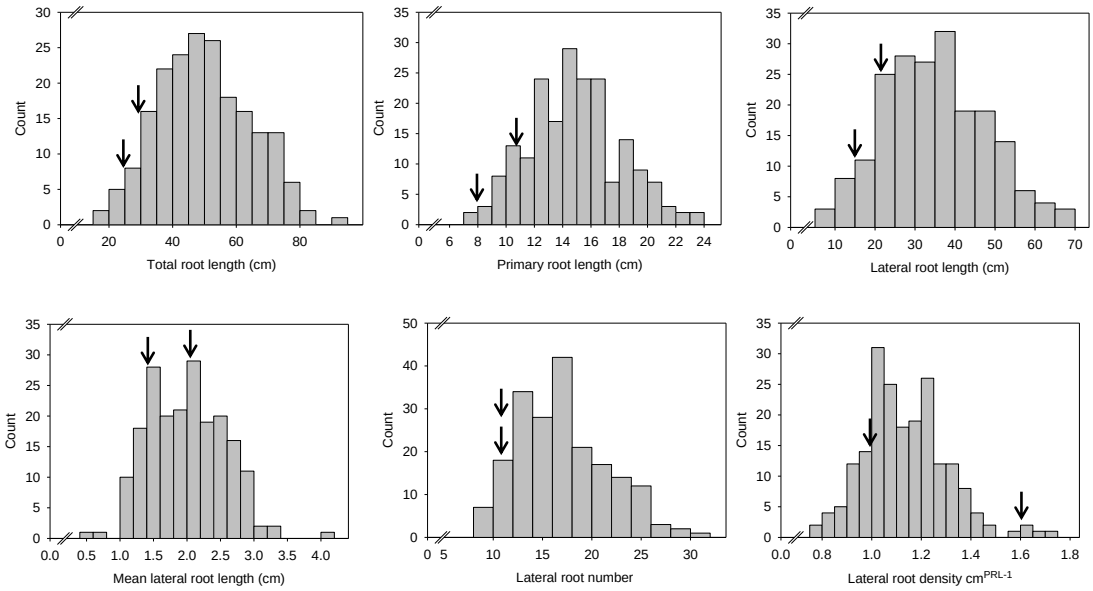
Linkage group	No. of markers	Length (cM)
1	48	26.86
2	70	26.14
3	55	19.37
4	31	16.88
5	39	22.45
6	51	23.76
7	67	20.81
8	38	19.79
9	64	31.41
10	54	15.78
11	14	40.92
12	34	51.07
13	116	64.13
14	73	52.19
15	56	45.98
16	20	36.57
17	81	30.03
18	54	38.30
19	45	47.11



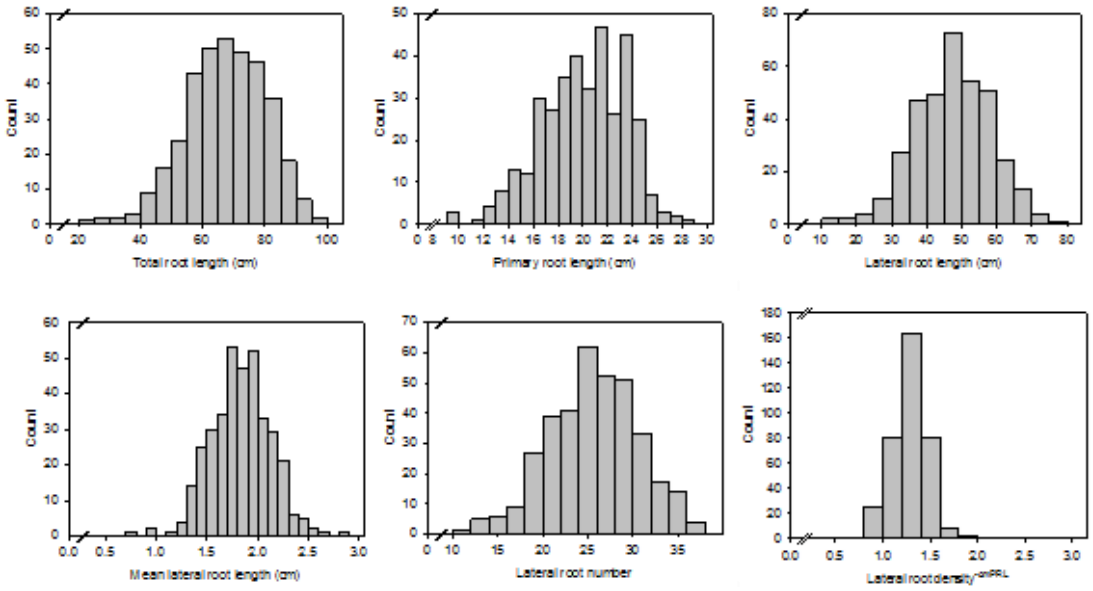
Supplementary figure 1. Candidate genotypes with the phenotypes; A. maximum- 5, 260, 369 and minimum- 280, 281, 340, 363 LRD, and B. maximum- 355 and minimum-73 MLRL, grown in 2 experiments (closed and open bars) for 14d in a 'pouch and wick' system in a ¼ strength Hoagland's solution. Bars = 1 SD.



Supplementary figure 2. frequency distribution of root traits in the TCDH population and parents (arrows) grown for 14 days under a ¼ strength Hoagland's solution in a 'pouch and wick' system.



Supplementary figure 3. frequency distribution of root traits in the TNDH population and parents (arrows) grown for 14 days under a ¼ strength Hoagland's solution in a 'pouch and wick' system.



Supplementary figure 4. frequency distribution of root traits in the ASSYST panel grown for 14 days in a 'pouch and wick' system.

Chapter 6

Genetic loci conferring a two-fold increase in magnesium concentration in *Brassica rapa* identified using a high-throughput forward genetic screen and bulked-segregant analysis

Thomas CL, Graham NS, Young S, Wilson L, O'Lochlainn S, King G, White PJ and Broadley MR

Abstract

Background and aims. There are substantial indications of a deficiency of mineral nutrients in modern Western diets. Genetic biofortification- breeding crops with greater mineral concentrations, is a sustainable approach to counter deficiencies. A high throughput screen of the leaf ionome of a *Brassica rapa* TILLING (Targeting Induced Local Lesions IN Genomes) population was undertaken in order to identify putative high magnesium (Mg) mutants. Markers associated with the loci underlying the mutation could be used for marker-assisted breeding of a high Mg Brassica vegetable crop.

Materials and methods. *B. rapa* mutagenized with EMS (ethyl methanesulfonate) (n =10,693) and WT (n = 300) seedlings were grown in a glasshouse and the 4th leaf sampled for ICP-MS analysis. Plants with concentrations of Mg between 3-5 SDs (standard deviation) of the global mean were selected as putative candidates. A high Mg mutation was confirmed in 6 successive generations and one line in the M_4 generation was back-crossed to the WT. Genomic DNA was pooled from sibling plants of two F_3 lines; which were homozygous recessive for the mutant phenotype and heterozygous for the WT phenotype. Bulked segregant analysis (BSA) using next-generation sequencing and SNP identification was performed on the lines.

Results and conclusion. In the $M_2 – F_3$ generations a high Mg accumulating mutant was identified, with a 1-2-fold increase in leaf Mg concentration ~2-3% DW (dry weight) compared to the wild-type ~1% DW. The mutant also had much greater concentrations of Na, Ca and S, and a lower concentration of K, compared to the wild type. Segregation ratios indicated that this was caused by a single recessive mutation. Analysis of SNP polymorphisms between the lines and a BLAST of candidate gene sequences to the genome sequence of *Arabidopsis* identified orthologous genes for K/ Na transport- *HKT1* and *CHX21*, and Mg transport- *MRS4/MGT6*. To our knowledge, no crop variety with high Mg accumulation, nor the underlying genes, has previously been identified.

Keywords: *Brassica rapa*, magnesium, mutant, accumulator, bulked segregant analysis, ion transporters.

Abbreviations: Mg (magnesium), Ca (calcium), Na (sodium), K (potassium), Mn (manganese), BSA (bulked segregant analysis), WT (wild type), EMS (ethyl methanesulfonate), SNP (single nucleotide polymorphism), TILLING (Targeting Induced Local Lesions IN Genomes).

Introduction

Humans require eight minerals in relatively large quantities- calcium (Ca), chloride (Cl), magnesium (Mg), nitrogen (N), phosphorus (P), potassium (K), sodium (Na) and sulphur (S). Ca and Mg are the most abundant group II elements in both plants and animals (Broadley *et al*., 2008). In humans, Mg is the second most abundant intracellular cation and the fourth most abundant element and regulates over 300 biochemical reactions (Hermans *et al*., 2013). Higher Mg intakes have been associated with reductions in systemic inflammation, blood pressure and metabolic syndrome, and hypomagnesemia has been identified in subjects with type II diabetes (Welch *et al*., 2009). Magnesium functions in plants are related to its capacity to interact with

nucleophilic ligands, it is the central molecule of a chlorophyll molecule and a bridging element for ribosomes. It is also essential for many enymes: RNA polymerases, ATPases, protein kinases, phosphatases, glutathione synthase and carboxylases (Shaul, 2002). Effects of Mg deficiency in plants include a breakdown in chlorophyll biosynthesis (Hermans *et al*., 2004; Hermans *et al*., 2010) and impairment in partitioning of carbon to sink organs (Hermans *et al*., 2004; Cakmak and Kirkby, 2008). Magnesium is phloem-mobile and easily translocated to sink organs- seeds and fruits (Karley and White, 2009).

There are substantial indications that concentrations of Mg in Western diets are deficient. Globally <1% of people are reported to be at risk of Mg deficiency (Kumssa *et al*., 2015). Yet, in developed countries such as the USA, the average magnesium daily intake has decreased from 475-500 mg to ~200 mg day^{-}1 from 1900 to 1992 (Grzebisz, 2011; Rosanoff, 2013). In the UK and USA, 9 and 14% of people respectively, consume quantities below the lowest reference nutrient intake (LRNI) levels and are at risk of physiological disorders, and much greater numbers have sub-optimal intake (White and Broadley, 2009; Broadley and White, 2010). A large-scale dietary intake survey in the USA found that 45% of people had intakes of Mg below the EAR (estimated average requirement, Fulgoni *et al*., 2011). Mg deficiency appears to be less prevalent in Africa, apparent in <4% of the entire continental population, although East African countries with a lower energy intake had a concomitant lower Mg supply (Joy *et al*., 2013; 2014). Current deficiency in mineral nutrients may in part result from ‘soil-dilution’ and ‘genetic-dilution’, whereby secondary nutrients become limited in intensive, high-yielding agriculture (Edmeades *et al*., 2004; Davis, 2009; Hermans *et al*., 2013). For example, archived wheat grains grown on the Rothamsted long term Broadbalk wheat experiment show reductions in Mg concentration of 19.6% in the modern high yielding varieties grown since 1968, compared to older varieties (Fan *et al*., 2008), similar observations have been made by Ficco *et al*. (2009). White and Broadley (2005) report significant declines in Mg concentrations in vegetable and fruit UK produce from the 1980s compared to the 1930s. Furthermore, in contrast to cereal grains, the production of pulses, fruit and vegetables which are rich sources of nutrients, have not kept pace with population growth in many countries (Welch *et al*.,

2002). And the milling process for flour leaves only 3-28% of the original mineral content (Fawcett *et al*., 1999).

Increasing human intake of essential mineral nutrients could be achieved through food fortification and supplementation approaches, however this is expensive and not widely available to those living in developing countries (Cakmak, 2008), and their health benefits are questionable (Elless *et al*., 2000). Therefore, agronomic biofortification of crops with essential nutrients through fertiliser application and/or genetic improvement are alternative approaches, which could complement one another. Supplementation of commercial fertilisers with selenium (Se) in Finland has been demonstrated to have a positive effect on the nutritive value of the whole food chain, from soil-plant-human and animal, and also to increase crop yield (Hartikainen, 2005). Soil and foliar fertilisation with zinc (Zn) increased grain Zn concentration and crop productivity in Turkey (Yilmaz *et al*., 1997). Although, the bioavailability of the applied mineral can be complicated by complex soil and plant physiological interactions (Stroud *et al*., 2010), and adverse environmental effects arise from excess fertilisation. Genetic improvement is a sustainable and economic solution to mineral and vitamin deficiencies (White and Broadley, 2005). Examples of genetic biofortification include the 'Golden Rice' variety, which has been significantly enhanced in vitamin A through transformation and has been bred into locally adapted varieties in Asia (Potrykus, 2008; http://www.goldenrice.org). A Broccoli variety with high glucoraphanin content and potential health benefits has been developed (Mithen *et al*., 2003) and commercialised as Beneforte broccoli (Seminis Vegetable Seeds, Inc).

However, naturally occurring diversity within germplasm for desired traits is often limited (Bus *et al*., 2011; Bhullar *et al*., 2013). Mutagenesis offers the possibility of artificially inducing such traits. The chemical mutagen EMS (ethyl methanesulfonate) induces point mutations at a high density, many of which could be stop-codons which are expected to produce a dramatic reduction in gene function (Stephenson *et al*., 2010). EMS mutagenisation has identified mutants with low linolenic acid in *B. rapa* and *B. napus* and *B. oleraceae* (Singer *et al*., 2014), in the *B. napus* mutant the *fad2* and *fad3* genes responsible for the mutation have since been cloned (Yang *et al*., 2012)

and the associated SNP markers patented for use in marker-assisted introgression (Hu *et al*., 2015).

Mineral nutrient concentrations including Mg are also often highest in Brassicas compared to other crops; broccoli and cauliflower (*B. oleracea*) have Mg concentrations of 0.6% DW, whereas in monocot species such as pea (*Pisum sativum*) and asparagus (*Asparagus officinalis*), and grain crops including maize (*Zea mays*) and common wheat (*Triticum aestivum*) the concentration is ~0.2% DW (Broadley *et al*., 2004). Similarly, Pennington and Fisher (2010) evaluated the nutritional components of 10 sub-groups of fruit and vegetables, and found that dark green leafy vegetables including Brassicas collard/kale (*B. oleracea*), mustard greens (*Brassica juncea*) and turnip greens (*B. rapa*), along with legumes (*Fabaceae*), contained the highest concentration of Mg and other nutrients, vitamins and antioxidants. Furthermore, the biovailability of nutrients is influenced by 'anti-nutrients' in the food e.g. phytate, phytic acid, oxalate and saturated fat (Bohn *et al*., 2004; Kumssa *et al*., 2015); oxalates are lower in kale (*B. oleracea*) and calcium absorption is much greater from this than in high-oxalate spinach (*Spinacea oleracea*) (Heaney *et al*., 1988; Heaney and Weaver, 1990). Diets rich in cruciferous vegetables have been associated with reduced incidence of cardiovascular related mortality and cancer (Bosetti *et al*., 2012; Traka *et al*., 2013). *Brassica* leaf and head factions are likely to be within the top three of 'vegetable' types consumed worldwide: production (tonnes) in 2013 was; 375m of potatoes, 163m of tomatoes, 94m of Brassica/cabbage including broccoli and cauliflower, 85m of onion, 37m of carrot and turnip, 25m of pumpkin/squash/gourd and less than 10m of lentils and pulses, this same trend is followed in Asia and Europe although not the Americas (FAOSTAT, 2016). Brassicas contribute ~10-15% of overall 'vegetable' consumption in 10 European countries, although in the UK and the Netherlands this was higher, and after fruiting vegetables, Brassica vegetables were consumed the most (EPIC, Agudo *et al*., 2002). Joy *et al* (2014) report food surveys from 46 African countries which show that Mg supply comes predominantly from cereal grains- 65%, although in Eastern Africa, vegetable and fruit may constitute a more important component of supply (Joy *et al*., 2013). Therefore, in Europe, Asia and East Africa it seems that there is significant potential to increase Mg intake and improve human health generally through Brassica vegetable consumption.

Brassica rapa (AA, 2n = 20) has wide genotypic and morphological variability in the form of several sub-species from different geographical regions, and is grown worldwide for the production of condiments, vegetables- Chinese cabbage, pak choi, mizuna, turnip roots and greens, vegetable oils and fodder crops. It has a fully sequenced genome (The *Brassica rapa* Genome Sequencing Project Consortium: Wang *et al*., 2011), and is the progenitor species of the A genome to *B. napus* the major oilseed crop. A TILLING (Targeted Induced Loci Lesions IN Genomes) population has been generated in *B. rapa* through EMS mutagenesis (RevGenUK: http://revgenuk.jic.ac.uk; Stephenson *et al*., 2010). Mutations in this population have identified the orthologue of the *Arabidopsis INDEHISCENT* gene controlling pod valve margin development (Girin *et al*., 2010) and the orthologue of the *Arabidopsis* Ca transporter *CAX1* (Ò Lochlainn *et al*., 2011). There is therefore much potential for comparative genomics and breeding in this species. To our knowledge, the genes underlying a high Mg accumulation, nor a high Mg crop variety have been identified, except a small 30% increase in tobacco (*Nicotiana benthamiana*) over-expressing the magnesium transporter *MGT1* (Deng *et al*., 2006). Furthermore, relatively little is known about the uptake and transport of Mg compared to other minerals such as Ca and K (Kobayashi and Tanoi, 2015). In the present study, a high throughput screen of this *B. rapa* TILLING population has identified a mutant Mg accumulator with ~300% Mg of the wild type. Bulked segregant analysis (BSA) has compared pools of DNA from individuals which show either high or low Mg concentration, but are arbitrary at other unselected loci (Michelmore *et al*., 1991). Next-generation sequencing and SNP identification has identified candidate causal genes for ion uptake and in-planta Mg, K and Na transport.

Materials and methods

Plant material

An inbred line of the *Brassica rapa* subsp. *Trilocularis (Yellow Sarson)* R-o-18 had been mutagenised using 0.4% ethyl methane sulfonate (EMS, Stephenson *et al*., 2010)

to develop a TILLING (Targeting Induced Local Lesions In Genomes) population, the population is used to screen for SNPs (Single Nucleotide Polymorphisms) within target genes. R-o-18 is self-compatible, rapid-cycling and can produce large numbers of seed. Seeds of the M_2 generation were acquired from *RevGen*UK (2007-09: http://revgenuk.jic.ac.uk/).

Growth conditions

Plants were grown at the Sutton Bonington Campus of the University of Nottingham (52°49'58.9"N, 1°14'59.2"W). The M_2 generation was grown in a single-skinned polytunnel (with a Visqueen Luminance Skin, Northern Polytunnels, Colne, UK) with no additional lighting or heating supplied and twice daily irrigation. Seeds were sown directly into fine-grade (<3 mm particle size) compost-based growing media (Levington Seed & Modular + Sand -F2S; Everris Ltd., Ipswich, UK) and plants were irrigated twice daily by hand. Following three weeks of vegetative growth, Vitafeed® 2-1-4 nutrient solutions (N-P-K: 16-8-32 + micronutrients; Vitax Ltd., Coalville, Leicestershire, UK) were applied to plants weekly at a rate of 3 g l-1. One sibling plant of each of 3,464 genotypes was sown per run and there were 3 runs in total. Pots were arranged in a randomised block design with 20 plants per tray, 23 trays per block, 2 blocks per bed, and 4 beds per run. In total 3 seeds of each genotype (n = 10,693) and 300 WT (wild type; R-o-18) were sown. Plants were covered by 380 x 900 mm micro-perforated pollination bags (Focus Packaging & Design Ltd, Brigg, UK) once inflorescences began to show to prevent cross pollination. M^3 seeds were harvested and stored at -10°c in a chilling cabinet.

Generations $M_3 – F_3$ and the corresponding R-o-18 WT were grown in a glasshouse with supplementary artificial lighting (Philips Master GreenPower SON-T 400 W bulbs controlled by Grasslin Uni 45 timer) used to maintain day lengths of 12 h light d-1. Seeds were sown directly into fine-grade (<3 mm particle size) compost-based growing media (Levington Seed & Modular + Sand -F2S; Everris Ltd., Ipswich, UK) and plants were irrigated twice daily by hand. Following three weeks of vegetative growth, Vitafeed® 2-1-4 nutrient solutions (N-P-K: 16-8-32 + micronutrients; Vitax Ltd., Coalville, Leicestershire, UK) were applied to plants weekly at a rate of 3 g l-1 via overhead irrigation. Plants were transplanted into individual 5 L pots (internal Ø

22.5 cm; height 18 cm) containing Levington M3 compost (Scotts Professional, Ipswich, UK) after leaf sampling and before flowering. Plants were covered by 380 x 900 mm micro-perforated pollination bags (Focus Packaging & Design Ltd, Brigg, UK) once inflorescences began to show to prevent cross pollination, except in the M_4 generation where all plants of line 19.6 were back-crossed to a WT plant, via emasculation and hand pollination. Seeds of each successive generation were harvested and stored at -10°c in a chilling cabinet.

Leaf sampling, digestion and ICP-MS (Inductively coupled plasma mass spectrometry) analysis

The 4th leaves were sampled at the rosette stage (typically 6-8 true leaves showing). They were dried in paper bags at 50°C for 48 h. Leaves of approximately 0.05-0.1g were hand-crushed to a powder within the bag and the exact weight recorded. Samples were digested using a microwave system comprising a Multiwave 3000 platform with a 48-vessel MF50 rotor (Anton Paar GmbH, Graz, Austria); digestion vessels were perfluoroalkoxy (PFA) liner material and polyethylethylketone (PEEK) pressure jackets (Anton Paar GmbH). Samples were digested in 2 mL 70% Trace Analysis Grade HNO_3, 1 mL Milli-Q water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, UK), and 1 mL H_2O_2, with microwave settings as follows: power = 1400 W, temp = 140°C, pressure = 20 Bar, time = 45 minutes. Two operational blanks and one sample of an external certified reference material (CRM) of Brassica leaf (IAEA-359 Cabbage; LGC, Teddington, UK) were included in each digestion run. Following digestion, each tube was made up to a final volume of 15 mL by adding 11 mL Milli-Q water and transferred to a universal tube and stored at room temperature. Leaf digestates were diluted 1-in-10 with Milli-Q water prior to elemental analysis.

The concentrations of 28 elements were obtained using ICP-MS (Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany); Ag, Al, As, B, Ba, Ca, Cd, Cr, Co, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, U, V, Zn. Operational modes included: (i) a helium collision-cell (He-cell) with kinetic energy discrimination to remove polyatomic interferences, (ii) standard mode (STD) in which the collision cell was evacuated, and (iii) a hydrogen collision-cell (H2-cell). Samples were introduced from an autosampler incorporating an ASXpress™ rapid uptake

module (Cetac ASX-520, Teledyne Technologies Inc., Omaha, NE, USA) through a PEEK nebulizer (Burgener Mira Mist, Mississauga, Burgener Research Inc., Canada). Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Sc (20 µg L-1), Rh (10 µg L-1), Ge (10 µg L-1) and Ir (5 µg L-1) in 2 % trace analysis grade HNO3 (Fisher Scientific UK Ltd). External multi-element calibration standards (Claritas-PPT grade CLMS-2; SPEX Certiprep Inc., Metuchen, NJ, USA) included Ag, Al, As, B, Ba, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti (semi-quant), U, V and Zn, in the range 0 – 100 µg L-1 (0, 20, 40, 100 µg L-1). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, Courtaboeuf, France) was used to create Ca, K, Mg and Na standards in the range 0-30 mg L-1. Boron, P and S calibration utilized in-house standard solutions (KH2PO4, K2SO4 and H3BO3). In-sample switching was used to measure B and P in STD mode, Se in H2-cell mode and all other elements in He-cell mode. Sample processing was undertaken using Qtegra™ software (Thermo Fisher Scientific) with external cross-calibration between pulse-counting and analogue detector modes when required.

Additional physiological and mineral measurements

In the F_3 generation 12 further plants of each F_3 line (19.6.1.1, 19.6.1.9 and R-o-18 WT) were grown as above. Six of the plants were sampled at the rosette stage and roots were washed and dried for mineral analysis (3 roots were pooled per sample to attain an adequate sample weight). The remaining six plants were grown until maturity and the 1st, 8^{th} and cauline leaves, immature pods/flowers, mature pods, stem and mature seeds were sampled for mineral analysis, a dry weight of the shoot, root and seed yield at maturity was made. The relative leaf chlorophyll content referred to as SPAD values (SPAD-502Plus, Konica Minolta, 2009) of each set of leaves were taken in 3 positions on the leaf and a mean was calculated.

Data analyses

In the M_2 generation the mineral concentrations of the 10,392 mutagenised and 300 WT leaves were compiled into a database. For each data-point, element-specific operational blank concentrations (ICP-run means) were subtracted on a per run basis, before correcting for sample weight. The LOD (limit of detection) was classed as 3*

standard deviation (SD) of the blank mean and samples with an Mg concentration less than this were removed. Outlier samples were then removed if they had Mg concentrations greater than 5* SD of the global elemental mean. Putative enhanced magnesium (Mg) concentration candidates were selected from the remaining samples which had a mean concentration greater than 3* SD of the global elemental mean. In the M_3 generation and onwards, from each data point element-specific operational blank concentrations (ICP-run means) were subtracted before correcting for sample weight, outliers were not apparent from visual data inspection.

T-tests were performed to calculate differences between the mutant and WT lines in concentrations of Mg, Ca, K, Na, S and Mn and in leaf chlorophyll (SPAD) measurements. All statistical analyses were conducted using GenStat 15th Edition (VSN International Ltd, Hemel Hempstead, UK).

Work-flow with successive generations M_2-F_3

Putative mutant candidate lines were selected from the M_2 generation which had concentrations of Mg > 3* < 5* SD of the elemental mean. In an initial run of the subsequent M_3 generation of these candidate lines, 20 sibling plants of each and the WT were grown. Only line 2343 displayed the enhanced Mg concentration observed in the previous generation in all progeny. In the subsequent M_4 generation of line 2343, 6 sibling plants of each of the remaining 18 lines and the WT were grown. Line 19 showed low variation, so all plants were back-crossed to the WT to generate a segregating line. In the subsequent F_1 generation 12 sibling plants of each of line 19.1 and 19.6 and the WT were grown. In the subsequent F_2 generation 12 sibling plants of each of lines 19.1.2, 19.1.4, 19.6.1 and 19.6.5 and the WT were grown. In the subsequent F^3 generation, 12 sibling plants of each of lines 19.6.1.1, 19.6.1.4, 19.6.1.9, 19.6.5.1 and 19.6.5.12 and the WT were grown. Leaves from the progeny of line 19.6.1; 19.6.1.1 and 19.6.1.9 and the WT were sampled for DNA extraction and the DNA from all siblings of the line were pooled for BSA (**Fig**. 1).

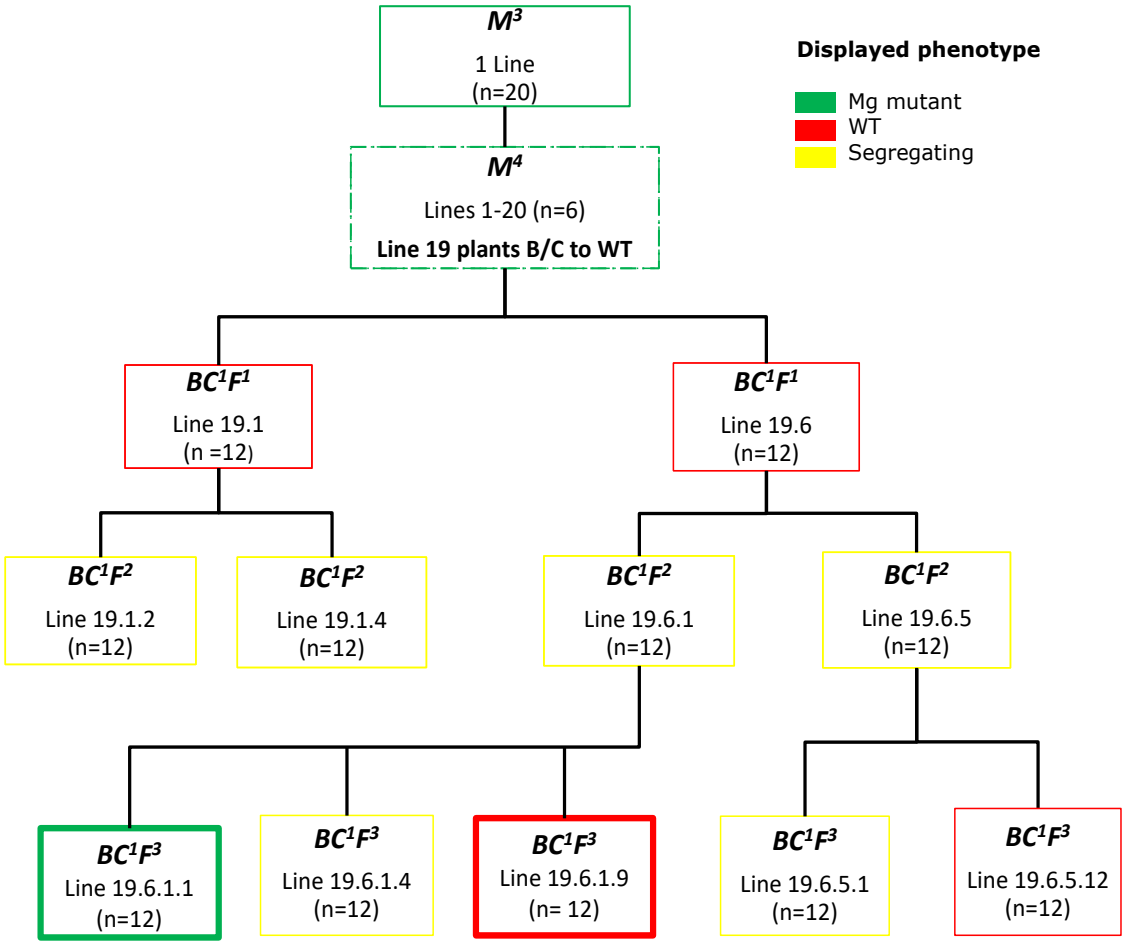


Figure 1. The plant lines grown in each successive generation; M3- F3 of mutant line 2343 and the phenotype they display. Green box = all mutant siblings displayed the mutant phenotype, red box = all mutant siblings displayed the WT phenotype, yellow box = mutant siblings were segregating for the mutant/WT phenotype. Broken line = mutant line 19 back-crossed to WT. Bold line = line used for bulked segregant analysis (BSA).

Bulked segregant analysis (BSA) and SNP (Single Nucleotide Polymorphisms) analysis in the F_3 generation

In the F_3 generation all 12 sibling plants of line 19.6.1.1 showed the mutant phenotype and all 12 sibling plants of line 19.6.1.9 showed the WT phenotype (Fig 3.F). The 5th leaf from each plant as well as from 12 R-o-18 WT plants were collected and immediately frozen in liquid nitrogen, they were stored at -80 in foil wrappers. The samples were later ground using a pestle and mortar in liquid nitrogen and transferred to eppendorf tubes and kept on liquid nitrogen. DNA was extracted from all plants

according to the protocol of the GeneJET Genomic DNA Purification Kit (ThermoFisher SCIENTIFIC, 2015: https://tools.thermofisher.com/content/sfs/manuals/MAN0012663_GeneJET_Genomic_DNA_Purification_UG.pdf). The DNA from each sibling plant of the lines were pooled.

Pooled samples were sequenced by TGAC (Norwich, UK) using an Illumina HiSeq2500 (Illumina, Inc), with 125bp paired end read metric. Each HiSeq lane generated at least 100 million pairs of reads. The sequences were aligned to the reference genome AENI01000000 (The Brassica rapa Genome Sequencing Project Consortium, 2011) on Genbank (http://www.ncbi.nlm.nih.gov/genbank).

SNP analysis comparing the mutant line with the sibling WT line was performed. Where the three genotypes were missing a SNP call at a particular position, the missing SNP was imputed from that of the *B. rapa* reference genome. WT SNPs were then removed from further analysis if they did not match those of R-o-18. The physical position of the SNPs was determined. In method 1, a count was made of the number of SNPs within each 100Mbp interval along the chromosome. In method 2, a count was made of the number of SNPs within a contig region. All analysis was performed using custom made R scripts. To identify *Arabidopsis* orthologous genes, the reference SNP sequence for the contig within the physical region with the greatest frequency of SNPs was BLASTED to the genome using GenBank (NCBI).

Results

M_2 generation candidates

In the M_2 generation there were 2,758 outliers with Mg concentrations lower than the LOD, of the remaining 7,634 samples there were 22 outliers with concentrations of Mg > 5* SD of the elemental mean (a concentration greater than 6% DW) these were removed from analysis. The Mg concentration of the remaining 7,612 mutant samples differed from the expected estimates in often being extremely high and some being

lower than expected (mean = 1.1% DW, range = 0.01-5.8% DW, SD = ± 0.3% DW), whereas the WT generally had concentrations within the 95% confidence interval (mean = 1.1% DW, range = 0.6–2.1% DW, SD = ± 0.2% DW, **Fig.** 2). Mutant candidate lines were selected from the M_2 generation which had concentrations of Mg > 3* < 5*SD of the elemental mean (a concentration > 2.3% < 6% DW), on this basis there were 83 putative candidate lines, but only 3 of which had set seed were selected, one line being 2343 which had an Mg concentration of 3% DW (**Fig**. 3A).

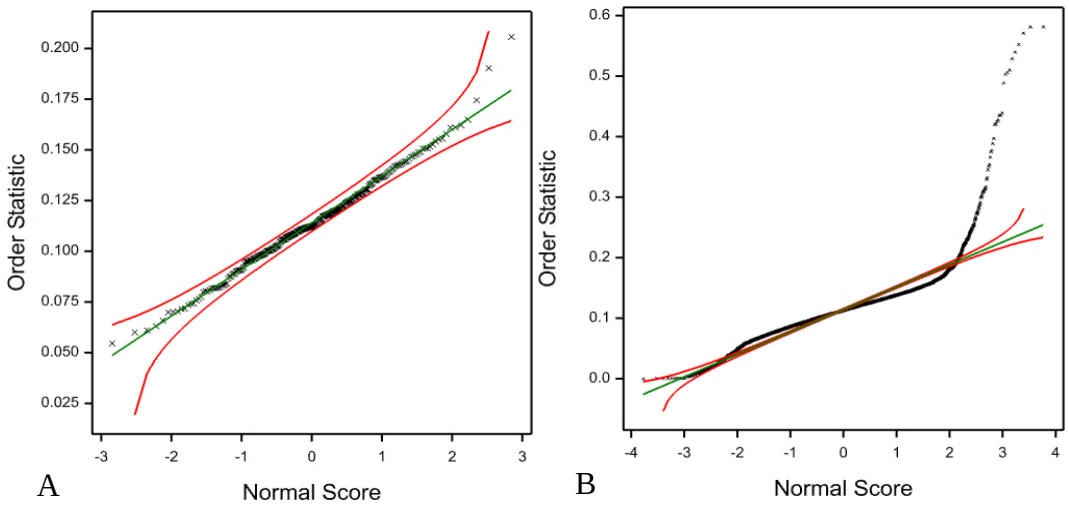


Figure 2. M_2 generation 4th leaf concentration (% DW) of Mg (y axis) in A. WT (n = 300) and B. mutants (n = 10,392) as a function of expected values (x axis). Green line= 1-1 reference line. Red lines = 95% simultaneous confidence bands. (NB. Values after removing outlier samples with Mg concentrations < LOD and > 5* SD of global mean).

Magnesium concentrations in future generations of the putative mutant

In the subsequent M_3 generation, only line 2343 displayed the enhanced Mg concentration observed in the previous generation in all 18 sampled progeny- ~2-3% DW, compared to ~1% DW in the WT, a 1.2-fold difference (t=8.6). The mutations appeared to be homozygous showing no obvious segregation (**Fig**. 3B). In the subsequent M_4 generation all 6 sibling plants of each of 18 lines showed enhanced Mg concentrations ~1.5-3% DW, compared to ~1% DW in the WT (t=19.4), again showing homozygous mutations (**Fig**. 3C). In this generation, all plants of line 19 were back-crossed to the WT to generate segregating lines. In the subsequent F_1 generation the 12 progeny of each of line 19.1 and 19.6 showed the WT phenotype with a Mg

concentration ~0.6% DW which was equivalent to the WT, confirming a successful back-cross and recessive mutant loci (**Fig**. 3D). In the subsequent F_2 generation the 12 progeny plants of each of lines 19.1.2, 19.1.4, 19.6.1 and 19.6.5 were segregating from 2:1 and 1:1 WT/mutant phenotype, with Mg concentrations ranging from ~0.5-3% DW, compared to ~1% DW in the WT (**Fig**. 3E). In the subsequent F_3 generation, progeny of line 19.6.1 showed the following phenotypes; 19.6.1.1 mutant phenotype- mean of 3.1% DW (t=13.0), 19.6.1.4 segregating phenotype- mean of 2.6% DW (t=6.4) and 19.6.1.9 WT phenotype- mean of 1.4% DW (t=0.4). Progeny of line 19.6.5 showed the following phenotypes; 19.6.5.1 segregating phenotype- mean of 2.2% DW (t=5.1) and 19.6.5.12 WT phenotype- mean of 1.1% DW (t=-2.5), compared to a mean of 1.3% DW in the WT (**Fig**. 3F). Therefore, F_3 sibling lines of line 19.6.1 consistently showed either the mutant or WT phenotype, and these lines were used for BSA.

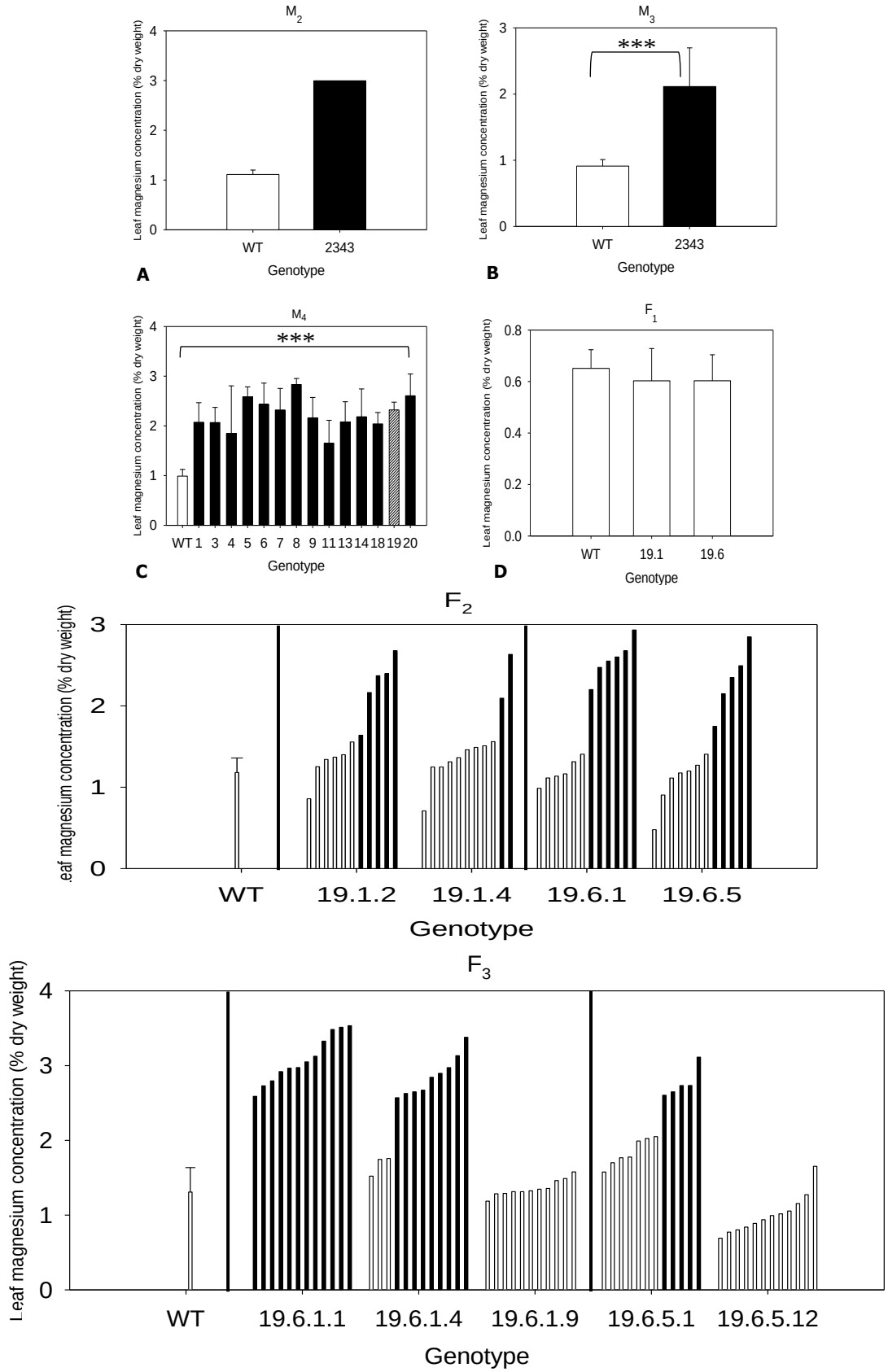


Figure 3. 4th leaf Mg concentration (% DW) of the wild type (WT) and mutant genotype lines in generations; A. M_2 (n = 300, 1) B. M_3 (n = 20 per line) C. M_4 (n = 6 per line) D. F_1 (n = 12 per line) E. F_2 (n = 12 per line) F. F_3 (n = 12 per line). White box = displaying the WT phenotype, black box = displaying the mutant phenotype, hashed box = back-crossed to WT in M_4. Error bars = 1 SD. *** = $p < 0.001$.

Other mineral concentrations in the F_3 generation leaf

The 4th leaf of the homozygous mutant line as compared to the WT also had greater concentrations of Ca- 2.9 and 2.1%, 0.7-fold difference (t=5.5), Na- 0.30 and 0.16%, 1-fold difference (t=5.3) and S- 1.8 and 1.2%, 0.8-fold difference (t=4.61), respectively, but a reduced concentration of K- 4.3 and 5.0%, 0.6-fold difference (t=-2.3) and Mn- 0.16 and 0.24%, 0.8-fold difference (t=-4.0), respectively (**Fig**. 4).

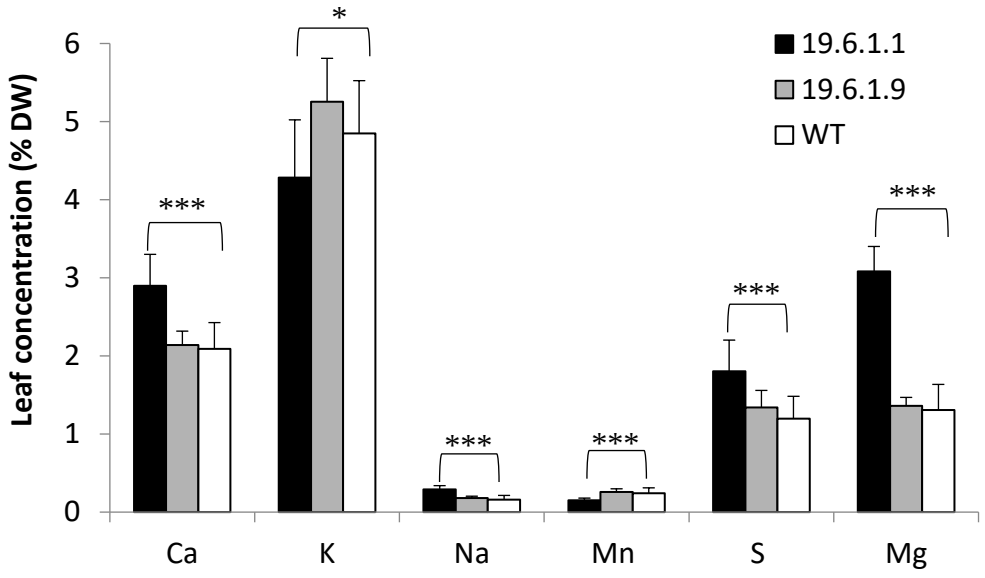


Figure 4. Mean 4th leaf concentration (% DW) in the WT, 19.6.1.9 and 19.6.1.1 lines (n=12), in the F3 generation of plants grown in a glasshouse. (NB Mn concentration displayed as an order of magnitude higher than actual concentration). Error bars = 1 SD. ***** = p <0.05,******* = p <0.001.

Mineral concentrations in the roots of the F_3 generation

The mineral concentration in the roots (**Fig**. 5) followed a similar pattern to the leaves, whereby the homozygous mutant compared to the WT had greater concentrations of Mg- 0.37 and 0.21%, 0.9-fold difference (t=64.2), Na- 0.51 and 0.25%, 1-fold difference (t=18.3) and S- 0.11 and 0.09%, 0.6-fold difference, and a lower concentration of Mn- 0.96 and 1.2%, 0.6-fold difference, respectively. Although, there was no difference between them in the Ca concentration. However, interestingly, K

concentration in the root as compared to the leaf was an order of magnitude greater in both genotypes, and conversely was higher in the mutant than the WT- 7.3 and 6.3%, respectively, 0.6-fold difference (t= 6.0).

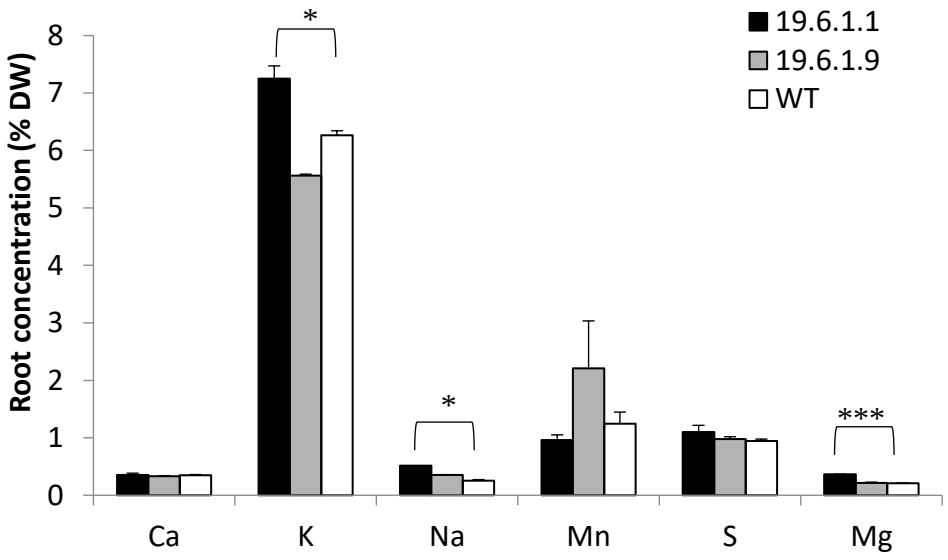


Figure 5. Mean mineral concentration (% DW) in the root of 14 day old plants of the WT, 19.6.1.9 and 19.6.1.1 lines (n=12), in the F3 generation of plants grown in a glasshouse. (NB. Mn concentration displayed as an order of magnitude higher than actual concentration). Error bars = 1 SD. * = p <0.05, *** = p <0.001.

Magnesium concentration and physiological measurements in other plant parts in the F_3 generation

The homozygous mutant as compared to the WT had greater Mg concentrations (% DW) in all leaves; 1st leaf- 3.6 and 1.5% (t = 10.0), 8th leaf- 1.6 and 0.9% (t = 6.1) and cauline leaf- 2.4 and 1.3% (t = 8.4), immature pods/flowers- 0.81 and 0.59% (t = 5.2), mature pods- 0.88 and 0.56% (t = 5.0), stem- 0.90 and 0.50% and seeds- 0.50 and 0.42% (t = 2.6), respectively (**Fig**. 6A). The homozygous mutant as compared to the WT had lower chlorophyll content in the leaves; 1st (t = -3.6), 4th and 8th (t = -2.7) (**Fig**. 6B), a lower dry shoot weight- 14.4 and 18.1g (t= -1.7), root weight- 0.61 and 1.0g and seed yield- 1.3 and 2.9g (**Fig**. 6C; **Fig**. 8), respectively.

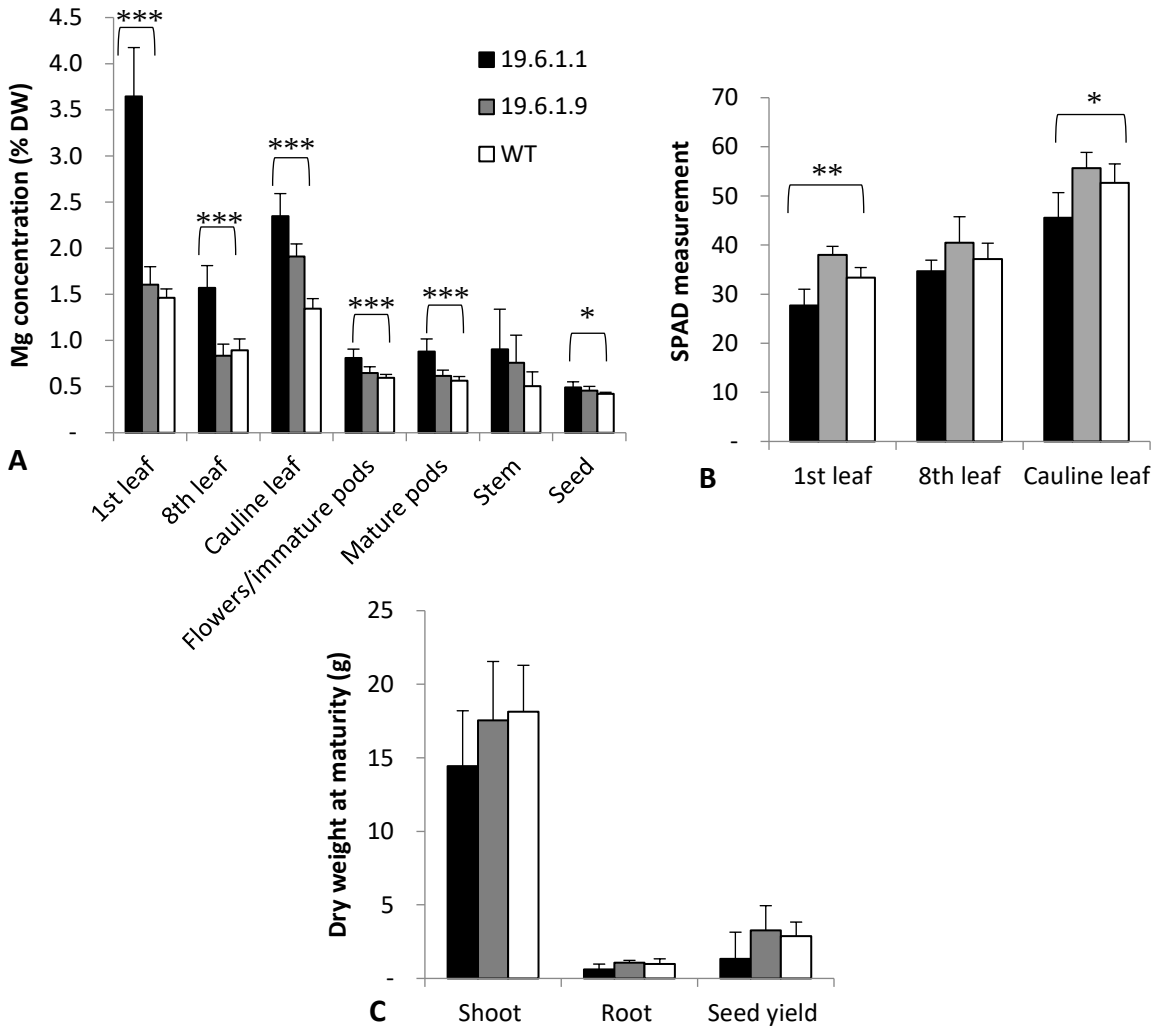


Figure 6. A. Mean Mg concentration (% DW), B. SPAD reading (chlorophyll measurement, no unit), C. shoot, root and seed yield dry weight at maturity, in the F3 generation of plants grown in a glasshouse. Error bars = 1 SD. * = p <0.05, ** = p <0.01, *** = p <0.001.

Bulked segregant analysis (BSA)

The greatest frequency of SNPs between the homozygous mutant and heterozygous sibling lines were within the 16,700-16,800 Kbp region at accession AENI01001670 on chromosome 2. In *Arabidopsis* the orthologue gene is *HKT1/ HIGH-AFFINITY K+ TRANSPORTER 1* (AT4G10310), a high-affinity K and low-affinity Na transporter expressed in xylem parenchyma cells. Using a slightly different method to assess SNP frequency, at the second highest region at accession AENI01003613 on chromosome 4 was the orthologue gene *CHX21*/*CATION/PROTON EXCHANGER21* (AT2G31910), an Na/H antiporter which regulates xylem and leaf Na and K concentration. At

accession AENI01008775 on chromosome 10 was the orthologue Mg transporter *MRS24*/*MGT6* gene, which is expressed in both the root and shoot and is known to increase Mg uptake (**Fig**. 7).

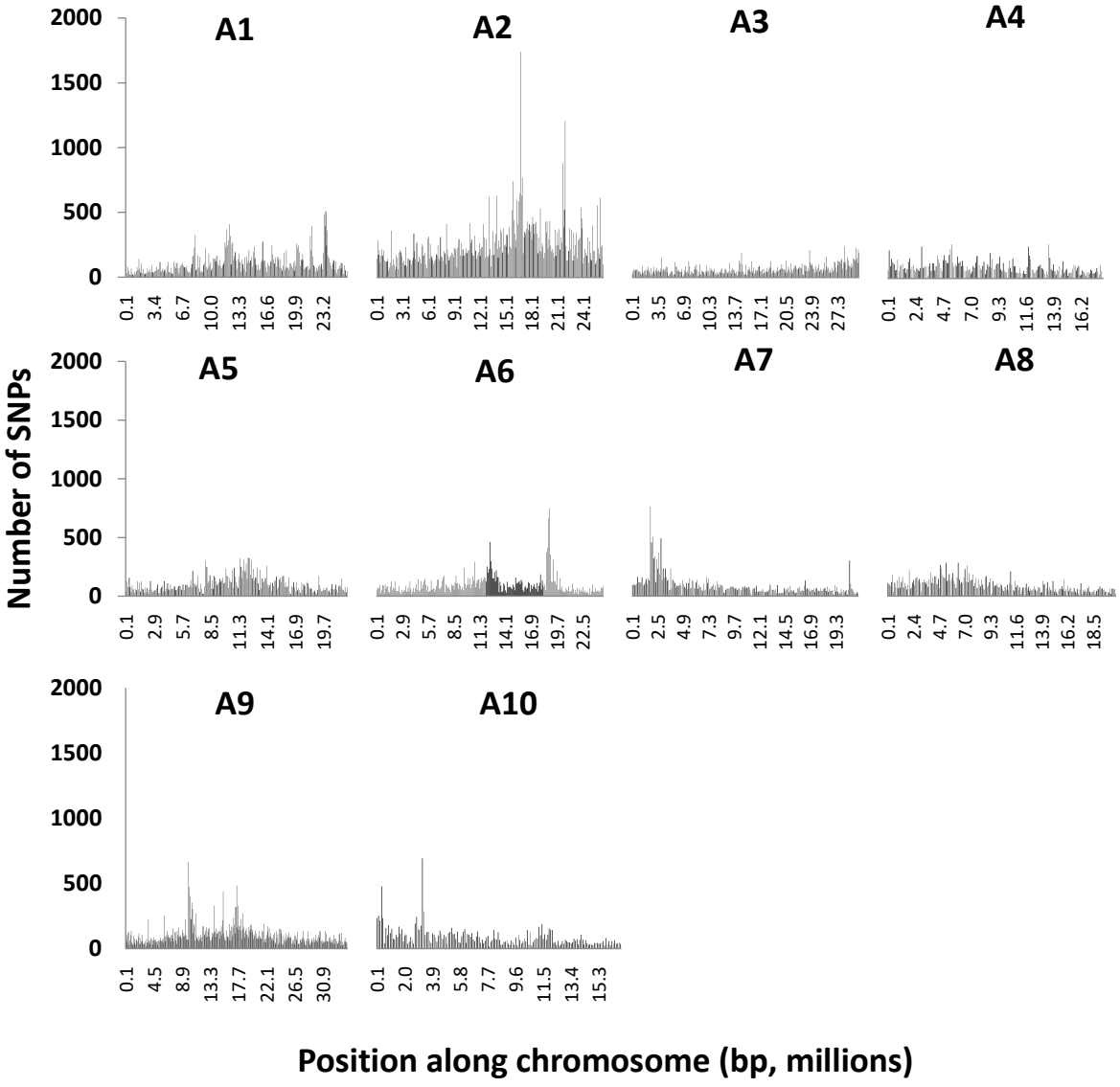


Figure 7. The number of SNPs between the F_3 generation homozygous mutant and segregating WT. From sequenced DNA acquired using BSA. Bars represent bins of 100 Kbp (kilo basepairs).

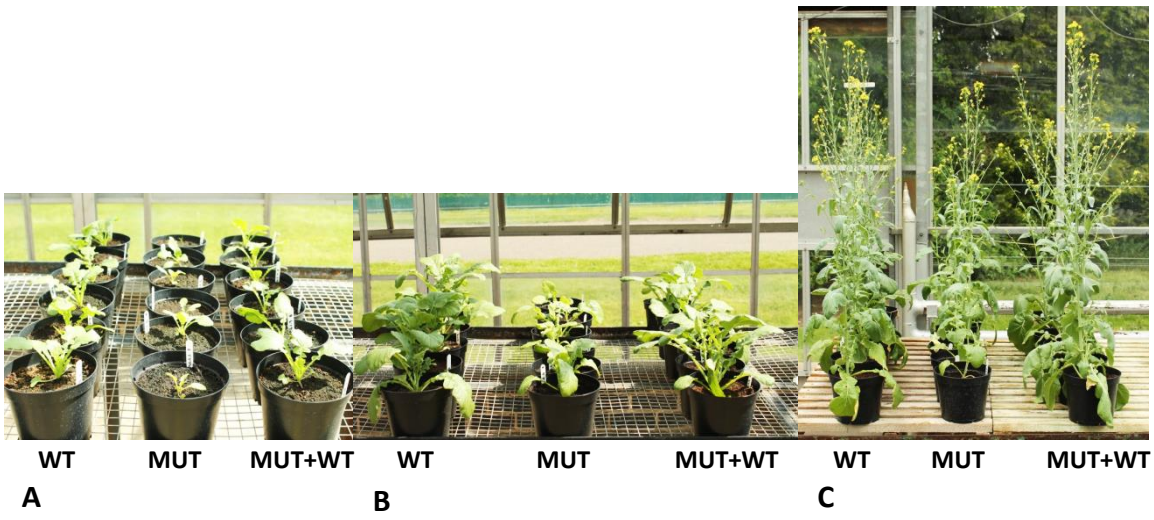


Figure 8. F_3 generation plants of WT, homozygous mutant and segregating WT lines at; A. 14 DAS (rosette stage) B. 25 DAS C. 50 DAS, in plants grown in a glasshouse.

Discussion

It has been observed here in *B. rapa* that the leaf Mg concentration of the wild type is ~1% DW with a range of 0.6–2.1%, whereas the mutant has a concentration of ~2-3% DW with a range of 0.01-5.8%, thus a 1-2- fold increase over the wild type in the mutant population. Concentrations of Mg in plant leaves are typically 0.3% DW, as observed in various dicot and monocot species growing naturally in the UK (Thompson *et al*., 1997) and in a controlled glasshouse study (Broadley *et al*., 2004). Dicots, in particular Brassicales- 0.5% DW and Caryophyllales- 0.8% DW, generally have greater leaf Mg concentrations compared to monocots e.g. Poales- 0.2% and Asparagales- 0.3%. Interestingly, the Caryophyllales also have a much greater shoot Mg: Ca ratio compared to other angiosperms (Broadley *et al*., 2004). In *B. oleracea* leaf Mg concentration has previously been observed; ranging from 0.4-0.8% DW in a diverse collection of 355 accessions (Broadley *et al*., 2008), a range of 0.2–0.4% DW in a diversity collection of hybrid broccoli heads (Farnham *et al*. (2000), and a range of 0.3-0.6% DW in 22 cultivars (Kopsell *et al*. (2005). In a *B. rapa* DH (doubled haploid) Chinese cabbage population, a leaf Mg range of 0.2-0.5 % DW was observed (Wu *et al*., 2008). Thus a larger variance than has previously been observed in natural

populations has been found here in the mutangenised plants, and demonstrates the advantage of mutagenisation for acquiring functional and desirable characteristics for crop improvement compared to utilising natural diversity.

Numerous genes controlling the transport of Mg have been identified. The most characterised Mg channel is the *CorA* protein first identified in the bacteria *Salmonella typhimurium* (Nelson and Kennedy, 1972). Ten homologs of this protein have been identified in *Arabidopsis*; including *MRS2* (*Mitochondrial RNA splicing 2*)/*MGT* (*Magnesium Transporters*, Li *et al*., 2001). These genes exhibit Mg/H+ anti-porter activity maintaining cytosolic Mg concentrations (Gerbert *et al*., 2009) and the physiological functions of most of these genes have been characterised. Other Mg transporters also identified in *Arabidopsis* are the *AtMHX1* (*Magnesium/Proton exchanger 1*) (Shaul *et al*., 1999); r*oot calcium channels* (*RCA*) in the plasma membrane which are permeable to a number of monovalent and divalent cations (Pineros *et al*., 1995); and the cyclic nucleotide-gated channel *AtCNCG1O* which transports K, Ca and Mg (Li *et al*., 2005; Guo *et al*., 2010). *CorA* homologs have also been identified in rice (*Oryza sativa*, Gerbert *et al*., 2009) and maize (*Zea mays*, Li *et al*., 2016). *Arabidopsis lyrata* genotypes growing naturally on serpentine (high Mg:Ca ratio) soil were mutated at loci for *MRS2* transporters compared to genotypes from non-serpentine soils (Turner *et al*., 2010). In an *Arabidopsis* mapping population *MRS2-2* and *MRS2-3* transporters co-located with QTL for seed Mg concentration (Waters and Grusak, 2008). Magnesium is more highly accumulated in the mesophyll as compared with epidermal leaf cells (Conn *et al*., 2011; Rios *et al*., 2012), and a number of the *MRS2* transporters have been shown to be more highly expressed in the mesophyll cells (Conn *et al*., 2011). More recently, the *MRS2-4*/*MGT6* transporter has been shown to respond to both low and high Mg concentrations in *Arabidopsis* (Mao *et al*., 2014; Oda *et al*., 2016), and its expression is highly induced in the root plasma membrane (Mao *et al*., 2014). This demonstrates the utility of transporters for increasing Mg uptake. However, as yet, no transporters have been demonstrated to significantly increase tissue Mg concentration (Conn *et al*., 2011), except a small 30% increase in tobacco (*Nicotiana benthamiana*) over-expressing *MGT1* (Deng *et al*., 2006). Using fast-neutron mutagenisation in *Arabidopsis*, a putative Mg accumulating mutant with 79% greater concentration than the WT was identified (Lahner *et al*.,

2003), although after further back-crossing this phenotype was lost (Tian *et al*., 2010). In *Brassica* species, shoot Mg concentration has been linked to QTL (quantitative trait loci) on chromosomes C08 in B. oleracea (Broadley *et al*., 2008), A08 in *B. rapa* (Wu *et al*., 2008), and in seeds of *B. napus* on A07 (Ding *et al*., 2010). In a diversity panel of *B. napus*, the SNPs most significantly associated for leaf Mg were found on chromosome C09 (Bus *et al*., 2014), and A09 and A02 (Alcock, 2016, pers comm).

As well as high Mg, the mutant also had greater leaf concentrations of Na- 1-fold, Ca- 0.8-fold and S-0.8-fold, but a lower concentration of K- 0.5-fold. Likewise, in the root, the mutant had greater concentrations than the wild type of all the aforementioned minerals, except for Ca. However, the difference in Mg concentration between the mutant and the wild type in the root was half that of the shoot. And in the root Na rather than Mg concentration differed more between the mutant and wild type. Interestingly, whereas the concentration of K in the leaf was lower in the mutant compared to the wild type, in the root the mutant concentration was 0.5-fold greater than the wild type. The proportional differences between the mutant and wild type in Na and Mn concentration were similar for the root and shoot. Therefore, the transport of the aforementioned ions into the root was enhanced in the mutant, however transport from the root to the shoot was inhibited for K, whereas for Mg and Ca it was enhanced. This perhaps indicates competitive inhibition between Mg/Ca and K transport from the root to the shoot. There is a strong correlation between the ability of a plant to accumulate Ca and its ability to accumulate Mg (White, 2001, 2005; Broadley *et al*., 2004; Bus *et al*., 2014). Magnesium, Ca and K can also competitively inhibit the uptake and transfer of one another (Oda *et al*., 2016).

The region with the greatest density of SNPs on chromosome 2 has a homologoue gene in *Arabidopsis- HKT1:1*. In *Arabidopsis* it is expressed in the root stele and in leaf vasculature. In the root it controls a higher uptake of Na compared to the wild type, but in the shoot regulates the retrieval of Na from the transpiration stream (Sunarpi *et al*., 2005), thereby reducing Na in the shoot and importance for reducing Na toxicity (Maser 2002a). In wheat (*Triticum* spp. Rubio *et al*., 1995) and yeast (Rubio *et al*., 1999) *HKT1* has also been shown to increase K selectivity and to reduce Na transport. In *Arabidopsis* there is a single-point mutation in *HKT1* compared to the gene found in wheat and yeast, which controls K-selectivity, and therefore

Arabidopsis does not have this K-selectivity component (Maser, 2002). Thus there is no evidence for the involvement of *HKT1* in K transport in *Arabidopsis* (Moller, 2009). All *HKT* homologues known from dicots have this point mutation, suggesting that *HKT1* functions mainly as an Na rather than K transporter in plants (Maser *et al*., 2002b). However, as observed here the K concentration in the roots was significantly enhanced over the wild type, perhaps indicating a role for *HKT1* in K uptake in Brassica and dicots generally. However, by contrast to the finding in *Arabidopsis*, in the mutant here Na in the shoot was significantly enhanced, perhaps indicating that *HKT1* was expressed in the root regulating both K and Na uptake, but unlike *Arabidopsis* the expression in the shoot vasculature regulating reduced Na transport from the root to the shoot was not involved. Na disturbs K uptake because of the similarities in their ionic radii and ion hydration energies (Yuan *et al*., 2015). It was also observed that Ca in the external solution decreased Na uptake in the wild type, and indicates that Ca blocks Na permeable channels in roots (Maser, 2002a). The magnesium concentration in the root and shoot of the *Arabidopsis HKT1* mutant was not observed to differ from the wild type (Maser *et al*., 2002a). Therefore, this gene does explain the high root Na and K, as well as the high leaf Na but low K phenotype, as well as an increased leaf Ca phenotype, which may be responding to Na stress, however it does not directly explain the high leaf Mg phenotype.

The homologous *Arabidopsis* gene *CHX21* was detected in the region with the second greatest frequency of SNPs on chromosome 4. In yeast it is known to be a high affinity K and low affinity Na transporter (Cellier *et al*., 2004). In *Arabidopsis* it is localised in the root plasma membrane and found to regulate a higher Na and K concentration into the stele and leaf (Hall *et al*., 2006). It has also been observed to have higher expression than the wild type in buds, flowers, stem and pollen, and is surmised to have a role in pollen development and function, through regulating K in the stele (Evans *et al*., 2012). Interestingly, although the mutant here had a significantly reduced concentration of K in the leaf compared to the wild type, it actually had significantly greater concentrations of K compared to the wild type in the pod, stem and seeds- a 0.6, 0.7 and 0.6-fold difference, respectively, likewise Na was consistently greater in the mutant than the wild type in all plant parts, except the seed (data not shown). Therefore, as with *HKT1*, it appears that *CHX21* has regulated an

increased Na concentration throughout the plant, and additionally increased K in reproductive organs.

MRS24/*MGT6* was detected in a region with a high density of SNPs on chromosome 10. It is a *CorA-like* gene family in *Arabidopsis* that encodes for Mg transport proteins. Constitutive expression of *MGT6* has been found in roots, stems and leaves (Gerbert *et al*., 2009; Mao *et al*., 2014). Under Mg sufficient conditions it is expressed only in root vascular tissue, controlling translocation toward the xylem. Whereas under Mg deficient conditions it is also highly expressed in the plasma membrane of root epidermal cells, including in root hairs, and improves uptake of Mg. No *MGT* family transporters have previously been shown to mediate uptake from the external environment. Whereas, in *Arabidopsis,* Oda *et al* (2016) found that *MGT6* was localised to the endoplasmic reticulum (ER), where it serves to monitor the Mg concentration in the cytosol and was expressed in the plasma membrane, although they suggest that the ER may signal the plasma membrane to increase Mg uptake when the concentration of Mg is low. Importantly, they also observed in mutants that *MGT6* was essential for Mg homeostasis under normal as well as low Mg conditions. Therefore, the mutant observed in the present study may overexpress this gene, signalling for a greater uptake of Mg.

In *Arabidopsis*, *SCHENGEN3* has been found to promote the fusion of CASP islands, CASPS are transmembrane-span proteins which form a ring-like domain in the endodermis and affect the spatial arrangement of the casparian strip. *SCHENGEN3* mutants were observed to have 1.5-2.1fold increase in the Mg concentration compared to the wild type, as well as 1.4-3 fold reduction in potassium concentration, caesium was also concentrated at higher levels, and Na and Mn were no different to the wild type (Pfister *et al*., 2014). The higher concentration of Mg and lower concentration of K matches the profile observed in the mutant here, although Ca and Na were not altered. It is likely that Ca reaches the xylem via the apoplast in regions where the casparian strip is disrupted, because transport through the symplast would pose problems for the Ca stress signalling processes (Karley and White, 2009). Stress conditions, such as salinity, are known to induce cytosolic Ca2+ accumulation (Hirshi, 1999). Although, a higher leaf Ca concentration was not observed in the mutation of

SHENGEN3. This gene could be a putative candidate, although it has not so far been identified in the mutant here.

Therefore, perhaps the mutation observed here regulates an increased uptake/transport across the root plasma membrane of multiple ions- Mg, Na, K, S, possibly through *HKT1*, *CHX21* and *MGT6*, all found to be expressed in the root. These ions are then transported to the shoot in similarly high concentrations, except K. Potassium may be inhibited because the expression of *HKT1* in the leaf vasculature which reduces Na concentration in the transpiration stream is not active, and Na competitively inhibits K transport, as does Mg and Ca concentration. Additionally, the shoot cytosolic Ca concentration may increase as a consequence of Na induced stress.

The mutant was observed to have reduced photosynthetic capacity as well as reduced overall biomass production and delayed development, as well as reduced fecundity. This could be both a consequence of a high mutation load, as only one generation of back-crossing was carried out. It could also be because of ion deficiency stress. Potassium is one of the major osmotica in phloem sap and is therefore essential for the transport of fixed carbon from photosynthetic tissues to sink tissues (Tian *et al*., 2010), Enhanced Na would also cause osmotic stress. Mutations that increase K, Mg or Ca concentrations in plant tissues substantially often have negative pleiotropic effects on either plant growth and/or concentrations of other essential mineral elements (Karley and White, 2009).

The SNP data is yet to be analysed in sufficient detail, therefore it is likely that the genetic mechanisms underlying this mutation are not fully elucidated. Particularly in that the segregation ratios indicate that the mutation is a single gene, in which case, the multiple genes found to be mutated are unlikely. Therefore, an improved method of SNP analysis will be performed which imputes the missing SNP calls in the mutant and WT sibling lines from R-o-18 rather than the reference genome. Future work would then involve confirming gene function through co-segregation analysis of phenotype and genotype or analysis of orthologous genes in *Arabidopsis* using gene knockouts. Once the causative gene has been identified further studies of its function can be performed including expression pattern, protein localisation and interacting partners. Most importantly, the benefits of this mutation for human health could be

realised by developing molecular markers for marker assisted breeding to transfer the allele to vegetable Brassicas.

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Chapter 7

General Discussion/ conclusion

This PhD project has screened the root architecture of more than 800 genotypes, >20,000 plants. This is probably a greater number of genotypes than has been screened previously in a single project. This took around 4 minutes per seedling/ 90 minutes per genotype, including all processing from start to finish. This shows the potential for high throughput root phenotyping. Having said that, at such a high throughput there was probably some loss of accuracy in the procedure, particularly in root measurement which used semi-automatic software, making errors difficult to verify and counter. The greatest coefficient of variation was observed for mean lateral root length, suggesting either that this trait is particularly responsive to environmental conditions, or that the measurements were less accurate. The latter may be the case, as using the same 'pouch and wick' system in wheat (*Triticum* spp.), grown until 9 days old, QTL for total root length explained ~60% of the phenotypic variation, indicating that this was a particularly heritable trait (Atkinson *et al*., 2015). The experimental design used in this study was also deliberately simplistic to enable such a high throughput, whereby a genotype was grown in a single run, with reference lines in each run to allow for normalisation of experimental effects, however the experimental effects across runs may not have been fully accounted for by this procedure. Consequently, there were quite low 'broad-sense hetitabilites' in the HTP system, ranging from 6% in mean lateral root length to 13% in lateral root density (Chapter 4). Furthermore, the field study indicated that heritabilities were not particularly different between the field and HTP system, and in fact root diameter in the field had the greatest heritability of all traits (chapter 2). Whereas, heritabilites of ~65% were found for root traits including root thickness and length in 28 day old rice grown in soil filled boxes (Macmillan *et al*., 2006). Broad sense heritabilities > 50% were found for total root length, root surface area and maximum root number in 20 day old *Brachypodium* grown in gellan-gum filled cylinders (Ingram *et al*., 2013). Nevertheless, this system

does enable screening at a throughput and speed which could not be achieved in the field without considerably more cost and time.

This study could have measured additional root traits, particularly those relating to topology such as the lateral root angle and overall shape of the root system, which may have given greater estimates of heritability, and be more relevant for nutrient acquisition. Convex hull and centre of mass, which indicate the shape of the overall root system and angle of the seminal roots have been found to have QTL which explained the most phenotypic variation in wheat seedlings (Atkinson *et al*., 2015). Traits observed to differ between rice genotypes were centroid (the vertical position of the centre of mass), bushiness (ratio of the max number of roots divided by the median number) and volume distribution (the ratio of the volume occupied by the top one third of the root system), Circumnutation (the tendency of roots to grow downwards through a helical axis) and gravitropism (the tendency of roots to grow downwards) can also impact on the spatial distribution of the entire root system (Clark *et al*., 2011). ROOTSCAPE is an allometric approach to root architecture analysis which integrates individual root traits to capture the overall configuration of the root system into a single element (Ristova *et al*. (2013). There are various software packages with the capacity to measure such topological traits, including RootNav (Pound *et al*., 2013), SmartRoot (Lobet and Draye, 2011) and WinRHIZO (Regent Instruments, Inc, 1996- 2015). Models use elementary developmental rules to predict the root structure under different conditions based on the observed static traits, (Fitter *et al*., 1991; de Dorlodot *et al*., 2007). Modelling root architecture has predicted that a herringbone structure, where the main branch has just one order of longer lateral roots, would be more efficient at accessing resources under nutrient deficient supply than a main root with multiple shorter branching orders (Fitter *et al*., 1991). Furthermore, identifying root traits important for nutrient acquisition may have been more successful had root growth been observed under a deficient rather than sufficient nutrient supply in this study.

The upstream maternal effects of seed size/resources have been observed to explain >50% of the variability in root growth in this HTP system, indicating that such young seedlings are dependent on their seed reserves (Chapter 4). The putative genes underlying the root trait QTL also indicate that upstream effects of the annual and

biennial life history of the genotype e.g. spring or winter growth habit, which affects the time to flowering and maturity may also be significant determinants of early root growth/lateral root density (Chapter 5). Therefore, the potential for identifying root specific genes is constrained by these factors and should be controlled for where possible. The loci underlying variation in lateral root density which was not found to correspond to life history traits i.e. GEM (GL2/GLABRA2 expression modulator) gene would be a potential target for stimulating lateral root growth directly.

There was a positive relationship between lateral root number/density/mean length and some leaf micro nutrient concentrations, particularly Mn, Zn and Fe, in both the field and meta-QTL analysis studies. Whereas, a greater lateral root number and length resulted in a biomass dilution effect of macro nutrients (chapter 5). Therefore, where nutrients are at a sufficient supply, root growth may actually reduce the leaf concentration of nutrients. Oilseed rape is most likely to suffer deficiency in the micro-nutrients boron, manganese and molybdenum (Yara, UK, 2015), indicating that these micro-nutrients might be at more limited supply, particularly where there is a high rate of conventional fertilisation. Therefore, in conventional agriculture where there is an adequate supply of macro-nutrients perhaps the focus should be on increasing micro-nutrient supply or uptake, particularly those important for human health. By contrast, most studies relating to genetic loci underlying root uptake consider P and N. Between 1990 and 2007, yields of various crop types grown in Europe increased by ~10%, whereas, the application of nitrogen fertiliser decreased by ~10. It was suggested that policies such as the Nitrate Directive can account for the decrease in fertiliser application (Levers *et al*., 2016). Therefore, despite lower fertiliser applications yield has continued to grow. The root growth of barley cultivars in Norway, bred over 100 years has been studied. It was found that root biomass-seminal root length and root dry weight has decreased over time, coinciding with increased application of fertilisers. However, in the most recent varieties, specific root length (SRL i.e. smaller root diameter) has increased, coinciding with the more recent laws specifying a below optimal application of fertilisers, and these most recent root changes have corresponded with an improved Harvest Index (HI, yield: biomass ratio, Bertholdsson and Brantestam, 2009). Thus, a greater fine root system has been shown to improve yield without any costs to above-ground biomass production. Therefore, if

the trend in a decrease in fertiliser application continues, fine root growth and traits such as lateral root density may be of more importance for crop production.

Genotypic variation in leaf macronutrient concentration in the ASSYST diversity panel observed here ranged from 2.0-fold for P, 2.1-fold for K, 3.0-fold for Ca, 2.6-fold for Mg, and 2.5-fold in S. Also in natural accessions; in data acquired from the ionomicshub, leaf concentrations of Ca, Mg and Zn varied by 3.3-fold, 3.7-fold and 7.2-fold, respectively (Conn *et al*., 2012); and ranged from 1.5- fold in Mg and Fe to 4.1-fold in Na (Baxter, 2012). In species from core germplasm collections, which had been grown under comparative conditions, within the dicots; chickpea, peanut, pea, bean and soybean, seed Ca concentration varied 3.7, 2.0, 9.1, 1.5 and 1.9-fold, and seed Mg concentration varied 2.4, 1.4, 2.3, 1.3, and 1.6-fold, respectively (White and Broadley, 2009). Whereas, in 10,000 mutagenized plants leaf mineral concentration compared to the wild type varied from -0.08-fold in Fe (iron) and Co (cobalt) to 4-fold in Na (Lahner et al., 2003). In the mutagenised *B. rapa* population studied here leaf Mg had a ~3-fold range. Therefore, it seems there is as much variation in mineral composition occurring naturally as there is within mutagenised populations, and thus good breeding potential.

Relatively few markers have been implemented in plant breeding programs despite many putative QTL being identified, often because the QTL has failed to predict the desired phenotype. This can result from inadequate refinement of the loci region, consequently recombination between the marker and gene of interest results. Preferably markers should be within <5 cM but ideally <1 cM away from the gene (Collard *et al*., 2005). However, with the low cost and high throughput of next-generation sequencing technologies it is now possible to sequence many more genotypes and therefore increase the number of markers substantially, perhaps making marker assisted breeding more feasible. Yet, plant breeder's rights impose restrictions on a farmer's ability to re-use sown seed produced by a commercial company, thus those who need them most have limited acces to improved varieties (Kuma and Sinah, 2015), and therefore cooperation between the private and public sectors will be important for applying genomic technologies to crop improvement (Khush, 2001).

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