NUTRIENT ACCUMULATION IN BRASSICA: IDENTIFICATION OF NEW TARGETS FOR CROP IMPROVEMENT AND BIOFORTIFICATION

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Abstract

Plants require at least 14 essential mineral nutrients in order to successfully complete their life cycle. Nitrogen, phosphorus and potassium are required in particularly large quantities, and there is an ever increasing demand for fertiliser inputs to maintain adequate concentrations in crops. Humans are made up of at least 35 mineral nutrients, most of which originate from the ingestion of plant material. It is likely that much of the world's population consumes insufficient quantities of numerous mineral nutrients, including calcium and magnesium.

Variation in nutrient concentration traits in a diversity population of *Brassica napus* was characterised. Leaf nitrate, phosphorus, potassium, calcium and magnesium data, along with markers developed through transcriptome sequencing, were subsequently used to determine associated quantitative trait loci. *Arabidopsis thaliana* and *Brassica* genotypes mutated in selected candidate genes were characterised for nutrient concentration traits. An additional, EMS mutagenesis-background genotype, previously shown to accumulate magnesium, was also investigated. Both physiological and genetic bases of the trait were considered, including through leaf spatial-distribution mapping of mineral elements, and analysis of bulked-segregant DNA sequence data.

Associative transcriptomics analyses identified numerous genetic loci and allelic variants for all traits investigated which may prove useful in marker-assisted selection strategies. Plants mutated in several calcium and magnesium accumulation candidate genes also have perturbed nutrient profiles. Characterisation of unique physiological traits in the magnesium accumulator mutant indicated drastic changes in tissue and cellular distribution of magnesium and phosphorus. Findings from this and other characterisation experiments enabled the identification of relatively few candidate genetic polymorphisms from DNA sequence data. This thesis has identified a number of new targets for controlling nutrient accumulation in *Brassica* spp. This knowledge could be applied in the development of novel crop varieties with greater nutrient-use efficiency and accumulation traits in order to decrease crop fertiliser requirements and reduce widespread human undernutrition.

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Abbreviations and Acronyms

ABC	ATP-binding cassette transporter
ACA	Autoinhibited Ca^{2+}-ATPase
AHDB	Agriculture and Horticulture Development Board
AT	Associative Transcriptomics
ATP	Adenosine triphosphate
BIP	Brassica Information Portal
Bra.MgA	*Brassica rapa* magnesium accumulator
CAX	Cation exchanger
CDS	Coding DNA sequence
CLC	Chloride channel
DEFRA	Department for Environment, Food and Rural Affairs
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
DW	Dry weight
EMS	Ethyl methanesulfonate
eQTL	Expression quantitative trait locus / loci
FAO	Food and Agriculture Organisation of the United Nations
FDR	False discovery rate
FLC	Flowering locus C
GEM	Gene expression marker
GM	Genetically modified
GWAS	Genome-wide association study
IC	Ion chromatography
ICP-MS	Inductively coupled plasma-mass spectrometry
KT	Potassium transporter
LD	Linkage disequilibrium
LRD	Lateral root density
LRL	Lateral root length
LRN	Lateral root number
LRNI	Lower reference nutrient intake
MGT	Magnesium transporter
MLRL	Mean lateral root length
NO_3^-	Nitrate
NRT	Nitrate transporter
OSR	Oilseed rape
PHT	Phosphate transporter
PIXE	Proton Induced X-ray Emission
PRL	Primary root length
PTH	Parathyroid hormone
QQ	Quantile-quantile
QTL	Quantitative trait locus / loci
REML	Restricted maximum likelihood
RIPR	Renewable Industrial Products from Rapeseed
RNA	Ribonucleic acid
RNI	Reference nutrient intake
RPKM	Reads per kilobase per million aligned reads
SegWT	Out-segregating wild-type
Seq	Sequencing
SNP	Single nucleotide polymorphism
SULTR	Sulphate transporter
TRL	Total root length
TSW	Thousand seed weight
USDA	United States Department of Agriculture
WHO	World Health Organisation
XRF	X-ray fluorescence
PCA	Principle components analysis
T-DNA	Transfer DNA
TILLING	Targeting induced local lesions in genomes
PCR	Polymerase chain reaction
NFT	Nutrient film technique
LOD	Limit of detection
CRM	Certified reference material
BC	Backcross

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Chapter 1. General Introduction

1.1 Nutritional Requirements of Higher Plants

All life on planet Earth is dependent on external inputs. These inputs can be split into two broad categories: energy and matter. Energy is required for metabolic activity within biological cells, as well as for growth and movement. It generally enters food webs through producing organisms such as photosynthesising plants, algae, or bacteria which harvest energy from the sun. This energy is then stored as sugars, thus making it biologically available to other species. Matter refers to all of the atomic material required to build, support and repair an organism. Most matter in biological systems is present as carbon (C), hydrogen (H) or oxygen (O) atoms. In plants, these elements make up approximately 90 % of dry matter (Broadley and White, 2005). Carbon and oxygen is largely sourced from carbon dioxide (CO_2) in the air, with hydrogen and further oxygen from water (H_2O), typically taken up from the soil.

Higher plants require at least 14 further essential mineral elements to complete their life-cycles (Broadley and White, 2010). These include nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P) 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; Kirkby, 2012). The shoot concentrations of these elements typically required for adequate plant health are summarised in Table 1.1. Plants also accumulate non-essential elements, some of which may have little or no effect on plant growth and development at the concentrations they occur in nature, and others 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).

1.2 Uptake of Nutrients into Plant Tissue

Most mineral elements are taken up in ionic form from the soil solution by plant roots. However, it is also possible for small quantities of certain elements to enter the plant through aerial tissue. For example, gasses such as ammonia (NH_3) and

sulphur dioxide (SO_2) can be taken up through open stomata in leaves, thus providing a source of N and S, respectively, to aerial plant parts (Eichert and Fernández, 2012). There is also evidence to suggest a number of other elements are able to enter through the cuticle or stomata if present in a dissolved solution, which may occur through atmospheric disposition or foliar fertilisation (Eichert et al., 2008). Element uptake from the soil via the roots is influenced by the available element concentrations, element speciation, microbial activity, pH, soil structure, water content and plant species or genotype. For instance, N_2 can only be taken up by plants capable of forming symbioses with N-fixing bacteria (Vance, 2002). Most other plants therefore rely on nitrate (NO_3^-) or NH_3 as a source of N.

There are typically great differences between mineral concentrations in the soil and the nutrient requirements of plants. Soil can contain large quantities of unnecessary or in some cases harmful elements, as well as toxins and viruses or other pathogens. Hence, plants have to be very selective about what they uptake and in what quantities. Movement of low-molecular-weight solutes including ions from the soil solution through individual root cell walls is largely a passive process. The cell wall of plant cells is made up of cellulose, cross-linking glycans, glycoproteins and pectin (Carpita and McCann, 2000). The cell wall network contains numerous pores which are small enough to prevent larger toxins and pathogens from entering the root tissue, whist being large enough to allow essential mineral ions to pass through without restriction. The main site of selectivity in the uptake of cations and anions is the plasma membrane. This comprises a lipid bilayer, which prevents the indiscriminate movement of ions from the apoplasm (areas within cell walls and intercellular spaces) to the cytoplasm. Instead, integral membrane proteins facilitate selective ion transport across the plasma membrane. Within cells, lipid membranes surrounding the various cellular components such as the endoplasmic reticulum, Golgi apparatus, mitochondria and plastids provide further barriers enabling unique transport of solutes required for specific functionality.

Table 1.1. Average concentrations of essential mineral elements in plant shoot dry matter. Adapted from Epstein (1965), Epstein and Bloom (2005), Brown et al. (1987), found in Kirkby (2012).

Element	Chemical symbol	µmol g^{-1} dw	mg kg^{-1} dw
Nitrogen	N	1,000	15,000
Potassium	K	250	10,000
Calcium	Ca	125	5,000
Magnesium	Mg	80	2,000
Phosphorus	P	60	2,000
Sulphur	S	30	1,000
Chlorine	Cl	3.0	100
Boron	B	2.0	20
Iron	Fe	2.0	100
Manganese	Mn	1.0	50
Zinc	Zn	0.3	20
Copper	Cu	0.1	6
Nickel	Ni	0.001	0.1
Molybdenum	Mo	0.001	0.1

1.3 Movement of Mineral Nutrients from Root to Shoot Tissue

Plant roots consist of multiple, concentric cell layers which make up specific tissue types. In dicotyledonous plants such as those of the *Brassica* genus, these typically include the epidermis, followed by the cortex, the endodermis, and the stele. The stele contains the vascular tissue of the root including the xylem and phloem, which are formed from meristematic tissue known as the procambium. The outer-most part of the stele is made up of a layer of cells called the pericycle, which are required for xylem loading and lateral root initiation (Beeckman and De Smet, 2014; Figure 1.1).

In order to transfer from root cells to shoot tissue, mineral nutrients first have to move radially across the root towards the stele. This can be achieved through either the apoplastic or the symplastic pathway. In the apoplastic pathway, solutes move through regions within the cell walls and intercellular space (Sattelmacher, 2001). In the symplastic pathway, solutes enter the cell cytoplasm through the plasma membrane, and then traverse the symplast through microscopic channels in cell walls called plasmodesmata which enable transport between neighbouring cells (Barberon

and Geldner, 2014). It is unclear to what extent most minerals travel through which pathway. However, perturbations in the cytoplasmic concentration of certain elements can have undesirable effects, and hence movement though this route must be carefully controlled.

Since selectivity of solute uptake is imperative for successful development, plants have developed mechanisms to prevent indiscriminate movement of solutes travelling through the apoplastic pathway from root to shoot tissue. The major site of this selectivity is the endodermis. From late in the elongation zone and throughout the differentiation zone of roots (Kamiya et al., 2015), movement of solutes into the stele is restricted by the Casparian strip, a band of lignified material which joins each endodermal cell and prevents uptake of solutes from the apoplast (Naseer et al., 2012). As root cells mature further, endodermal cells are further differentiated through the deposition of suberin lamellae all around the cellular surface, thus providing an additional barrier to solute transport to the stele (Schreiber et al., 1999). Where such barriers exist solutes must enter cells through the plasma membrane and continue their route to the xylem through the symplast (Barberon and Geldner, 2014). It has been postulated that an entirely apoplastic route to the xylem is possible in regions where Casparian strips are not fully formed, such as in root apical tissue and in regions where lateral roots emerge (Barberon et al., 2016). Since cytosolic concentrations of certain nutrients, such as calcium (Ca), must be carefully regulated, this route could be highly relevant for transport of such nutrients to the xylem without disrupting cellular function (White, 2001). However, since a lack of selectivity in these regions could lead to uptake of toxic elements or pathogens, it is likely that alternative selection mechanisms exist. For example, is has been reported that endodermal cells in contact with developing lateral root primordia undergo drastic morphological changes in order to avoid significant gaps in the selection barrier during emergence (Robbins et al., 2014). Once movement to the stele is achieved, solutes enter non-living xylem vessels through ion channels or uniporters via the plasma membrane of xylem parenchyma cells (Tester and Leigh, 2001). They then travel along this pathway to the shoot, driven by hydrostatic root pressure and the gradient in water potential from root to shoot cells, where they are unloaded.

Transport of mineral nutrients to non-transpiring tissues such as fruits, seeds and tubers largely occurs by redistribution of leaf minerals through the phloem. However, there is significant variation in phloem mobility between mineral nutrients, and this can affect concentrations in tissues that are predominantly phloem fed. Among the nutrients readily transported in the phloem are K and Mg (Karley and White, 2009; White and Broadley, 2009). Several mineral nutrients have intermediate mobility, including Fe, Zn, Cu and I (Muramatsu et al., 1995; Welch and Shuman, 1995; Rengel, 2001; Garnett and Graham, 2005). One of the least phloem-mobile elements is Ca, which is largely bound to cell walls in plant cells. Hence, concentrations of Ca in non-transpiring tissues are generally very low (White and Broadley, 2003).

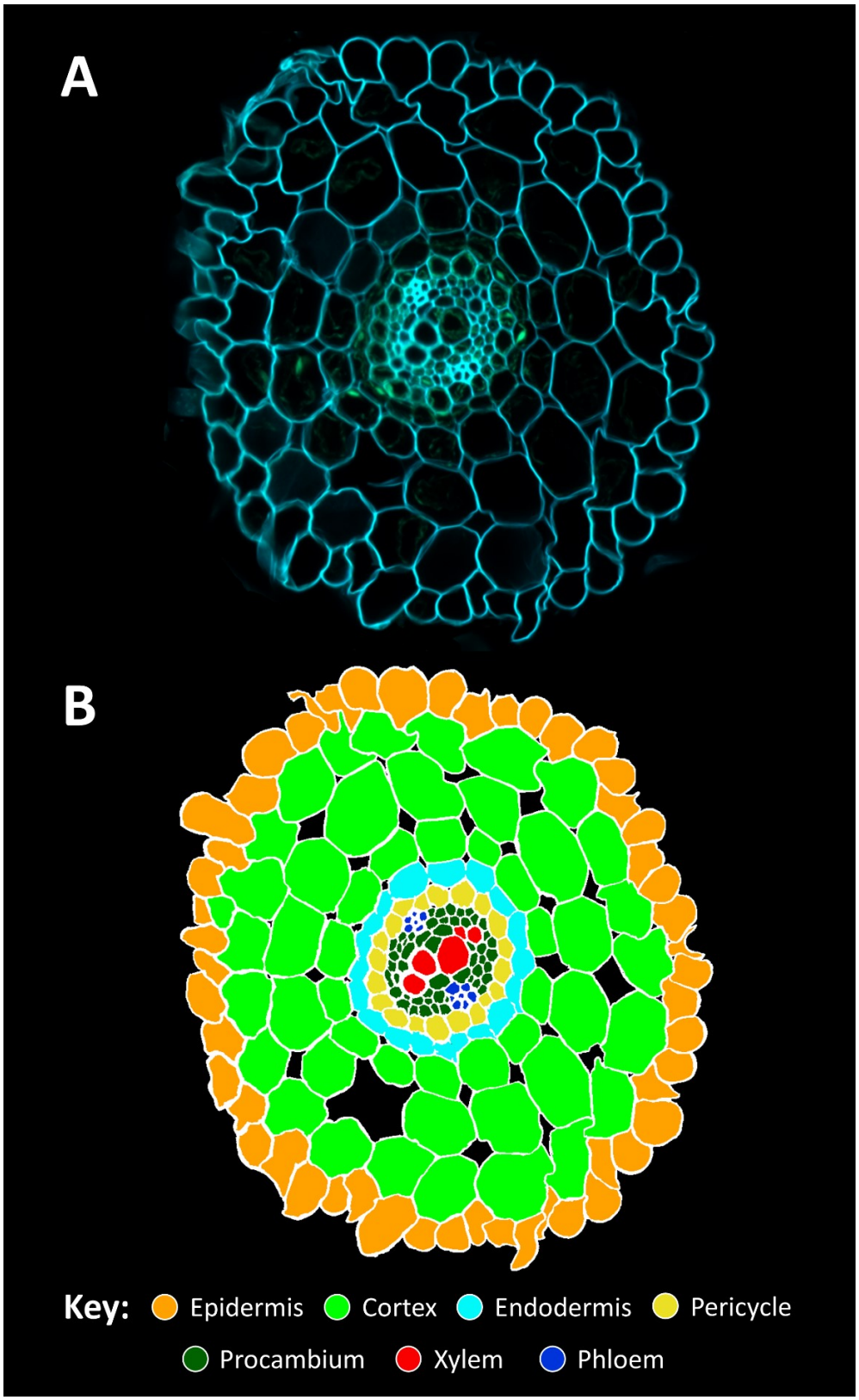


Figure 1.1. A: Confocal laser scanning microscopy image (Eclipse Ti CLSM, Nikon Instruments) of mature root tissue from 7 day old Brassica rapa *sectioned to 120 µm and stained with calcofluor white (0.3 mg ml^{-1} H_2O for 60 seconds) to highlight cell walls. B: Skeletonised schematic based on image in A. Colour coded to represent concentric tissue layers. Skeleton thresholding and image optimisation carried out in GIMP 2.8.16 (https://www.gimp.org/).*

1.4 Factors and Symptoms Involved in Plant Mineral Deficiencies

1.4.1 General Factors Influencing Mineral Deficiencies

Plant mineral nutrient deficiencies generally occur when there is an insufficient exogenous supply of available nutrient and this can lead to serious limitations in plant development. The prevalence of specific nutrient deficiencies can vary depending on soil type and condition. In heavily farmed soil, nutrients including N, P and K can become rapidly depleted through intensive crop production, and if not replaced through chemical fertilisation or other means, this can greatly hinder future crop yield. Certain soil types are also naturally prone to maintaining low concentrations of certain essential elements. For example, serpentine soils (normally derived from serpentinite rock) typically have a very low Ca to Mg ratio which can prevent adequate Ca nutrition (Walker et al., 1955). Serpentine soils also typically have low concentrations of other essential nutrients including N, P and K, as well as high concentrations of heavy metals, which may lead to toxicity symptoms in certain plants.

Soil pH can have a huge influence on nutrient availability; in alkaline soils, the availability of P, Zn, Fe, Mn, Cu and B is very low (Dolar and Keeney, 1971; Peterson and Newman, 1976; White and Broadley, 2009; Cerozi and Fitzsimmons, 2016). Some nutrients such as S are also highly unavailable in more acidic soils. Soil pH can also influence the chemical form of nutrients in the rhizosphere, which particularly influences the uptake of elements that can only be transported across the plasma membrane in specific forms. For instance, at higher pH levels, there is a decrease in the concentration of $H_2PO_4^-$, the substrate of the proton-coupled phosphorus symporter in the plasma membrane (Hendrix, 1967). High levels of rainfall can also decrease available concentrations of soil-mobile elements which may be washed deep within the soil.

Of the essential plant micronutrients, most are generally present in sufficient quantities in the soil for adequate nutrition. As well as this, under shorter term periods of nutrient unavailability, many elements can be remobilised from less crucial

parts of a plant to areas where they are needed most via the phloem. However, deficiencies in these elements can still occur, as can deficiencies in phloem immobile elements such as Ca, and this can have significant economic consequences. Below, the roles of specific plant mineral nutrients, mechanisms controlling their uptake, and symptoms associated with deficiency are discussed further.

1.4.2 Nitrogen

Nitrogen is an integral constituent of proteins, nucleic acids, chlorophyll, co-enzymes, phytohormones and secondary metabolites. The primary form of N in aerobic soils is nitrate (NO_3^-). There are two principle nitrate transporter (NRT) gene families known as *NRT1* and *NRT2*, comprising at least 53 and 7 members respectively (Tsay et al., 2007). A major gene of the former family, *NRT1.1*, encodes a protein expressed in the root tip that can act as both a high- and low-affinity transporter depending on the phosphorylation status of threonine 101 (Liu et al., 1999; Liu and Tsay, 2003). This protein also has roles in activating the expression of other nitrate-related genes through nitrate sensing (Ho et al., 2009). After entering cells, nitrate is reduced to nitrite and then ammonium, after which it can be assimilated into amino acids and other compounds (Xu et al., 2012). Any excess nitrate enters the vacuole where it remains as a store for any future requirements, under the control of chloride channel (CLC) protein family member CLCa (De Angeli et al., 2006).

Nitrogen is found throughout all tissue types (Dechorgnat et al., 2011), and hence deficiencies in this nutrient can cause serious developmental defects. Such defects include stunting, narrow leaves, and chlorosis, with the latter starting in older leaves as N is remobilised to newer leaves via the phloem (Hawkesford et al. 2012). In order to prevent these symptoms, N fertiliser is used widely across the world. In 2016, global N fertiliser demand was 113 million tonnes, and demand is estimated to be increasing by 1.4 % annually. It is estimated that around 58 % of the increase in demand between 2014 and 2018 will have come from Asia, indicating the spread of crop fertilisation to developing countries. However, only 8 % of the increase is expected to have come from Africa, which suggests potential gains in crop production in this area could be realised if fertilisation is more widely adopted (FAO, 2015).

1.4.3 Phosphorus

Like N, P also has significant functions as a structural element, with important roles as a component of nucleic acids and in the phospholipid bilayer of cell membranes. It is also involved in numerous other processes such as photophosphorylation which enable the synthesis of ATP as an energy store, and the transport of other nutrients around the plant. Phosphorus is taken up by plants in the orthophosphate forms $H_2PO_4^-$ and HPO_4^{2-} which are typically available at concentrations of 0.1 to 10 µM in the soil solution (Hinsinger, 2001). In *Arabidopsis thaliana*, four major phosphate transporter (*PHT*) gene families have been described, known as *PHT1*, *PHT2*, *PHT3*, and *PHT4*. They encode integral membrane proteins, and are responsible for acquiring inorganic phosphate from the soil and transferring it between tissues (Raghothama and Karthikeyan, 2005). Members of the *PHT1* gene family are predominantly expressed in epidermal cells and in the outer cortex of the root, suggesting their role as primary mediators of phosphate uptake from the soil (Nussaume et al., 2011).

Due to its importance in plant structural development, and in providing ATP for various cellular processes, P deficiency can lead to slower plant development, which can have huge impacts for crop productivity. Phosphorus fertiliser demand was around 43 million tonnes globally in 2014, and demand is thought to be growing by 2.2 % every year (FAO, 2015). Despite some recent interest in more renewable P sources including struvite from wastewater (Antonini et al., 2012), the vast majority of phosphorus fertilisers are sourced from rock phosphate reserves (Cordell et al., 2009). Whilst recent estimates predict these reserves to remain available for longer than previous estimates of only 50-100 years (Van Vuuren et al., 2010; Scholz et al., 2013; Ulrich and Frossard, 2014), it is still a limited global resource, and techniques for maintaining adequate concentrations of P in crop plants whilst reducing applied P fertiliser may become increasingly important in years to come.

1.4.4 Potassium

Potassium is the most abundant cation within the plant cell. Potassium ions (K^+) are responsible for balancing the excess negative charge associated with amino and

nucleic acids (Maathuis and Sanders, 1996), thus providing the correct ionic environment for various metabolic processes in the cytosol. Potassium is also involved in enzyme activation, protein synthesis, and osmoregulation. It has a further role in photosynthesis, through regulating proton pumps that affect the flaccidity of guard cells, thus controlling their opening and closure (Hawkesford et al., 2012). Within cells, K^+ influx / efflux is tightly controlled in order to maintain constant cytosolic concentrations. Plants absorb K^+ ions through K^+ channels and membrane bound transport proteins in root cells. In *A. thaliana*, two high-affinity transporters, HIGH-AFFINITY K^+ TRANSPORTER 5 (HAK5) and *ARABIDOPSIS* K^+ TRANSPORTER 1 (AKT1) are thought to co-ordinate most K^+ uptake (Hirsch et al., 1998;Pyo et al., 2010). Other channels and transport proteins are also involved, including low-affinity transporters which function when exogenous K^+ supply is higher (Maathuis and Sanders, 1996).

Potassium deficiency symptoms include browning and curling of leaves and slower development of all tissues. Like N, K is a phloem mobile element, and as such, symptoms generally appear in older leaves first as K is remobilised to younger plant tissue. Potassium fertiliser demand was around 31 million tonnes in 2014, with demand forecast to increase by around 2.6 % annually over the next few years (FAO, 2015).

1.4.5 Sulphur

Despite some atmospheric SO_2 being available for uptake by stomata for use in aerial plant tissues, the largest source of S is from the soil. The major source of sulphur available for plant uptake is as the divalent sulphate anion SO_4^{2-}. As such, the most prevalent S-metabolite transporters in plants are sulphate transporters of the SULTR family (Smith et al., 1995). The activity of two members, *SULTR1;1* and *SULTR1;2*, appears to be particularly important in uptake of SO_4^{2-} from soil to root. Both encode proteins expressed at the root surface, with the former acting as a high-affinity sulphate transporter, and the latter contributing to SO_4^{2-} uptake under stressed conditions (Yoshimoto et al., 2002). *SULTR1;2* is also thought to vary in expression in

response to metabolic demand, and hence may be important in not only uptake but regulation of S concentrations within plant tissues (Rouached et al., 2008).

Sulphur is a constituent of the amino acids cysteine and methionine, and hence is crucial for building proteins (Brosnan and Brosnan, 2006). It is important for maintaining photosynthesis, as it is found in an enzyme required for the formation of chlorophyll. Further to this, S is a key building block of glucosinolates. These are characteristically bitter compounds found in some dicotyledonous plant families including the Brassicaceae, and are thought to have evolved as a defence against pests and herbivory (Kopriva et al., 2015). Where insufficient quantities of SO_4^{2-} are present, shoot growth is reduced, and there is a decrease in the leaf concentrations of sulphur dependent compounds including chlorophyll, proteins and glucosinolates. It has been observed that an overall move away from burning coal in power plants has led to decreases in the deposition of atmospheric sulphur emissions in agricultural fields (David et al., 2016). It is hence likely that more fertilisers will be required to maintain plant sulphur nutrition in future.

1.4.6 Calcium

In plants, most Ca is extracellular, where it is a key strengthening component in cell walls. It is also important in plant cell signalling through changes in cytosolic Ca^{2+} concentrations which coordinate numerous developmental and environmental stress responses (McAinsh and Pittman, 2009). It is thought that a combination of the apoplastic and symplastic pathways are used to transport Ca through the root to the stele (White and Broadley, 2003). Since the concentration of Ca in the cytoplasm must be maintained at sub-micromolar concentrations in order to prevent cytotoxicity, the apoplastic pathway offers clear advantages. However, Ca delivery to the xylem through this route alone is likely limited to the extreme root tip and regions in which lateral roots are being initiated. In these regions, the Casparian strip is absent or not fully formed. In other areas of the root, the Casparian strip prevents apoplastic transport past the endodermis. At this point, Ca can enter root endodermal cells through plasma membrane localised Ca^{2+}-permeable ion channels. It is then pumped through the symplast towards cells within the stele by Ca^{2+} ATPases or Ca^{2+} / H^+-antiporters (White, 2001).

Generally, there are sufficient concentrations of Ca in the soil for adequate plant nutrition. However, since cells rely on Ca influx via the xylem, short term unavailability can have serious consequences for developing tissues as Ca cannot be remobilised. Ca deficiencies can lead to weaknesses in plant cells as a direct result of less Ca being available to maintain cell walls. This can particularly affect tissues with low transpiration rates such as fruits and young, expanding leaves which can lead to costly deficiency symptoms. In young leaves, this can present itself as “tipburn”, a discolouring and desiccation of the ends of leaves. In fruits such as tomato, apple and cherry, insufficient Ca supplies can lead to cracking, as a result of cell wall weaknesses, and can cause blossom end rot (White and Broadley, 2003).

1.4.7 Magnesium

In plants, Mg is vital for photosynthesis, ATP and protein synthesis, and generation of reactive oxygen species (Cakmak and Yazici, 2010). Around 10 % of the total Mg content of mature leaves is bound to chlorophyll, in which it forms the central atom of the molecule (Wilkinson et al., 1990). In ATP synthesis, Mg acts by forming a bridging element between ADP and ATP synthase (Igamberdiev and Kleczkowski, 2015). As well as this, the substrate for ATPases, which are required to breakdown and release energy from ATP, is Mg-ATP and not free ATP, and hence Mg must be bound to ATP in order for it to be biologically useful (Hawkesford *et al.* 2012). Magnesium is taken up from the soil as the divalent cation Mg^{2+}. Influx across the plasma membrane is largely co-ordinated by members of the MGT / MRS2 family of transport proteins (Lenz et al., 2013). Since cytosolic Mg^{2+} concentrations are generally lower than those found in soil solutions, it is also possible for Mg^{2+} to enter root cells through Mg^{2+}-permeable cation channels (Karley and White, 2009).

Mg is a phloem-mobile element, and is readily translocated to fruit, seeds, and tubers (White and Broadley, 2009). Under low exogenous supply, Mg is remobilised to younger tissues, causing chlorosis symptoms to form starting in the older leaves first. Under longer term deficiencies, leaf margins can also start to become necrotic. Mg is less available to most plants under strongly acidic soil conditions, and its availability

is also reduced where high concentrations of K are present (Guo et al., 2016). Magnesium also binds less willingly to soil particles than other cations, and hence is prone to leaching. This is a particularly important factor to consider for plant cultivation in shallow or coarse soils (Wilkinson, et al., 1990).

1.4.8 Micronutrients

Whilst plant micronutrient deficiencies are generally quite low in prevalence under the majority of growth conditions, they can occur in nature and lead to reductions in crop productivity. The most common crop micronutrient deficiency, which has been observed in various soil types across the world, is Zn. Crop Zn deficiencies are particularly common when grown in low pH soils. In plants, Zn is an essential component of thousands of proteins. Deficiency can lead to root apex necrosis, chlorosis, bronzing, and reduction in growth (Broadley et al., 2007). Further crop deficiencies in B, Fe, Mn and Mo have been observed, and hence these are also worthy of consideration in agronomic practices and crop breeding strategies. As a component of cell walls, B deficiencies can lead to stunting, and so adequate levels of B in the soil are important for early crop establishment. Iron and Mn, among other roles, are involved in photosynthesis and/or formation of chlorophyll and hence deficiencies in these nutrients can lead to chlorosis and poorer productivity. Molybdenum is essential for the conversion of nitrate to ammonia for use in amino acid synthesis. For this reason, Mo deficiency symptoms are very similar to those observed under N deficiency (Maathuis and Diatloff, 2013).

1.5 Scope for Breeding for Improved Nutrient Use Efficiency in Crop Plants

In terms of crop productivity, nutrient use efficiency may be defined as yield per unit input. It relates not only to a plant's ability to uptake nutrients from the growth medium, but also on its ability to adequately mobilise nutrients to required tissues and then to use them effectively. There are typically high levels of variation in shoot mineral concentrations at all taxonomic levels including at the levels of order, family, species, and even between different genotypes of individual species. At higher taxonomic levels, such variation is commonly due to differences in environments that ancestral individuals adapted to. For instance, the order Caryophyllales contains

around 35-40 % of all known halophytic species of angiosperms (Flowers *et al.* 2018), defined as plants which grow in areas of high salinity. Species within this order typically have much higher tissue concentrations of Mg than most other angiosperms (Broadley et al., 2004), and some have also been shown to hyper-accumulate Na under non-saline conditions (White et al., 2017). The storage of Na may be an adaptation to coping with higher salt concentrations in ancestral growth conditions. When looking at levels of variation in specific nutrients across taxonomic levels, it becomes clear that certain nutrients vary more at particular levels than others. For instance, there is a higher proportion of variation in shoot Ca and Mg concentration at the levels of order and family than lower taxonomic levels, whereas variation in shoot N and P concentrations is largely a species-level trait (Thompson et al., 1997; Broadley et al., 2004). Despite this, there are often high levels of heritable variation across all mineral nutrients within crop species, indicating the potential for utilising this natural variation in traditional breeding strategies. For instance, within a diversity set of 376 *Brassica oleracea* genotypes, shoot Ca and Mg concentrations were observed to vary 2- and 2.3-fold, respectively, and these traits were found to be highly heritable and hence under strong genetic control (Broadley et al., 2008). In a similar population of *B. oleracea*, shoot P concentration was found to vary around 5-fold between 355 genotypes, and correspondingly calculations of P use efficiency were found to vary drastically (Hammond et al., 2009). Unsurprisingly, commercial cultivars generally had higher P use efficiency scores, likely as a result of strong artificial selection pressures for higher crop yield. Nevertheless, there were some non-commercial varieties that had higher P use efficiency scores, indicating the possibility of utilising traditional breeding techniques for further improvements.

Whilst traditional breeding may prove useful in improving nutrient accumulation and use efficiency in crop species, it is a slow process that often takes a significant number of years for meaningful outputs to be produced. A complementary approach may be to identify genetic markers for improved nutrient use efficiency, and even better, genes which directly influence nutrient uptake and transport. Many resources have been put into such approaches in recent years, such as quantitative trail loci (QTL) analysis of mapping populations based on two parent individuals. In the study of

Broadley *et al.* (2008), this method led to the discovery of a number of QTL associated with shoot Ca and Mg concentration in *B. oleracea*, particularly on chromosomes C8 and C9. A similar approach led to the identification of QTL for shoot P and P use efficiency in *B. oleracea* on chromosomes C3 and C7 (Hammond et al., 2009).

It is also useful to consider not only genes but physiological traits that may influence nutrient uptake and use efficiency. Root architecture can hugely affect nutrient availability, and is especially important for the uptake of elements that are less mobile in the soil such as P. For instance, it has been shown that root traits including overall root architecture (Lynch, 1995; Liao et al., 2001; Miller et al., 2003) root hair length (Bates and Lynch, 2000), and total root length (Ma et al., 2003) respond to external P concentrations and can influence P uptake in a range of environments. Reduced allocation of carbon to root structures via increased aerenchyma and reduced cortical cell file formations (Lynch, 2011) and smaller root diameter (Hufnagel et al., 2014) may allow some plants more efficient access to larger soil volumes, and thereby water and nutrients. The identification of such physiological traits and the available variation within crop species could allow selection of varieties more suited to particular environments, and targeted breeding to further improve these traits. Regardless of the method used to improve nutrient use efficiency in crop species, it is clear that this is a necessary goal within agricultural research in the current global condition. The perhaps over-quoted, but profound estimate of human population increase to around nine or ten billion people by 2050 is a stark reminder of this. Coupled with depleting supplies of rock fertilisers on Earth, this illustrates the requirement to develop new techniques to ensure sufficient food production to adequately feed and nourish the population.

1.6 Nutritional Requirements of Humans

Humans must get all of the energy they require for proper metabolic functioning, growth and movement from food. Estimates of energy requirements for humans vary considerably, reflecting the huge variation in energy needs between people based on age, gender, body weight, levels of activity, and genetics. In 2001, the Food and Agriculture Organization of the United Nations (FAO) put together a detailed report

with the aim of estimating human energy requirements based on many of these parameters (FAO, 2001). Men between the ages of 18 and 60 were reported to require between around 2,100 and 4,500 kilocalories (kcal) per day, with lower requirements reported as age increased, and higher requirements reported as physical activity level and weight increased. For women between the ages of 18 and 60, these estimates were lower, ranging from around 1,650 to 3,850 kcal per day, with the main effectors of energy requirement being physical activity level and weight. For men and women over the age of 60, estimates of energy requirement were lower, ranging from 1,700 to 3,600 kcal for men and 1,550 and 3,150 kcal for women. Again, these estimates were higher for groups with higher physical activity levels, and those with higher body weights. For children between the ages of 6 and 18, estimates of daily energy requirements varied hugely, reflecting the physical changes children undergo during development. These were as low as 1,225 kcal per day for girls aged 6-7 with light physical activity levels, and as high as 3,925 kcal per day for boys aged 17-18 undergoing heavy physical activity levels (FAO, 2001).

As well as energy, humans require a vast range of minerals to build tissue and function correctly, and the majority of these also come from food. A total of 96% of the weight of the human body is made up of the four elements oxygen, hydrogen, carbon and nitrogen. The remaining 4 % is made up of at least 35 essential or non-essential elements (Sardesai, 2003). Human essential minerals can be split into macrominerals, which are typically present between around 0.25 and 1.8 % of body weight, and microminerals, typically present from trace levels, to around 0.006 % of total body weight. The macrominerals include Ca, P, S, K, Cl, Na, and Mg, and the microminerals include Fe, Zn, fluorine (Fl), Si, Cu, iodine (I), Mn, Se, Mo, chromium (Cr), and cobalt (Co). The average quantity of each of these minerals in the human body is summarised in Table 1.2.

Typical terminology used to indicate levels of nutrients required per person per day include Reference Nutrient Intake (RNI) and Lower Reference Nutrient Intake (LRNI). RNI refers to the daily, dietary quantity of a given nutrient that is sufficient to satisfy the needs of 97.5% of people in a group. If the average intake of a group is below this,

it is likely that some individuals will have intakes below their requirement. LRNI refers to the daily, dietary quantity of a given nutrient that is sufficient to satisfy the needs of only 2.5% of the population. Intake below this value over a sustained period of time can be considered a problem for the group. The UK Department of Health in 1991 published a list of RNI and LRNI values for humans. These values are still relevant today, and are currently used by Public Health England and the Food Standards Agency for assessing the prevalence of malnutrition in the UK. The daily requirements of males and females grouped by age of seven mineral nutrients commonly lacking in human diets (Fe, Ca, Mg, K, I, Se and Zn) are summarised in Table 1.3 and Table 1.4 (Department of Health, 1991; adapted from National Diet and Nutrition Survey, 2016).

Table 1.2. Average mineral makeup of the human body expressed as percentage body weight and total quantity of mineral element in a typical adult weighing 70 kg. Adapted from Sardesai (2003).

Element	Chemical symbol	% body weight	Total in an adult weighing 70 kg (g)
Calcium	Ca	1.78	1,250
Phosphorus	P	0.96	670
Sulphur	S	0.25	175
Potassium	K	0.19	130
Chlorine	Cl	0.15	105
Sodium	Na	0.14	100
Magnesium	Mg	0.04	25
Iron	Fe	0.006	4
Zinc	Zn	0.003	2
Fluorine	F	0.002	1
Silicon	Si	0.001	0.7
Copper	Cu	0.0001	0.08
Iodine	I	0.00004	0.03
Manganese	Mn	0.00002	0.02
Selenium	Se	0.00002	0.015
Molybdenum	Mo	0.00001	0.009
Chromium	Cr	0.00001	0.008
Cobalt	Co	Trace	Trace

Table 1.3. Reference Nutrient Intakes (RNI) and Lower Reference Nutrient Intakes (LRNI) of seven human mineral nutrients for males by age (Department of Health, 1991; adapted from Public Health England, 2018)

Mineral (unit of measurement per day)		**Age group (years)** 1-3	4-6	7-10	11-14	15-18	19-50	51-64	65-74	75+
Males										
Iron (mg/d)	RNI	6.9	6.1	8.7	11.3	11.3	8.7	8.7	8.7	8.7
	LRNI	3.7	3.3	4.7	6.1	6.1	4.7	4.7	4.7	4.7
Calcium (mg/d)	RNI	350	450	550	1000	1000	700	700	700	700
	LRNI	200	275	325	480	480	400	400	400	400
Magnesium (mg/d)	RNI	85	120	200	280	300	300	300	300	300
	LRNI	50	70	115	180	190	190	190	190	190
Potassium (mg/d)	RNI	800	1100	2000	3100	3500	3500	3500	3500	3500
	LRNI	450	600	950	1600	2000	2000	2000	2000	2000
Iodine (µg/d)	RNI	70	100	110	130	140	140	140	140	140
	LRNI	40	50	55	65	70	70	70	70	70
Selenium (µg/d)	RNI	15	20	30	45	70	75	75	75	75
	LRNI	7	10	16	25	40	40	40	40	40
Zinc (mg/d)	RNI	5.0	6.5	7.0	9.0	9.5	9.5	9.5	9.5	9.5
	LRNI	3.0	4.0	4.0	5.3	5.5	5.5	5.5	5.5	5.5

Table 1.4. Reference Nutrient Intakes (RNI) and Lower Reference Nutrient Intakes (LRNI) of seven human mineral nutrients for females by age (Department of Health, 1991; adapted from Public Health England, 2018)

Mineral (unit of measurement per day)		**Age group (years)** 1-3	4-6	7-10	11-14	15-18	19-50	51-64	65-74	75+
Females										
Iron (mg/d)	RNI	6.9	6.1	8.7	14.8	14.8	14.8	8.7	8.7	8.7
	LRNI	3.7	3.3	4.7	8.0	8.0	8.0	4.7	4.7	4.7
Calcium (mg/d)	RNI	350	450	550	800	800	700	700	700	700
	LRNI	200	275	325	450	450	400	400	400	400
Magnesium (mg/d)	RNI	85	120	200	280	300	270	270	270	270
	LRNI	50	70	115	180	190	150	150	150	150
Potassium (mg/d)	RNI	800	1100	2000	3100	3500	3500	3500	3500	3500
	LRNI	450	600	950	1600	2000	2000	2000	2000	2000
Iodine (µg/d)	RNI	70	100	110	130	140	140	140	140	140
	LRNI	40	50	55	65	70	70	70	70	70
Selenium (µg/d)	RNI	15	20	30	45	60	60	60	60	60
	LRNI	7	10	16	25	40	40	40	40	40
Zinc (mg/d)	RNI	5.0	6.5	7.0	9.0	7.0	7.0	7.0	7.0	7.0
	LRNI	3.0	4.0	4.0	5.3	4.0	4.0	4.0	4.0	4.0

1.7 Malnutrition in Human Populations

1.7.1 Terminology and estimates of overall undernutrition

There are a number of terms used in defining causes of energy and nutritional deficiencies in humans, some of which are often used interchangeably which can lead to confusion. Henceforth, the definitions described by FAO *et al.* (2017) will be used. These are listed in Table 1.5.

Table 1.5. List of terms used in defining energy and nutritional deficiencies in humans as described by FAO et al. *(2017).*

Term	Definition
Food Insecurity	A situation that exists when people lack secure access to sufficient amounts of safe and nutritious food for normal growth and development and an active and healthy life. It may be caused by unavailability of food, insufficient purchasing power, inappropriate distribution or inadequate use of food at the household level. Food insecurity, poor conditions of health and sanitation and inappropriate care and feeding practices are the major causes of poor nutritional status. Food insecurity may be chronic, seasonal or transitory.
Macronutrients	Refers to the proteins, carbohydrates and fats available to be used for energy.
Malnutrition	An abnormal physiological condition caused by inadequate, unbalanced or excessive consumption of macronutrients and/or micronutrients. Malnutrition includes undernutrition and overnutrition as well as micronutrient deficiencies.
Micronutrients	Vitamins, minerals and other substances that are required by the body in small amounts.
Overnutrition	A result of excessive food intake relative to dietary nutrient requirements.
Undernourishment	A state, lasting for at least one year, of inability to acquire enough food, defined as a level of food intake insufficient to meet dietary energy requirements.
Undernutrition	The outcome of poor nutritional intake in terms of quantity and/or quality and/or poor absorption and/or poor biological use of nutrients consumed as a result of repeated disease. It includes being underweight for one's age, too short for one's age (stunted), dangerously thin for one's height (wasted) and deficient in vitamins and minerals (micronutrient malnutrition).

Due to variation in estimates of human energy requirements, estimates of the prevalence of undernourishment also vary greatly and can be prone to error. For instance, estimates of undernourishment in 12 countries in Sub-Saharan Africa conducted by the International Food Policy Research Institute (IFPRI) were approximately 50 % higher than estimates calculated by the FAO in the same countries during the same period (de Haen et al., 2011). Part of the issue with such estimates is likely to come from the method of measurement, which is generally derived from measuring food supply and availability (Stein, 2014). Many estimates fail to take into account access based on financial means, food preferences, food waste, or are performed at only a country-wide level without taking into account intra-country variation. The most accurate estimate of undernutrition in humans is likely to be FAO's Prevalence of Undernutrition. This is based on not only food supply, but also food consumption and energy requirements across many demographics. This measurement is also used to monitor progress towards the first Millennium Development Goal of eradicating extreme poverty and hunger. FAO estimates of worldwide undernourishment were 815 million in 2016 (FAO *et al.*, 2017). This was an increase of 38 million people from 2015, and was the first year in which world hunger appeared to be on the rise after a long decline. The highest levels of food insecurity appear to be in the African continent. At 27.4 % of the population, this is almost four times higher than levels of food insecurity in any other region in 2016.

Though levels of undernourishment are incredibly high, even more concerning are the levels of undernutrition in terms of nutrient intake. For instance, over 2 billion people are likely to be at risk of undernutrition in a number of mineral nutrients including Ca, Mg, I, Fe, Se Zn and vitamins such as vitamin A (Kumssa et al., 2015a; Kumssa et al., 2015b). Undernutrition can be caused by overreliance on a single crop species that does not have a complete, appropriate nutrient profile for adequate human nutrition. This is typically the case in a number of developing countries which rely on subsistence farming. For example, in Malawi and Zambia, around 50 % of dietary calorie intake is from maize grain alone (Chilimba et al., 2011). Micronutrient undernutrition can also be caused by cultural preferences for certain food types. One example of this is an increased preference for processed foods in higher income

countries, in which processing may lead to lower concentrations of certain mineral nutrients. Below, the functions of two human mineral nutrients, Ca and Mg, are discussed, along with symptoms commonly associated with deficiency, and the prevalence of undernutrition.

1.7.2 Functions of Calcium and Symptoms and Prevalence of Undernutrition in Humans

In the human body, Ca is the fifth most abundant element after carbon, hydrogen, oxygen and nitrogen. Typically adults contain around 18 g Ca per kg of body weight (Sardesai, 2003), approximately 99 % of which is found in bones and teeth. The remaining Ca is found in extracellular fluid, soft tissues, and as a component of various membrane structures. Despite relatively low concentrations of Ca in these tissues, its importance cannot be overestimated. Cells use their internal Ca supplies to regulate a number of metabolic processes, including vascular and muscular contraction and dilation, and nerve transmission (Catharine, 2011). Calcium is also required to activate secretion of small molecules such as insulin from the pancreas (Draznin, 1988), and has necessary roles in the blood clotting response (Lovelock and Porterfield, 1952). At a cellular level, Ca concentrations are normally kept low by ATP-dependent Ca pumps, and fluxes are created as needed by the regulated release of Ca from the endoplasmic reticulum. Calcium must also be present at appropriate concentrations at a tissue level. To this end, blood Ca concentration is regulated to around 100 mg L^{-1}, with only low levels of variation between sexes and across the lifespan of a human adult. This is controlled by three hormones: parathyroid hormone (PTH), active vitamin D, and calcitonin. PTH both reduces loss of Ca from kidneys, and stimulates Ca release from bone, acting to restore blood Ca to normal following a loss in concentration. PTH also stimulates conversion of vitamin D to its active form, which proceeds to induce synthesis of a Ca-binding protein that increases intestinal Ca absorption (Goldstein, 1990). Calcitonin counteracts PTH, and becomes active when blood Ca levels increase (Carter and Schipani, 2006).

During long term Ca undernutrition, release of Ca from bone mass cannot be properly replaced, which can lead to serious consequences if Ca intake is not increased. In

young children, when bone is developing and there is a large demand for Ca for bone growth, undernutrition can lead to hypocalcaemia and rickets (Pettifor et al., 1981). In adults, such conditions are less common. However, longer term undernutrition can lead to an increased risk of osteoporosis (Nordin, 1997). Low levels of blood Ca can also occur in some situations, particularly in new-born infants and those suffering from rickets. This can lead to a condition called tetany, characterised by muscle pain, involuntary muscle contractions, numbness, and/or tingling, the latter particularly in hands and feet (Williams et al., 2011). Due to the role of vitamin D in the uptake of Ca from the intestine, Ca undernutrition symptoms can occur in the absence of sufficient vitamin D (Kennel et al., 2010). Hence, it is also important to consider vitamin D nutrition when attempting to remedy Ca undernutrition symptoms. It is also worthy of note that not all dietary Ca is bioavailable. For instance, phytate, an anti-nutrient found in high concentrations in grains, can form insoluble complexes with Ca and prevent its uptake in the intestine (Guéguen and Pointillart, 2000). For this reason, diets based on single staple crops are likely to contain insufficient quantities of bioavailable Ca.

Based on food supply data, it was estimated that around half of the human population worldwide was at risk of Ca undernutrition in 2011, with significant risks across all continents (Kumssa et al., 2015b). Whilst the prevalence of this risk appears to be decreasing (undernutrition risks ~25 % lower in 2011 than in 1992), a shockingly high number of people are still likely to be living with insufficient dietary Ca. The highest risks of Ca undernutrition were observed in Africa and Asia, with undernutrition risks in some countries exceeding 75 % at the national level. This is consistent with a study on mineral nutrient supplies in Africa specifically, which found that Ca undernutrition risks in 16 African countries exceeded 95 % in 2009 (Joy et al., 2014). Undernutrition in these regions is likely to be linked to decreased availability of animal products through financial means or otherwise, which were found to be the best source of dietary Ca (Kumssa et al., 2015b).

1.7.2 Functions of Magnesium and Symptoms and Prevalence of Undernutrition in Humans

The human body typically contains approximately 0.4 g Mg per kg (Maguire and Cowan, 2002; Sardesai, 2003). Around 53 % of this is found in bone, and a further 27 and 19 % is found in muscle and soft tissues respectively (Elin, 1988). The remaining Mg is found in red blood cells and serum. Most Mg is found within cells, where it acts as a cofactor in over 300 enzymatic reactions. It acts as a counter-ion for ATP, and hence ATP-dependent reactions also depend on suitable concentrations of cellular Mg (Swaminathan, 2003). In this role, Mg is crucially necessary for the synthesis of nucleic acids and proteins, as well as in muscle contraction and relaxation and regulation of the heart's rhythm. There is also evidence to suggest that Mg has a role in insulin secretion, and lower cellular-Mg contents have been observed in diabetics (Barbagallo and Dominguez, 2007). Magnesium competes with Ca for membrane binding sites, and hence may act as a natural antagonist, helping to maintain low, resting, intracellular Ca concentration (Swaminathan, 2003). Finally, Mg has various structural roles, maintaining the conformation of nucleic acids, and forming part of the structure of proteins and mitochondria (Jahnen-Dechent and Ketteler, 2012).

Similar to Ca, Mg homeostasis is controlled by the intestine, bones and kidneys. Magnesium can be stored in bone tissue to be released as required, and any excess Mg is excreted by the kidneys and in the faeces (Jahnen-Dechent and Ketteler, 2012). Though the evidence is less supportive as in the case of Ca, it is likely that Mg homeostasis is at least in part also controlled by PTH (Vetter and Lohse, 2002; Swaminathan, 2003). Magnesium is maintained within a narrow concentration range in the serum (Saris et al., 2000), and when dietary Mg drops, it is thought that PTH stimulates Mg release from bone, as well as increased absorption from the gut (Zofkova and Kancheva, 1995).

Whilst short term Mg undernutrition is unlikely to lead to severe consequences, symptoms can start to appear if greater concentrations of dietary Mg are not consumed. Early symptoms can include loss of appetite, nausea, vomiting, weakness,

and depression (Hashizume and Mori, 1990). These are fairly non-specific, and hence Mg deficiency is rarely detected in these early stages, as observed in a study on Mg status in hospital patients (Whang and Ryder, 1990). As Mg deficiency progresses, symptoms become more pronounced. These can involve muscle contractions and cramps, seizures, and an abnormal heart rhythm (Hashizume and Mori, 1990). These latter symptoms are perhaps linked to less Mg being available to complex with ATP. It is thought that increasing dietary Mg concentrations can reduce the risk of cardiovascular disease and diabetes among other diseases (Bo and Pisu, 2008).

Studies on the prevalence of Mg undernutrition in humans are limited. Further to this, those that have been conducted tend to vary considerably in undernutrition estimates, potentially due to differences in methodology. For instance, intake surveys have previously suggested that a large proportion of the UK and USA adult population is at risk of Mg undernutrition, with 25.3 million or around 9 % of the combined population consuming Mg concentrations below the LRNI (Broadley and White, 2010). The National Diet and Nutrition Survey carried out on behalf of Public Health England and the Food Standards Agency roughly agrees with this observation, with around 14 and 11 % of men and women respectively between the ages of 19 and 64 found to intake Mg at levels below the LRNI. More striking from this survey was the proportion of children between the ages of 11 and 18 with Mg intakes below the LRNI; 27 and 50 % for boys and girls respectively (Public Health England, 2018). However, a global study which investigated Mg availability based on food supply data at national levels, estimated that only 0.26 % of the worldwide population was at risk of Mg undernutrition in 2011. Despite some variation, Mg undernutrition risks in all continents were around this same, low order of magnitude (Kumssa et al., 2015a). This study is also in agreement with a study that looked into supply data in Africa specifically, which similarly concluded the risk of Mg undernutrition was low, at less than 4 % across the continent (Joy et al., 2013).

The reasons for the discrepancy between Mg undernutrition risks calculated through dietary surveying and supply data are unclear, though a few suggestions can be put forward. Firstly, dietary supply data does not account for food processing and waste

at the household level, and it is likely that not all of the Mg that is available enters the diet. This data also doesn't consider distribution within countries, and between groups of different socio-economic classes. It is also worth considering inhibitors of Mg adsorption such as phytate, which can considerably reduce the bioavailability of a number of mineral nutrients. Such "anti-nutrients" are not fully considered in the aforementioned studies, and we might expect that Mg undernutrition risks are higher than reported. Magnesium undernutrition risks are also likely to be greater in higher-income countries in which there is a preference for consuming processed foods, as Mg is among the nutrients commonly lost in processing (Swaminathan, 2003; Broadley and White, 2010; Kumssa et al., 2015a). Hence, whilst the worldwide prevalence of Mg undernutrition may not be as high as for other mineral nutrients including Ca, it is clear that there are likely to be significant risks of Mg undernutrition in a number of human populations.

1.8 Symptoms and Prevalence of Calcium and Magnesium Undernutrition in Livestock Animals

It is not just in humans that Ca and Mg undernutrition can be problematic. Deficiency symptoms have been observed in a number of other animal species, particularly in livestock. Calcium undernutrition can often occur when diets consist of low Ca cereals. This can have serious consequences in terms of animal health and productivity. Whilst lactating, cattle require very high levels of dietary Ca, up to around 73 g per day in dry matter intake (DMI). Sheep tend to require lower quantities, between around 1 and 10 g per day in DMI (Suttle et al., 1991; Suttle, 2010). In cattle, Ca undernutrition can lead to a condition commonly known as 'milk fever', which can affect production of milk, longevity and fertility (McCaughan, 1992). Symptoms commonly associated with milk fever can include difficulties standing and, in severe cases, loss of consciousness. Calcium metabolism in livestock animals is controlled by similar mechanisms to those in humans, and homeostasis tends to be controlled by PTH, calcitonin and vitamin D. However, because milk-production can drain Ca from the plasma at such a quantity (McAdam and O'Dell, 1982), it is unlikely that sufficient Ca can be released from bone matter into the plasma quick enough to counter low dietary supplies of Ca. The prevalence of milk fever typically ranges from

around 1-10 % in different populations of cattle, increasing with the age of the cow. However, under calving, this can rise to a risk of incidence of over 25 % (DeGaris and Lean, 2008).

As well as Ca, Mg can be significantly lost during lactation; 1 kg milk can contain around 0.12 g Mg (McCaughan, 1992). If this exceeds the quantity of Mg absorbed from DMI, hypomagnesemia can occur. Cattle Mg requirements are typically lower than Ca requirements, ranging from 2.3 to 29.4 g d^{-1} depending on weight, age and lactation status (Suttle, 2010). However, high dietary K concentrations can negatively impact the absorption of Mg, and this can further increase the risk of hypomagnesemia. Hence, it is also important to consider the K / Mg ratio when estimating risk of magnesium undernutrition in livestock (Cakmak, 2013). Hypomagnesemia can lead to a loss of appetite, lowered milk production, and a condition known as 'grass tetany' (McCaughan, 1992). In the latter case, symptoms starts with signs of nervous apprehension, progressing to violent convulsions, coma and death if not treated rapidly. Hypomagnesemia is common in grazing livestock, as levels of Mg tend to be low in forage grasses unless sufficient fertiliser is applied (Grunes and Welch, 1989).

1.9 Potential Remedies for Mineral Nutrient Deficiencies

There are a number of strategies currently being employed and developed to counter human and animal mineral nutrient deficiencies, each with their own advantages and disadvantages. These include dietary supplementation, i.e. providing nutrients from non-food sources to supplement normal dietary intakes; fortification of food products, i.e. adding nutrients in chemical form to food products as they are being processed; and biofortification of crops, i.e. ensuring larger quantities of available nutrient are present in edible crop tissue prior to processing. The latter, can be achieved either using fertilisers, or through genetic selection for genotypes predisposed to the accumulation of desired mineral nutrients. These strategies are discussed in further detail below.

1.9.1 Dietary Supplementation

Dietary supplements include any consumed products that provide additional nutrients that are missing from the diet, or consumed in insufficient quantities. They can come in the form of tablets, capsules, powders, liquids, or supplement bars (NHS, 2011). The effectiveness of dietary supplementation is unclear, with some sources stating clear benefits in alleviating undernutrition, whilst others argue lesser effects, or even negative consequences resulting from excessive intakes which may occur when nutrient sufficiency has already been achieved (Rautiainen et al., 2016). For instance, it has been shown that dietary supplementation in lower income countries can positively affect cognitive development of children, particularly when multiple nutrients are administered (Ip et al., 2017). Supplementation can also reduce the risk of low birth rate when administered to pregnant women in lower income countries, though seems unsuccessful in reducing perinatal mortality (Kawai et al., 2011). A further success story of supplementation is that of vitamin A, deficiencies in which can lead to blindness and mortality in children. Supplementation using vitamin A capsules have been shown to significantly decrease such symptoms (UNICEF, 1999). However, looking into the use of supplements in higher income countries reveals a contrasting scenario. A large-scale study on older women in the USA showed that most supplements used did not affect mortality rate. Worryingly, some of the supplements used, particularly Fe, were associated with *higher* overall risks of mortality (Mursu et al., 2011). A global review on dietary supplementation and disease prevention also concluded that among most people in more developed countries, adequate nutrition can be achieved through diet alone, and that supplementation is unlikely to have additional benefits (Rautiainen et al., 2016).

Overall the efficacy of dietary supplementation is likely to depend on the nutrient being administered, as well as the nutritional state of the consumer. Current evidence suggests that supplementation with many nutrients is unnecessary or even potentially harmful for most people in higher income countries where a good diet is likely to provide sufficient nutrition. Supplementation with certain vitamins and minerals can be useful in alleviating nutrient deficiencies in lower income countries

where adequate nutrition may be difficult to achieve based on food supply. However, in such countries, supplementation may not be the most cost effective option, requiring appropriate social infrastructures, stable political policies and continued investment, as well as behaviour changes in those taking the supplements (White and Broadley, 2009). Whilst this may be a suitable approach for some nutrients such as vitamin A, for which a single, high dose can increase stores for 4-6 months, it will be more difficult to implement in the case of more water-soluble vitamins and minerals, which require more frequent consumption (Allen *et al.*, 2006). Hence, it is clear that other methods of alleviating undernutrition are important to consider.

1.9.2 Food Fortification

Food fortification or enrichment is the process of adding nutrients to foodstuffs that are otherwise not present or present in concentrations deemed inadequate. It is often used to increase the nutritional value of staple foods to provide large scale benefit to populations, and is mandatory for certain foods in many countries. For instance, UK regulations state that all white bread flour must be fortified with calcium carbonate, iron, nicotinic acid (or nicotinamide) and thiamin (UK Parliament, 1998). A number of less well developed countries have also recently legislated mandatory fortification, including maize fortified with iron, folic acid, vitamin A and Zn in Malawi, Rwanda, Kenya and Nigeria (WHO, 2016). Fortification of salt with iodine is also a common practice in a number of countries, and has been successful in reducing iodine deficiencies, even over relatively short-term administration (Jooste et al., 2000). The former examples can be considered as mass fortification, an approach that is normally used at the national level. More targeted approaches have been considered in the past, and these may be more economically feasible, providing that those set to benefit most from fortification can be accurately identified. For instance, as described in a systematic review, Fe, I, Zn, vitamin A, B-vitamins and vitamin C provided through a range of fortified foods to school children in both more and less well developed countries consistently improved micronutrient status and reduced anaemia prevalence, with varying levels of positive effects on morbidity, growth, and cognitive ability (Best et al., 2011).

Whilst fortification is a good strategy for tackling widespread nutrient deficiencies for certain nutrients, it is not suitable for all situations in all locations. Fortification techniques require long-term financial input, which isn't always available. This can be especially problematic in the case of nutrients needed at higher concentrations such as Ca. Issues can also arise due to chemical restraints caused by certain combinations of inorganic sources of mineral nutrients. For example, large amounts of Ca in fortified food can inhibit absorption of Fe and Zn (Allen *et al.*, 2006). Difficulties can also arise in more rural areas of lower income countries in which subsistence farming is common. By relying on their own produce as dietary staples, these communities inadvertently miss out on fortified products that may be available in more urban locations. Whilst further efforts to target such communities may prove useful, other techniques may prove more effective and financially viable.

1.9.3 Biofortification

Biofortification is a technique which can be employed to increase concentrations of nutrients in edible parts of crop plants during their growth (White and Broadley, 2009). This can be achieved using fertilisers to increase nutrients available for uptake by roots or shoot tissue (the latter in the case of foliar application of certain nutrients). This approach, known as agronomic biofortification, has proven useful in alleviating nutrient deficiencies in humans. For instance soil or foliar applications of Se has had beneficial effects on the delivery of Se to the human diet in Finland (Eurola et al., 1991; Hartikainen, 2005). However, the efficacy of this technique can vary depending on the mineral and crop targeted and environmental conditions. For instance, whilst Zn fertiliser application is generally considered an appropriate method of increasing Zn in crop plants, mixed results have been observed in cereal crops, with different success rates in observed in different soil types (Cakmak, 2008; White and Broadley, 2009). Also important to note is that whilst Zn fertilisation can be successful in increasing shoot Zn concentrations in most environments, it doesn't always reach the desired tissue type. For example, in rice, most fertiliser-Zn appears to be stored in vegetative tissue, and grain-Zn concentrations seem to be mostly unchanged (Wissuwa et al., 2008). Hence, it is important to consider not only nutrient uptake in biofortification strategies, but also allocation to edible tissues. This is likely

to be less of an issue in crops in which vegetative tissue is consumed, as in the case of leafy vegetables such as those in the *Brassica* genus. Nevertheless, application of fertilisers that do not significantly increase the economic value of the crop are unlikely to be utilised by poorer communities, despite these groups commonly considered to be at the highest risk of nutrient deficiency.

An alternative approach to biofortification is through targeted crop breeding (Graham et al., 2001; Bouis, 2003). This approach, termed genetic biofortification, focusses on developing crop varieties that are genetically predisposed to uptake or synthesise larger quantities of nutrients, make them more bioavailable, and/or mobilise them to edible tissues in greater quantities. Crops that are able to accumulate essential human nutrients in the absence of fertilisation do not require continuous financial investment to positively impact human nutrition, as is the case for agronomically biofortified crops, chemically fortified foods, and long-term supplementation. Hence, such varieties are likely to be a much better solution to nutrient deficiencies in lower income communities. These communities typically cannot afford sufficient crop fertilisation, and have less access to nutrient supplements or fortified foods, either for financial reasons or distance from amenities. Genetic biofortification is a particularly viable solution where sufficient levels of heritable variation exist in crop species. Much of the variation in plant concentrations of minerals often lacking in human diets occurs at or above the taxonomic level of order. For instance, bread wheat grain Ca and Mg concentrations range from 250-730 and 920-1430 mg kg^{-1} respectively, whereas in leaves of a core collection of *Brassica oleracea*, Ca and Mg concentrations range from 14,300-39,200 and 4,000-9,600 mg kg^{-1} respectively (White and Broadley, 2009). For this reason, it is important to consider which crop is best to use as a 'vehicle' for the delivery of certain nutrients. In populations that rely more heavily on a single staple crop, such a choice may be unavailable. However, there is still typically around 2-3 fold variation in mineral nutrient concentrations present within many crop species, and hence conventional breeding techniques can be employed to cross favourable varieties in terms of nutrient accumulation with those that perform well in the field at a given location.

Whilst traditional breeding is a viable option for generating biofortified crop varieties, such techniques are typically slow, requiring numerous generations of crossing before a suitable variety is available. Complementary to this approach is the identification of genetic markers for a given trait, which can be tracked through the breeding process and allow more targeted selection of genotypes. Identification of genetic loci controlling the uptake or accumulation of nutrients could also lead to deciphering genes which control mineral accumulation. This could open up the possibility of creating novel alleles which are able to increase accumulation of human and animal nutrients in edible tissues at greater quantities than would otherwise be possible through more traditional breeding methods. Whilst regulation of genetically modified (GM) crops have hindered such advances in the last 60 or so years (Dubock, 2014), this may change in years to come. Non-GM approaches to create novel alleles are also available, including untargeted chemical mutagenesis followed by screening of regions of interest for useful alleles. In practice, both agronomic and genetic biofortification are likely complementary approaches. Whilst fertilisation of a field may not lead to adequate accumulation of a given nutrient in the desired tissue, genetically biofortified varieties are still limited by the concentration of the nutrient in the soil. In situations where soil nutrient concentrations are lacking, well considered fertiliser use coupled with a crop genotype that can successfully mobilise nutrients into edible tissue may prove to be the most effective option.

One example of a biofortified crop that has shown significant efficacy in reducing nutrient deficiency is orange-fleshed sweet potato bred to survive in sub-Saharan Africa. Most varieties historically available in this region are white-fleshed, lacking the vitamin A precursor beta-carotene. Since around 1995, efforts to breed orange-fleshed sweet potato that can be grown in sub-Saharan African conditions have only been increasing, and it has been shown that consumption of such varieties can successfully reduce vitamin A deficiencies among young children (Low et al., 2017). Much of this work has taken place by HarvestPlus, a group dedicated to delivering biofortified varieties of crop plants to communities in developing countries. Other crop species that have been and are being developed by HarvestPlus include maize, cassava, beans, rice, wheat and pearl millet, thus representing the needs of a large

range of crop systems. Traits most commonly considered are vitamin A, Zn and Fe concentrations in edible plant parts, as deficiencies in these nutrients are amongst the most widespread in developing nations (Bouis et al., 2011). However, it is not only in developing nations that biofortified crops could reduce human undernutrition. The effects of biofortifying horticultural produce including vegetables, fruits and potatoes on human nutrition in the UK was previously modelled. Based on current dietary behaviours, it was revealed that biofortifying all such produce by 50 % with Ca, Mg and K could bring intakes of these elements above the LRNI for a further 0.3, 2.0 and 4.1 million UK adults, respectively (Broadley and White, 2010). For the latter two nutrients, this improvement was greater than the effect of consuming two extra portions of vegetables per day. In reality, biofortification combined with the consumption of two extra portions of vegetables a day represents the best approach, which would likely reduce Ca, Mg and K undernutrition in UK adults by 60, 75 and 70 %, respectively.

Currently, there are limited examples of real-world application of biofortification in more developed countries. However, worthy of mention are a number of varieties of broccoli which have been selectively bred for elevated shoot glucoraphanin concentrations. Termed ‘Beneforte Broccoli’, the higher levels of glucoraphanin in these varieties have been linked to lower plasma concentrations of LDL-cholesterol (Armah et al., 2015). These have been developed over the last 20 years using naturally available variation, following hybridisation between heading broccoli (*Brassica oleracea*) and the high glucoraphanin wild-relative *B. villosa* (Faulkner et al., 1998). Subsequent work has been performed to develop these varieties further, and they are now available for purchase in UK supermarkets (Traka et al., 2013). Whilst progress towards the development of commercially viable varieties was slow, this demonstrates the efficacy of using naturally available variation for increasing concentrations of essential human nutrients in crops, which could be exploited further if suitable, additional variation is identified and utilised.

1.10 Brassica*: a globally important crop genus*

With thousands of varieties, encompassing a large range of crop growth habits suitable for cultivation in many different environments, the *Brassica* genus is among the most important taxa in terms of global food production and economy. One species, *B. oleracea*, comprises crop types including cabbage, broccoli, cauliflower, kale, collards, Brussels sprouts and kohlrabi, thus representing the phenotypic diversity present within this group. *Brassica rapa* is another important crop species within this group, comprising turnip varieties, as well as varieties more widely cultivated in Asian countries including napa cabbage (Chinese cabbage), pak choi, and mizuna (a leafy crop cultivated in Japan). World production of vegetables of these species in 2016 was over 96 million tonnes (FAO, 2018). For comparison, world production of wheat in the same period was 749 million tonnes. This is around eight-fold higher than *Brassica* production. However, wheat has a very different function within the human diet, functioning as a staple calorie crop. Hence, despite the apparent large difference in production between these two crop types, production of *Brassicas* worldwide is still incredibly large. A further economically important crop species within the *Brassica* genus is *B. napus*, comprising crop types including the vegetable swede (or rutabaga), and oil types commonly known as oilseed rape or canola. Among crops in the UK, oilseed rape production is consistently the third highest behind wheat and barley, with a production of 1.8 million tonnes in 2016 (DEFRA, 2017). *Brassica* oilseeds are also of high importance globally. Of the 31 million tonnes of oilseed crops produced in Europe in 2016, 20 million tonnes came from oilseed rape and turnip rape (Eurostat, 2017). Among oilseeds worldwide, rapeseed had the second highest production in the growing season of 2016/17 behind soybean at 71 million tonnes. It is also consistently ranked the third most important source of vegetable oil worldwide behind palm and soybean, with oil production of 28 million tonnes in 2016-17 (USDA, 2018). Oilseed Brassicas also have an additional use; the remains from pressed seeds make a high quality, protein-filled meal which is ideal for livestock feed. Whilst currently used widely, there is further interest in replacing soybean meal (a similar, secondary product from soybean) with rapeseed meal in some systems, as it has been shown to be an adequate substitute

(Huhtanen et al., 2011; Martineau et al., 2013). Forage-specific *B. napus* varieties also exist, and remaining leafy tissue from swede varieties also provides a suitable livestock feed. *Brassica napus* has only very recently reached high importance in global agriculture. This is following intensive breeding programmes over the last fifty or so years leading to the development of varieties with lower seed concentrations of bitter compounds known as glucosinolates, and toxic erucic acid (Allender and King, 2010).

Brassica napus is a polyphyletic, allopolyploid species of relatively recent origin, thought to have originated from multiple, spontaneous hybridisation events between *B. rapa* and *B. oleracea* around 7,500 years ago (Nagaharu, 1935; Allender and King, 2010; Chalhoub et al., 2014). Due to the amphidiploid nature of *B. napus*, it has a full set of diploid chromosomes from each of *B. rapa* (A genome; AA) and *B. oleracea* (C genome; CC). Intriguingly, a third *Brassica* species, *B. nigra* (black mustard; B genome; BB), has also historically formed hybridisations with *B. rapa* and *B. oleracea*. This has led to the formation of two further amphidiploid species: *B. juncea* (brown mustard; AABB) and *B. carinata* (Ethiopian mustard; BBCC), each of which comprise oilseed and vegetable crop types. *Brassica juncea* is particularly important as an oilseed crop in India, though higher levels of potential toxins including sinapine (Kajla et al., 2017) have likely limited global popularity of these varieties. The genetic relationship between *B. rapa*, *B. nigra* and *B. oleracea* and the amphidiploid hybrid species is summarised in Figure 1.2.

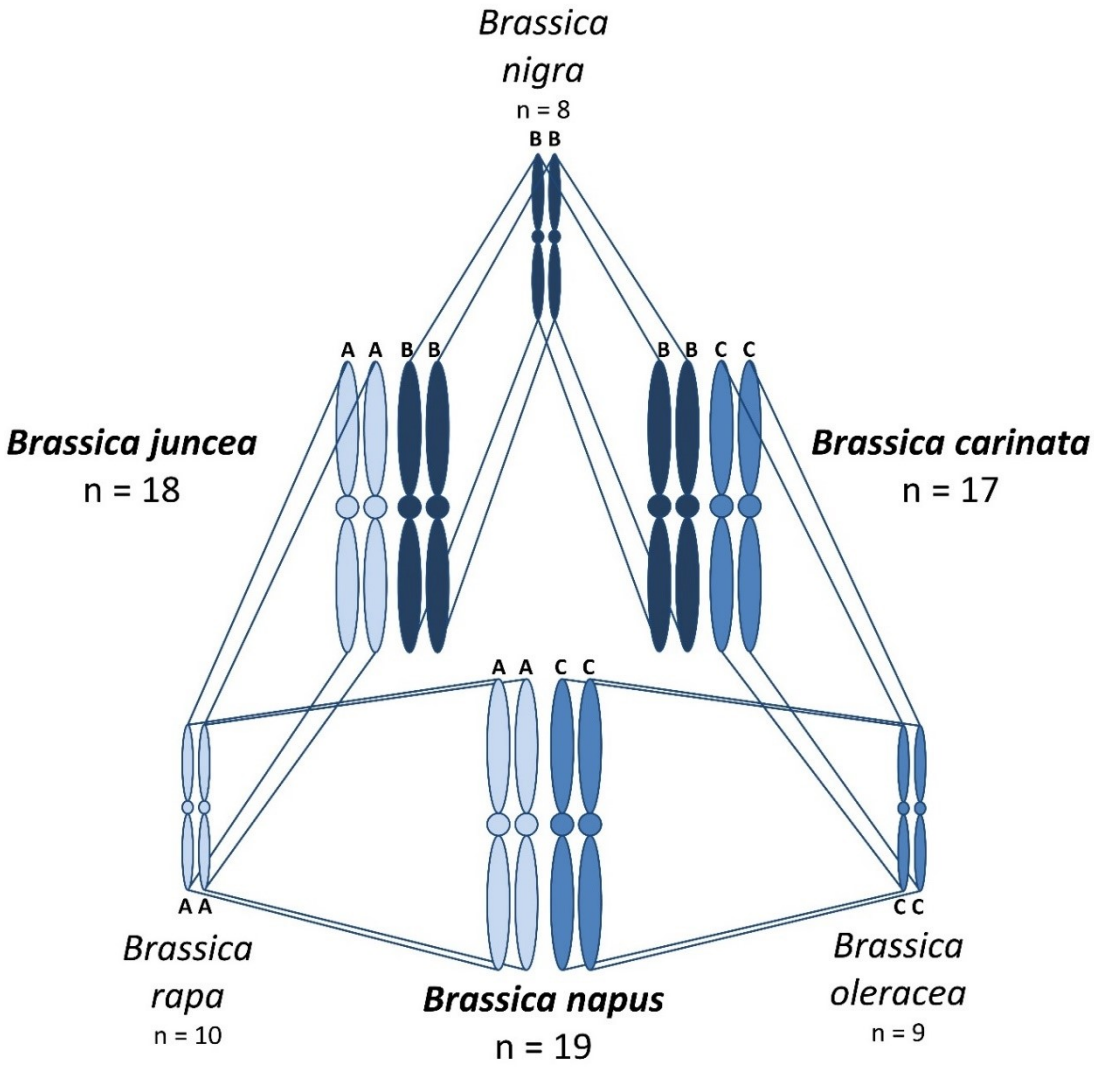


Figure 1.2. Summary of the genetic relationship between Brassica rapa*,* B. nigra*, and* B. oleracea *and the interspecific hybrid species* B. juncea*,* B. napus *and* B. carinata*, each of which possess a full set of diploid chromosomes from both parent species . Adapted from Nagaharu (1935).*

Oilseed rape yields have shown relatively little improvement in recent history. For instance, UK oilseed rape yields have consistently averaged around three tonnes per hectare (t ha^{-1}) between 1990 and 2014, despite estimated potential yields of 6.5 t ha^{-1} (AHDB, 2015). This is much lower than average UK wheat yields of around 8 t ha^{-1}. This is perhaps unsurprising, given the recent development of oilseed rape varieties suitable for widespread agricultural use. However, this also suggests that there is significant scope for improvement in this crop, either through traditional breeding practices or marker-assisted selection. Due to the relative genetic complexity of *B. napus* and other polyploid *Brassicas*, the identification of loci important for crop production has proven difficult in the past. However, recent and ongoing advances and cost reductions in sequencing and mapping technologies and have improved the feasibility of such studies (Trick et al., 2009). QTL have previously been identified for seed oil and erucic acid content (Qiu et al., 2006), flowering time (Raman et al., 2013), seedling ionomic traits (Bus et al., 2014) and root architecture (Zhang et al., 2016; Wang et al., 2017). Whilst these may prove useful in breeding strategies for improved crop productivity, there is still a long way to go towards identifying specific genes controlling these traits. Whilst still a difficult task, a benefit of studying traits in polyploid *B. napus* is the potential for any findings to be readily transferred into *B. rapa* and *B. oleracea*, due to significant synteny between gene models in each of these species.

Relative to other groups, *Brassica* crops have previously been shown to accumulate large concentrations of a number of mineral nutrients essential for human and animal health. Commercial varieties of *B. oleracea*, for example, can accumulate leaf DW concentrations of up to 338 mg kg^{-1} Fe, 122 mg kg^{-1} Zn, 35,000 mg kg^{-1} Ca, and 8,300 mg kg^{-1} Mg (Broadley et al., 2008). Similar concentrations of Zn and Fe were also observed in dry leaves of *B. rapa* in a core collection (Wu et al., 2007; White and Broadley, 2009). In a diversity population of *B. napus* varieties encompassing oilseed and vegetable types, seedling shoot Ca was found to occupy a similar order of magnitude as in *B. oleracea*, between around 20,000 and 35,000 mg kg^{-1} DW. Seedling shoot Mg, Fe and Zn were typically around 4,000, 60 and 50 mg kg^{-1} DW, respectively (Bus et al., 2014). *Brassica* shoot Ca and Mg concentrations in particular

are much higher than those found in many other crop species including wheat as discussed earlier. For this reason, *Brassica* crops, particularly leafy varieties, are a good vehicle for delivering mineral nutrients to humans.

Despite relatively large concentrations of Ca, Mg and other mineral elements in *Brassica* crops, there are significant levels of variation in these traits. For instance, shoot Ca and Mg concentrations within a diversity set of 376 accessions and 74 modern F_1 hybrid cultivars of *B. oleracea* were found to vary 2- and 2.3-fold respectively (Broadley et al., 2008). Similar levels of variation were previously found in a *B. napus* diversity set (Bus et al., 2014). In both of the former studies, both shoot / leaf Ca and Mg concentrations were found to be highly heritable traits. Hence, there is significant scope for increasing available, edible-tissue concentrations of these mineral elements in commercial *Brassica* varieties through conventional breeding techniques. Such an approach might be complemented by the identification of QTL controlling such traits, which could aid through enabling marker-assisted selection. An understanding of the genetic and physiological mechanisms of shoot Ca and Mg accumulation could also lead to varieties being developed that are capable of accumulating much higher concentrations of these nutrients than would otherwise be possible through more conventional techniques. Previous work carried out includes genetical genomics analyses of a mapping population of *B. rapa* which led to the discovery of a number of expression quantitative trait loci (eQTL) that responded to altered Ca supply (Graham et al., 2014). Determination of eQTL hotspots allowed associated candidate genes to be identified. Notably, these included orthologues of *Arabidopsis thaliana* Ca^{2+} transporter genes *CATION EXCHANGER 1* (*CAX1*) and *AUTOINHIBITED* CA^{2+} *ATPASE, ISOFORM 8* (*ACA8*). Further work in the same study characterised allelic variants in the former gene in ethyl methanesulfonate mutagenised genotypes of *B. rapa*, and found that one variant was able to accumulate 64 % more leaf Ca than its respective wild-type. Given that such techniques are capable of identifying genes controlling nutrient accumulation in *Brassica*, they may prove useful for the future development of biofortified varieties capable of reducing widespread nutrient deficiencies in humans.

1.11 Aims of Study and Format of Thesis

The research presented in this thesis has two main aims. The first aim is to identify genetic loci controlling the uptake of nutrients generally considered limiting in *Brassica* crop production. Identification of such loci may aid in the development of new crop varieties which are able to grow more vigorously under lower exogenous nutrient supply, thus expanding the range of suitable growth environments and reducing fertiliser inputs. The main nutrients considered here are N, P and K. The second aim is to identify genetic loci controlling the uptake and accumulation of mineral nutrients essential for human and animal nutrition into edible parts of *Brassica* plants. This may enable biofortified crop varieties to be developed, which in turn could help to reduce widespread human mineral deficiencies. The elements of particular focus here are Ca and Mg, two elements discussed in this introduction which have high importance in human nutrition and are likely to be lacking in diets across the world. The initial hypothesis for this work is that there are large amounts of variation in mineral concentration traits between diverse varieties of *Brassica* spp. that could be made use of in breeding strategies. Previous work in *B. oleracea*, as mentioned above, is indicative of this, although detailed work in further species including *B. napus* is yet to be performed to fully confirm this. It is also hypothesised that these traits are highly heritable and amenable to genetic dissection. If this is proven to be the case, it will enable the identification of quantitative trait loci and subsequently genes controlling trait variation, so long as suitable genetic markers are made use of. Thus, this will enable fulfilment of the two major aims described above.

Towards both of these aims, Chapter 2 comprises a published journal article entitled: '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'. In this study, huge levels of variation are detected in root architecture and seed and leaf ionomic traits across 387 genotypes of *B. napus*, indicating the scope for breeding for increased nutrient-use efficiency and accumulation traits using naturally available germplasm. Of particular importance to this thesis is the establishment of species-

wide variation in leaf mineral concentration traits in *B. napus*, which are utilised in transcriptome-based association studies in subsequent chapters.

Chapter 3 consists of a submitted journal article entitled: 'Species-wide variation in shoot nitrate concentration, and genetic loci controlling nitrate, phosphorus and potassium accumulation in *Brassica napus* L.'. In this chapter, the generation of leaf nitrate (NO_3^-) data across the same diversity population described in chapter two is presented. This data, along with leaf P and K concentration data presented in chapter two are then used to perform individual, transcriptome-based association studies, hereafter referred to as 'Associative Transcriptomics' (Harper et al., 2012; Havlickova et al., 2018). This technique makes use of RNA sequence data made available for all accessions in the *B. napus* diversity population (He et al., 2016). This allows analyses based on both single nucleotide polymorphisms (SNPs) and gene expression markers (GEMs) to be performed, in order to identify genetic loci responsible for the control of measured traits. From these associations, a series of candidate genes likely to be directly involved in the control of one or more of the characterised traits are identified, many of which may be useful in crop breeding strategies for increased nutrient-use efficiency.

Chapter 4 is a published journal article entitled 'Identification of Candidate Genes for Calcium and Magnesium Accumulation in *Brassica napus* L. by Association Genetics'. This chapter again uses data presented in Chapter 2 in Associative Transcriptomics analyses, here to identify loci controlling Ca and Mg accumulation. Numerous, highly associated loci are detected for these two mineral traits, many of which co-localise. Several convincing candidate genes are also identified, many of which have previously been shown to influence shoot Ca and / or Mg concentrations. Of those candidates which had not previously been characterised, genotypes of *Arabidopsis thaliana* (a closely related, model plant species) mutated in orthologous genes are analysed for perturbations in leaf mineral traits.

Chapter 5 is a direct continuation of the work described in Chapter 4. In this chapter, *MAGNESIUM TRANSPORTER 7* (*MGT7*), one of the most convincing candidate genes

likely responsible for the association of a locus on chromosome C2 with leaf Mg concentration, is further characterised. This gene was previously proposed to express a root-expressed, high-affinity Mg transporter in *A. thaliana* (Gebert et al., 2009). Here, an ethyl methanesulfonate (EMS) mutagenised *B. rapa* genotype identified as a 'knock-out' mutant in this gene is grown along with wild-type plants under a range of exogenous Mg concentrations in order to elucidate possible responses to the mutation. Results suggest a partially conserved role of *MGT7* in Mg homeostasis in *Brassica* spp.

In Chapter 6, a genotype of *B. rapa* previously found to accumulate around 200 % greater shoot Mg concentrations than wild-type plants (Thomas, 2017) is characterised further. Such a trait could hugely impact efforts to reduce dietary Mg deficiencies in humans and livestock if bred into commercially viable crop cultivars. To improve understanding of the physiological bases of this trait, bulked leaf samples of Mg accumulator and wild type plants grown under varying exogenous Mg concentrations were analysed for variation leaf mineral concentrations and variation in photosynthetic activity. Tissue and cellular level Mg and P spatial distributions were also mapped through micro-Proton Induced X-ray Emission (micro-PIXE) and micro-X-ray fluorescence (micro-XRF) spectrometry. This revealed specific trends in nutrient distribution unique to the Mg accumulator. The genetic basis of this trait was also investigated through analysis of DNA sequence data acquired from Mg accumulator and wild-type plants. A combined consideration of likely physiological mechanisms controlling the trait and SNPs in genetic loci between Mg accumulator and wild-type plants has enabled the identification of relatively few genes which may control this trait. If confirmed, these would rapidly allow generation of crop varieties with greater abilities to accumulate Mg in shoot tissue.

Finally, Chapter 7 presents a general discussion, in which the scope of the research presented in this thesis is evaluated. Under particular consideration are the potential applications of the findings described henceforth, which largely revolve around improving crop productivity and increasing concentrations of available human nutrients in edible crop tissue. The *B. rapa* Mg accumulator genotype is a highly

promising target for the latter application. It is likely that relatively little further work is required to introgress this trait into high-yielding *Brassica* crop cultivars, which could help to alleviate nutrient deficiencies in humans in years to come.

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Chapter 2. 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

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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. CLT, TDA and MRB generated figures and wrote the manuscript. All other authors contributed to project conception and advised on data analysis.

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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

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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

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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).

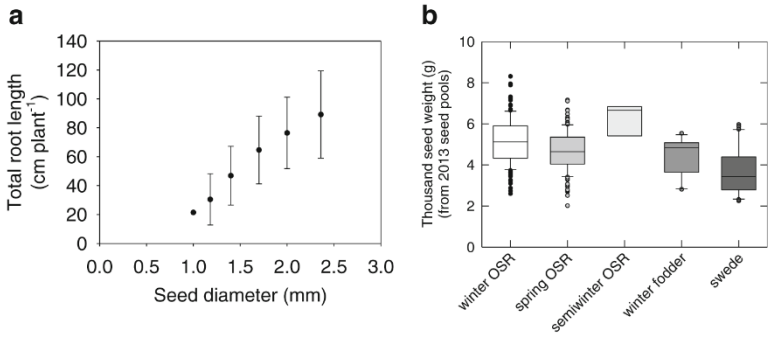


Fig. 1 a Total root length (TRL) as a function of *Brassica napus* seed diameter from a 'pouch and wick' system. Data represent mean ± standard error of the mean for all seedlings grown, from seeds with diameters of 1.18, 1.40, 1.70, 2.0 and 2.36 mm; $n = 44$, 1349, 2055, 2242 and 1059, respectively, 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

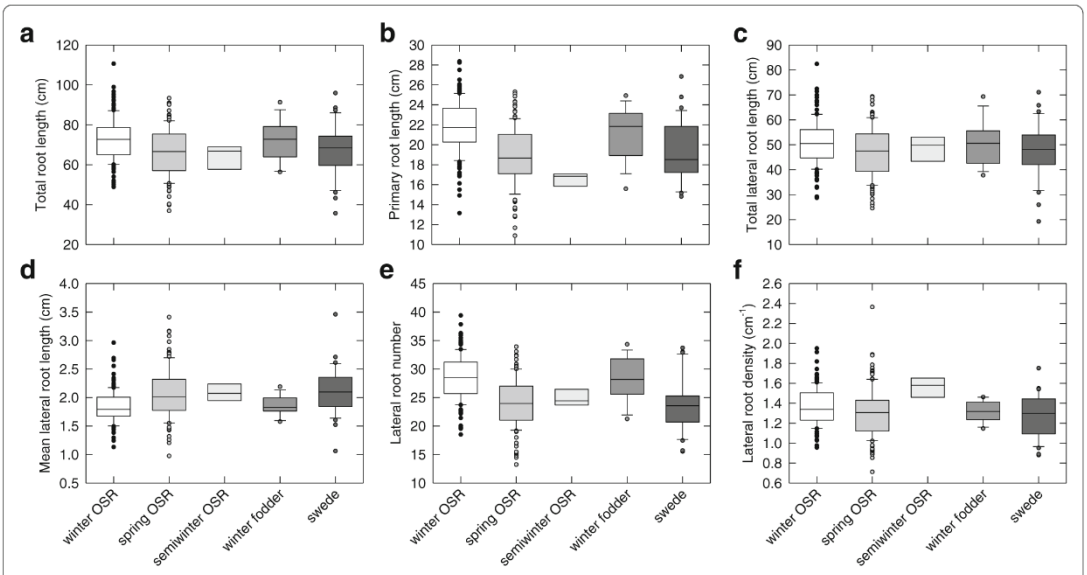


Fig. 2 Root traits of *Brassica napus* grown in a 'pouch and wick' system. Data are means of up to 319 genotypes, including winter OSR ($n = 142$), spring OSR ($n = 124$), semiwinter OSR ($n = 7$), winter fodder ($n = 14$) and swede ($n = 32$) habits. Boxes represent the mid two quartiles with the median 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],

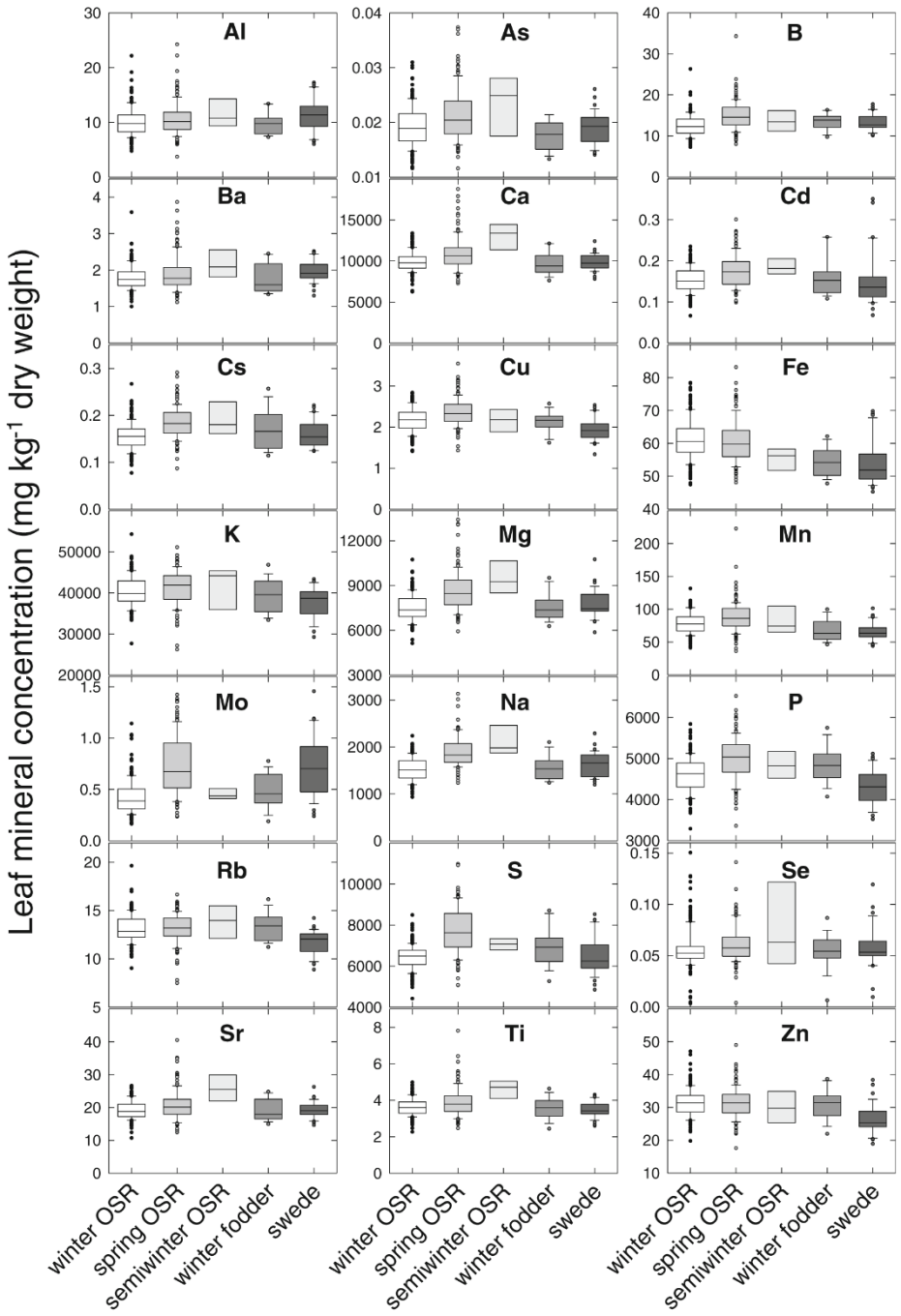


Fig. 3 Leaf mineral concentrations of *Brassica napus* grown in compost. Data are means of up to 385 genotypes, including winter OSR ($n = 163$), spring OSR ($n = 127$), semiwinter OSR ($n = 7$), winter fodder ($n = 15$) and swede ($n = 35$) habits. Boxes represent the mid two quartiles with the median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

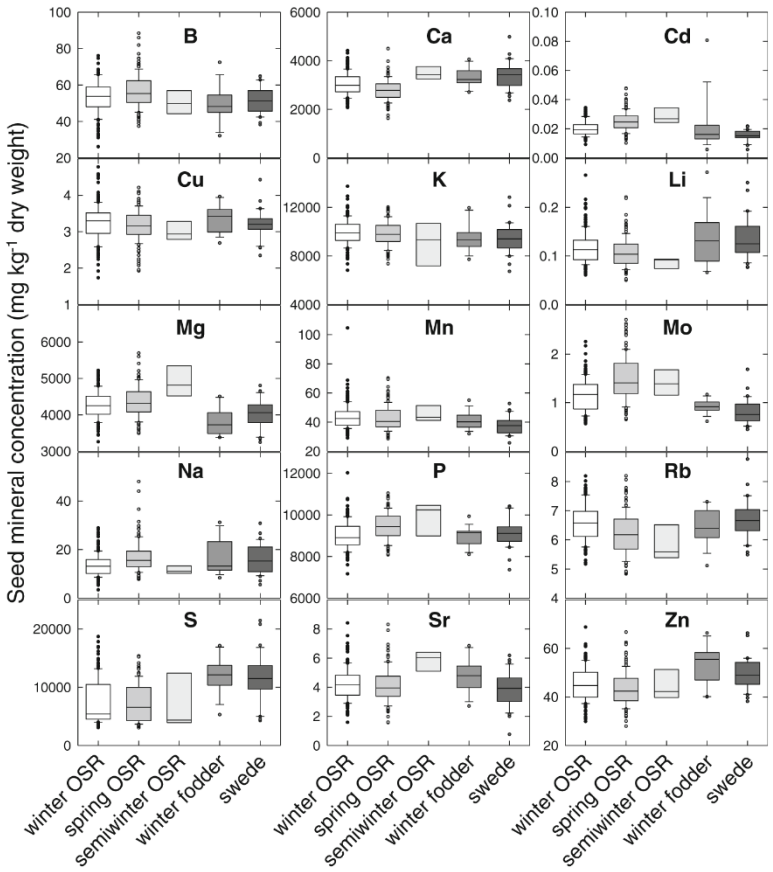


Fig. 4 Seed mineral concentrations of *Brassica napus* grown in compost. Data are means of up to 380 genotypes, including winter OSR ($n = 162$), spring OSR ($n = 127$), semiwinter OSR ($n = 7$), winter fodder ($n = 15$) and swede ($n = 31$) habits. Boxes represent the mid two quartiles with the median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

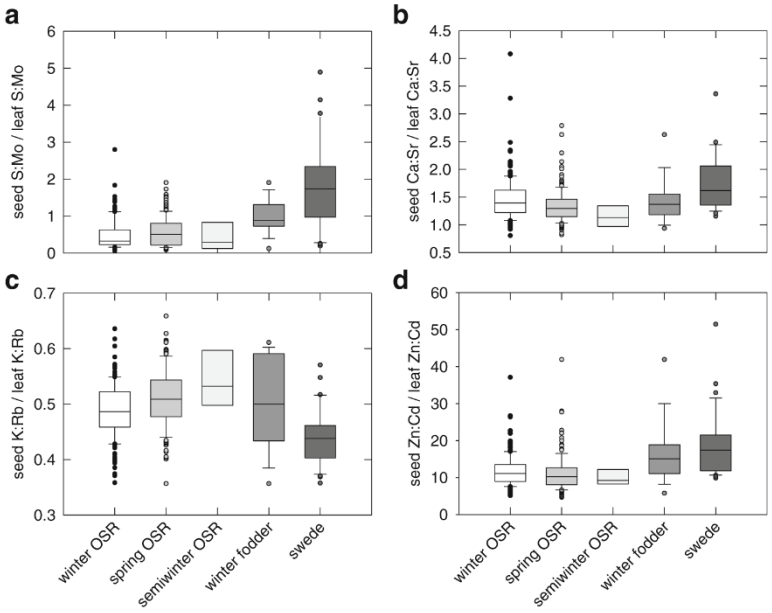


Fig. 5 Leaf and seed element concentration ratios of *Brassica napus* grown in compost. Data are means of up to 385 and 380 genotypes, for leaf and seed concentrations, respectively, including winter OSR ($n = 163$), spring OSR ($n = 127$), semiwinter OSR ($n = 7$), winter fodder ($n = 15$) and swede ($n = 35$) 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

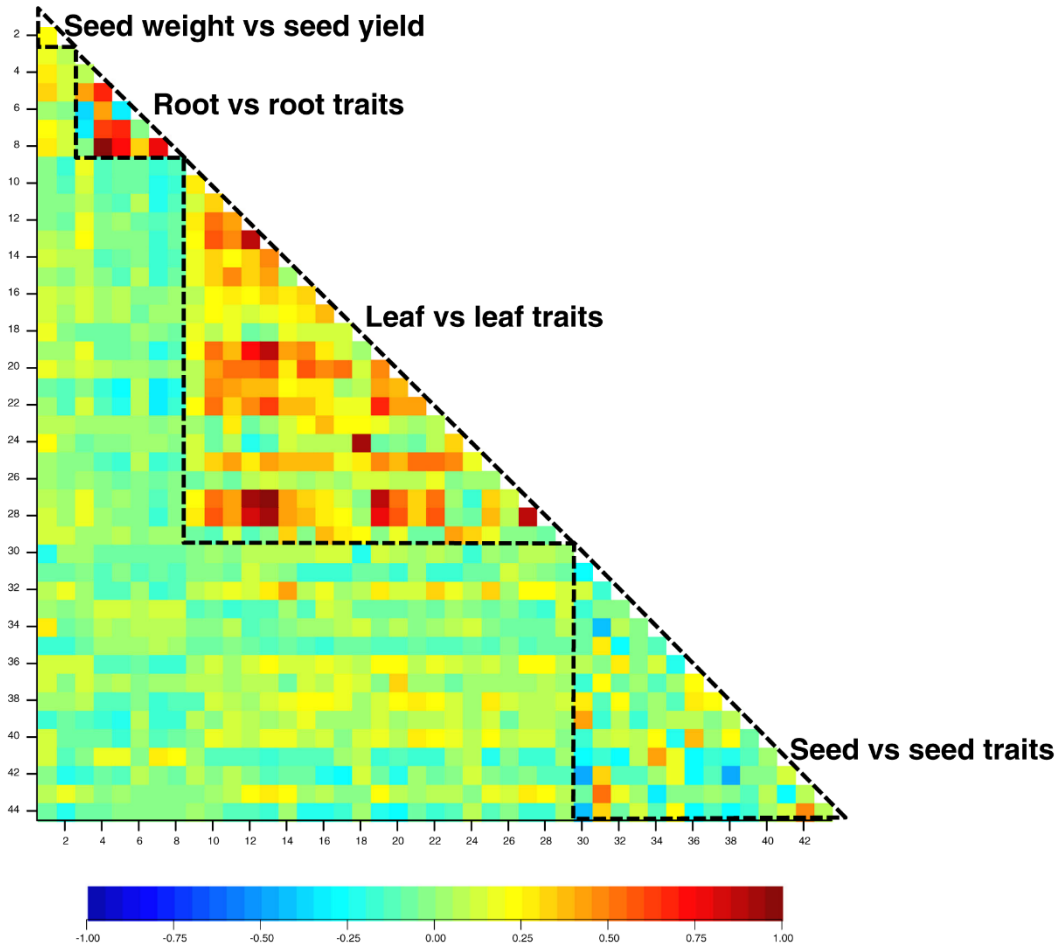


Fig. 6 Pair-wise correlations of all 44 traits; TSW and seed yield, root morphology and leaf and seed mineral composition traits in *Brassica napus* genotypes. Plants were grown in a 'pouch and wick' system (root traits, up to 319 genotypes) or compost (leaf and seed traits; up to 385 and 380 genotypes, 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

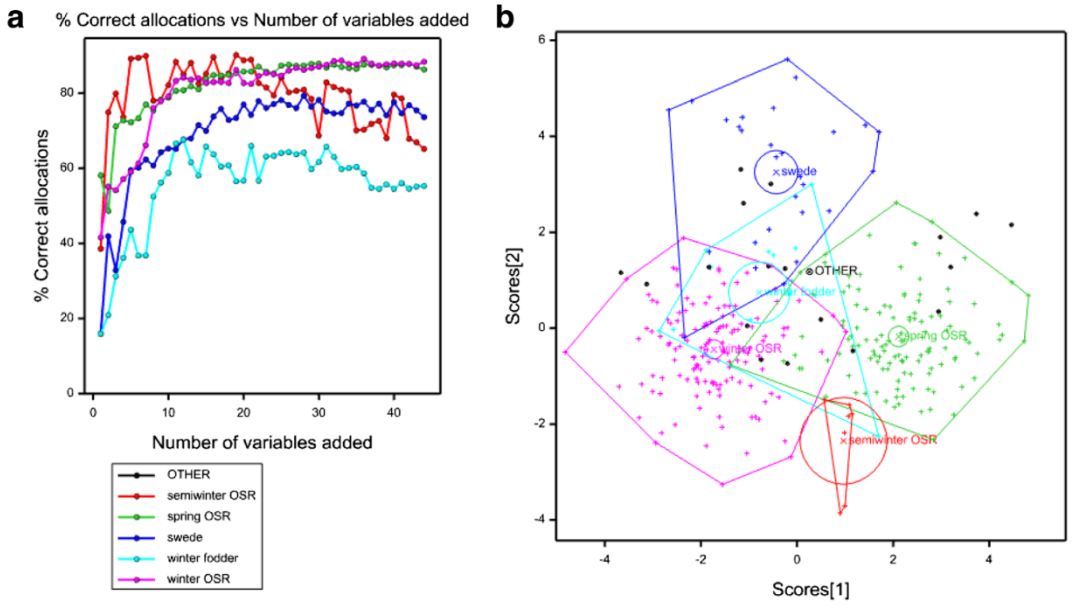


Fig. 7 Step-wise discriminant analysis using the variate set of all 44 traits; root morphology, TSW and seed yield and leaf and seed mineral composition traits combined. **a** Specificity plot showing the proportion of genotypes of each 'crop habit' correctly allocated to each group, at each step of 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.

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Chapter 3. Species-wide variation in shoot nitrate concentration, and genetic loci controlling nitrate, phosphorus and potassium accumulation in *Brassica napus* L.

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Species-Wide Variation in Shoot Nitrate Concentration, and Genetic Loci Controlling Nitrate, Phosphorus and Potassium Accumulation in *Brassica napus* L.

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Large nitrogen, phosphorus and potassium fertilizer inputs are used in many crop systems. Identifying genetic loci controlling nutrient accumulation may be useful in crop breeding strategies to increase fertilizer use efficiency and reduce financial and environmental costs. Here, variation in leaf nitrate concentration across a diversity population of 383 genotypes of *Brassica napus* was characterized. Genetic loci controlling variation in leaf nitrate, phosphorus and potassium concentration were then identified through Associative Transcriptomics using single nucleotide polymorphism (SNP) markers and gene expression markers (GEMs). Leaf nitrate concentration varied over 8-fold across the diversity population. A total of 455 SNP markers were associated with leaf nitrate concentration after false-discovery-rate (FDR) correction. In linkage disequilibrium of highly associated markers are a number of known nitrate transporters and sensors, including a gene thought to mediate expression of the major nitrate transporter NRT1.1. Several genes influencing root and root-hair development co-localize with chromosomal regions associated with leaf P concentration. Orthologs of three ABC-transporters involved in suberin synthesis in roots also co-localize with association peaks for both leaf nitrate and phosphorus. Allelic variation at nearby, highly associated SNPs confers large variation in leaf nitrate and phosphorus concentration. A total of five GEMs associated with leaf K concentration after FDR correction including a GEM that corresponds to an auxin-response family protein. Candidate loci, genes and favorable alleles identified here may prove useful in marker-assisted selection strategies to improve fertilizer use efficiency in *B. napus*.

Keywords: associative transcriptomics, GWAS, nitrogen, nutrient use efficiency, suberin, casparian strip, fertilizer, diversity population

INTRODUCTION

The plant macronutrients nitrogen (N), phosphorus (P) and potassium (K) are required in large amounts by higher plants, which typically contain approximately 1.5% N, 0.2% P, and 1% K on a dry weight (DW) basis (Hawkesford et al., 2012). Large concentrations of soil N are found in organic matter, the majority of which is in the form of peptides and proteins (Jones et al., 2002). Whilst largely unavailable to plants directly (Dechorgnat et al., 2011), there is evidence to suggest that plants can acquire organic N through root uptake of free amino acids (Näsholm et al., 2009). These can be made available through the breakdown of peptides and proteins by soil micro-organisms, and may be the dominant form of N in some high latitude ecosystems (Miller and Cramer, 2004). However, the major source of N to plants in aerobic, agricultural soils is thought to be nitrate (NO_3^-), which represents <2% of soil N (Dechorgnat et al., 2011). Similarly, although total soil P content is generally considered to be relatively high, bioavailable P is present at much lower concentrations (White and Hammond, 2008). This is largely due to the ability of P to form insoluble complexes with cations including calcium (Ca), magnesium (Mg), iron (Fe), and aluminum (Al; Hinsinger, 2001). The availability of P in soils is also thought to be declining due to soil degradation, which is estimated to have affected over half of global agricultural land, and the majority of agricultural land in Africa (Lynch, 2011). The concentration of K in soils can vary greatly depending on soil type, moisture, and chemical composition (Maathuis and Sanders, 1996). Much of the K available to crops is present in the soil solution, which represents only 0.1–0.2% of total soil K (White, 2013). Whilst further sources of K from exchangeable and non-exchangeable fractions of the soil are generally available to replenish soil solution K, typically 90–98% of total soil K remains effectively inaccessible to crop plants.

Due to the highly intensive nature of modern agriculture, bioavailable supplies of N, P, and K in arable soils can quickly become depleted (Jones et al., 2013). This can lead to the manifestation of symptoms including stunting, narrow leaves, and chlorosis under N insufficiency, inhibited shoot growth under P insufficiency, and retarded growth and shoot necrosis under K insufficiency (Hawkesford et al., 2012). In order to prevent such symptoms, huge quantities of fertilizer are applied to soils every year. In 2016, worldwide N fertilizer demand was 112 million tons, and demand is estimated to be increasing by 1.5% annually (FAO, 2017). Global demand for P and K fertilizer in the same year were around 42 and 33 million tons, with demand expected to increase by 2.2 and 2.4% per year, respectively (FAO, 2017). Fertilizer use is expensive, driving up costs to both the farmer and the consumer. It can also be harmful to the environment by contributing to greenhouse gas emissions, of which agricultural fertilizer use is the main source of atmospheric N_2O release (Snyder et al., 2009). There is also a need to reduce excessive anthropogenic nutrient inputs to aquatic ecosystems through agricultural runoffs in order to reduce eutrophication and protect drinking water supplies (Conley et al., 2009). For example, in order to return to within Earth's critical boundary set for excessive P in waterways, it is estimated that daily food supply per capita would have to drop below one tenth of the present supply under current agricultural practices and diets (Kahiluoto et al., 2013). Thus, a significant challenge in modern agriculture is the generation of new varieties better able to uptake and utilize available nutrients, in order to improve yields, whilst reducing fertilizer inputs.

Brassica napus L. is a crop species of global importance, encompassing oilseed varieties, commonly known as oilseed rape or canola, as well as vegetable crop types including swede or rutabaga, and leafy types including fodder and kale (Allender and King, 2010). Among major oilseeds, production of oilseed rape is the second largest behind soybean, with an estimated production of 74.28 million tons between April 2017 and April 2018 (USDA, 2018). It is also the third largest source of vegetable oil globally behind palm and soybean, with world supply predicted to be 28.84 million tons for the same period. Of this, approximately 10 million tons is estimated to be produced by the European Union, in which oilseed rape is the primary source of vegetable oil (European Commission, 2018). Only following intensive breeding programmes to reduce concentrations of toxic erucic acid and bitter glucosinolates from seed oil over approximately the last 50 years has *B. napus* reached global importance as a source of high quality vegetable oil (Allender and King, 2010). Hence, it is likely that there is still extensive scope for improvements in nutrient use efficiency in *B. napus*. For instance, N use efficiency in oilseed rape was estimated to be less than half that of barley and winter wheat (Berry et al., 2010).

There is significant potential for such improvements to be made through conventional breeding, as reflected by the variation in nutrient concentration traits previously observed (Ding et al., 2010; Bus et al., 2014; Koprivova et al., 2014; Thomas et al., 2016). For instance, in the study of Koprivova et al. (2014), leaf nitrate and phosphate concentrations varied 83- and 5-fold respectively across a population of 99 field-grown genotypes of *B. napus*. Approximately 2-fold variation was also observed in both leaf P and K concentration traits in a larger population of *B. napus* grown in compost (Thomas et al., 2016). However, efforts to breed for increased nutrient use efficiency could also benefit from the identification of genes controlling nutrient accumulation, which would allow a more targeted approach to crop improvement. In *Arabidopsis thaliana*, the genetic control of NO_3^-, P and K uptake is relatively well understood. Two gene families known as *NRT1* and *NRT2* are largely thought to co-ordinate NO_3^- supply (Tsay et al., 2007), four major phosphate transporter (*PHT*) gene families have been described, known as *PHT1*, *PHT2*, *PHT3*, and *PHT4* (Raghothama and Karthikeyan, 2005), and K uptake is thought to be largely co-ordinated by two high-affinity transporters, HIGH-AFFINITY K^+ TRANSPORTER 5 (*HAK5*) and ARABIDOPSIS K^+ TRANSPORTER 1 (*AKT1*; Hirsch et al., 1998; Pyo et al., 2010). Despite being closely related to *A. thaliana*, an understanding of the genetic basis of NO_3^-, P and K accumulation in *B. napus* specifically is limited (Bouchet et al., 2016). Whilst work to identify genetic loci controlling anion homeostasis (Koprivova et al., 2014) and shoot mineral concentrations (Bus et al., 2014) in *B. napus* has been performed previously, further work is required to pinpoint

specific genes and pathways that have the largest influence on such traits.

A genome-wide association study (GWAS) performed with leaf anion concentration data from 84 genotypes in the study of Koprivova et al. (2014) led to the discovery of a number of significantly associated markers, indicating a genetic component of leaf anion concentration traits. An updated, diversity population of 383 genotypes of *B. napus* has since been compiled and RNA-sequenced (Thomas et al., 2016; Havlickova et al., 2017). This forms an excellent foundation for Associative Transcriptomics (Harper et al., 2012), a technique that focuses on the analysis of transcribed sequences across diversity populations to identify high-resolution loci influencing complex traits. The use of RNA sequence data allows the development of markers based on both single-nucleotide polymorphisms (SNPs) and transcript abundance (gene-expression markers; GEMs). Gene expression levels may be particularly important in the control of traits in polyploid species such as *B. napus* in which gene duplication may have led to unequal expression (Adams et al., 2003). This technique has recently been used to dissect the genetic control of leaf Ca and Mg accumulation in *B. napus* (Alcock et al., 2017). Multiple genetic loci were highly associated with leaf Ca and Mg concentration, and numerous closely linked genes were identified as suitable candidates for their control. *Arabidopsis thaliana* plants mutated in orthologs of candidate genes were shown to have perturbed nutrient concentration traits, thus providing further evidence for their role in trait control and indicating the efficacy of this approach.

In this study, variation in leaf NO_3^- concentration across a diversity population of 383 genotypes of *B. napus* was characterized by ion chromatography. This is likely to reflect most of the species-wide variation in this trait. Ion chromatography presented a high-throughput method suitable for rapid processing of the large number of samples associated with using a diversity population. As the major source of N to crops grown in aerobic soils, NO_3^- is also likely to be a suitable measure of N status in *B. napus*. Leaf NO_3^- data generated here, along with leaf P and K concentration data previously described in the same population (Thomas et al., 2016), was then utilized in Associative Transcriptomics analyses to identify single nucleotide polymorphisms (SNPs) and gene expression markers (GEMs) associated with these traits, as well as allelic variants which differ in NO_3^-, P and K accumulation, and genes in linkage disequilibrium (LD) likely to be responsible for their control.

MATERIALS AND METHODS

Growth of Plant Material

This study makes use of the Renewable Industrial Products from Rapeseed (RIPR) diversity population of inbred lines of *Brassica napus* genotypes (Thomas et al., 2016). A subset of 383 genotypes were selected, comprising 169 winter-, 123 spring-, and 11 semiwinter-oilseed rape (OSR), 27 swede, six fodder, three kale and 44 of unspecified growth types. All plant growth took place at the Sutton Bonington Campus of the University of Nottingham (52°49′58.9″N, 1°14′59.2″W) in compost in a designed experiment in a polytunnel as described previously (Thomas et al., 2016). Briefly, seeds were all sown into a fine-grade, medium nutrient compost (N144, P73, K239 ppm added; pH 5.3-6; Levington Seed & Modular + Sand -F2S; Everris Ltd., Ipswich, United Kingdom) in propagation trays and allowed to establish in a glasshouse with venting set to 15°C and supplementary lighting used to maintain day lengths of 12 h, for approximately 12 weeks. Plants were then transferred to individual 5 L pots containing a coarse, medium nutrient compost (N200, P150, K200 ppm added; pH 5.3-5.7; Levington C2; Scotts Professional, Ipswich, United Kingdom) and placed into single skinned polytunnels with no supplementary lighting or heating. Compost nutrient concentrations were deemed sufficient for plant growth prior to leaf sampling. Pots were arranged in a randomized block design of five replicate blocks, each split into 12 sub-blocks with 36 pots randomly allocated within. Sixteen reference genotypes were included within each replicate block to allow more accurate normalization. Pots were watered by automatic irrigation three times daily.

Characterization of Leaf Nitrate, Phosphorus and Potassium Concentrations

Leaves were sampled from all plants at the rosette stage (typically 6–8 true leaves showing) approximately 20 weeks after sowing as described previously (Thomas et al., 2016). This was after a period of vernalisation of all plants, but prior to stem extension in preparation for flowering, which enabled as close synchronization of growth stages as reasonably possible between genotypes of different growth habits (winter, spring OSR, etc.). Typically three leaves were taken from each plant and freeze dried for 48–60 h (CHRIST Alpha 2–4 LD freeze dryer; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). For each plant, all sampled leaves were pooled and homogenized in liquid N_2 using a pestle and mortar and stored at −80°C prior to analyses. Since plants typically only had 6–8 true leaves at sampling stage, and sampling took place prior to stem extension in preparation for flowering, this is likely to reflect total shoot nutrient status. Leaf NO_3^- concentration was measured using an ion chromatography (IC) system, following minor variations to the method described in Herschbach et al. (2000). For each dried, homogenized sample, 20 mg was weighed and transferred to a 1.5 mL Eppendorf tube (Eppendorf AG, Hamburg, Germany), to which 1.5 mL of Milli-Q water (18.2 MΩ cm; Fisher Scientific UK Ltd, Loughborough, United Kingdom) containing 20 mg of insoluble polyvinylpolypyrrolidone (PVPP) was added. This was incubated at 4°C for 60 min with frequent mixing, then at 90°C for 15 min in a water bath, prior to centrifugation at 5,000 RPM for 15 min at 4°C in an Eppendorf 5180 centrifuge (Eppendorf). The resulting clear supernatant was transferred into a 0.5 mL anion sample tube (PolyVials; Thermo Fisher Scientific). This was then analyzed by anion exchange chromatography using a Dionex™ ICS-1100 Ion Chromatography System, using IonPac AS14 analytical columns (Thermo Scientific, Waltham, MA, United States). Flow rate was 1.4 mL min^{-1} and temperature was set to 30°C. For each sample, injection volume was 50 μL. Eluents used were Na_2CO_3 (Fisher) and $NaHCO_3$ (Fisher) at 2.7 mM and 1.8 mM, respectively, prepared in 2 L deionised water. Standards

used were sodium fluoride, sodium chloride, potassium nitrate, potassium di-hydrogen orthophosphate, potassium sulfate, and di-sodium DL-malate prepared to 1,000 ppm then diluted to between 10 and 150 ppm across six standard samples. The instrument was primed for 20 min prior to analysis. Leaf P and K concentrations in all genotypes were previously characterized by inductively coupled plasma-mass spectrometry (ICP-MS; Thomas et al., 2016). Briefly, a further 200 mg of dry, homogenous sample was digested in 2 mL 70% v/v Trace Analysis Grade HNO_3, 1 mL Milli-Q water, and 1 mL H_2O_2 in a microwave system (Multiwave 3,000; Anton Paar GmbH, Graz, Austria). Leaf digestates were diluted 1-in-5 using Milli-Q water, and then analyzed by ICP-MS with operational modes including (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).

The relative contribution of genotypic and non-genotypic variance components underlying variation in leaf NO_3^-, P and K concentration was calculated using a REML procedure in GenStat (17th Edition, VSN International Ltd, Hemel Hempstead, United Kingdom). For leaf NO_3^- concentration, genotype and experimental sources of variation were classed as random factors according to the model [IC_analysis_run_number + (genotype*replicate)]. For leaf P and K concentration traits, a further model was used [replicate + (replicate/sub-block) + genotype + (replicate/genotype)]. In the former model (for leaf NO_3^- concentration), sub-block aliased with IC_analysis_run_number due to the order of sample analysis, and hence was largely accounted for without being specifically included in the model. Similarly, polytunnel was taken into account by replicate in each of the two models, since replicates were organized by distinct blocks within the two polytunnels used. For each analysis, genotype was subsequently added as a fixed factor to estimate normalized genotype-means. Standard deviations between genotypes within each of six defined crop habits (spring-, winter- and semiwinter-OSR, fodder, kale, and swede) as well as pairwise, Pearson correlation coefficients between leaf NO_3^-, P and K concentration data and two-sided tests of correlations against zero were calculated in GenStat (17th Edition).

Leaf nitrate, phosphorus and potassium normalized genotype-means utilized in this study are included in **Supplementary Table 1** and at the Brassica Information Portal (BIP; https://bip.earlham.ac.uk; The Earlham Institute, Norwich, United Kingdom). Leaf nitrate concentration data as well as previously analyzed leaf mineral concentration data including phosphorus and potassium data used here are also available at DOIs 10.5281/zenodo.1321978 and 10.5281/zenodo.59937 respectively.

Transcriptome Sequencing, SNP Identification and Quantification of Transcript Abundance

Plant growth conditions, sampling techniques, RNA extraction, quality checking and Illumina transcriptome sequencing were described previously (He et al., 2017). For each genotype, RNA-sequence data was mapped onto ordered *Brassica* A and C genome-based pan-transcriptomes developed by He et al. (2015) using methods described by Bancroft et al. (2011) and Higgins et al. (2012). SNP positions with a read depth below 10, base call quality below Q20, or missing data above 25% among all genotypes, were excluded from alignment, as were SNP positions for which >3 alleles were called. Across the 383 genotype panel, 46,307 single SNPs and 309,229 hemi-SNPs were detected and scored. Transcript abundance was quantified and normalized as reads per kb per million aligned reads (RPKM) for each genotype. Of the 116,098 coding DNA sequence (CDS) models in the pan-transcriptome, significant expression (mean >0.4 RPKM) was detected for 53,889. Transcriptome sequences are deposited within the Sequence Read Archive (Leinonen et al., 2011) under accession number PRJNA309367. Clustering of genotypes based on SNP data was previously carried out, and a dendogram visualization can be found in Havlickova et al. (2017).

Associative Transcriptomics

Association analyses using SNPs and GEMs were carried out in R 3.2.0 (R Core Team, 2015) as previously described by Harper et al. (2012) with the modifications recently described by Havlickova et al. (2017). Inference of population structure by Q-matrix was obtained by Population Structure Inference using Kernel-PCA and Optimization (PSIKO; Popescu et al., 2014; highest likelihood subpopulation $k = 2$). SNP-based analyses were performed using a compressed mixed linear model approach (Zhang et al., 2010) implemented in the GAPIT R package (Lipka et al., 2012). GEM-based analyses were performed using fixed-effect linear modeling in R with RPKM values and PSIKO-inferred Q-matrix data as the explanatory variables and trait score the response variable. Coefficients of determination (R^2), constants and significance values were output for each regression. Genomic control (Devlin and Roeder, 1999) was applied to the GEM analysis when the genomic inflation factor was observed to be >1 to correct for spurious associations. Manhattan plots reporting $-\log_{10}p$-values for each marker for each trait were generated using graph functions in R. SNPs with low second allele frequency (<0.01) were filtered from the dataset prior to plot generation. In total 256,397 SNPs and 53,889 GEMs were plotted. SNP markers for which genetic mapping to the appropriate genome could not be confirmed due to sequence similarity are plotted in gray, and appear in both *Brassica* A and C sub-genomes. False Discovery Rate (FDR; Benjamini and Hochberg, 1995) and Bonferroni (Dunn, 1961) corrections were used to set significance thresholds at $p < 0.05$. QQ plots for all association analyses are included as **Supplementary Figures 2–7**.

Candidate Gene Selection

Genome browsers comprising sequences of *B. rapa* (A genome, Chiifu-401-41; Wang et al., 2011) and *B. oleracea* (C genome, TO1000DH3; Parkin et al., 2014) at Ensembl Plants (Kersey et al., 2016) as well as ordered *Brassica* pan-transcriptome data (He et al., 2015) were used to determine genes that fell within visually-determined association peaks and were within estimated LD decay (∼1–2 cM on average; Ecke et al., 2010)

of SNPs associated with measured traits. All genes that fulfilled these requirements were considered in candidate gene selection. Predicted gene function, inferred using ortholog annotations from closely related *Arabidopsis thaliana* held by The Arabidopsis Information Resource (TAIR; Huala et al., 2001), gene expression data at The Bio-Analytic Resource for Plant Biology (Waese and Provart, 2017) and ionomic data at the Purdue Ionomics Information Management System (PIIMS; Baxter et al., 2007) was used to select for genes most likely contributing to the control of leaf NO_3^-, P and/or K concentration. For candidate genes derived from GEM analyses, only genes corresponding to GEMs directly associated with the measured traits were considered.

Lead-Marker Allelic Effects on Leaf Nitrate, Phosphorus and Potassium Concentration

For SNP-based association analyses, the allelic effects of lead-markers on leaf NO_3^- and P concentration traits were determined. Lead-markers were defined as the most highly associated SNP markers within visually-determined association peaks for a given trait that had a second allele frequency above 0.01 and had been correctly mapped to the appropriate *B. napus* sub-genome. For each lead-marker, leaf NO_3^- or P concentration data were plotted separately for each allele at the SNP locus. Box plots representing allelic effects were created in SigmaPlot (13th Edition, Systat Software Inc., San Jose, CA, United States). For leaf K concentration, scatter plots representing marker expression effects on the trait were plotted for all markers with $-\log_{10}p$-values greater than the FDR corrected significance threshold ($p = 0.05$) as well as markers corresponding to described candidate genes (**Table 1**). Scatter plots and coinciding linear regression lines were generated in SigmaPlot. r^2-values and respective significance values were calculated in GenStat.

RESULTS

Oilseed Rape Varieties Have the Highest Mean Leaf Concentrations of Nitrate, Phosphorus and Potassium

Mean leaf NO_3^- concentration varied 8.34-fold between genotypes, ranging from 368 to 3070 mg kg^{-1} dry weight (DW; **Figure 1**). When compared between crop types, the highest average leaf NO_3^- concentrations were observed in oilseed rape (OSR) varieties, particularly semiwinter and spring OSR types. Ranked highest to lowest in terms of leaf NO_3^- concentration, the crop-types fall in the order of semiwinter OSR, spring OSR, winter OSR, fodder, swede, and kale, with average leaf NO_3^- concentrations of 1,775, 1654, 1,129, 1,090, 890, and 637 mg kg^{-1} DW, respectively. Standard deviations within these groups were 255, 556, 363, 282, 377, and 182, respectively. Large amounts of variation were observed within both winter and spring OSR types; 6.34- and 4.92-fold variation, respectively (**Figure 1**). Leaf P and K concentration data were reported previously (Thomas et al., 2016). Briefly, leaf P and K concentrations varied 1.98- and 2.07-fold from 3,290 to 6,526 mg kg^{-1} DW and 26,228 to 54,329 mg kg^{-1} DW, respectively, between all 383 genotypes. Mean leaf P concentration was highest in spring OSR types, followed by fodder, semiwinter OSR, winter OSR, kale, and finally swede types. Mean leaf K concentration was highest in semiwinter OSR, followed by spring OSR, winter OSR, swede, fodder, and finally kale types. Pearson correlation coefficient between leaf NO_3^- and K was 0.45; between leaf NO_3^- and P was 0.32; and between leaf P and leaf K was 0.25 (all $p < 0.001$). The range of concentrations of each nutrient followed approximately a normal distribution across the diversity population (**Supplementary Figure 1**).

Genes Controlling Root Suberin Synthesis and Nitrate and Ammonium Transport Are Within LD Decay of Markers Associated With Leaf Nitrate Concentration

A total of 455 and 10 SNP markers were significantly associated with leaf NO_3^- concentration after FDR and Bonferroni correction respectively ($p = 0.05$). This was reduced to 415 and 9 SNP markers, respectively, after filtering out markers with low second allele frequency (SAF; < 0.01). The majority of associated markers fell within visually-determined association peaks. The two most defined association peaks are located on chromosomes A1 and A3 and co-localize with one and four of the 10 SNPs above the Bonferroni corrected significance threshold, respectively ($p = 0.05$). Further, notable association peaks are located on chromosomes A4, A9, A10, C1, C3 C4, C5, C8, and C9 (**Figure 2A**). There were no GEMs that fell above FDR or Bonferroni corrected significance thresholds. However, less distinct association peaks were visually-determined on chromosomes A2, C2, and C9 (**Figure 2B**). By searching genetic loci within linkage disequilibrium (LD) decay (∼1–2 cM on average; Ecke et al., 2010) of SNP-based association peaks associated with leaf NO_3^- concentration, 15 candidate genes were identified (**Table 1**). These were orthologous to nine unique *Arabidopsis thaliana* genes, with multiple, paralogous copies of some candidate genes within LD decay of associated markers. Within LD of the association peak on chromosome A1 lies Cab024292.1, an ortholog of *A. thaliana* AT4G24020.1. This gene encodes NIN (nodule inception)-LIKE PROTEIN 7 (NLP7), a putative transcription factor thought to be responsible for sensing and responding to exogenous nitrate concentrations (Castaings et al., 2009). A further ortholog of *NLP7* was found within the syntenous region of chromosome C1 (Bo1g037430.1). Within the association peak on chromosome A3 lies Cab002472.4, an ortholog of *A. thaliana* AT5G10140.4. This encodes FLOWERING LOCUS C (*FLC*), a transcription factor responsible for repressing flowering prior to sufficient cold treatment (Sheldon et al., 2000). The most highly associated GEM on chromosome A3 also corresponds to Cab002472.4. Further candidate genes include seven members of the NITRATE TRANSPORTER 2 (*NRT2*) gene family (paralogous copies of *NRT2.1*, Cerezo et al., 2001; *NRT2.3*, Orsel et al., 2002; *NRT2.4*, Kiba et al., 2012), three genes encoding ABC transporters thought to be required for suberin synthesis in roots (*ABCG2*; and paralogous copies of *ABCG20*; Yadav et al., 2014), a gene encoding a high-affinity ammonium transporter (*AMT2*; Sohlenkamp et al., 2002), and a chloride channel (*CLCa*) which

TABLE 1 | Candidate genes within estimated linkage disequilibrium decay of lead-markers associated with leaf nitrate, phosphorus, and/or potassium concentration.

Trait	Candidate gene	*Arabidopsis ortholog*	*E*-value	Putative function	Linkage group	Lead-marker ID
Leaf NO_3^- concentration	Cab024292.1	AT4G24020	0.0	NIN-LIKE PROTEIN 7; involved in regulation of nitrate assimilation	A1	Cab024343.4:2028:T
Leaf NO_3^- concentration	Cab002472.4	AT5G10140	5×10^{-33}	FLOWERING LOCUS C; MADS-box transcription factor family protein	A3	Cab003105.1:423:C
Leaf NO_3^- concentration	Cab035003.1	AT2G37360	7×10^{-312}	ATP-BINDING CASSETTE G2; required for root suberin synthesis	A4	Cab034977.1:588:G
Leaf NO_3^- concentration	Cab034940.1	AT2G38290	2×10^{-186}	AMMONIUM TRANSPORTER 2	A4	Cab034977.1:588:G
Leaf NO_3^- concentration	Cab000535.1	AT3G53510	5×10^{-167}	ATP-BINDING CASSETTE G20; required for root suberin synthesis	A9.1	Cab000410.2:940:G
Leaf NO_3^- concentration	Cab014282.1	AT1G08090	5×10^{-252}	NITRATE TRANSPORTER 2.1	A9.2	Cab014338.1:34:T
Leaf NO_3^- concentration	Cab014283.1	AT1G08090	3×10^{-291}	NITRATE TRANSPORTER 2.1	A9.2	Cab014338.1:34:T
Leaf NO_3^- concentration	Cab017152.1	AT5G60780	3×10^{-270}	NITRATE TRANSPORTER 2.3	A10	BnaA10g13370D:657:T
Leaf NO_3^- concentration	Cab017154.1	AT5G60770	3×10^{-286}	NITRATE TRANSPORTER 2.4	A10	BnaA10g13370D:657:T
Leaf NO_3^- concentration	Bo1g037430.1	AT4G24020	0.0	NIN-LIKE PROTEIN 7; involved in regulation of nitrate assimilation	C1	Bo1g036990.1:1395:C
Leaf NO_3^- concentration	Bo4g145580.1	AT5G40890	0.0	CHLORIDE CHANNEL A; involved in nitrate accumulation in vacuoles	C4	Bo4g143900.1
Leaf NO_3^- concentration	Bo8g082890.1	AT3G53510	0.0	ATP-BINDING CASSETTE G20; required for root suberin synthesis	C8	Bo8g082080.1:192:A
Leaf NO_3^- concentration	Bo9g147020.1	AT5G60780	2×10^{-173}	NITRATE TRANSPORTER 2.3	C9	Bo9g143680.1:1029:T
Leaf NO_3^- concentration	Bo9g146990.1	AT5G60770	8×10^{-164}	NITRATE TRANSPORTER 2.4	C9	Bo9g143680.1:1029:T
Leaf NO_3^- concentration	Bo9g147000.1	AT5G60770	0.0	NITRATE TRANSPORTER 2.4	C9	Bo9g143680.1:1029:T
Leaf P concentration	Cab013031.3	AT4G16480	2×10^{-297}	INOSITOL TRANSPORTER 4	A1	Cab013058.2:573:G
Leaf P concentration	Cab012953.1	AT4G17230	7×10^{-252}	SCARECROW-LIKE 13; required for the regulation of hypocotyl elongation	A1	Cab013058.2:573:G
Leaf P concentration	Cab023424.2	AT3G08500	1×10^{-137}	MYB83; transcription factor required for activation of lignin synthesis	A5	Cab023469.1:384:C
Leaf P concentration	Cab022857.1	AT3G54700	8×10^{-262}	PHOSPHATE TRANSPORTER 1.7	A6	Cab022858.1:2706:G
Leaf P concentration	Cab022780.1	AT1G50420	1×10^{-131}	SCARECROW-LIKE 3; involved in control of gibberellin-mediated plant growth	A6	Cab022858.1:2706:G
Leaf P concentration	Cab022771.1	AT1G50600	1×10^{-265}	SCARECROW-LIKE 5; transcription factor likely to be involved in plant growth	A6	Cab022858.1:2706:G
Leaf P concentration	Bo2g020730.1	AT5G20240	6×10^{-116}	PISTILLATA; MADS-box transcription factor involved in floral development	C2	Bo2g017990.1:1221:G
Leaf P concentration	Bo2g018220.1	AT5G19600	0.0	SULFATE TRANSPORTER 3.5	C2	Bo2g017990.1:1221:G
Leaf P concentration	Bo4g182590.1	AT2G34440	1×10^{-64}	AGAMOUS-LIKE 29; transcription factor likely involved in floral development	C4	Bo4g182560.1:642:G
Leaf P concentration	Bo4g182170.1	AT2G33770	2×10^{-125}	PHOSPHATE 2 / UBC24; involved in phosphate starvation response	C4	Bo4g182560.1:642:G
Leaf P concentration	Bo4g185840.1	AT2G38940	4×10^{-116}	PHOSPHATE TRANSPORTER 1.4	C4	Bo4g182560.1:642:G
Leaf P concentration	Bo4g184590.1	AT2G35740	6×10^{-137}	INOSITOL TRANSPORTER 3	C4	Bo4g182560.1:642:G
Leaf P concentration	Bo8g108330.1	AT1G12240	0.0	BFRUCT4; plays a role in mobilizing sucrose to sink organs and in root elongation	C8	Bo8g108620.1:687:A
Leaf P concentration	Bo8g108240.1	AT1G12360	2×10^{-54}	KEULE; regulates cytokinesis related vesicle trafficking and root hair formation	C8	Bo8g108620.1:687:A
Leaf P concentration	Bo8g108070.1	AT1G12560	1×10^{-111}	EXPANSIN A7; ex pressed in root hair cells and involved in elongation	C8	Bo8g108620.1:687:A
Leaf P concentration	Bo8g107860.1	AT1G12950	2×10^{-96}	ROOT HAIR SPECIFIC 2; positively mediates root hair elongation	C8	Bo8g108620.1:687:A
Leaf P concentration	Bo9g166650.1	AT5G13580	0.0	ATP-BINDING CASSETTE G6; required for root suberin synthesis	C9.1	Bo9g166760.1:437:A
Leaf P concentration	Bo9g166210.1	AT5G14040	4×10^{-143}	PHOSPHATE TRANSPORTER 3.1	C9.1	Bo9g166760.1:437:A
Leaf P concentration	Bo9g173370.1	AT5G10140	9×10^{-73}	FLOWERING LOCUS C; MADS-box transcription factor family protein	C9.2	Bo9g173750.1:963:A

(Continued)

TABLE 1 | Continued

Trait	Candidate gene	*Arabidopsis ortholog*	*E*-value	Putative function	Linkage group	Lead-marker ID
Leaf K concentration	Cab024257.1	AT4G23640	0.0	POTASSIUM TRANSPORTER 3; also required for tip growth of root hairs	A1	GEM_Cab024257.1
Leaf K concentration	Cab001225.1	AT2G04850	2×10^{-200}	Au × in-responsive family protein	A3	GEM_Cab001225.1
Leaf K concentration	Cab002494.2	AT2G30320	3×10^{-207}	Pseudouridine synthase family protein	A3	GEM_Cab002494.2
Leaf K concentration	Cab037725.3	AT1G05940	9×10^{-237}	CATIONIC AMINO ACID TRANSPORTER 9	A9	GEM_Cab037664.1
Leaf K concentration	Cab007712.1	AT5G10740	2×10^{-151}	Protein phosphatase 2C family protein	A10	GEM_Cab007712.1
Leaf K concentration	Bo9g003730.1	AT4G01080	5×10^{-50}	TRICHOME BIREFRINGENCE-LIKE 26	C9	GEM_Bo9g003730.1
Leaf K concentration	Bo9g002280.1	AT4G00360	0.0	CYTOCHROME P450, FAMILY 86, SUBFAMILY A, POLYPEPTIDE 2	C9	GEM_Bo9g002280.1
Leaf K concentration	Bo9g001030.1	AT1G05940	6×10^{-143}	CATIONIC AMINO ACID TRANSPORTER 9	C9	GEM_Bo9g002280.1
Leaf K concentration	Bo9g171810.1	AT5G10740	3×10^{-47}	Protein phosphatase 2C family protein	C9	GEM_Bo9g171810.1

Putative functions obtained from annotation data at The Arabidopsis Information Resource (Huala et al., 2001).Sequences of both Brassica and A. thaliana genes available at Ensembl Plants (Kersey et al., 2016). Expect (E) value between coding DNA sequences of Brassica candidate genes and A. thaliana orthologs generated using BLAST functions at Ensembl Plants.

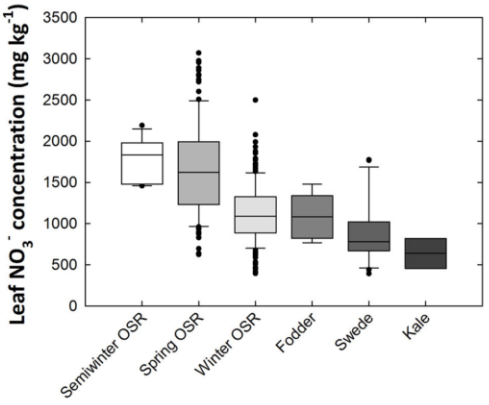


FIGURE 1 | Leaf nitrate concentrations of *Brassica napus* plants grown in compost. Data are means of 339 genotypes for which crop growth type had been assigned including 169 winter-, 11 semiwinter-, and 123 spring-oilseed rape (OSR), 27 swede, six fodder and three kale types. Boxes represent the mid two quartiles with the median drawn; whiskers above and below the boxes indicate the 90 and 10th percentiles with any outliers shown. Boxes organized by mean leaf nitrate concentration between crop growth types from highest (left) to lowest (right).

is thought to be responsible for NO_3^- accumulation in vacuoles (De Angeli et al., 2006; **Table 1**).

Differences in gene expression between oilseed crop-type groups were observed for five candidate genes. Specifically, elevated transcript abundance of Cab034940.1 (*AMT2*) and Bo1g037430.1 (*NLP7*) was observed in semiwinter compared to spring and winter OSR genotypes ($p < 0.01$) and lower transcript abundance of Bo4g145580.1 (*CLCa*) and both Cab017152.1 and Bo9g147020.1 (both homeologous copies of *NTR2.3*) was observed in winter compared to spring OSR genotypes ($p < 0.05$, 0.001, and 0.001, respectively). For lead-markers within selected association peaks, genotype-specific allelic variants in the RIPR diversity population were identified and leaf nitrate concentration between variant and reference alleles were compared. The allelic variant was defined as the second most frequent allele in the population after the reference base call. Lead-markers for each association peak investigated are summarized in **Table 1**. For lead-markers within association peaks on chromosomes A1, A3, A4, A9 (labeled A9.1 as two association peaks referred to in this chromosome in **Table 1**), A10, C1, C8, and C9, the allelic variant was associated with a 1.86-, 1.93-, 2.06-, 1.84-, 1.24-, 1.85-, 1.92-, and 1.73-fold increase in leaf NO_3^- concentration respectively (**Figure 3**; all p values < 0.001).

Genes Controlling Root-Hair Development Among Candidates for Leaf Phosphorus Concentration

There were no SNPs or GEMs that associated with leaf P concentration above the level of Bonferroni or FDR corrected significance thresholds. However, a number of SNP markers had notably higher $-\log_{10}p$ values than the majority of markers, and many of these formed visually-determinable association peaks (**Figure 2C**). The most distinct of these are present on chromosomes A1, A5, C8, and C9. Further, less prominent association peaks were detected on chromosomes A6, C2, and C4. A total of 19 candidate genes were identified within LD decay of associated markers at these loci (**Table 1**). Most notable candidate genes are four genes involved in root or root hair development on chromosome C8. These are orthologous to *A. thaliana* AT1G12240.1 (*BFRUCT4*), AT1G12360.1 (*KEULE*), AT1G12560.1 (*EXPANSIN A7*), and AT1G12950.1 (*ROOT*

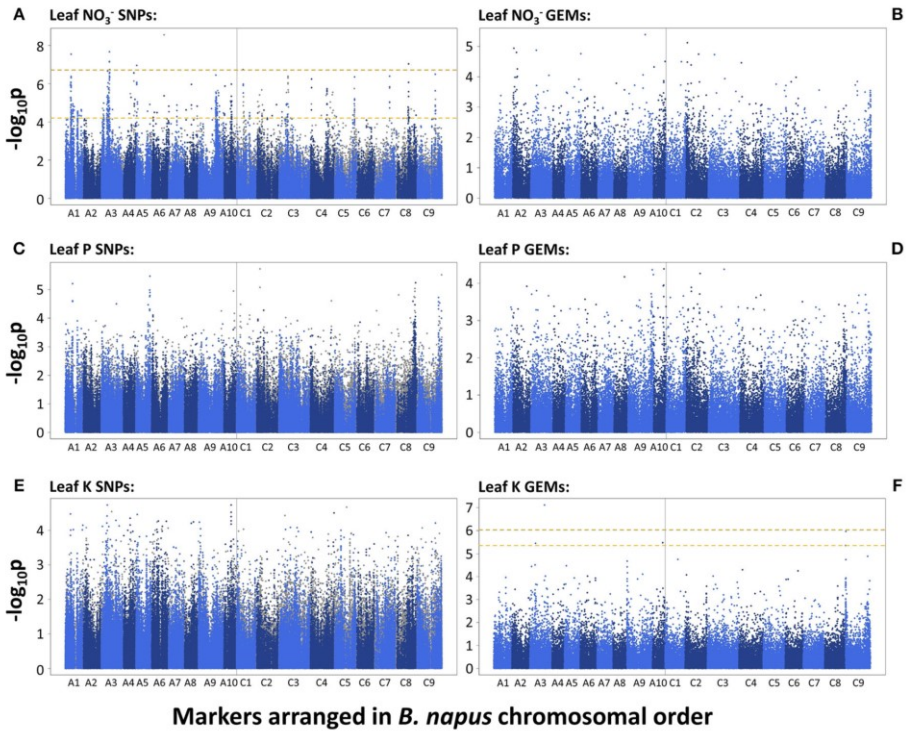


FIGURE 2 | $-\log_{10}p$-values of SNPs and GEMs associated with leaf nitrate concentration (**A**,**B**, respectively), leaf phosphorus concentration (**C**,**D**, respectively) and leaf potassium concentration (**E**,**F**, respectively) in order of markers within the *Brassica napus* pan-transcriptome. Upper, gold, dashed line represents Bonferroni corrected significance threshold; lower, yellow, dashed line represents FDR corrected significance threshold ($p = 0.05$).

HAIR SPECIFIC 2). An additional outstanding candidate is a Bo9g166650, an ortholog of *A. thaliana* AT5G13580.1. This encodes an ABC transporter of the same family as those within LD of markers associated with leaf NO_3^- concentration and this member is also thought to be required for root suberin synthesis. Further candidates include three genes encoding SCARECROW-LIKE proteins (3, 5, and 13) which are transcription factors involved in the control of developmental processes during the plant life cycle, as well as three genes encoding flowering time regulators, six transporter-protein encoding genes, a gene involved in response to low phosphate, and a transcription factor which may have a role in lignin synthesis (*MYB83*; Zhong and Ye, 2012; **Table 1**). It is also worthy of note that two of the GEMs most highly associated with leaf P concentration (chromosomes A10 and A3, ranked 1 and 11th, respectively; **Figure 2D**) correspond to distinct, orthologous copies of *A. thaliana* AT5G10140.4 (*FLC*). The latter of these is identical to the gene within LD of the association peak identified for leaf NO_3^- concentration described above. For all eight loci within which candidate genes were identified, box plots were generated to visualize the effect of allelic variant at lead-marker position on leaf P concentration (**Figure 4**). For lead-markers within association peaks on chromosomes A5, A6, C2, C4, and the former peak on C9 (C9.1), the allelic variant was associated with a 1.13-, 1.06-, 1.15-, 1.09-, and 1.14-fold increase in leaf P concentration compared to the reference allele, respectively ($p < 0.001$, $p = 0.010$, $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively). For lead-markers within association peaks on chromosomes A1, C8, and the latter peak on chromosome C9 (C9.2), the allelic variant was associated with a 1.09-, 1.18-, and 1.15-fold decrease in leaf P concentration compared to the reference allele (all $p < 0.001$).

The Most Highly Associated GEM For Leaf Potassium Concentration Corresponds to an Auxin Responsive Family Protein

No SNPs, but a total of 1 and 5 GEMs were associated with leaf K concentration above the level of Bonferroni and FDR corrected significance thresholds, respectively, after genomic control (**Figures 2E,F**). The GEM above both the significance thresholds was located on chromosome A3 and corresponds to

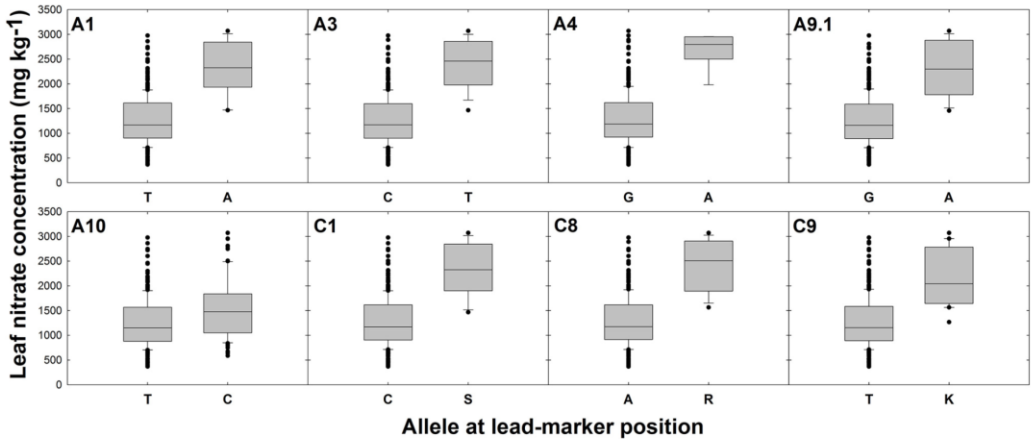


FIGURE 3 | Effects of reference allele and most frequent allelic variant at lead-marker loci on leaf nitrate concentration. Each panel refers to a specific association peak; lead-markers at each peak are listed in table 1. Boxes represent the mid two quartiles with the median drawn; whiskers above and below the boxes indicate the 90 and 10th percentiles with any outliers shown. A, T, G, and C alleles represent adenine, thymine, guanine and cytosine nucleotide calls, respectively. S, R, and K alleles represent Strong (C or G), purine (A or G) or Keto (C or T) nucleotide calls, respectively, thus called due to unresolvable variation in closely related genes. Differences in leaf nitrate concentration between alleles at each loci significant at $p < 0.001$.

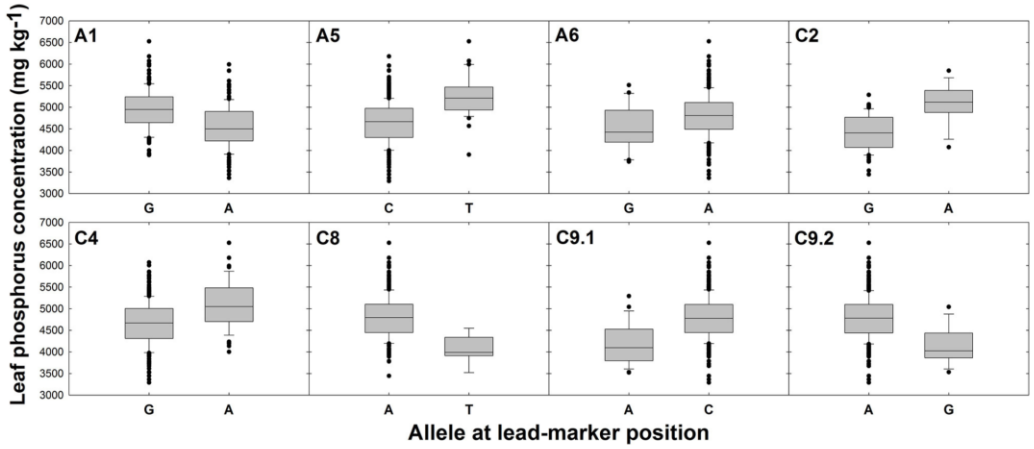


FIGURE 4 | Effects of reference allele and most frequent allelic variant at lead-marker loci on leaf phosphorus concentration. Each panel refers to a specific association peak; lead-markers at each peak are listed in table 1. Boxes represent the mid two quartiles with the median drawn; whiskers above and below the boxes indicate the 90 and 10th percentiles with any outliers shown. A, T, G, and C alleles represent adenine, thymine, guanine and cytosine nucleotide calls, respectively. Differences in leaf phosphorus concentration between alleles at each loci significant at $p < 0.001$ except locus on chromosome A6; $p = 0.010$.

Cab001225.1, an ortholog of *A. thaliana* AT2G04850.1 which encodes an auxin-responsive family protein. Of the remaining four GEMs over the FDR corrected significance threshold, a further marker falls within chromosome A3, one within chromosome A10, and two within chromosome C9 (**Figure 2F**). Unlike results from SNP-based analyses, for which any gene within LD decay of associated markers could be the cause of the variation observed in the trait, genes underlying GEMs are more likely to be directly involved in trait control. For this reason, genes under all five of the aforementioned GEMs were considered as likely candidate genes (**Table 1**). However, two visually-determined association peaks were located toward

the beginning of chromosomes A9 and C9, and hence genes within LD of these association peaks were also considered. Within LD decay of the more highly associated markers within these loci are the genes Cab037725.3 and Bo9g001030.1, both orthologous to *A. thaliana* AT1G05940.1, a gene that encodes a cationic amino acid transporter. Two further candidate genes with relatively high $-\log_{10}p$-values in the corresponding GEM were considered; Cab024257.1 and Bo9g171810.1. The former of these is orthologous to *A. thaliana* AT4G23640.1 which encodes a potassium transporter family protein. The latter is paralogous to the gene underlying the GEM over the FDR corrected significance threshold on chromosome A10. The effects of expression levels of the GEMs underlying each of these candidate genes is modeled in **Figure 5**. For GEMs underlying genes Cab024257.1, Cab001225.1, Cab002494.2 and Cab037664.1, an increase in expression was associated with an overall increase in genotype-specific mean leaf K concentration ($r^2 = 0.059$, 0.098, 0.075, and 0.068 respectively, all $p < 0.001$). For GEMs underlying genes Cab007712.1, Bo9g003730.1, Bo9g002280.1 and Bo9g171810.1, an increase in expression was associated with an overall decrease in genotype-specific mean leaf K concentration ($r^2 = 0.067$, 0.075, 0.059 and 0.064 respectively, all $p < 0.001$; **Figure 5**).

DISCUSSION

Species-Wide Variation in Leaf Nitrate Concentration Indicates Scope for Breeding for Increased N Uptake and use Efficiency via Traditional Means

Leaf NO_3^- concentration varied over 8-fold among the 383 genotypes in the RIPR diversity population, indicating large amounts of species-wide variation. The variation observed here is lower than the 83-fold variation shown previously in a population of 84 similar genotypes of *B. napus* grown under field conditions (Koprivova et al., 2014). After converting FW measurements to DW for comparison, assuming a 90% leaf water content, genotypes in the study of Koprivova et al. (2014) had estimated minimum and maximum leaf NO_3^- concentrations of 192 and 15,935 mg kg^{-1} DW, respectively. Hence, values from the present study occupy similar orders of magnitude to those measured previously. The lower variance between genotypes observed here is likely linked to the age of the plants, which were sampled after approximately 4 months of growth. In the earlier study of Koprivova et al. (2014), they were sampled after only 8 weeks. It could also be linked the growth conditions, which in the present study comprised compost-filled pots under controlled irrigation placed in polytunnels, unlike the field conditions used in the earlier study. Despite these differences, it is clear that large amounts of species-wide variation in shoot NO_3^- concentration exist in *B. napus*, irrespective of growth condition or plant age. This knowledge could be applied in traditional breeding strategies for increasing N uptake and use efficiency, perhaps under low exogenous NO_3^- conditions. It is worth bearing in mind that a low leaf NO_3^- concentration may be indicative of a high N use efficiency. There are multiple definitions of N use efficiency including yield per unit of N input, amount of N in plant per unit of N input, and shoot weight per unit of shoot N (Good et al., 2004). If a crop has relatively low shoot concentrations of NO_3^- but still yields highly, it may possess traits that allow it to prosper under lower exogenous NO_3^- supply. Hence it is essential that genotype-specific yield be considered as well as leaf NO_3^- concentration in selecting suitable varieties for breeding. It is also important to consider the limitations of sampling plants grown under only a single exogenous N supply at a single developmental time point, as performed in this study, in the assessment of variation in N use between genotypes. It is possible that further variation could be uncovered if plants were grown and sampled under N-limiting conditions. This could lead to the identification of genotypes suitable for breeding novel crop cultivars that are more vigorous in low N conditions than current, widely cultivated germplasm. It may also lead to the identification of genetic loci controlling leaf NO_3^- accumulation under low exogenous supply, which may be particularly useful in more targeted breeding strategies.

Identification of Genes Encoding Nitrate and Ammonium Transporters Within LD of Leaf Nitrate Association Peaks Likely Linked to Altered Expression Between Genotypes in the Diversity Population

A total of eight genes encoding putative NO_3^- transporters and one gene encoding a putative ammonium (NH_4^+) transporter were identified within LD decay of markers highly associated with leaf NO_3^- concentration. Seven of the eight genes encoding NO_3^- transporters are members of the NITRATE TRANSPORTER 2 (*NRT2*) family and fall within three distinct loci on chromosomes A9 (two orthologs of *Arabidopsis thaliana NRT2.1*), A10 (orthologs of *A. thaliana NRT2.3* and *NRT2.4*) and C9 (further orthologs of *A. thaliana NRT2.3* and *NRT2.4*; **Table 1**). The latter two loci are in regions of known shared sequence homology (Chalhoub et al., 2014), indicating that copies of these genes on both *B. napus* sub-genomes are likely to be important for leaf NO_3^- accumulation. Each of these genes encode high-affinity NO_3^- transporters, transcript abundances of which are high under low exogenous NO_3^- concentrations (Orsel et al., 2002). *NRT2.3* forms a highly interesting candidate, since transcript abundance of both paralogous copies discussed above is lower in winter compared to spring OSR genotypes. This may contribute to the typically lower leaf NO_3^- concentrations observed in winter compared to spring OSR genotypes. Tissue specific gene expression analysis of *A. thaliana NRT2.3* revealed expression in both root and shoot tissue throughout developmental stages (Orsel et al., 2002). Intriguingly, whilst *A. thaliana NRT2.3* shows a nitrate inducible expression pattern in shoots, root gene expression appears to be constitutive (Okamoto et al., 2003). However, to our knowledge, specific, functional characterization of *NRT2.3* is yet to be performed, and so the importance of this is currently unclear. The eighth putative nitrate transporter gene encodes CHLORIDE CHANNEL-A (*CLCA*). Not only was this gene found to mediate nitrate accumulation in plant vacuoles in

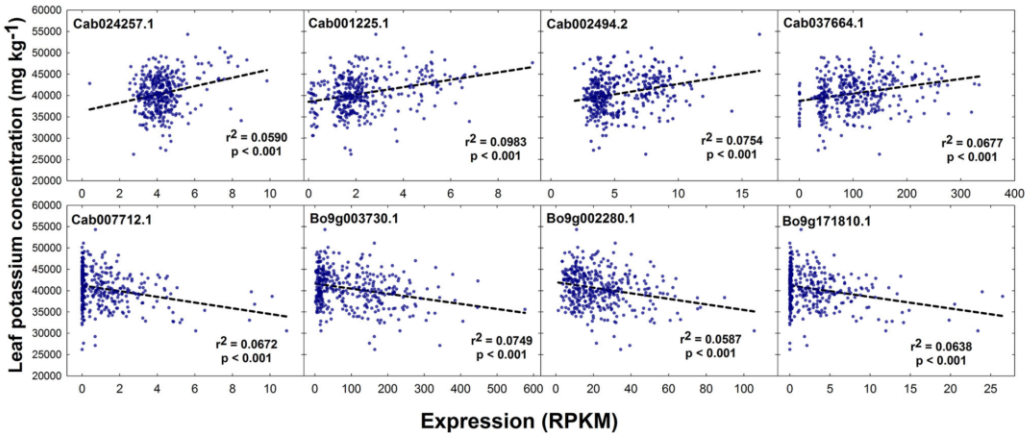


FIGURE 5 | Effect of leaf transcript abundance of genes underlying candidate GEMs on leaf potassium concentration. Transcript abundance was quantified and normalized as reads per kb per million aligned reads (RPKM) for each genotype. r^2 values and respective significance values calculated in GenStat are shown.

A. thaliana (De Angeli et al., 2006), but it was also identified as a contributor to the control of leaf NO_3^- homeostasis in a previous association analysis in *B. napus* (Koprivova et al., 2014). Thus, this is a very convincing candidate gene for controlling leaf NO_3^- concentration. The gene encoding an ammonium transporter, specifically AMMONIUM TRANSPORTER 2 (*AM2*; **Table 1**), was identified on chromosome A4, close to a SNP at which locus allelic variation confers 2.1-fold variation in leaf NO_3^- concentration (**Figure 3**). The encoded protein is thought to be localized to the plasma membrane, and experiments in *A. thaliana* indicate that it is a high-affinity transporter, expression of which is repressed by higher exogenous ammonium nitrate concentrations (Sohlenkamp et al., 2002). Whilst it seems clear that NH_4^+, and not NO_3^- is the substrate for this protein, it is likely that changes in NH_4^+ uptake efficiency will impact on leaf NO_3^- concentration and hence this association is perhaps unsurprising.

A further, highly interesting candidate for leaf NO_3^- concentration is NIN (nodule inception)-LIKE PROTEIN 7 (*NLP7*). Allelic variation in the nearby lead-marker within the strong association peak on chromosome A1 confers 1.9-fold variation in leaf NO_3^- concentration (**Figure 3**). *NLP7* has recently been shown to modulate expression of the major NO_3^- sensor and transporter encoding gene *NRT1.1* (Zhao et al., 2018). As a NO_3^- sensor, NRT1.1 appears to enable detection of NO_3^--rich soil patches, leading to subsequent proliferation of roots in these areas (Bouguyon et al., 2016). This protein can also act as a dual-affinity transporter, depending on the phosphorylation status of a threonine residue within the protein, and is active at a range of exogenous NO_3^- concentrations (Parker and Newstead, 2014). The association of SNP markers within LD decay of *NLP7* provides further evidence of its downstream role in NO_3^- uptake in *B. napus*. This gene provides an excellent candidate for further study, allelic variants in which may provide a suitable route toward improvement of NO_3^- uptake and N fertilizer use efficiency in *B. napus*.

The association of SNP markers close to the genes described here is indicative of variation in expression of these genes across the diversity population, which may subsequently impact on NO_3^- uptake and leaf accumulation. Whilst the corresponding GEMs were not associated with the trait, it is important to state that the GEMs used in this study are based on transcript abundance in young leaves. Hence, genotype-specific differences in the expression of genes in roots or at different growth stages are likely to be masked. Similarly, variation in the expression of genes that are specifically expressed in root tissue, and not expressed in leaves, will not be reflected here. Thus, further work will be required to confirm differences in expression of such genes between genotypes in the RIPR diversity population.

Orthologs of Three ABC Transporters Required for Root Suberin Synthesis Likely Contribute to Nitrate and Phosphorus Homeostasis

Three and one gene(s) encoding putative ABC transporters were identified close to SNPs highly associated with leaf NO_3^- and P concentration respectively (**Table 1**). These are orthologous to the three unique *A. thaliana* ABC-2 transporters G2 (AT2G37360), G6 (AT5G13580) and G20 (AT3G53510). All of these genes are expressed in roots and thought to be required for the transport of aliphatic polymer precursors of suberin or related structural components of the Casparian strip (Yadav et al., 2014). These genes also appear to be required for synthesis of an effective suberin barrier in seed coats, since *A. thaliana* plants mutated in these genes had increased seed permeability to tetrazolium red dye (Yadav et al., 2014). Suberin is considered to be a major component of the Casparian strip in roots which forms

a chemical barrier restricting extracellular transport of water and dissolved nutrients to the vascular tissue (Baxter et al., 2009). This enables greater control of solute transport between plant tissues. *Arabidopsis thaliana* triple mutants in the aforementioned ABC transporter encoding genes were previously characterized and root systems were found to be more permeable to water as well as the solutes sodium chloride (NaCl) and potassium nitrate (KNO_3; Yadav et al., 2014). The authors also deduced that whilst the mutant plants were able to make suberin, its structure was altered compared to wild-type plants. That all three of these genes were identified close to SNPs highly associated with leaf NO_3^- and P concentration in this study provides further evidence for the role of suberin in the control of nutrient uptake in *B. napus*.

Flowering Time Is an Important Marker for Leaf Nitrate and Phosphorus Concentration

Within LD decay of highly associated markers for leaf NO_3^- and P concentration, were a number of genes thought to control flower development. These include orthologs of *A. thaliana* FLOWERING LOCUS C (*FLC*), a flowering time regulator, which were detected close to association peaks for leaf NO_3^- and P concentration on chromosomes A3 and C9, respectively, as well as orthologs of PISTILLATA (*PI*), and AGAMOUS-LIKE 29 (*AGL29*) close to association peaks for leaf P concentration on chromosomes C2 and C4, respectively (**Table 1**). Allelic variation at the lead-marker close to *FLC* on chromosome A3 is associated with 1.9-fold variation in mean leaf NO_3^- concentration (**Figure 3**). Allelic variation close to the *FLC* ortholog on chromosome C9 is also associated with 1.2-fold variation in leaf P concentration (**Figure 4**). Interestingly, orthologs of *FLC* and other flowering time regulating genes were previously found within LD decay of association peaks for leaf Ca and Mg concentration (Alcock et al., 2017). Expression of *FLC* directly influences flowering time by suppressing activation of flowering until after sufficient cold treatment (vernalisation; Sheldon et al., 2000). It has previously been demonstrated that winter cultivars of *B. napus*, which have higher vernalization requirements, have greater *FLC* transcript abundance than spring cultivars, which have lower vernalization requirements (Tadege et al., 2001). In the present study, leaf NO_3^- concentration was found to vary between spring and winter crop types. Similarly, leaf P concentration data used for association analyses here was previously shown to vary between winter and spring crop types (Thomas et al., 2016). It is possible that the associations with flowering time regulator genes observed here and in previous studies are linked to spurious correlations between leaf nutrient concentration and flowering time between different crop types. However, it was previously shown that leaf N concentrations decline with leaf expansion during crop development (Liu et al., 2018). Hence, it is also possible that expression levels of *FLC* and other flowering time related genes could influence leaf nutrient concentrations indirectly by changing the duration of vegetative growth. Whilst further work will be required to elucidate the mechanisms controlling this association, the tendency of flowering time genes to associate with multiple nutrient traits indicates that they are suitable markers for leaf mineral concentration traits in *B. napus*.

Root Hair Development Genes Are Particularly Important for Leaf Phosphorus Accumulation

No SNPs or GEMs were significantly associated with leaf P concentration above the FDR corrected significance threshold in this study. This indicates that unlike leaf NO_3^- concentration, leaf P concentration is under relatively low genetic control, or at the very least, that there are low levels of genetic variation that associate with variation in the trait. This is surprising, as variance components analysis in this population previously showed that 20% of the variation in leaf P concentration was associated with genotype, indicating a moderate genetic component (Thomas et al., 2016). However, this result is consistent with a previous association study in a similar population of *B. napus*, in which although heritability of shoot P concentration was estimated at 0.43, no significant marker associations were detected for the trait (Bus et al., 2014). It is possible that the markers used in both of these studies did not fully reflect genetic variation controlling shoot P accumulation. Despite this, a number of notable association peaks were detected in the Manhattan plot generated from SNP-based association data (**Figure 2C**). Allelic variation in lead-marker positions within these loci were also associated with variation in leaf P concentration (**Figure 4**), hence indicating that genes within LD of these markers are likely to contribute to control of the trait.

The three phosphate transporter encoding genes PHOSPHATE TRANSPORTER (*PHT*) 1.4, 1.7 and 3.1 were located close to highly associated SNP markers on chromosomes C4, A6, and C9, respectively. The former two genes appear to encode high-affinity transporters for external phosphate uptake (Shin et al., 2004; *PHT1.7* function inferred by sequence similarity), and thus their proximity to markers associated with leaf P concentration is unsurprising. The latter transporter is expressed in mitochondria, and appears to modulate plant salt stress responses (Zhu et al., 2012). Hence, whilst it remains an interesting candidate to consider, it is less likely than the former transporters to be the causative gene within its respective association peak. A further candidate which may have a direct influence on phosphate uptake is PHOSPHATE 2, otherwise known as UBIQUITIN-CONJUGATING ENZYME 24 (Huang et al., 2013). This gene has been shown to be involved in the response to exogenous phosphate concentration through degradation of PHOSPHATE TRANSPORTER 1 (PHO1) under phosphate sufficiency (Liu et al., 2012). PHO1 is crucial for phosphate loading to the xylem and subsequent transport to the shoot (Hamburger et al., 2002). Hence, alterations in expression or protein structure of PHOSPHATE 2 may provide a suitable avenue for improving P-fertilizer use efficiency, through altering the plant's response to exogenous phosphate supplies.

A number of candidate genes that may contribute indirectly to the accumulation of phosphorus were also identified, including genes within LD of SNPs highly associated with leaf P concentration on chromosome C8 that play a role in root

system architecture. The genes, *EXPANSIN A7,* and *ROOT HAIR SPECIFIC 2* are both crucial for root hair elongation; *A. thaliana* mutants in these genes have shorter root hairs (Won et al., 2009; Lin et al., 2011). A further gene identified in close proximity, known as *KEULE*, plays a role in cytokinesis through proper vesicle tethering, which among other things is required for polar growth of root hairs (Wu et al., 2013). Root hairs have previously been linked to phosphorus acquisition, which increase in length and number under low exogenous supply or deficiency (Lynch, 2011; Wang et al., 2018). It has also recently been shown that the expression of auxin-inducible transcription factors is increased in root hairs and that auxin accumulates in root hair zones under low exogenous P conditions, hence promoting root hair elongation (Bhosale et al., 2018). It is clear that root hair traits can influence P accumulation by plants, and there is genetic variation in this trait that could be exploited to improve P acquisition and P fertilizer use efficiency (Wang et al., 2018).

Markers used for association analyses in this study were developed from leaf mRNA-sequence data. Whilst SNPs called from this data form suitable markers for identifying genetic differences between genotypes across all tissue types and developmental stages, GEMs based on leaf transcript abundance are only suitable for assessing genetic variation within young leaf tissue. Many of the candidate genes for leaf P concentration are predominantly involved in regulating root development and root specific transport. It is possible that stronger trait/marker associations would have been identified if more root-specific markers, such as root transcript abundance, had been utilized. Whilst significantly further work is required in order to generate sequence data required for calling such markers, this may be a suitable next step that could lead to the identification of many more molecular markers for nutrient acquisition in *B. napus* in the future.

Allelic-Variant Analysis Indicates Scope for Marker-Assisted Selection for Improved Nitrate and Phosphorus Use Efficiency

Investigation into the effects of allelic variation at lead-marker positions for leaf NO_3^- concentration indicated that huge differences in trait values between genotypes can be detected whilst only considering few, individual SNPs. For instance, a SNP in the lead-marker within an association peak on chromosome A4 was shown to confer over 2-fold mean variation in leaf NO_3^- concentration between genotypes (**Figure 3**). Significant differences in leaf P concentration between allelic variants were also observed. However, the observed effects were of a lower magnitude than those observed for leaf NO_3^- concentration. For instance, the greatest fold change between allelic variants was only 1.15, for lead-markers on chromosomes C2 and C9 (**Figure 4**). It is thus likely that there is still significant variation in the genetic basis of leaf P accumulation to be uncovered. This seems particularly convincing considering huge differences in P use efficiency previously identified within a diverse population of *B. oleracea* genotypes grown under varying exogenous P supply (Hammond et al., 2009) As discussed above, such variation could be realized by looking into root gene expression, as this data was not reflected by the leaf mRNA-sequence-based GEMs used in analyses here. It may also be worth quantifying P concentrations in specific cellular compartments. Under P sufficient conditions, up to 95% of intracellular P can be found in the vacuole (Yang et al., 2017). Whilst measuring vacuole-specific P concentrations across all 383 genotypes in the RIPR diversity population represents a difficult task, this could be a better measure of P status between genotypes, enabling identification of variation more closely associated with P use efficiency. Despite this, the identification molecular markers that associate with significant variation in leaf NO_3^- and P concentration here may prove useful in marker-assisted selection strategies to improve nutrient accumulation or use efficiency in *B. napus*. This could accelerate the development of suitable crop varieties for a changing world, which is likely to demand greater yield from crop plants whilst reducing fertilizer inputs.

Shoot Gene Expression Is Likely to Play a Role in the Control of Potassium Homeostasis

As with leaf phosphorus concentration, no SNP markers were significantly associated with leaf K concentration in the RIPR diversity population. Similarly, no associations were identified for shoot K concentration in an association study in a different population of 509 *B. napus* genotypes (Bus et al., 2014). Thus, it is possible that leaf K concentration is under lower genetic control than leaf NO_3^- concentration. However, there were several GEMs that had $-\log_{10}p$-values over the FDR corrected significant threshold. The GEM most highly associated with leaf K concentration is orthologous to *A. thaliana* At2g04850. Whilst relatively little is known about this gene, data from the SMART web-based tool (Schultz et al., 1998) indicates that it is membrane bound, and that it contains a Cytochrome b-561 / ferric reductase transmembrane domain. This gene is also thought to be auxin-responsive. Auxin is a plant hormone that is responsible for orchestrating multiple growth and development processes (Swarup and Bennett, 2003). Hence, it is possible that expression of this gene is related to development stage, which may correlate with leaf K concentration. Whilst the link between this gene and leaf K concentration is currently unclear, this was by far the most highly associated GEM, and hence is worthy of further consideration. A further GEM of interest underlies a gene orthologous to *A. thaliana*, *POTASSIUM TRANSPORTER 3* (*KT3*), also known as *TINY ROOT HAIR 1* (Daras et al., 2015). Whilst not associated with leaf K concentration above the FDR corrected significance threshold, the GEM corresponding to this gene is relatively highly associated with the trait, and shoot gene expression is positively correlated (**Figure 5**). Experiments in *A. thaliana* showed that KT3 knockout lines, which have significantly smaller root hairs, have a reduced rate of K transport, although not enough for the plants to develop deficiency symptoms (Rigas et al., 2001). The presence and length of root hairs have also been correlated with increased K acquisition among plant species (White, 2013; Hinsinger et al., 2017). Thus, genetic variation in this trait might be exploited to improve K acquisition and K fertilizer use efficiency. It is

perhaps surprising that further known K^+ transporters were not identified by association analyses performed here. However, this may be a product of using leaf mRNA-sequence data to develop GEMs. As discussed above with respect to leaf P concentration candidates, such markers do not reflect root gene expression differences between genotypes. Within LD decay of co-localizing GEM association peaks on chromosomes A9 and C9 are also genes orthologous to *A. thaliana* AT1G05940.1, a gene that encodes a cationic amino acid transporter. This may play a role in K acquisition within *B. napus*. However, association peaks in the same loci were detected for seed glucosinolate content (Lu et al., 2014). Over approximately the last 50 years, intensive breeding programmes have been undertaken to reduce seed glucosinolate concentrations in *B. napus* (Allender and King, 2010). Due to such strong selection pressures, it is possible that a number of other traits were perturbed in the process, and hence the association peaks on chromosomes A9 and C9 detected here may be a result of unconscious co-selection.

It is clear from SNP and GEM association analyses that transcript abundance forms a better marker for elucidating genes controlling leaf K concentration than sequence polymorphisms between genotypes. This also consistent with results from a previous study in *B. napus* in which no SNPs were associated with leaf K concentration (Bus et al., 2014) and in spinach in which only two SNPs associated with leaf K concentration, whereas a greater number of associations were observed for other minerals (Qin et al., 2017).To our knowledge, this is the first time that leaf transcript abundance has been used as a molecular marker to identify loci controlling leaf K accumulation. However, correlations between transcript abundance of candidate genes and leaf K concentration were relatively low ($r^2 < 0.1$; **Figure 5**). Whilst the use of such genes in marker assisted selection strategies may be possible, the identification of genes that have a greater influence over trait control is desirable. It is possible that examining the effects of root gene expression across the population would lead to the discovery of further such genes. Root traits have previously been correlated with plant K acquisition (White, 2013; Hinsinger et al., 2017), and genotype-specific gene expression within this tissue is likely to reflect variation in K uptake and consequently leaf K concentration. However, root gene expression data across a diversity population of *B. napus* is not available at this time. Despite the limitations of using shoot gene expression data for the determination of genes controlling leaf K concentration, a number of convincing candidate genes have been identified, expression of which could be modified to alter K accumulation and use efficiency.

CONCLUSIONS AND PERSPECTIVE

This study has identified large amounts of variation in leaf NO_3^- concentrations across 383 genotypes of *B. napus* grown in controlled conditions. This likely reflects the majority of species-wide heterogeneity in the trait and demonstrates the scope for breeding for increased NO_3^- use efficiency through traditional techniques. This study has also determined genetic loci and allelic variants associated with leaf NO_3^-, P and K concentrations in *B. napus*. Several convincing candidate genes that may affect these traits either directly or indirectly have also been identified. These include genes which relate to suberin synthesis and root hair development, both of which are likely to be amenable traits for improving uptake and use efficiency of multiple nutrients (White et al., 2013). Characterizing variation in traits related to suberin synthesis and root hair development across genotypes of the RIPR diversity population would likely add to the understanding of their function and how they can be applied in crop breeding strategies. Whilst numerous candidate genes for leaf accumulation of NO_3^-, P and K have been discussed here, each of these were largely identified using functional data available for *Arabidopsis thaliana* orthologous genes. It is likely that there are many other genes associated with the traits measured here that are yet to be identified, including genes within association peaks in which no candidate genes were identified. Some of these could represent novel targets for the control of nutrient uptake and distribution in *B. napus*, functions of which are yet to be revealed. Further investigation into Associative Transcriptomics data produced here may help to elucidate these in the near future. Allelic variants associated with variation in leaf NO_3^-, P and K concentration identified here also provide an excellent resource for marker-assisted selection. Together, these results make significant progress in the understanding of nutrient homeostasis in *B. napus*, which may prove useful in the generation of more nutrient and fertilizer use efficient crop varieties.

DATA AVAILABILITY

Transcriptome sequences used in Associative Transcriptomics analyses are deposited within the Sequence Read Archive (Leinonen et al., 2011) under accession number PRJNA309367. Raw, Associative Transcriptomics outputs supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MB, IB, PW, TA, and NG conceived the project and contributed to experimental design. TA analyzed leaf nitrate and Associative Transcriptomics data. LH and ZH prepared functional genotypes and performed Associative Transcriptomics. LW prepared leaf samples and carried out ion chromatography analyses. TA and NG wrote the manuscript. All authors contributed to and have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01487/full#supplementary-material

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Chapter 4. Identification of Candidate Genes for Calcium and Magnesium Accumulation in Brassica napus L. by Association Genetics

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Identification of Candidate Genes for Calcium and Magnesium Accumulation in *Brassica napus* L. by Association Genetics

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Calcium (Ca) and magnesium (Mg) are essential plant nutrients and vital for human and animal nutrition. Biofortification of crops has previously been suggested to alleviate widespread human Ca and Mg deficiencies. In this study, new candidate genes influencing the leaf accumulation of Ca and Mg were identified in young *Brassica napus* plants using associative transcriptomics of ionomics datasets. A total of 247 and 166 SNP markers were associated with leaf Ca and Mg concentration, respectively, after false discovery rate correction and removal of SNPs with low second allele frequency. Gene expression markers at similar positions were also associated with leaf Ca and Mg concentration, including loci on chromosomes A10 and C2, within which lie previously identified transporter genes *ACA8* and *MGT7*. Further candidate genes were selected from seven loci and the mineral composition of whole *Arabidopsis thaliana* shoots were characterized from lines mutated in orthologous genes. Four and two mutant lines had reduced shoot Ca and Mg concentration, respectively, compared to wild type plants. Three of these mutations were found to have tissue specific effects; notably reduced silique Ca in all three such mutant lines. This knowledge could be applied in targeted breeding, with the possibility of increasing Ca and Mg in plant tissue for improving human and livestock nutrition.

Keywords: associative transcriptomics, GWAS, *Brassica napus*, calcium, magnesium, biofortification, nutrient use efficiency

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INTRODUCTION

Calcium (Ca) and magnesium (Mg) are essential plant nutrients and vital for human and animal nutrition (Broadley and White, 2010; White and Brown, 2010). In plants, most Ca is extracellular, where it is a key strengthening component in cell walls (Grusak et al., 2016). It also has an important role in plant-cell signaling. Calcium enters root cells through a variety of Ca^{2+}-permeable cation channels (White and Broadley, 2003; Karley and White, 2009; White, 2015). The opening of these channels must be tightly controlled, as changes in cytosolic Ca^{2+} concentrations coordinate numerous developmental and environmental stress responses (McAinsh and Pittman, 2009). The accumulation of Ca at tissue- and cellular-levels is dependent on the expression of transport

proteins (Conn and Gilliham, 2010; Rios et al., 2012). After uptake from soil by roots, Ca travels via either apoplastic or symplastic pathways to the xylem, through which, in the form of either Ca^{2+} or complexed with organic acids, it is transported to the shoot. Calcium is immobile in the phloem, and as such, tissues with low transpiration rates (including fruits, seeds, and tubers) often have low Ca concentrations (Karley and White, 2009). Among plant nutrients, Ca is required in relatively large amounts. However, concentrations vary amongst taxa, typically ranging from ∼0.1 to 4.4% dry matter (Broadley et al., 2003). Calcium deficiencies are relatively rare in field-grown crops, but can occur in crops grown in acidic or leaching prone soils. Where Ca supply is insufficient to meet growth requirements, costly symptoms can ensue. For instance, fruits lacking in Ca are prone to cracking, as a direct result of weakness in the cell wall (White, 2015).

Magnesium is essential for photosynthesis, forming the central atom of chlorophyll molecules. It also has a key role in protein synthesis by functioning as a bridging element for the aggregation of ribosome subunits, as well as in photophosphorylation and generation of reactive oxygen species in plants (Cakmak and Yazici, 2010). Magnesium is taken up by roots as Mg^{2+}. Control of influx across the plasma membrane is dominated by members of the MGT/MRS2 family of transport proteins and potentially Mg^{2+}-permeable cation channels (Karley and White, 2009; Lenz et al., 2013). One member of the MGT gene family in *Arabidopsis thaliana*, *MAGNESIUM TRANSPORTER 1* (*MGT1*), encodes a protein localized to the plasma membrane (Li et al., 2001), suggesting its importance in the import and/or export of Mg in cells. Like Ca, Mg is transported from root to shoot cells through the xylem either as Mg^{2+} or complexed with organic acids. However, Mg is a phloem-mobile element, and is readily translocated to fruit, seeds, and tubers (White and Broadley, 2009). Shoot Mg concentrations are typically lower than shoot Ca concentrations across plant taxa, and vary between ∼0.1% to ∼1.0% dry matter (White et al., 2015).

In humans and animals, Ca is associated with the formation and metabolism of bone as well as being crucial for mediating vascular contraction and vasodilation, muscle function, nerve transmission, intracellular signaling, and hormonal secretion (Catharine, 2011). Based on food supply data, it was estimated that half of the population worldwide was at risk of Ca deficiency in 2011, with significant deficiency risks across all continents (Kumssa et al., 2015b). Magnesium is needed for over 300 biochemical reactions. It helps to maintain muscle function, prevents an irregular heartbeat, and is involved in protein synthesis (Yardley, 2009). Based on food supply data, <1% of the global population appeared to be at risk of dietary Mg deficiency in 2011 (Kumssa et al., 2015a). However, these data do not account for inhibitors of Mg adsorption, household waste, or distribution within countries and it is likely that significant deficiency risks exist within some populations. Magnesium deficiency risks are also likely to be greater in higher-income groups consuming processed foods, because Mg is among the nutrients commonly lost in processing (Swaminathan, 2003; Broadley and White, 2010; Kumssa et al., 2015a). Biofortification of crops has been previously suggested as a suitable approach for alleviating human deficiencies in a number of mineral nutrients, including Ca and Mg (White and Broadley, 2005; White and Broadley, 2009).

Previous analyses of variation in mineral concentrations across a wide range of plant species have shown that tissue Ca and Mg concentrations are inherently high in Brassicaceae compared most other taxa (Broadley et al., 2004; White et al., 2015). These traits have proven to be heritable in *Brassica oleracea* (Broadley et al., 2008), *B. rapa* (Graham et al., 2014), and *B. napus* (Thomas et al., 2016). Thus, *Brassica* spp. are potentially good targets for understanding genetic bases of leaf Ca and Mg accumulation, and for potentially increasing dietary intakes of Ca and Mg in humans and animals. Expression quantitative trait locus (eQTL) analyses in *B. rapa* previously led to the discovery of Ca responsive genes which may prove useful in marker-assisted selection for increased Ca concentration in shoot tissue (Graham et al., 2014). These include orthologs of *A. thaliana* Ca^{2+} transporter genes *CATION EXCHANGER 1* (*CAX1*) and *AUTOINHIBITED* CA^{2+} *ATPASE, ISOFORM 8* (*ACA8*), and subsequent work showed that allelic variants of the former gene in *B. rapa* influenced Ca accumulation. *B. napus* includes oilseed types, swedes and fodder crops, and is widely cultivated globally. It is an amphidiploid species that likely originated from multiple spontaneous hybridizations between *B. rapa* (A genome; turnip rape) and *B. oleracea* (C genome; cabbage, kale) and contains a full set of chromosomes from each (Iniguez-Luy and Federico, 2011; Chalhoub et al., 2014). This complexity has previously hindered the genetic study of this and other polyploid crops. However, recent and ongoing advances in sequencing and genome mapping technologies have allowed the rapid genotyping of multiple accessions at a fraction of the cost of older technologies. This has improved the feasibility of using a large diversity population over traditional mapping populations in genetic studies of crop species (Trick et al., 2009).

Associative transcriptomics (Harper et al., 2012) focuses on the analysis of transcribed sequences (mRNA-seq) across diversity populations to identify high-resolution loci influencing complex traits. An advantage using of RNA over DNA sequences in association studies is the ability to develop markers based on both single-nucleotide polymorphisms (SNPs) and transcript abundance (gene-expression markers; GEMs; Harper et al., 2012). Gene expression levels may be particularly important in the control of traits in polyploid species in which gene duplication may have led to unequal expression (Adams et al., 2003). Associative transcriptomics has been recently used in *B. napus* to identify genes underlying control of seed glucosinolate content (Harper et al., 2012; Lu et al., 2014) and anion homeostasis (Koprivova et al., 2014). The former two studies utilized panels of 84 and 101 genotypes, respectively. Despite the relatively small population sizes, a number of loci associated with seed glucosinolate concentrations were identified. Most notable associations include loci containing orthologs of *A. thaliana HIGH ALIPHATIC GLUCOSINOLATE 1* and *3* (*HAG1* and *HAG3*), known to regulate aliphatic glucosinolate biosynthesis (Sønderby et al., 2010). Koprivova et al. (2014), also made use of the panel of 84 genotypes and identified a number of loci associated with leaf nitrate, phosphate, and sulfate. Within

these loci were a number of clear candidate genes, including a calcium-activated chloride channel previously shown to control nitrate levels in *A. thaliana* (De Angeli et al., 2006) which was associated with leaf nitrate concentration and a hypothetical phosphate/phosphoenolpyruvate translocator associated with leaf phosphate concentration.

Leaf Ca and Mg concentrations were previously characterized in a diversity population of ∼400 genotypes of *B. napus* in a broad-spectrum mineral analysis (Thomas et al., 2016). This population is likely to capture most of the species-wide variation, comprising oilseed, swede and fodder types. In this study, we perform associative analyses on this data using transcriptome sequences from 383 genotypes to identify genes influencing Ca and Mg accumulation. Candidate genes could be applied in marker assisted breeding in this and other *Brassica* crops, with the possibility of improving nutrient use efficiency of the crop and increasing available nutrients in edible plant tissue for improving human and livestock nutrition.

MATERIALS AND METHODS

Characterization of Leaf Ca and Mg Concentration

This study used the Renewable Industrial Products from Rapeseed (RIPR) diversity population of inbred lines of *Brassica napus* genotypes (Thomas et al., 2016). These were developed from the ERANET-ASSYST consortium diversity population (Bus et al., 2011, 2014; Körber et al., 2012, 2015) with further lines included. A subset of 383 genotypes were selected, comprising 160 winter-, 127 spring-, and seven semi-winter-oilseed rape (OSR), 35 swede, 15 winter fodder, and 39 exotic/unspecified habits. These were previously characterized for leaf mineral concentrations by inductively coupled plasma-mass spectrometry (ICP-MS) of polytunnel-grown plants sampled at the rosette stage (typically 6–8 true leaves showing; Thomas et al., 2016). The full leaf mineral dataset is available at the Brassica Information Portal (BIP[1]; The Earlham Institute, Norwich, United Kingdom) and at doi: 10.5281/zenodo.59937.

Associative Analyses

Transcriptome Sequencing and Population Structure Analysis

Extraction of RNA, quality checking and Illumina transcriptome sequencing were carried out as described by He et al. (2016). Tissue samples for RNA extraction were prepared from second true leaves, harvested when they reached ∼3 cm in diameter. RNA-seq data from each accession was mapped using methods described by Bancroft et al. (2011) and Higgins et al. (2012) onto ordered *Brassica* A and C genome-based pan-transcriptomes developed by He et al. (2015). Transcriptome sequencing was performed by the Earlham Institute (formerly The Genome Analysis Centre; Norwich, United Kingdom). Across the 383 accession panel, 46,307 single SNPs and 309,229 hemi-SNPs were detected and scored of which 256,397 SNPs had a population second allele frequency (saf) > 0.01. Transcript abundance was quantified and normalized as reads per kb per million aligned reads (RPKM) for each accession for 116,098 coding DNA sequence (CDS) models of the pan-transcriptome reference. Significant expression (mean > 0.4 RPKM) was detected for 53,889 CDS models. Inference of population structure by Q-matrix was obtained by Population Structure Inference using Kernel-PCA and Optimization (PSIKO; Popescu et al., 2014). A heatmap illustrating the relatedness of all genotypes in this study can be found in Supplementary Figure 1. Transcriptome sequences are deposited within the Sequence Read Archive (Leinonen et al., 2011) under accession number PRJNA309367.

Associative Transcriptomics

Associative transcriptomics was performed using SNPs, Q-matrix and trait data in a compressed mixed linear model approach (Zhang et al., 2010) implemented in the GAPIT R package (Lipka et al., 2012) in R 3.2.0 (R Core Team, 2015). The association analysis between gene expression markers (GEMs) and traits was performed by using fixed effect linear modeling in R with RPKM values and Q-matrix data as the explanatory variables and trait score the response variable using custom designed scripts (Harper et al., 2012; Havlickova et al., in press). Coefficients of determination (R^2), constants and significance values were calculated for each regression. Manhattan plots were generated using graph functions in R. SNPs with low second allele frequency (<0.01) were filtered from the dataset prior to generating plots. In total 256,397 SNPs and 53,889 GEMs were plotted. False discovery rate (FDR; Benjamini and Hochberg, 1995) and Bonferroni (Dunn, 1961) corrections were used to set significance thresholds at $P < 0.05$. Due to sequence similarity between *B. napus* A and C genomes, assignment to a specific genome was not possible for all SNP markers; such markers are plotted in gray and appear in both positions on Manhattan plots. See Supplementary Figures 2–5 for quartile–quartile (QQ) plots of each association analysis.

Candidate Gene Identification

Ordered pan-transcriptome data based on *Brassica* A and C genomes from *B. rapa*, *B. napus*, and *B. oleracea* CDS gene models (He et al., 2015) were used to identify candidate genes. Candidate genes were selected based on *Arabidopsis thaliana* annotated functions of *Brassica* orthologs within estimated linkage disequilibrium (LD) decay of significantly associated markers (around 1–2 cM on average; Ecke et al., 2010). Further information relating to candidate gene predicted function was obtained from genome browsers comprising sequences of *B. rapa* (A genome, Chiifu-401-41; Wang et al., 2011) and *B. oleracea* (C genome, TO1000DH3; Parkin et al., 2014) at Ensembl Plants (Kersey et al., 2016). *A. thaliana* functional information were obtained from The Arabidopsis Information Resource (TAIR; Huala et al., 2001). Further resources used to aid with selection of candidates included *A. thaliana* gene expression data at The Bio-Analytic Resource for Plant Biology (Waese and Provart, 2017) and ionomic data at the Purdue Ionomics Information Management System (PIIMS; Baxter et al., 2007).

[1]https://bip.earlham.ac.uk/

Experiments Using *Arabidopsis thaliana* Mutants

Plant Material and Genotyping

Seed of 15 *Arabidopsis thaliana* mutant lines representing 10 candidate genes were acquired from the Nottingham Arabidopsis Stock Centre (Nottingham, United Kingdom). These comprised SALK (Alonso et al., 2003) and SAIL (Sessions et al., 2002) T-DNA lines and are summarized in **Table 1**. *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used as the wild type control in all experiments. Plants were by genotyped for homozygous T-DNA insertions by conventional PCR. Genotyping primers are summarized in **Table 1**. Left border primers used were SALK LBb1 and SAIL LB1 for SALK and SAIL lines, respectively.

Preliminary Phenotyping

Seeds from homozygous mutant lines and Col-0 were sterilized in bleach, then washed in H_2O and 70% ethanol prior to sowing on plates containing 1% agar containing 0.4 g L^{-1} Hoagland's solution (Hoagland and Arnon, 1950; ¼ strength). Plates were stored in darkness at 4°C for 24 h, and then moved to a controlled environment growth chamber set to 23°C (∼30 W m^{-2} continuous light). After 7 days, plants were transferred to pots containing Levington M3 compost (ICL Specialty Fertilizers, Ipswich, Suffolk, United Kingdom) plus T34 biocontrol (Fargro Ltd., Arundel, West Sussex, United Kingdom) and placed on flow benches in a glasshouse with 18°C heating, venting at 20°C, with 16-h supplementary lighting (76 W m^{-2}). Flow bench automatic irrigation operated once daily. After 10 days of establishment, six plants of each line were chosen randomly and transferred to individual wells in 16 well trays in a six block, using a one-way randomized design generated in GenStat (17th edition; VSN International, 2014) in which plants of each line were represented once per block and randomized within each block (Supplementary Table 1). In total, each line was represented six times. ARACON systems (Betatech BVBA, Gent, Belgium) were used to keep plants separate. At mid-flowering, whole shoots were harvested by cutting them below the rosette. Shoots were dried at 50°C for at least 2 days, and then crushed by hand within paper bags. Shoot subsamples (∼0.10 g DW) 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, United Kingdom), and 1 mL H_2O_2 with microwave settings as follows: power = 1,400 W, temp = 140°C, pressure = 2 MPa, time = 45 min. Two operational blanks and duplicate samples of certified reference material (CRM; Tomato SRM 1573a, NIST, Gaithersburg, MD, United States) 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 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 broad-spectrum elemental analysis by ICP-MS as described previously (Thomas et al., 2016). 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. The CRM Ca and Mg recovery averaged 99 and 89%, respectively.

Tissue Partitioning Experiment

Based on results from preliminary phenotyping, lines At2g13610.2, At5g07320.2, and At5g48650.2 were found to have significantly lower shoot Ca or Mg concentrations than wild type plants and hence were selected for further characterization. Individual seed from these lines and Col-0 were sown into 12 well trays containing Levington M3 compost plus T34 biocontrol

TABLE 1 | Summary of *Arabidopsis thaliana* T-DNA insertion lines acquired for characterisation including primers used for genotyping.

Line Name	SALK/SAIL code	NASC stock code	Forward primer	Reverse primer
At2g05120.1	SALK_119762	N619762	TTCTGGAGAAACAAGGTCCAA	ATGGCAGCAAGTTTTTCACC
At2g13610.1	SALK_074250	N574250	CGATTTGCCGAAAAGAAAAA	GTTTCCTCCACCGTAAGCAA
At2g13610.2	SALK_074250C	N681303	CGATTTGCCGAAAAGAAAAA	GTTTCCTCCACCGTAAGCAA
At2g45660.1	SALK_138131	N638131	GGTTCTTCCTTTCGCAGAGA	CCACAAAAGGCCAATCAAAT
At5g03960.1	SALK_138382	N638382	TGGTTGAGGAAGCAAGAAGG	TGTGCTCTGCCTCCTTTGTA
At5g06530.1	SALK_024391	N524391	TTCCCCAAAGGTATCGATTCTA	TCGAACAACTGGGATTGACA
At5g06530.2	SALK_076250	N576250	TTCCCCAAAGGTATCGATTCTA	CGGGCATTTGATAGCACTTT
At5g07320.1	SALK_037517	N537517	CGCTGCATATGAAACGCTAA	TCAATGATCGCAACAAAACAA
At5g07320.2	SALK_037517C	N683966	CGCTGCATATGAAACGCTAA	CCATAAAAATATATGTCCCAATTTCA
At5g08670.1	SALK_083107	N583107	CGATGTTCCCAACATTTGAA	AACAGAGACCGGCGAGACTA
At5g10520.1	SALK_019299	N519299	TATTTCATGCACGGCATTGT	GGGTTGGAAATGTGGAAGAA
At5g10520.2	SALK_053754	N553754	CCGTTTCGTCTTCTCACCAT	ACATGGTGAGGCCAGTTCTC
At5g14040.1	SALK_105845	N605845	CCCTTACTTTTCGGAGCATTC	TTGCACTTGACGAGATCGAG
At5g48650.1	SALK_027468	N527468	GCGGTAGCTGAGGGTACATC	CCACCATCAAGCCAAAGACT
At5g48650.2	SAIL_64_G12	N803057	GCCCAATAGGCAAACAAATG	AAGTCTGGGACCAACAATGG

NASC stock code shown for reordering.

and placed on flow benches in a glasshouse with 18°C heating, venting at 20°C, with 16-h supplementary lighting (76 W m^{-2}). Flow bench automatic-irrigation operated once daily. After successful establishment, 12 plants per genotype were selected randomly and transplanted into individual 9 cm pots. These were arranged in a 12 block, one-way randomized design generated in GenStat in which each genotype was represented once per block and genotypes randomized within each block (Supplementary Table 2). At mid-flowering (40 days after sowing), entire shoots were harvested. Shoots were partitioned into rosette leaves, stem, cauline leaves, and siliques. Tissue samples were dried at 50°C for 6 days, and then samples from plants in blocks 1–4, 5–8, and 9–12 were pooled into the four genotypes to ensure enough sample was available for mineral analysis. Pooled samples were crushed by hand, and then microwave digested prior to mineral analysis by ICP-MS as described above. Digestates were diluted 1-in-10 prior to mineral analysis. The recovery of Ca and Mg from the CRM averaged 96 and 88%, respectively.

Statistical Analyses

Data from experiments using *A. thaliana* mutants were analyzed using one-way ANOVA in GenStat (17th edition; VSN International, 2014) with block design included in the model. Tissue Ca and Mg concentration data were analyzed separately in each case, and tissue types were analyzed using separate ANOVA tests in the tissue partitioning experiment. For the preliminary phenotyping experiment, six replicate plants were analyzed for each genotype. For the tissue partitioning experiment, three samples, each comprising pooled samples from four replicate plants, were analyzed for each genotype. Means of different *A. thaliana* genotypes were compared using Least Significant Difference (LSD) functions in GenStat with differences considered significant at $P < 0.05$. Further LSD tests were conducted at $P < 0.01$ and $P < 0.001$ levels.

RESULTS

Variation in Leaf Ca and Mg Concentration in the RIPR Diversity Population

The leaf concentrations of 21 mineral elements including Ca and Mg in the RIPR diversity population were previously determined by Thomas et al. (2016). Leaf Ca concentrations varied over threefold across the population, from 5,838 mg kg^{-1} to 18,752 mg kg^{-1}. Leaf Mg concentrations were of a similar order of magnitude and varied over twofold, from 5,118 mg kg^{-1} to 13,429 mg kg^{-1}. The frequency distribution of these two elements approximated a normal distribution (Supplementary Figure 6). Leaf Ca and Mg concentrations were among the highest, positively correlated elements measured across genotypes and tissues, with an r-value of 0.87 ($P < 0.001$). Leaf Ca and Mg concentrations varied between crop type, with higher concentrations of both elements in leaves of spring and semi-winter OSR than in winter OSR, winter fodder, and swede types.

Associative Transcriptomics Suggest Flowering Time Regulators Are Important Markers for Leaf Ca and Mg Concentrations

To identify candidate loci, SNPs and GEMs were used separately in analyses. A total of 1,295 and eight SNPs were found to be significantly associated with *B. napus* leaf Ca concentration after FDR and Bonferroni corrections, respectively. After removing SNPs with low second allele frequency, this was reduced to 247 and five SNPs, respectively, across all chromosomes. Visually determined association peaks on Manhattan plots were observed on chromosomes A3, A6, A7, A10, C2, C3, and C9 (**Figure 1A**). The most well defined peak was located on chromosome A10 and contained four out of the five SNPs above the Bonferroni corrected significance threshold ($P = 0.05$). The fifth SNP above this threshold fell in a peak on chromosome C9, in a region known to share sequence homology with parts of chromosome A10 (Chalhoub et al., 2014). A total of 5,557 and 141 GEMs were identified as significantly associated with leaf Ca concentration after FDR and Bonferroni corrections, respectively (**Figure 1B**). Notable peaks were observed on chromosomes A2 and C2. Single, associated GEMs were found at similar locations to SNP association peaks on chromosomes A3 and C2. The *A. thaliana* ortholog of *B. napus* genes corresponding to both these GEMs is At5g10140, which encodes FLOWERING LOCUS C (FLC), a transcription factor important for controlling flowering time (Michaels and Amasino, 1999). A further associated GEM was found in a region of chromosome A10, close to a SNP peak associated with leaf Ca concentration. Other single GEMs associated with leaf Ca concentration were observed on chromosomes A5, A6, C4, and C6. *B. napus* genes corresponding to these GEMs on chromosomes A5 and C4 are orthologous to *A. thaliana* At2g45660, which encodes SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1), another flowering time regulator (Lee et al., 2000).

The SNP and GEM associations for leaf Mg concentration were similar to those described for leaf Ca concentration. A total of 1,012 and one significant SNP(s) were found after FDR and Bonferroni corrections, respectively. After removing SNPs with low second allele frequency, this was reduced to 166 and zero SNPs, respectively, across all chromosomes. Of these 166 SNPs, 86 were identical to SNPs identified as significantly associated with leaf Ca concentration after FDR correction and removal of SNPs with low second allele frequency, indicating the potential of similar mechanisms to partly regulate accumulation. Visually determined association peaks largely co-localized with those associated with leaf Ca concentration, specifically on chromosomes A3, A7, A10, C2, C3, and C9 (**Figure 1C**). The most well defined peak was again on chromosome A10, and as before, a region on chromosome C9 with sequence homology to this region also contained associated SNPs. An association peak on chromosome A2 was also particularly well defined, containing 15 SNPs above the FDR corrected significance threshold. A total of 12,973 and 1,489 GEMs were identified as significantly associated with leaf Mg concentration after FDR and Bonferroni corrections, respectively, across all chromosomes

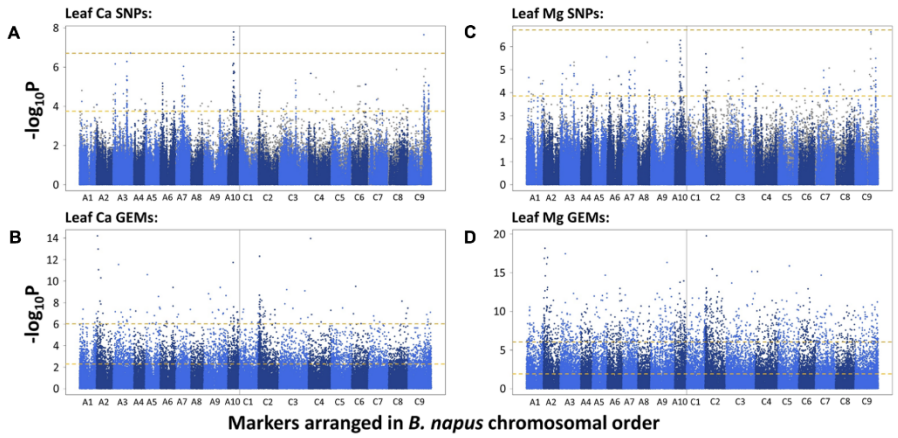


FIGURE 1 | $-\log_{10}$P values of SNPs and GEMs associated with leaf Ca concentration (**A,B**, respectively) and leaf Mg concentration (**C,D**, respectively) in order of markers within the *B. napus* pan-transcriptome. Upper, gold, dashed line represents Bonferroni corrected significance threshold; lower, yellow, dashed line represents FDR corrected significance threshold ($P = 0.05$).

(**Figure 1D**). Of these, 5,160 and 131 were also significantly associated with leaf Ca concentration after FDR and Bonferroni corrections, respectively. Notable peaks were again observed on chromosomes A2 and C2. The most highly associated GEMs on A3 and C2 were identical to those associated with leaf Ca concentration which correspond to *A. thaliana FLC*, and an associated GEM on C4 is identical to the GEM associated with leaf Ca concentration corresponding to *SOC1*.

Genes Encoding Previously Identified Ca and Mg Transporters Are within Linkage Disequilibrium of Highly Associated Markers for Leaf Ca and Mg Concentration

Linkage disequilibrium describes the non-random association of alleles at different loci (Slatkin, 2008). Genes located physically near to each are generally inherited together, and hence are often in very strong LD. It is therefore feasible that any number of genes within LD of SNPs significantly associated with a trait may be controlling such associations. Based on associative transcriptomics results, seven loci were focussed on for the identification of candidate genes. These comprised regions of chromosomes A2, A3, A5, A6, A10, C2, and C4. A total of 17 *B. napus* candidate genes orthologous to 15 *A. thaliana* genes are summarized in **Table 2**. Four candidate genes were selected based on direct GEM hits as described above. These are Cab002472.4 and BnaC02g00490D (on chromosomes A3 and C2, respectively) encoding orthologs of *A. thaliana* At5g10140 (*FLC*), and Cab025356.1 and Bo4g024850.1 (on chromosomes A5 and C4, respectively) encoding orthologs of *A. thaliana* At2g45660 (*SOC1*). One and two candidate genes on chromosomes A2 and C2, respectively are orthologous to *A. thaliana MAGNESIUM TRANSPORTER 7* (*MGT7/MRS2-7*). This was previously characterized in *Arabidopsis thaliana* as an Mg transporter important for Mg uptake at low external concentrations (Gebert et al., 2009). A further candidate gene on chromosome A10 is orthologous to *A. thaliana AUTOINHIBITED* CA^{2+}*-ATPASE, ISOFORM 8* (*ACA8*). This was previously characterized as a plasma membrane-localized Ca^{2+} transporting ATPase in *A. thaliana* (Bonza et al., 2000) and was identified as Ca responsive in *B. rapa* (Graham et al., 2014). The functions of the remaining nine candidate genes were selected based on sequence homology and annotations of *A. thaliana* orthologs and are either uncharacterized, or have not previously been experimentally shown to be involved in plant Ca or Mg accumulation (**Table 2**). These and At2g45660 (*SOC1*) were used for the selection of *A. thaliana* mutants.

Four Mutant *A. thaliana* Lines Have Reduced Shoot Ca and Mg Concentration Compared to Wild Type and Effects Are Tissue Specific

Shoot Ca concentrations in a preliminary *A. thaliana* phenotyping experiment varied threefold between individual plants, from 8,684 to 26,387 mg kg^{-1} dry weight (DW; Supplementary Table 3). Much of this variation was observed within genotypes, with the largest variation observed in lines At5g7320.1 and At5g08670.1. Four mutant lines had significantly lower mean shoot Ca concentrations than wild type plants. These were At2g13610.2 ($P < 0.05$), At5g07320.2 ($P < 0.01$), At5g08670.1 ($P < 0.05$) and At5g48650.2 ($P < 0.01$; **Figure 2A**). Leaf Mg concentrations varied less, with twofold variation from 8,189 to 16,186 mg kg^{-1} DW observed between individual plants. Much of this variation was between genotypes. Two

mutant lines had lower mean shoot Mg concentration than wild type plants. These were At2g13610.1 ($P < 0.05$) and At5g48650.2 ($P < 0.05$; **Figure 2B**). Based on these data, lines At2g13610.2, At5g07320.2, and At5g48650.2 were selected for characterization of tissue specific leaf Ca and Mg concentration.

Calcium concentrations varied over eightfold between tissues and pooled tissue samples, ranging from 4,998 mg kg^{-1} DW in stems to 40,536 mg kg^{-1} DW in cauline leaves (**Figures 3A–D** and Supplementary Table 4). Cauline and rosette leaf Ca concentrations were similar, ranging from 32,966 mg kg^{-1} DW in rosette leaves to 40,536 mg kg^{-1} DW in cauline leaves. Mean silique Ca concentrations were lower in lines At2g13610.2, At5g07320.2, and At5g48650.2 than wild type plants ($P < 0.01$; **Figure 3B**). Mean stem Ca concentrations were lower in lines At5g07320.2 and At5g48650.2 than wild type plants ($P < 0.05$; **Figure 3C**). Mean cauline leaf Ca concentration was lower in line At5g48650.2 than wild type plants ($P < 0.05$; **Figure 3D**). Mg concentrations varied over ninefold between tissues and pooled samples, ranging from 2,608 mg kg^{-1} DW in stems to 23,999 mg kg^{-1} DW in rosette leaves (**Figures 4A–D**). Cauline leaf and rosette leaf Mg concentrations had a similar range, from 18,026 to 21,328 mg kg^{-1} DW and from 19,240 to 23,999 mg kg^{-1} DW, respectively. Lines At2g13610.2 and At5g48650.2 had lower mean silique Mg than wild type plants ($P < 0.05$; **Figure 4B**). Line At5g48650.2 also had lower mean stem Mg than wild type plants. Finally, and comparable with results from cauline leaf Ca concentration analysis, mean cauline leaf Mg concentration was lower in line At5g48650.2 than in wild type plants ($P < 0.05$; **Figure 4D**).

In summary, data from *A. thaliana* experiments identified four and two mutant lines with lower shoot Ca and Mg concentrations than wild type plants, respectively, and three of these mutations have tissue specific phenotypes. The main tissue specific effects were observed in silique tissue, with lower silique Ca concentrations in all three mutant lines investigated in the tissue partitioning experiment.

TABLE 2 | Summary of candidate genes selected from associative transcriptomics outputs.

Candidate gene	Chromosome	*A. thaliana* ortholog	Putative function
Cab036107.1	A2	At5g09690	Mg transporter – MGT7
Cab039480.1	A2	At5g03960	IQ-domain – IQD12
Cab002472.4	A3	At5g10140	Flowering locus – FLC
Cab001235.1	A3	At2g05120	Nucleoporin – NUP133
Cab001274.1	A3	At2g13610	ABC transporter – ABCG5
Cab025356.1	A5	At2g45660	Suppressor of overexpression of CO – SOC1
Cab007043.1	A6	At5g48650	Nuclear transport factor – NTF2
Cab017470.1	A10	At5g57110	Ca transporting ATPase – ACA8
BnaC02g00490D	C2	At5g10140	Flowering locus – FLC
Bo2g007260.1	C2	At5g06530	ABC transporter – ABCG22
Bo2g008580.1	C2	At5g07320	ATP-Mg/Pi transporter – APC3
Bo2g009200.1	C2	At5g08670	Mitochondrial ATP synthase beta-subunit
Bo2g009480.1	C2	At5g09710	Mg transporter – MGT7
Bo2g009490.1	C2	At5g09690	Mg transporter – MGT7
Bo2g009910.1	C2	At5g10520	ROP binding protein kinase – RBK1
Bo2g011650.1	C2	At5g14040	Phosphate transporter – PHT3;1
Bo4g024850.1	C4	At2g45660	Suppressor of overexpression of CO – SOC1

Putative functions obtained from The Arabidopsis Information Resource (TAIR; Huala et al., 2001).

DISCUSSION

SNP Based Association Analyses Identify Novel and Confirm Pre-determined Candidate Loci for Leaf Ca and Mg Concentrations

Leaf Ca concentration was highly associated with loci on chromosomes A3, A6, A7, A10, C2, C3, and C9 (**Figure 1A**). Similar loci were associated with leaf Mg concentration, specifically in regions of chromosomes A3, A7, A10, C2, C3, and C9 (**Figure 1C**). The most highly associated SNP for leaf Ca concentration was located on chromosome A10 (**Figure 1A**). This co-localizes with associated markers on C9 and markers on A10 and C9 for leaf Mg concentration. Co-localization of association peaks and associated markers for both mineral elements is unsurprising, as leaf Ca and Mg concentration data used in this study were very highly correlated ($r = 0.87$, $P < 0.001$; Thomas et al., 2016) and may reflect the relative lack of selectivity between these and other group II elements during accumulation within the plant (White, 2001). Such correlations between shoot Ca and Mg concentration have been previously shown in *B. oleracea* (Broadley et al., 2008) and a number of other angiosperm species (Broadley et al., 2004). Bus et al. (2014) previously investigated the genetic control of shoot ionome traits across 505 lines of *B. napus* using 3,910 SNPs in association analyses. Results showed two associations at a locus on chromosome C9 for shoot Ca and Mg concentration with a further association on chromosome C7 for shoot Ca concentration. The detection of an association locus on C9 is consistent with co-localized associations identified in this study. These results are also consistent with earlier findings by Broadley et al. (2008) who identified significant QTL for shoot Ca and Mg

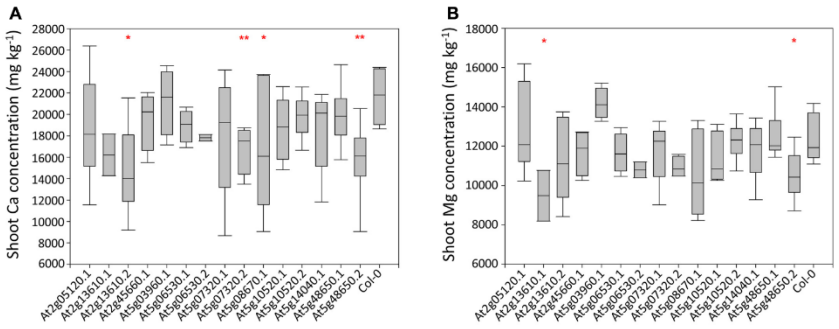


FIGURE 2 | Shoot Ca **(A)** and Mg **(B)** concentrations across 15 mutant *A. thaliana* lines and wild type (Col-0) plants. Boxes represent the mid two quartiles with the median drawn; whiskers are the 95% confidence limits. Single and double stars above boxes represent significance at $P < 0.05$ and $P < 0.01$, respectively, compared to wild type (Col-0) plants.

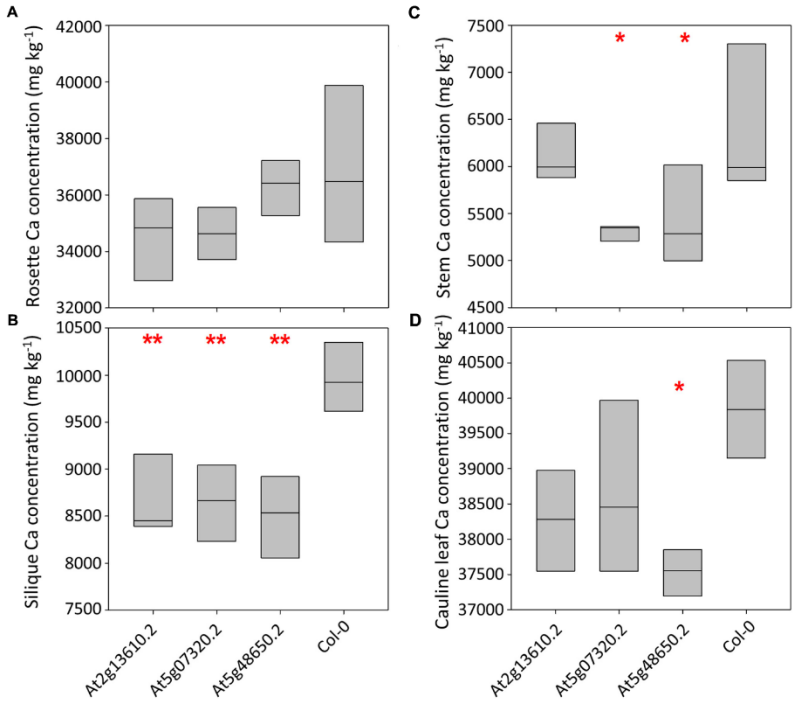


FIGURE 3 | Rosette leaf **(A)**, silique **(B)**, stem **(C)** and cauline leaf **(D)** Ca concentrations across three mutant *A. thaliana* lines and wild type (Col-0) plants. Boxes represent full range of values with the median drawn. Single and double stars above boxes represent significance at $P < 0.05$ and $P < 0.01$, respectively, compared to wild type (Col-0) plants.

in *B. oleracea* on chromosomes C2, C6, C7, C8, and C9. Together, these results indicate the importance of loci on chromosomes A10 and C9 for Ca and Mg accumulation. The QTL identified for shoot Mg in *B. oleracea* on chromosome C2 by Broadley et al. (2008) is also consistent with findings in the present study that a locus on C2 is highly associated with leaf Mg concentration in *B. napus*. However, further work is required to confirm whether the loci are in close proximity to one another. To our

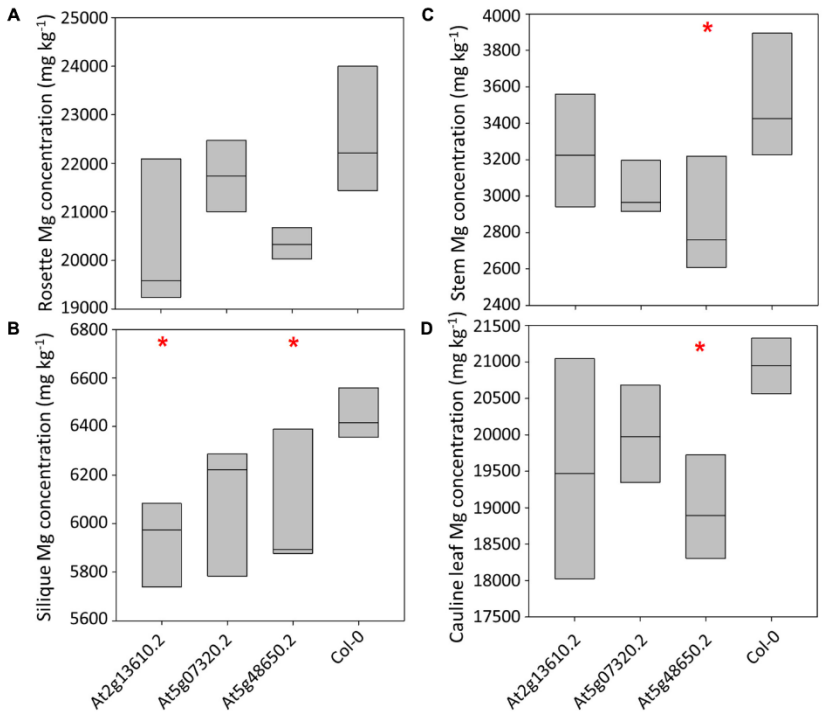


FIGURE 4 | Rosette leaf **(A)**, silique **(B)**, stem **(C)** and cauline leaf **(D)** Mg concentrations across three mutant *A. thaliana* lines and wild type (Col-0) plants. Boxes represent full range of values with the median drawn. Single and double stars above boxes represent significance at $P < 0.05$ and $P < 0.01$, respectively, compared to wild type (Col-0) plants.

knowledge, the remaining loci identified in this study have not been previously identified as important QTL for leaf Ca and Mg concentration in *Brassica* spp.

FLC and SOC1 GEM Associations May Be Linked to Variation in Leaf Ca and Mg Concentrations between Spring and Winter *B. napus* Types in the RIPR Panel

Gene-expression marker analyses associated markers corresponding to *FLC* and *SOC1* with both leaf Ca and Mg concentrations. In *A. thaliana*, FLC is a repressor of flowering (Michaels and Amasino, 1999) and it has been previously shown that FLC transcript concentration correlates with vernalization requirements (Sheldon et al., 2000). Expression levels of *SOC1* also correlate with flowering time in *A. thaliana*; in lines which flower later, *SOC1* expression is very low (Lee et al., 2000). It is thought that *SOC1* expression is repressed by *FLC*, indicating the tight regulatory links between these genes and flowering time. Leaf Ca and Mg concentration data used in this study were obtained from analysis of plants in the RIPR panel (Thomas et al., 2016) which includes a large number of spring and winter *B. napus* varieties. Thomas et al. (2016) observed differences in leaf Ca and Mg concentrations between these types, with higher mean concentrations of both leaf Ca and Mg in spring OSR compared to winter OSR and winter fodder types. Since winter OSR types are generally considered to have longer vernalization requirements than spring types, it is possible that the association of *FLC* and *SOC1* with leaf Ca and Mg concentration observed in this study was a result of differences in vernalization requirement between these groups rather than direct genetic control of Ca and Mg uptake. It is worth noting that the association of GEMs with a trait does not indicate the causative polymorphism/s, only genes in which expression level is associated with variation in the trait. The causative polymorphism/s may lie in the promotor sequence of such genes, or localize somewhere upstream in the pathway. Hence, in the case of the flowering time genes identified here, it is unclear whether or not the observed associations with leaf Ca and Mg concentration are directly caused by changes in expression of *FLC* and *SOC1*. Despite this, their expression appears to be a suitable marker for the concentrations of these elements in *B. napus*. Further to this, the concentrations of a number of other mineral elements measured in the study of Thomas et al. (2016) were found to vary between crop types with typically different

flowering times and vernalization requirements. Most notably, leaf concentrations of Mo, Na, P, and S were higher in spring OSR than winter OSR types. This suggests that flowering time, or the upstream mechanisms leading to changes in flowering time, has an effect on the concentrations of a number of nutrients in *B. napus*, though the pathway/s that lead to these differences remain unclear.

ACA8 and MGT7 Are among Genes within Linkage Disequilibrium of Associated Loci

Identification of high-resolution loci influencing leaf Ca and Mg concentrations enabled locus-specific exploration of the *Brassica* pan-transcriptomes and other genome resources for candidate genes within LD of SNPs. LD is especially relevant to the efficacy of associative transcriptomics in the absence of a marker in a trait-controlling gene. LD decays relatively quickly in *B. napus* (Ecke et al., 2010; Harper et al., 2012), and this helps to reduce the number of possibilities when searching for candidate genes. However, in this study, typically hundreds of genes were still within previously estimated LD decay (around 1–2 cM on average; Ecke et al., 2010) of most candidate loci. Fortunately, well annotated browsers of *Brassica* A and C genomes are available at Ensembl Plants (Kersey et al., 2016), which enabled rapid identification of nearby genes in the reference sequences with links to functional annotation of *A. thaliana* orthologs.

Most notable genes identified using this workflow include an ortholog of *A. thaliana ACA8* near markers associated with leaf Ca on chromosome A10 and two orthologs of *A. thaliana MGT7* near markers associated with leaf Mg on chromosomes A2 and C2. *ACA8* encodes a Ca^{2+} transporting ATP-ase localized to the plasma membrane (Bonza et al., 2000). A *B. rapa* ortholog of *A. thaliana ACA8* was previously identified under an eQTL hot spot on chromosome A3 (Graham et al., 2014). The eQTL associated with this gene was defined as Ca-responsive, i.e., the direction of the eQTL changed under high Ca supply. The *A. thaliana* ortholog of *ACA8* was further investigated *in silico* in the same study using publically available phenotypic data at the PIIMs database (Baxter et al., 2007). This led to the identification of *ACA8* T-DNA knockout mutants with greater shoot Ca concentrations than control plants in over 50% of mutant samples, indicating the ability of this gene to influence Ca accumulation in *Brassica*. *MGT7* is a member of the MGT/MRS2 Mg transport family. This was previously characterized as a key transporter for Mg uptake at low external Mg concentrations by Gebert et al. (2009). *Arabidopsis thaliana* T-DNA knockout mutants were severely retarded in development when grown at low external Mg concentrations, but were visually unaffected when grown at higher external Mg concentrations. Both *ACA8* and *MGT7* are very promising candidate genes for the control of Ca and Mg accumulation in *B. napus*. The presence of these genes within LD of highly associated SNPs demonstrates the effectiveness of associative transcriptomics in candidate gene identification. Since *ACA8* and *MGT7* knockout mutants had previously been characterized in *A. thaliana*, they were not included in further experiments in this study.

Arabidopsis thaliana Mutant Phenotyping Reveals New Candidates for Ca and Mg Accumulation

The preliminary *A. thaliana* phenotyping experiment identified four mutant lines with lower shoot Ca concentrations and two with lower shoot Mg concentrations than wild type plants. The most notable of these was At5g48650.2, the only line in which both shoot Ca and Mg was affected. The gene mutated in this line encodes NUCLEAR TRANSPORT FACTOR 2 (NTF2). This protein is proposed to function in the import of RAN, a multifunctional GTPase involved in nucleocytoplasmic transport (Zhao et al., 2006). It is the first time that it has been characterized with a shoot Ca and Mg phenotype in *A. thaliana*. Further investigation of this line showed that it had lower Ca and Mg concentration than wild type plants in all tissues except rosette leaves, suggesting it could be a promising candidate for manipulating the translocation of Ca and Mg to specific tissues in crop plants. At5g07320 encodes the ATP-Mg/Pi transporter APC3. Despite being annotated as an Mg/Pi transporter, mutants in this gene were only found to have reduced shoot Ca concentration. Tissue specific characterisation of this line showed silique and stem Ca concentrations were lower than wild type plants. This suggests that, at least in these conditions, the effects of the mutation are limited. However, effects at different external Ca or Mg concentrations might be different. A further line mutated in a gene encoding an ABC transporter was found to have both lower Ca and Mg concentrations in siliques compared to wild type plants. Identifying candidate genes controlling silique nutrient traits is particularly important in *B. napus*, which is mostly grown for the harvest of seeds which have a secondary use in animal feed. All *A. thaliana* experiments in this study took place using a high-nutrient compost. This could have masked the phenotypes of mutations in a number of candidate genes which may have been able to maintain normal Ca and Mg concentrations due to sufficient soil concentrations. In addition, it is possible that the mutations characterized here would show greater defects in shoot Ca and Mg concentrations when grown in nutrient limiting conditions. As well as this, all plants in both *A. thaliana* experiments were harvested at a single growth stage and other phenotypes might be seen at other growth stages. Despite this, four candidate genes analyzed here have proven to be potential targets for altering Ca and Mg concentrations in *B. napus*. These are orthologs of the *Arabidopsis thaliana* genes At2g13610, At5g07320, At5g08670, and At5g48650.

SUMMARY AND POTENTIAL APPLICATIONS

In this study, we have identified a number of genetic loci associated with leaf Ca and Mg concentration in *B. napus*. Within these loci, several novel candidate genes together with genes previously shown to influence or respond to Ca and

Mg concentrations in this and closely related *Brassica* spp. were localized. Most well defined loci included regions on chromosomes A2, A10, C2, and C9, close to the known Ca and Mg transporters *ACA8* and *MGT7*. Experiments in *A. thaliana* T-DNA knockouts confirmed that a further four candidate genes influence shoot Ca and Mg concentrations. This study used *B. napus* associative transcriptomics followed by an *A. thaliana* T-DNA knockout workflow to identify and test candidate genes quickly and efficiently. Due to similar phylogeny, genes characterized here in *A. thaliana* are likely to have additive effects in *B. napus*. However, further study of candidate genes in *B. napus* is required to confirm *A. thaliana* gene functions observed here and in previous studies are conserved. Both *ACA8* and *MGT7* are good targets for this, especially since *ACA8* has previously exhibited Ca-responsiveness in *B. rapa* (Graham et al., 2014), and since the effects of mutations in *A. thaliana MGT7* are so marked. Selection of *B. napus* genotypes with different alleles of target genes may lead to improved ability to grow in the presence of low soil Ca or Mg concentrations. The development of high Ca and Mg accumulating lines in edible portions of *Brassica* spp. also has the potential to reduce nutrient deficiencies in humans and livestock across the world.

AUTHOR CONTRIBUTIONS

MB, IB, PW, TA, and NG conceived the project and contributed to experimental design. TA analyzed associative transcriptomics data and performed and analyzed *A. thaliana* mutant experiments. LH and ZH prepared functional genotypes and performed associative transcriptomics. TA and NG wrote the manuscript. All authors contributed to and have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.01968/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 5. Characterisation of *Brassica rapa* MGT7, a putative magnesium transporter

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Author contributions:

TDA performed genotyping of *Brassica rapa* mutant plants, and cultivated and analysed plants in magnesium response experiments. MRB and NSG oversaw project management and data analysis. TDA analysed data, generated figures and wrote the chapter.

5.1 Abstract

Magnesium (Mg) is an essential plant mineral nutrient, though understanding of genetic and molecular mechanisms controlling its uptake and distribution lags behind that of other mineral elements. Associative transcriptomics analysis has previously led to the identification of multiple, quantitative loci underlying the control of leaf Mg accumulation in *Brassica napus*. Within linkage disequilibrium of association peaks on two chromosomes were three orthologues of *Arabidopsis thaliana MAGNESIUM TRANSPORTER 7* (*MGT7*). Thus, these form suitable candidate genes for understanding Mg uptake in *Brassica* spp. and increasing the efficiency of Mg use and distribution. Here, variation in leaf mineral concentrations under multiple exogenous Mg concentrations between a *B. rapa mgt7* mutant genotype and wild-type controls was characterised. All genotypes responded to exogenous Mg concentration; as Mg supply increased, so proportionately did leaf Mg concentration. Under the two lowest Mg supplies investigated, plants mutated in *B. rapa mgt7* accumulated 31 and 25 % greater concentrations of leaf Mg, respectively, than plants of an out-segregating wild-type line. However, no effects on leaf mineral concentration were observed at the two higher exogenous Mg supplies. That the effects of the mutation were only observed at low exogenous Mg supply is consistent with previous studies in *A. thaliana* which indicated that AtMGT7 is important for Mg uptake when only low concentrations are available in the growth media. However, the direction of the effects were opposite to that expected, suggesting that other genes may play a role in Mg uptake under low supply. Further work is required to confirm possible changes in gene expression of other *MGT* family genes within *B. rapa* plants mutated in *MGT7*. However, this study indicates that *MGT7* is important for Mg transport in *B. rapa*, and may be amenable to manipulation in order to improve Mg accumulation and use efficiency in *Brassica* crop cultivars.

5.2 Introduction

Magnesium (Mg) is an essential plant nutrient which is required for photosynthesis, ATP and protein synthesis, and generation of reactive oxygen species (Cakmak and Yazici, 2010). Magnesium is taken up from the soil as the divalent cation Mg^{2+}. Influx of Mg^{2+} into root cells and around plant tissues is thought to be largely controlled by members of the MGT / MRS2 family of transport proteins (Lenz et al., 2013). However, despite the importance of Mg in plant nutrition, knowledge regarding Mg homeostasis mechanisms lags behind that of other mineral elements (Waters, 2011). As well as this, efforts to dissect the molecular basis of Mg uptake and distribution in plants have largely taken place in the model plant *Arabidopsis thaliana* (Schock et al., 2000; Li et al., 2001; Drummond et al., 2006; Li et al., 2008; Mao et al., 2008; Chen et al., 2009; Gebert et al., 2009; Conn et al., 2011), with only very few studies having been conducted in crop species including in rice (Tanoi et al., 2011; Chen et al., 2012; Saito et al., 2013) and maize (Li et al., 2016; Li et al., 2017). Thus, further work is needed in order to confirm putative Mg uptake mechanisms in species more amenable to food production, as well as to identify novel processes that could be manipulated towards crop improvement.

Whilst investigation into specific molecular mechanisms controlling Mg uptake and accumulation in crop plants is limited, several quantitative trait loci (QTL) have been previously identified in maize (Simic et al., 2012), wheat (Hussain et al., 2017) and *Brassica* spp. (Broadley et al., 2008; Bus et al., 2014; Alcock et al., 2017) through QTL mapping and genome wide association analysis. In the latter study (Alcock et al., 2017; chapter 4), 17 candidate genes for leaf Mg concentration in *Brassica napus* were identified within linkage disequilibrium of association peaks identified across seven chromosomes. Among these were three orthologues of *A. thaliana MAGNESIUM TRANSPORTER 7* (*AtMGT7*; AT5G09690), which fell within homeologous regions of chromosomes A2 and C2. These correspond to paralogous genes within both *B. napus* sub-genomes, derived from historical hybridisation events between closely related progenitor species *B. rapa* and *B. oleracea* (Chalhoub et al., 2014).

The *MGT7* gene has been studied previously in *A. thaliana*, and results suggest that it is a highly important member of the MGT family, with significant roles in Mg^{2+} transport in plants (Mao et al., 2008; Gebert et al., 2009). Two splice-variants have been identified and characterised, each of which contain two, closely spaced, trans-membrane domains. These appear to be highly conserved in all MGT proteins in *A. thaliana* and in homologous proteins in a number of species. Indeed, proteins containing this putative Mg^{2+} transporter region have been identified in all domains of life, including in archaea, eubacteria, and in all eukaryote kingdoms (Knoop et al., 2005). Work performed by Mao et al. (2008) suggests that the primary splice variant of *AtMGT7* is expressed in all tissues, and that the second splice variant only appears to be expressed in root and flower tissue. However, it seems likely that the latter variant is not a functional Mg^{2+} transporter, as it was unable to complement a bacterial mutant lacking Mg^{2+} transport activity. The functional splice variant functions at the range of sub-millimolar Mg^{2+} concentrations in the medium, which is within the Mg concentrations observed in some soil types (Karley and White, 2009). Hence, it is possible that this protein is involved in influx of Mg to root cells from the soil (Mao et al., 2008).

Further evidence for the role of AtMGT7 in root Mg uptake from the growth medium was previously revealed through hydroponic based experiments performed by Gebert et al. (2009). Under standard growth conditions, three individual knockout lines of *Atmgt7* had no obvious phenotypes, suggesting genetic redundancy within the gene family. However, under low exogenous Mg supply (0.05 mM Mg^{2+}), growth of plants mutated in *Atmgt7* was severely retarded. Normal growth was restored when Mg supply was increased to 0.15 mM (Gebert et al., 2009). Data derived from the Ionomics Hub (Baxter et al., 2007) indicated no perturbations in shoot Mg concentration in two out of three *Atmgt7* T-DNA knockout genotypes, and only a 4 % reduction in shoot Mg concentration in the third. However, it remains to be seen whether shoot Mg concentration phenotypes are present in mutated genotypes under the low exogenous Mg supply used in the study of Gebert et al. (2009).

In this study, a genotype of *B. rapa* mutated in a gene orthologous to *A. thaliana MGT7* was investigated in order to elucidate the role of this gene in Mg^{2+} transport in a crop species. Based on data derived from *EnsemblPlants* (Zerbino et al., 2018) there appear to be 15 functional members of the *MGT* gene family in *B. rapa* orthologous to genes within *A. thaliana*. Historic genome triplication with the Brassiceae tribe has led to the existence of up to three copies of each single-copy *A. thaliana* gene within this group (Lysak et al., 2005; Parkin et al., 2005). This is true for a number of the *MGT* family genes in *B. rapa*, including *MGT9* for which three paralogues are present, and *MGT1*, *MGT4*, *MGT5* and *MGT6* for which two paralogues of each are present. Whilst there are also two copies of *MGT7* in the *B. oleracea* genome, there is only one copy apparent in *B. rapa*, thus reducing complexity of studying this gene in this species. Effects of an effective knockout mutation in *B. rapa mgt7* are studied here under a range of exogenous Mg concentration treatments. Identifying mechanisms controlling Mg uptake and accumulation in *B. rapa* are likely to be informative of mechanisms employed within the entire *Brassica* genus. These could be manipulated to increase the efficiency of Mg use and uptake from the soil in crop cultivars.

5.3 Materials and Methods

5.3.1 Plant material

A genotype of *Brassica rapa* subsp. *trilocularis* (Yellow Sarson; R-o-18) descended from a population previously subjected to ethyl methanesulphonate (EMS) mutagenesis (Stephenson et al., 2010) was the primary material used in this study. This was obtained from the RevGenUK TILLING service at the John Innes Centre (Norwich, UK; http://revgenuk.jic.ac.uk). All genotypes of the *B. rapa* TILLING population are likely to have a high mutation load (approximately 10,000 mutations per plant; Stephenson et al., 2010). The genotype acquired was of plant line ji30263-a, which putatively includes a G > A mutation within the gene Bra028621. This gene is an orthologue of *Arabidopsis thaliana MAGNESIUM TRANSPORTER 7* (*MGT7*). Line ji30263-a was of particular interest as the putative mutation within Bra028621 falls at nucleotide position 511, which is at the almost invariably conserved GT splice donor site (Senapathy et al., 1990) at the 5' end of intron 4 (Figure 5.1; sequence data derived from *EnsemblPlants* (Zerbino et al., 2018). The deletion of this splice site should result in intron 4 remaining in mature mRNA. This intron contains stop codons within all possible reading frames, and thus the mutation within ji30263-a is likely to result in a truncated protein from this position, including the absence of the conserved Mg^{2+} transmembrane transporter region of MGT7 (Figure 5.1). This splice site is also upstream of a 15 amino acid deletion detected in an alternative splice variant in the orthologous gene in *A. thaliana* (Mao et al., 2008). Hence protein truncation from this position is likely to lead to a loss of function of both potential splice variants in *B. rapa*. The mutated *B. rapa* genotype, along with an out-segregating wild-type line derived from the same mutant background (see section 5.3.2 below) was used in the experiments described here. The reference, R-o-18 parent of the *B. rapa* TILLING population was used as a further wild-type control.

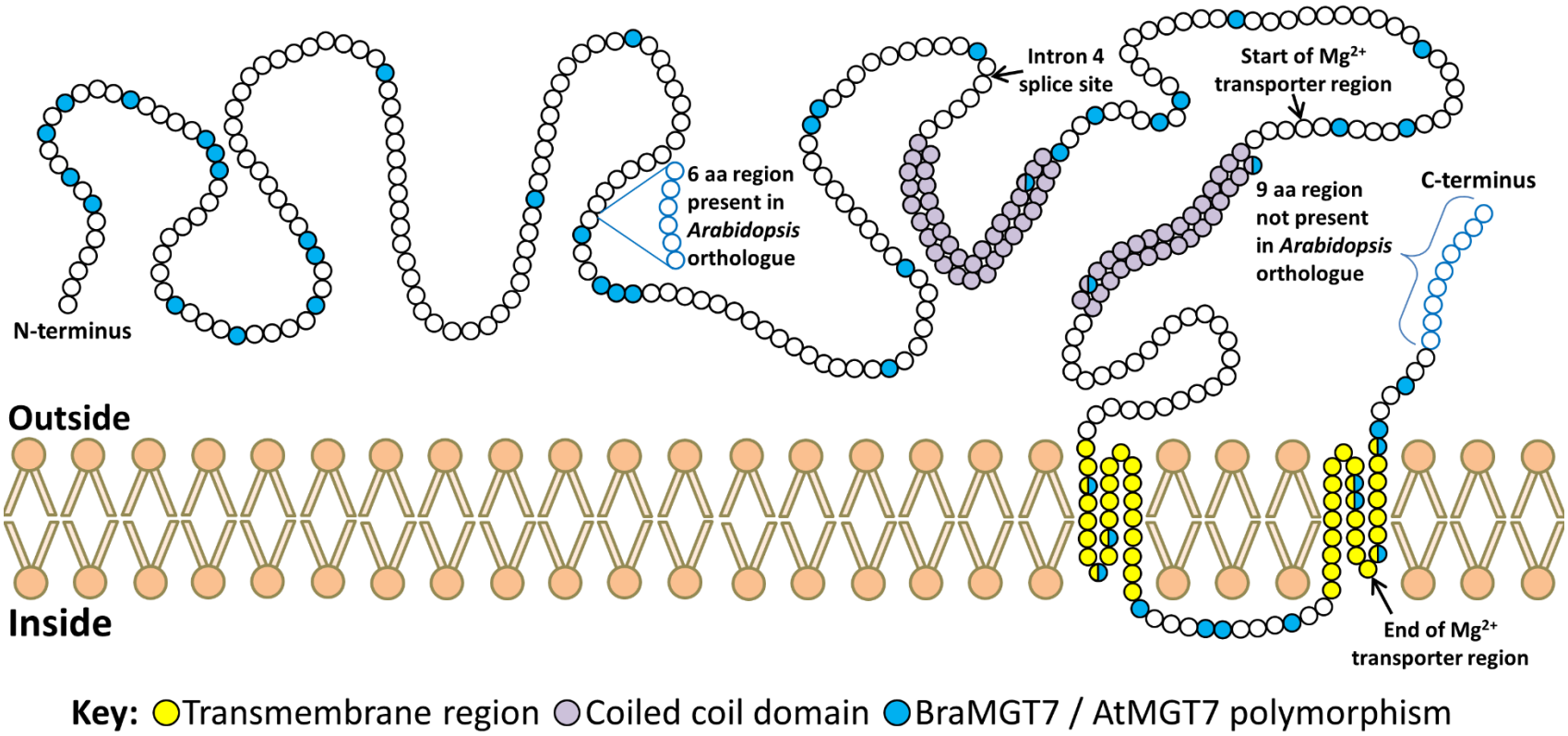


Figure 5.1. Putative structure of the Brassica rapa *MGT7 protein encoded by gene Bra028621. Phospholipid bilayer shown to indicate position of protein inside and outside of the cell. All other circles represent amino acids. Solid blue circles indicate amino acid polymorphism between* B. rapa *and* Arabidopsis thaliana *MGT7 inferred from data at Ensembl Plants (Zerbino et al., 2018). Yellow and purple circles represent transmembrane and coiled coil domains, respectively, inferred from TMHMM2.0 (Krogh et al., 2001) and SMART (Letunic and Bork, 2018). Conserved Mg^{2+} transporter region inferred from eggNOG (Jensen et al., 2008). Position of intron 4 shown to indicate site of putative protein truncation.*

5.3.2 Genotyping of *Brassica rapa* mutant lines by sequencing

Approximately 20 M_3 seeds of line ji30263-a were acquired from the RevGenUK TILLING service. Due to the high heterozygote to homozygote ratio previously described in M_2 generation plants of this population (Stephenson et al., 2010), it was assumed that resulting M_3 plants would have segregating genotypes at most mutated loci, including at the loci of interest. Thus, 12 seeds of each of ji30263-a and R-o-18 were initially sown in order to identify plants homozygous for the mutation within Bra028621. Seeds were sown on 4th December 2015 directly onto homogenised, high nutrient compost (Levington M3, ICL Specialty Fertilizers, Ipswich, Suffolk, United Kingdom) in 130 x 100 mm (750 mL) pots and placed into a glasshouse at the Sutton Bonington campus of the University of Nottingham (52°49'58.9" N, 1°14'59.2" W). Plants were subject to 18 hour supplementary lighting and heating set to 20°C during the day (venting at 22°C) and 18°C at night (venting at 20°C; controlled by Priva Integro control system, Hortech Systems Ltd., Spalding, UK). Watering was daily by hand as required.

After 38 days of growth, leaf tissue was taken from all plants and DNA was extracted using standard lab protocols (Supplementary File 5.1). A 1671 bp region of the Bra028621 gene was subsequently amplified by conventional PCR in a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) using *Bra.mgt7-wr_L* and *Bra.mgt7-wr_R* primers (Table 5.1). Reagents used were JumpStart Taq DNA polymerase with 10X reaction buffer (with $MgCl_2$) and Deoxynucleotide Mix, 10 mM (Sigma-Aldrich, St. Louis, MO, United States). PCR conditions were as recommended by the manufacturer, with annealing temperature set to 57°C and extension time set to 2 minutes. PCR amplicons were purified using Wizard SV Gel and PCR Clean-Up System (A9281; Promega, Madison, WI, USA). DNA concentrations of purified PCR products were quantified using a NanoDrop ND-1000 (NanoDrop Technologies, Montchanin, DE, USA) and each diluted to a concentration of 10 ng DNA μL^{-1}. For each sample, 15 µL of diluted, purified PCR product was aliquoted into a 1.5 mL Eppendorf tube (Eppendorf, Hamburg, Germany) along with 2 µL of *Bra.mgt7-ji30263-a_seq* primer (10 µM; Table 5.1). Samples were then submitted to the Eurofins Genomics TubeSeq

Service (Eurofins Scientific, Luxembourg) to be cycle sequenced on ABI 3730XL sequencing machines. Quality read lengths of approximately 400 bp starting roughly from the *Bra.mgt7-ji30263-a_seq* primer binding position were obtained for each sample.

From this initial screen, no seed-bearing plants were identified as homozygous for the mutation at nucleotide position 511 in gene Bra028621. A total of 20 confirmed heterozygous seeds of the M_4 generation were sown on 14th February 2017 under the same conditions described above. DNA was extracted 44 days after sowing, and a region of Bra028621 amplified by PCR, purified, diluted, and sent to be sequenced, as before. Seeds were harvested from 15 out of 20 plants. Seeds from a healthy looking, confirmed homozygous mutant and a confirmed out-segregant were selected for use in subsequent experiments. These genotypes are hereafter referred to as *Bra.mgt7* and SegWT, respectively.

Table 5.1. Primers used for genotyping mutation in gene Bra028621 within ji30263-a from the RevGenUK TILLING service at the John Innes Centre.

Primer name	Sequence
Bra.mgt7-wr_L	GGAGGAGTTTCAGAGACGCT
Bra.mgt7-wr_R	AAACAAGGCTGCGCAAAATG
Bra.mgt7-ji30263-a_seq	CGTTTGAATTTCGGGCGCTA

5.3.3 Magnesium response experiment: effects of exogenous magnesium concentration on leaf mineral composition

5.3.3.1 Growth of plant material using Nutrient Film Technique

Plant growth took at the Sutton Bonington campus of the University of Nottingham (52°49'58.9" N, 1°14'59.2" W) between 28th June and 9th August 2017. All growth took place in a glasshouse with no supplementary lighting or heating (venting at 22°C, controlled by Priva Integro control system, Hortech Systems Ltd., Spalding, UK). Seeds of *Bra.mgt7*, SegWT and R-o-18 lines were sown into 25 x 25 x 40 mm rockwool plugs (Grodan, Roermond, The Netherlands) soaked with half strength Hoagland's

solution (Hoagland's No. 2 Basal Salt Mixture; Sigma-Aldrich, Darmstadt, Germany) buffered to pH 6. Rockwool plugs were kept moist with deionised water throughout 14 days of plant growth.

Following plant establishment, the rockwool plugs were transferred to a hydroponic system based on the nutrient film technique (NFT) as described previously (Broadley et al., 2003). The system comprised eight individual gullies, each custom built from flat-bottomed, 80 - 120 mm wide, trapezoid, PVC guttering (John A Stephens Ltd., Nottingham, UK) covered with flat, PVC strips. Each gully was 4 m long, with 10, equally spaced holes cut into the top 3 m of the PVC strip covers. Each gully was fed by an individual, 84 L water tank (Really Useful Box; Key, Verwood, UK), with nutrient solutions pumped to the upper end of the gully by an Aquarius Universal Classic 1500 Pump (Hortech Systems Ltd.). All fixtures and fittings for pipe work were supplied by Hortech Systems Ltd. Gullies were laid horizontally at a slight incline (~2°), such that nutrient solutions would trickle down to the lower end of the gully, under the influence of gravity, to be collected back into the gully-specific water tank and recycled. Solution flow rate was maintained at approximately 3 L per minute, which ensured a thin layer of nutrient solution at the bottom of each gully. Each gully's water tank was filled with 30 L deionised water into which mineral nutrient salts were added. Each solution contained 2 mM $Ca(NO_3)_2$, 2 mM NH_4NO_3, 0.75 mM H_2SO_4, 0.5 mM KOH, 0.25 mM KH_2PO_4, 0.1 mM FeNaEDTA, 30 µM H_3BO_3, 10 µM $MnSO_4$, 3 µM $CuSO_4$, 1 µM $ZnSO_4$ and 0.5 µM Na_2MoO_4. Each water tank also contained varying concentrations of $MgCl_2$, which formed 4 Mg treatments. These were 0.0375 mM, 0.075 mM, 0.375 mM and 0.75 mM. Each nutrient solution was buffered to pH 6 with NaOH. Each exogenous Mg treatment was represented by two separate gullies, considered as blocks, and treatments were randomly allocated to each gully within the glasshouse (Supplementary Table 5.1). Within each gully, three plants per genotype were allocated based on a two-way design generated in GenStat (17th Edition, VSN International, Hemel Hempsted, UK), such that each genotype was represented three times per gully, and six times per Mg treatment (Supplementary Table 5.1). After 14 days of growth, nutrient solutions in each water tank were replaced with freshly made solutions buffered to pH 6. These were replaced again

after a further 9 days (earlier solution replacement required due to increased transpiration rate of plants at later growth stage).

5.3.3.2 Sampling, digestion and mineral analysis of leaf samples

After 28 days of growth under NFT conditions, 4th leaves (counting from the base of each plant, excluding cotyledons) were sampled from each plant. The first 6 cm of each leaf (typically ~12 – 18 cm) were cut and disregarded. The following 5 cm of each leaf was then cut for mineral analysis. Leaf samples from each plant were placed into individual paper bags, and dried in an incubator (Gallenkamp Economy Incubator with Fan, Size 2) set to 50°C for five days. After this period, dry leaf material was homogenised within paper bags by hand, and then subsamples of approximately 0.1 grams per sample were aliquoted (actual weights recorded) for tissue digestion prior to mineral analysis. Digestion of subsamples was carried out as described previously (Thomas et al., 2016; chapter 2). Briefly, subsamples were placed into separate vessels of perfluoroalkoxy (PFA) liner material (Anton Paar GmbH) with 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. They were then digested in a Multiwave 3000 platform with a 48-vessel MF50 rotor (Anton Paar GmbH, Graz, Austria) with settings of: power = 1400 W, temperature = 140 °C, pressure = 2 MPa, time = 45 minutes. All samples were digested over two separate runs, with two operational blank (containing digestion reagents but no plant material) and two certified reference material (CRM; Tomato SRM 1573a, NIST, Gaithersburg, MD, USA) samples included in each run. Following digestion, each digestate 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.

Immediately prior to mineral analysis, all digested samples were further diluted 1 in 10 with Milli-Q water. The concentrations of 31 mineral elements were then obtained using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany) as described previously (Thomas et al. 2016; chapter 2); B, Na, Mg, P, S, K, Ca, Ti, Li, Be, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Ag, Cd, Cs, Ba, Tl, Pb, U. Operational modes included: (i) a helium collision-cell with kinetic energy discrimination to remove polyatomic

interferences, (ii) standard mode in which the collision cell was evacuated, and (iii) a hydrogen collision-cell. After raw ICP-MS data was obtained, mean element-specific operational blank concentrations were subtracted from each data point, and then values were normalised to measurements of mg element per kg dry leaf material (mg kg^{-1} DW). 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 0.1 g. The elements Be, Se, Ag, Cs, Tl and U had mean leaf concentrations below LODs, and hence were disregarded from subsequent analyses. Of the elements with specific certified reference values; B, Na, Mg, P, S, K, Ca, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Se, Mo, Cd, Cs, Ba and U, CRM recovery was 126, 79, 94, 118, 121, 105, 99, 91, 92, 89, 100, 127, 74, 87, 88, 96, 120, 119, 101, 105, 323, 100, 73, 101 and 43 %, respectively. Due to the larger than expected recovery of Mo, this element was also removed from further analysis.

5.3.4 Statistical analysis and graph generation

All statistical analyses comparing mineral concentration data between genotypes and treatment were carried out in GenStat using one-way ANOVAs in which each exogenous Mg treatment and mineral was analysed in a separate analysis. Genotype was considered a treatment, with mineral concentration data considered the Y-variate. Block design was included in each analysis. Mean mineral concentration values between genotypes were compared using Least Significant Difference (LSD) functions in GenStat at $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels. Trends between treatments and genotype mineral concentrations were calculated using regression functions. All results figures excluding spatial distribution maps were generated in SigmaPlot (13^{th} edition; Systat Software Inc., San Jose, CA, USA).

5.4 Results and Discussion

5.4.1 Homozygous mutant and out-segregating wildtype lines of *Bra.mgt7* identified through sequencing

Of 12 seeds originally sown of *Brassica rapa* TILLING line ji30263 (RevGenUK, John Innes Centre, Norwich, UK), presumed segregating for a mutation in *MGT7* (Bra028621), nine plants germinated and established. DNA was successfully extracted from these lines and submitted for short-read sequencing in order to deduce allelic variants of *MGT7*. However, only two plants yielded seed at quantities deemed sufficient for subsequent experiments. Of these, one was confirmed heterozygous for the mutation in *MGT7*, and the other confirmed an out-segregating wild-type line. Since a confirmed homozygous mutant was required, twenty seeds of the confirmed heterozygous plant were subsequently sown, leading to M_4 plants. Sixteen plants successfully established, enabling DNA extraction for further sequencing. Of these, four plants were confirmed homozygous for the mutation in *MGT7*, nine were confirmed heterozygous, and two were confirmed out-segregating wild-types. The genotype of the final plant was inconclusive. Seeds were collected from all plants, and seeds (M_5) from the highest yielding, confirmed homozygous mutant and out-segregating wild-type plant were used in subsequent experiments. These are referred to hereafter as *Bra.mgt7* and SegWT, respectively.

5.4.2 *Brassica rapa* responds to exogenous Mg concentration

The effect of exogenous magnesium (Mg) concentration on 4th leaf Mg concentration in three genotypes of *B. rapa* (*Bra.mgt7*, SegWT and R-o-18) was examined by ICP-MS. Mean leaf Mg concentrations observed in *Bra.mgt7* under 0.0375, 0.075, 0.375 and 0.75 mM exogenous Mg concentration treatments were 1649, 1903, 5686 and 8921 mg kg^{-1} DW, respectively. In SegWT, mean leaf Mg concentrations were 1262, 1518, 5449 and 8586, respectively. In R-o-18, mean leaf Mg concentrations were 1520, 2158, 5760 and 9614, respectively (Figure 5.2; Supplementary Table 5.2). This corresponds to variation in leaf Mg concentration of 5.41-fold in *Bra.mgt7*, 6.80-fold in SegWT and 6.33-fold in R-o-18 across the 20.0-fold variation in exogenous Mg supply analysed here. This also corresponds to significant trends between exogenous

Mg concentration and 4th leaf Mg concentration in *Bra.mgt7* (r^2 = 0.925, p < 0.001), SegWT (r^2 = 0.971, p < 0.001) and R-o-18 (r^2 = 0.977, p < 0.001; Figure 5.3). Thus, a response of increased leaf Mg concentration with increasing exogenous Mg supply was observed in all *B. rapa* genotypes analysed here.

The Mg response observed here is consistent with that observed in *B. rapa* previously in which leaf Mg concentration was found to vary 3-fold across 15-fold variation in supplemented Mg concentrations, with greater leaf Mg concentrations measured in plants grown under higher Mg supply (Rios et al., 2012). In the present study, there was a 20-fold difference between exogenous Mg concentrations in the highest and lowest treatments used. This may explain the greater variation in leaf Mg concentration observed here than in the former study. Regardless, it is clear that concentrations of Mg in leaves of *B. rapa* are highly dependent on exogenous supply. Interestingly, *Bra.mgt7* exhibited the same Mg response as the two wild-type genotypes. This indicates that transport mechanisms other than those encoded by *MGT7* are responsible for increasing shoot Mg concentration in response to exogenous supply. These may act alone, or in parallel with MGT7 in wild-type plants. *Arabidopsis thaliana MGT* genes cloned into yeast mitochondrial constructs were previously used to assess rates of Mg^{2+} transport across biological membranes (Gebert et al., 2009). Magnesium uptake efficiency was particularly high in *MGT1, MGT2 and MGT7* complemented yeast mitochondria. However, there was a clear response to exogenous Mg concentration observed in all of the *MGT* genes studied. Hence, the Mg response observed here may be due to the activity of a multiple MGT proteins, as well as transporters from other gene families.

Previous studies showed that whole plant Mg concentration also increased with increasing $MgCl_2$ concentration in rice grown in a hydroponic system, even at up to 30 mM Mg (Kobayashi et al., 2005). However, soil type may play a bigger role in rice Mg accumulation than Mg application rate, as apparent from a study that applied $MgSO_4$ to rice plants in grown in two different field sites (Choudhury and Khanif, 2001). Similarly, application of $MgSO_4$ to wheat plants grown in the field revealed positive trends between Mg applied and shoot and grain Mg concentration in some

field sites but not others, indicating a significant role of other environmental factors (Craighead and Martin, 2001). The effect of applied Mg to maize plants grown in the field was also marginal (Abunyewa and Mercer-Quarshie, 2004). Thus, it seems that the response to exogenous Mg supply in *Brassica* crops is larger than that observed in a number of other crop species. However, analysis of the effects of Mg applied to *B. rapa* plants grown under field conditions will be required to confirm this.

There were no significant trends between exogenous Mg supply and concentrations of any other mineral in leaves of *Bra.mgt7*. However, in SegWT, a negative interaction between exogenous Mg supply and leaf K concentration was observed (r^2 = 0.411, p < 0.001). There were also slight negative interactions observed between exogenous Mg supply and leaf Na concentration in SegWT (r^2 = 0.164, p < 0.05) and R-o-18 (r^2 = 0.34, p < 0.01; Figure 5.3). The negative interaction between Mg supply and leaf K concentration is consistent with antagonistic effects previously reported between these two elements (Guo et al., 2016). However, the effects on leaf K concentration observed here was fairly marginal compared to the effects on leaf Mg concentration, and unlikely to lead to negative effects on productivity. The negative interaction between Mg supply and leaf Na concentration may also be due to antagonistic effects between these two elements. Application of Mg has previously been suggested to alleviate Na toxicity through blockade of a Na^+ uptake channel (Kinraide et al., 2004).

5.4.3 Under low exogenous Mg supply, *Bra.mgt7* accumulates greater leaf Mg concentrations than out-segregating wild-type plants

At the lowest exogenous Mg concentration (0.0375 mM), mean 4th leaf Mg concentration was 31 % higher in *Bra.mgt7* than in SegWT plants (p < 0.001; Figure 5.2). Similarly, mean leaf Mg concentration was 25 % higher in *Bra.mgt7* than SegWT at the second lowest exogenous Mg concentration (0.075 mM; p < 0.001; Figure 5.2; Supplementary Table 5.2). However, no differences in leaf Mg concentration were detected between *Bra.mgt7* and SegWT at the two higher exogenous Mg treatments (0.375 and 0.75 mM). As well as this, leaf Mg concentrations were not significantly higher in *Bra.mgt7* than R-o-18 in any treatment, and were actually 12 % lower at an exogenous Mg concentration of 0.075 mM (p < 0.01). Interestingly, mean leaf Mg

concentrations were also 17 and 30 % lower in SegWT than R-o-18 at exogenous Mg concentrations of 0.0375 ($p < 0.01$) and 0.075 ($p < 0.001$), respectively (Figure 5.2; Supplementary Table 5.2).

That the effects of the mutation in gene Bra028621 were only observed at relatively low exogenous Mg concentrations is consistent with putative role of MGT7 in Mg uptake at low Mg supply (Gebert et al., 2009). In fact, the concentrations at which a Mg phenotype was observed at here in *B. rapa* (0.0375 and 0.075 mM) are similar to that under which a phenotype was observed previously in *A. thaliana* (0.05 mM). If MGT7 functions as a Mg transporter under low exogenous Mg supply, it would be reasonable to assume that an effective knockout mutation, such as that within *Bra.mgt7*, would lead to a lower shoot Mg phenotype under low Mg supply. However, the direction of the effect of the mutation in *Bra.mgt7* on leaf Mg concentration was opposite to that expected. This could be due to an increase in expression of further *MGT* genes in *Bra.mgt7* in place of the faulty MGT7 protein. This response may have overcompensated for the perturbed Mg transport functionality, and led to an overall increase in leaf Mg uptake and accumulation in leaves compared to SegWT at the lower two exogenous Mg treatments.

Between *Bra.mgt7* and SegWT, there were no differences in leaf concentrations of any other element under any exogenous Mg treatment, excluding 15 and 19 % lower leaf concentrations of Na and Cu in *Bra.mgt7* at 0.0375 mM exogenous Mg, respectively ($p < 0.05$ for both), and a 14 % lower leaf S concentration in *Bra.mgt7* at 0.075 mM exogenous Mg ($p < 0.05$; Supplementary Table 5.2). In *A. thaliana*, there is evidence to suggest that a secondary splice variant of *AtMGT7*, comprising a 15 amino acid deletion between L^{278} and E^{292}, may play a role in the transport of other divalent cations including copper (Cu^{2+}) and cadmium (Cd^{2+}; Mao et al., 2008). If similar splice variants exist in *B. rapa*, it is likely that neither function correctly in *Bra.mgt7*, due to the location of the mutation leading to a putative, truncated protein. The primary splice variant contains two coiled coil regions, whilst the latter only contains one. Comparing the activity of the two splice variants, it seems reasonable to suggest that the coiled coil domain in this protein is crucial for Mg^{2+}

selectivity, since the latter splice variant exhibit little in the way of Mg^{2+} transport in *A. thaliana* (Mao et al., 2008). That leaf Cu concentration was perturbed in *Bra.mgt7* at the lowest exogenous Mg treatment analysed here provides further evidence of this gene's role in the movement of non-Mg divalent cations. This is also consistent with data obtained from the Ionomics Hub (Baxter et al., 2007) which indicated roughly a 10 – 20 % reduction in shoot Cu concentration in two out of three *mgt7* knockout genotypes in *A. thaliana*. Interestingly, a 10 – 20 % reduction in shoot K concentration was also observed in all *Atmgt7* mutant genotypes, though this was not observed in *Bra.mgt7*. The lower leaf Na concentration coupled with elevated leaf Mg concentration in *Bra.mgt7* compared to SegWT at 0.0375 mM is consistent with putative antagonistic effects between the uptake of these two elements by plants, as discussed above.

Since SegWT exhibited lower leaf Mg concentrations than R-o-18 under the lowest two Mg treatments studied, and *Bra.mgt7* exhibited more similar leaf Mg concentrations to R-o-18, it is apparent that the mutation in Bra028621 in *Bra.mgt7* effectively rescues negative effects presumably caused by a high background mutation load in the mutant background genotypes. The mechanisms controlling this are currently unclear, as there are likely to be around 10,000 mutations in each line of the *B. rapa* TILLING population, approximately 700 of which could lead to amino acid changes in proteins (Stephenson et al., 2010). However, backcrosses to the wild-type reference line, R-o-18, could dramatically reduce this mutation load within a few generations, and potentially lead to a plant genotype with greater Mg uptake ability.

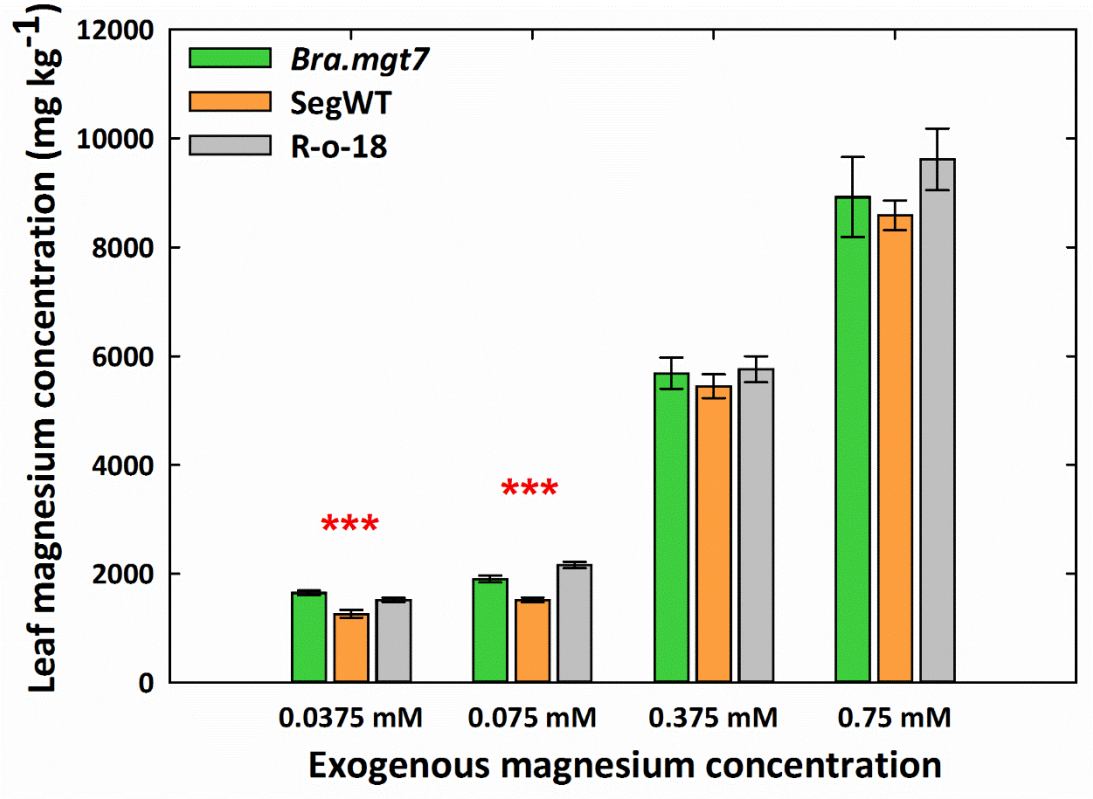


Figure 5.2. Mean concentrations of magnesium (Mg) in leaves of 42 day old (post seed sowing, including 28 days under varying Mg treatments) Bra.mgt7*, out-segregating wild-type* (SegWT) *and R-o-18 plants grown under four exogenous Mg concentrations in a hydroponic system (n = 6 for all genotypes at all treatments except* Bra.mgt7 *at 0.075 and 0.75 mM Mg, and R-o-18 at 0.075 and 0.375 mM Mg, n = 5). Error bars represent standard error of the mean. Stars above groups (***) represent significant differences in leaf Mg concentrations between* Bra.mgt7 *and SegWT plants at* p < *0.001.*

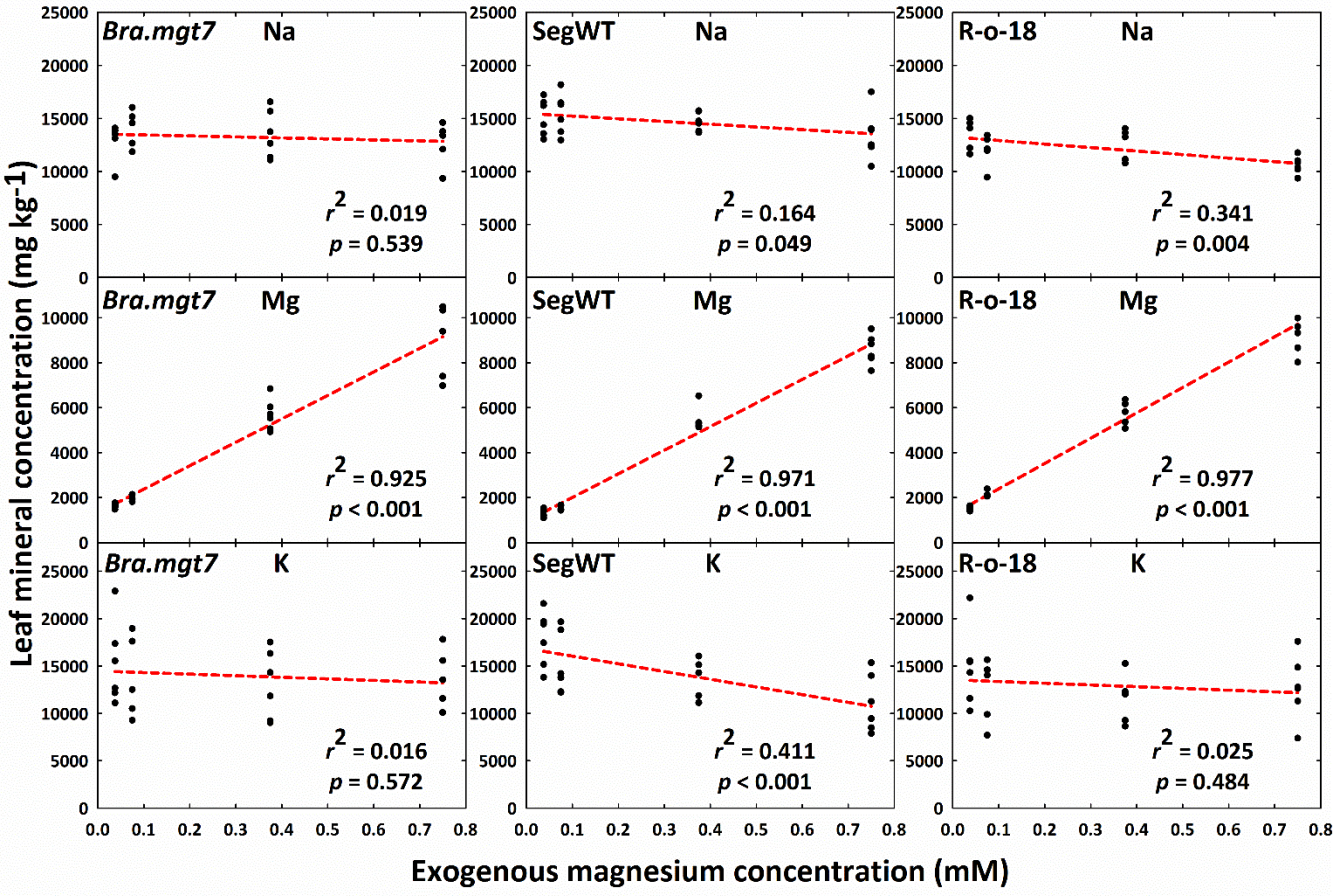


Figure 5.3. Trends between exogenous magnesium (Mg) supply and leaf sodium (Na), Mg and potassium (K) concentration in leaves of 42 day old (post seed sowing, including 28 days under varying Mg treatments) Bra.mgt7*, out-segregating wild-type* (SegWT) *and R-o-18 plants.* r^2 *and respective* p *values for each correlation generated using regression functions in GenStat* (17th Edition, VSN International, Hemel Hempsted, UK).

5.4.4 Relatively low effects of mutation in *Brassica rapa* MGT7 may be due to genome plasticity and genetic redundancy in the Brassica genus

There were clear differences in leaf Mg concentration observed between *Bra.mgt7* and SegWT under the two lowest exogenous Mg concentrations studied here. However, despite acting in the opposite direction to that expected, the effects of the mutation in the *B. rapa MGT7* gene did not have nearly the same effect as that observed previously in *A. thaliana* knockout mutants. Notably, in *A. thaliana*, plants mutated in *MGT7* were severely retarded in development under low exogenous Mg supply (Gebert et al., 2009). Under all exogenous Mg concentrations at which plants of *Bra.mgt7* were grown here, no effects on plant development, productivity, or overall health were observed. This is in spite of a lower exogenous Mg concentration having been used in one treatment in this study than in the study on *A. thaliana*.

In *A. thaliana*, there appear to be nine functional members of the *MGT* gene family (Waters, 2011). However, based on sequence homology, there are at least 15 orthologues in *B. rapa*. It is also evident that there are multiple, paralogous copies of many of these genes that only exist in single-copy form in *A. thaliana*, as expected due to historical genome triplication (Lysak et al., 2005). Most notably, there are three separate copies of *MGT9* in *B. rapa*. Whilst there is only one apparent orthologue of *AtMGT7* in *B. rapa*, due to genome plasticity within the *Brassica* genus (Lukens et al., 2004), it is possible that other members of the *MGT* gene family are able to, in one form or another, compensate for loss of function in *MGT7*. This is an especially convincing hypothesis, given that the effect of the mutation in *Bra.mgt7* was actually a greater leaf Mg concentration than SegWT under low exogenous Mg supply. Good candidates for this are *MGT1* and *MGT3*, both of which appear to be plasma membrane expressed within *A. thaliana* (Li et al., 2001;Alexandersson et al., 2004). Alternatively, it is possible that *MGT7* is fairly redundant in *B. rapa* in terms of function. Given that effects of a mutation in this gene were observed in this study, this seems unlikely. However, further work will be required to deduce expression patterns of this and other *MGT* genes in *B. rapa*. This will provide further insights into Mg homeostasis mechanisms in this crop species.

5.4.5 Characterisation of differentially expressed genes in *Bra.mgt7* may lead to identification of genes for improved Mg uptake efficiency at low exogenous Mg concentration

Since *Bra.mgt7* had elevated leaf concentrations of Mg under low exogenous supply, it is possible that one or more genes were upregulated in this genotype in order to compensate for any negative effects of the mutation. As discussed above, it is possible that further members of the *MGT* gene family may be responsible for the observed effects. Hence, it is worth performing expression analyses on all genes of the *B. rapa MGT* family, at a range of exogenous Mg concentrations and in both mutant and wild-type lines. There was no evidence for magnesium-dependent regulation of any of the *MGT* genes observed in *A. thaliana* (Gebert et al., 2009). However, different effects may be observed in *B. rapa*, particularly in the case of a loss-of-function mutation. Gene expression may be particularly important in the control of traits in genomes that have undergone duplication and thus have a high level of genetic redundancy (Adams et al., 2003). It is therefore not unreasonable to suggest that a loss-of-function mutation in one gene may lead to the expression of further genes of high sequence homology that were otherwise silenced in wild-type plants. It is also possible that one or more genes that are not of the *MGT* family exhibit a change in expression under the mutation in *Bra.mgt7*. Hence, the analysis of changes in expression of all genes under varying exogenous Mg supply in both mutant and wild-type plants, perhaps through RNA-sequencing, is also worthy of consideration. This may also lead to the identification of novel Mg uptake, translocation, and accumulation controlling mechanisms, or reveal new roles for genes in *B. rapa* that have not previously been considered.

5.5 Summary

This study has investigated the effects of an effective knockout mutation in Bra028621, a putative Mg transporter encoding gene in *Brassica rapa*. Whilst previously characterised as a Mg transporter that plays a significant role in plant development under low exogenous Mg supply in *Arabidopsis thaliana*, this represents the first effort, to our knowledge, to confirm gene function in a *Brassica* crop species. Results presented here indicated perturbed leaf Mg concentration in the mutant genotype (*Bra.mgt7*) compared to an out-segregating wild-type line (SegWT) under relatively low exogenous Mg supply, though there was no effect observed on plant development. The relatively small effects of the mutation observed in *B. rapa* compared to *A. thaliana* are indicative of partial genetic redundancy within the *B. rapa MGT* gene family. However, the elevated leaf Mg concentrations observed in *Bra.mgt7* may be amenable to manipulation to increase Mg accumulation and uptake efficiency under low exogenous supply. A number of backcrosses to background plants will be required to properly realise potential benefits of the mutation in *Bra.mgt7* compared to un-mutagenised wild-type plants. Nevertheless, further work on this gene may help to elucidate mechanisms controlling Mg uptake, use-efficiency, and accumulation into edible tissues in crop species.

5.6 References

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5.7 Supplementary Information

Supplementary File 5.1. DNA Extraction Protocol

1. Take a small leaf sample in an Eppendorf tube
2. Freeze in liquid-N and grind with micropestle
3. Add 600 µL of DNA Extraction Buffer (see below)
4. Vortex for 5 seconds and spin at 13,000 RPM for 5 minutes
5. Transfer 500 µL of supernatant to a new tube
6. Add 500 µL of isopropanol
7. Spin at 13,000 RPM for 10 minutes
8. Discard supernatant
9. Wash pellet in 250 µL 70% ethanol
10. Air dry pellet
11. Re-suspend DNA in 50 µL water

DNA Extraction Buffer:

Reagent	Quantity required in 100 mL solution (mL)
1 M TrisHCl (pH 7.5)	20
4 M NaCl	6.25
0.5 M EDTA	5
10 % SDS	5

If difficulty dissolving EDTA ensues, add NaOH

Chapter 6. Breeding high magnesium Brassicas: physiological and genetic characterisation of a *Brassica rapa* magnesium accumulator

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Author contributions:

TDA cultivated, sampled and analysed all plants used in the experiments described here. PP contributed to preparation of plant tissue prior spatial distribution analysis and advised on spatial distribution methodology. CLT cultivated earlier generations of the *Brassica rapa* magnesium (Mg) accumulator, leading to the germplasm used here. JPH performed SNP mapping and contributed to identification of candidate loci controlling the high Mg trait. PJW contributed to project conception and experimental design. MRB and NSG oversaw project management and data analysis. TDA analysed data, generated figures and wrote the Chapter.

6.1 Abstract

Magnesium (Mg) is an essential human nutrient, deficiencies in which can lead to fatigue, muscle spasms, hypertension, and cardiovascular disease. Magnesium undernutrition is widespread, particularly in higher income countries where processed foods containing less Mg are consumed more widely. Biofortification of crops with Mg, using breeding or fertilisers, has previously been proposed as a method to increase dietary Mg intakes for millions of people. An ethyl methanesulfonate (EMS) mutagenised population of *Brassica rapa* was previously screened for perturbations in leaf mineral concentrations through broad-spectrum, inductively coupled plasma-mass spectrometry (ICP-MS). From this, a genotype that accumulated Mg in shoots and roots at concentrations approximately 200 and 60 % higher, respectively, than in wild-type plants was identified. Here, the physiological and genetic bases of this trait is examined further. The *B. rapa* Mg accumulator responds to exogenous Mg concentration; as Mg supply increases, it accumulates Mg at greater concentrations than wild type-plants. The spatial distribution of Mg in leaves at both the tissue and cellular level was examined through micro-proton induced X-ray emission and micro-X-ray fluorescence spectrometry. All cell types within leaves of the Mg-accumulating genotype showed greater concentrations of Mg than in corresponding tissues of wild-type plants, with particularly elevated concentrations in epidermal cells. At a cellular level, much of the increased Mg in the Mg-accumulating genotype accumulated in apoplastic or intercellular regions, with only small increases observed within mesophyll cells. DNA was previously extracted and sequenced from bulked-segregant samples of the Mg-accumulating genotype and wild-type plants. Fine-mapping reveals multiple candidate single nucleotide polymorphisms (SNPs) and genes that may be controlling the observed traits. Work performed thus far indicates potential for breeding the Mg-accumulating trait into edible *Brassica* crop cultivars, to contribute to increasing human dietary Mg intakes.

6.2 Introduction

Magnesium (Mg) is an essential human nutrient. Within cells, Mg acts as a cofactor for over 300 enzymatic reactions, and as a counter-ion for ATP. Within these roles, Mg is necessary for the synthesis of nucleic acids and proteins, muscle contraction and relaxation, and regulation of the heart's rhythm (Yardley, 2009). Large reserves of Mg are found within bones, and these can be partially accessed in order to maintain suitable blood plasma concentrations for necessary biological processes to continue (Castiglioni et al., 2013). However, under long-term deprivation of dietary Mg, or Mg undernutrition during development, severe consequences can ensue. Early symptoms can include loss of appetite, nausea, vomiting, weakness, and depression, progressing to muscle tremors and cramps, seizures, and an abnormal rhythm of the heart (Hashizume and Mori, 1990; Jahnen-Dechent and Ketteler, 2012). Increasing dietary Mg can reduce the risk of these symptoms, and also confer protection against cardiovascular disease and diabetes (Bo and Pisu, 2008).

Magnesium undernutrition is widespread, particularly in higher income countries where processed foods containing less Mg are consumed more widely (Cakmak, 2013). For instance, intake surveys have suggested that a large proportion of the UK and USA adult population is at risk of Mg undernutrition, with 25.3 million (~ 9 %) of the combined adult population consuming Mg at concentrations below the lowest recommended nutrient intake (LRNI; Broadley and White, 2010). Recent data from the National Diet and Nutrition Survey (NDNS) carried out on behalf of Public Health England and the Food Standards Agency is consistent with this observation, which estimated that 14 and 11 % of men and women, respectively, between the ages of 19 and 64 in the UK have Mg intakes below the LRNI. More striking was the proportion of children between the ages of 11 and 18 with estimated Mg intakes below the LRNI; 27 and 50 % for boys and girls respectively (Public Health England, 2018). Magnesium deficiencies are also a problem in animals. In grazing ruminants, hypomagnesemia-related conditions such as grass tetany or staggers have long-been recognised (Suttle, 2010; Schonewille, 2013). In Europe, 1-4% of cows are affected but rates can be 20-30% within individual herds (Suttle, 2010; NADIS, 2018). In sheep,

20-40 hypomagnesemia outbreaks occur in a typical year in the UK (Suttle, 2010). Hypomagnesemia can occur at the onset of lactation due to increased Mg requirements; this can often be accompanied by, or confused with, hypocalcaemia (milk fever) which affects ~ 7-8% of UK cows (Suttle, 2010).

Human and animal dietary intakes of mineral nutrients can be increased through biofortification, a technique in which nutrient concentrations in edible parts of crop plants are increased during plant growth (White and Broadley, 2009). This can be achieved using fertilisers to increase nutrients available for plant uptake (termed agronomic biofortification), or through targeted crop breeding (termed genetic biofortification (Graham et al., 2001; Bouis, 2003). It was shown previously that increasing concentrations of essential human nutrients in crop plants by 50 % can drastically reduce the prevalence of human undernutrition (Broadley and White, 2010). For instance, in the UK alone, this would bring Mg intakes above the LRNI for a further 2 million adults. Among crop species, those within the *Brassica* genus present a good target for biofortification. This genus includes a wide range of crop types consumed throughout the world, by both humans and animals, including cabbage, broccoli, cauliflower, kale, Brussels sprouts and kohlrabi (*B. oleracea*; Liu et al., 2014), oilseed rape and swede / rutabaga (*B. napus*; Allender and King, 2010) and pak choi, turnip and Chinese cabbage (*B. rapa*; Wang et al., 2011). Production of *Brassicas* is also very high among vegetable crops; world production of cabbage in 2016 was 71 million tonnes (FAO, 2018). Relative to other groups, *Brassica* crops have also been shown to accumulate high levels of a number of mineral nutrients. Commercial varieties of *B. oleracea*, for instance, can accumulate leaf DW concentrations of up to 35,000 mg kg^{-1} calcium (Ca), and 8,300 mg kg^{-1} Mg (Broadley et al., 2008). Thus, they are a suitable vehicle for delivering mineral nutrients to humans and animals.

Naturally occurring diversity within species for desired traits is often limited (Bus et al., 2011; Bhullar and Gruissem, 2013). However, this can be overcome using mutagenesis, which offers the possibility of introducing novel, favourable allelic variants that are otherwise unavailable. A *Brassica rapa* genotype with shoot and

root magnesium concentrations approximately 200 and 60 % higher respectively than wild-type plants was recently identified through broad-spectrum mineral analyses of an ethyl-methanesulfonate (EMS) mutagenised population (Thomas, 2017). If bred into economically viable Brassica cultivars, this trait could have profound implications for alleviating magnesium undernutrition in the UK and elsewhere. *Brassica* crop varieties with elevated levels of the glucosinolate, glucoraphanin, have previously been developed and recently become commercially available under the brand Beneforté® Broccoli (Faulkner et al., 1998; Traka et al., 2013). Diets rich in glucosinolates and their derivatives have been associated with reduced incidence of cardiovascular disease and various cancers (Juge et al., 2007), and hence this cultivar is likely to confer significant health benefits. However, to our knowledge, a commercially viable *Brassica* cultivar with elevated concentrations of a mineral nutrient, or crop cultivar of any group biofortified with Mg has not previously been developed. The *B. rapa* Mg accumulator described here provides an excellent opportunity for this to be realised.

In this study, the physiological and genetic bases conferring the increase in shoot Mg concentration in the *B. rapa* Mg accumulator are dissected. An increased understanding of the mechanisms controlling the observed traits will pave the way towards generating high Mg vegetable varieties that could reduce human Mg undernutrition globally.

6.3 Materials and Methods

6.3.1 Descent of the *Brassica rapa* Mg accumulator and out-segregating wild-type lines

An inbred genotype of *Brassica rapa* subsp. *trilocularis* (Yellow Sarson; R-o-18) descended from a population that had previously been subjected to ethyl-methanesulfonate (EMS) mutagenesis to induce lesions across the genome (Stephenson *et al*., 2010) was used in this study. Ancestry of this genotype is summarised in Figure 6.1. The genotype was selected on the basis of previous observations of a high leaf magnesium (Mg) phenotype as described henceforth. Following self-fertilisation of M_1 EMS mutagenised seeds, M_2 plants were grown and analysed for variation in leaf mineral concentrations as described previously (Thomas, 2017). Briefly, seeds of 3,464 mutagenised genotypes were sown into fine-grade compost (Levington Seed & Modular + Sand -F2S; Everris Ltd., Ipswich, UK), in triplicate, and grown in a single-skinned polytunnel with no additional lighting or heating. Plants were watered twice daily by automatic irrigation. At the rosette stage (typically 6-8 fully expanded true leaves showing), the fourth leaf of each plant was sampled, dried, homogenised by hand, and digested in nitric acid, hydrogen peroxide and water in a Multiwave 3000 microwave system (Anton Paar GmbH, Graz, Austria). Digestates were then analysed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany) as described previously (Thomas et al. 2016; chapter 2) for concentrations of 28 elements: 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.

Following identification of an M_2 genotype with elevated leaf Mg concentration, plants of this genotype were self-fertilised by covering with 380 x 900 mm micro-perforated pollination bags (Focus Packaging & Design Ltd, Brigg, UK). Seed was collected and sown (M_3 generation) in order to confirm heritability of the observed trait through further ICP-MS analysis. Plants of $M_3 – F_3$ generations were grown in glasshouse conditions with supplementary artificial lighting. Seeds were sown directly into fine-grade, compost (Levington Seed & Modular + Sand -F2S; Everris Ltd.,

Ipswich, UK) and plants were irrigated twice daily by hand. All M_3 plants showed the elevated leaf Mg concentration trait, and seed from self-fertilised plants of this generation was collected and sown. Subsequent M_4 plants were backcrossed to un-mutagenised R-o-18 background plants to reduce unwanted-mutation load. No plants of the F_1 generation following this backcross (BC_1F_1) showed the elevated leaf Mg concentration trait. However, after self-fertilisation, those of the following generation (BC_1F_2) segregated at approximately a Mendelian segregation ratio.

Self-fertilisation of BC_1F_2 plants with the elevated leaf Mg concentration trait led to the generation of BC_1F_3 plants which consistently showed the trait. This line has been subsequently maintained across two further generations used for experiments described here, and is hereafter referred to as the *B. rapa* magnesium accumulator (Bra.MgA). Self-fertilisation of BC_1F_2 plants that did not show the elevated leaf Mg concentration trait led to the generation of out-segregating BC_1F_3 plants which consistently did not show the trait. This line has also been maintained across two further generations and is hereafter referred to as the out-segregating wild-type (SegWT). This genotype is likely to contain a similar mutation load to Bra.MgA, but without the specific mutation that resulted in the high leaf Mg phenotype. The SegWT line is therefore considered a suitable wild-type control genotype, which is used in all experiments, along with un-mutagenised *Brassica rapa* subsp. *trilocularis* (R-o-18) plants used as a further control genotype. A second backcross has recently been initiated between BC_1F_4 Bra.MgA plants and un-mutagenised R-o-18 background plants (Figure 6.1).

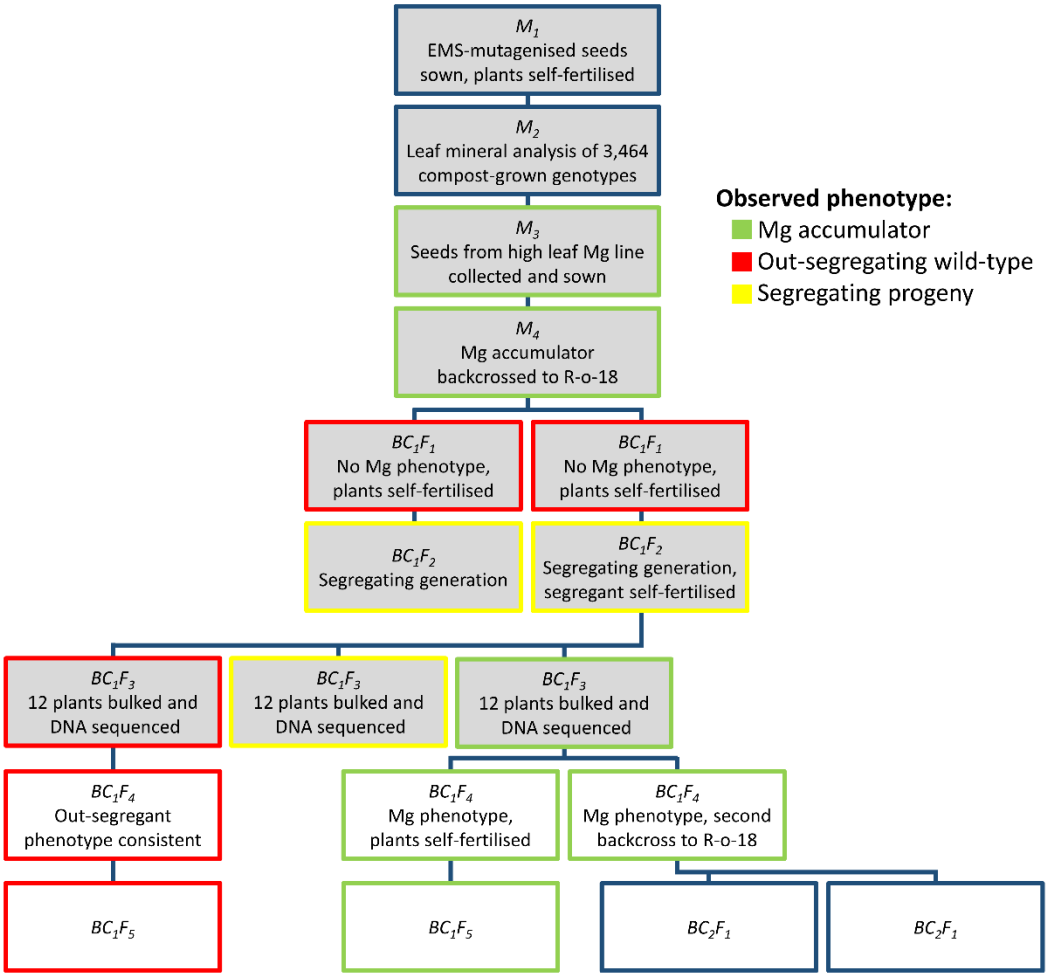


Figure 6.1. Brassica rapa *magnesium (Mg) accumulator and out-segregating wild-type workflow from original, EMS mutagenised seed stocks. Boxes outlined in green, yellow or red represent phenotypes observed among all progeny of a plant from the previous generation; green = high leaf Mg phenotype, red = leaf Mg concentration similar to that of wild-type plants, yellow = segregating leaf Mg phenotypes. Grey-background boxes refer to work performed prior to the work described in this thesis.*

6.3.2 Characterisation of leaf mineral concentration of *BC_1F_4* plants

6.3.2.1 Growth of plant material

Plant growth took place at the Sutton Bonington campus of the University of Nottingham (52°49'58.9" N, 1°14'59.2" W). Backcrossed *F_4* (*BC_1F_4*; Figure 6.1) seeds of Bra.MgA, SegWT and R-o-18 lines were sown directly onto homogenised, high nutrient compost (Levington M3, ICL Specialty Fertilizers, Ipswich, Suffolk, United Kingdom) in 165 x 130 mm (2000 mL) pots. Seeds were sown on 30th June 2017, and 20 plants were cultivated per genotype. All growth took place in a glasshouse with no supplementary lighting or heating (venting at 22°C, controlled by Priva Integro control system, Hortech Systems Ltd., Spalding, UK). All plants were watered daily by hand as required. Pots were randomly distributed on a single bench (Supplementary Table 6.1). Prior to opening of flowers, all plants were individually covered with 380 x 900 mm micro-perforated pollination bags (Focus Packaging & Design Ltd, Brigg, UK) in order to prevent cross-pollination. Watering was reduced post-flowering, and seed was collected from dry pods by hand and cleaned with a custom-built seed aspirator.

6.3.2.2 Sampling, digestion and mineral analysis of leaf samples

After 45 days of growth, whole 6th leaves were taken from each plant, placed into labelled paper bags, and then dried in an incubator (Gallenkamp Economy Incubator with Fan, Size 2) set to 50°C for three days. After this period, dry leaf material was homogenised within paper bags by hand, and then subsamples of approximately 0.1 grams per sample were aliquoted (actual weights recorded) for tissue digestion prior to mineral analysis. Digestion of subsamples was carried out as described previously (Thomas et al., 2016; chapter 2). Briefly, subsamples were placed into separate vessels of perfluoroalkoxy (PFA) liner material (Anton Paar GmbH) with 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. They were then digested in a Multiwave 3000 platform with a 48-vessel MF50 rotor (Anton Paar GmbH, Graz, Austria) with settings of: power = 1400 W, temperature = 140 °C, pressure = 2 MPa, time = 45 minutes. All samples were digested over two separate runs, with two operational blank and two

certified reference material (CRM; Tomato SRM 1573a, NIST, Gaithersburg, MD, USA) samples included in each run. Following digestion, each digestate 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.

Immediately prior to mineral analysis, all digested samples were further diluted 1 in 10 with Milli-Q water. The concentrations of 31 mineral elements were then obtained using inductively coupled plasma-mass spectrometry (ICP-MS; Thermo Fisher Scientific iCAPQ, Thermo Fisher Scientific, Bremen, Germany) as described previously (Thomas et al. 2016; chapter 2); B, Na, Mg, P, S, K, Ca, Ti, Li, Be, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Ag, Cd, Cs, Ba, Tl, Pb, U. 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).

After raw ICP-MS data was obtained, mean element-specific operational blank concentrations were subtracted from each data point, and then values were normalised to measurements of mg element per kg dry leaf material (mg kg^{-1} DW). 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 0.1 g. The elements Be and Ag had mean leaf concentrations below LODs, and hence were disregarded from subsequent analyses. Of the elements with specific certified reference values; B, Na, Mg, P, S, K, Ca, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Se, Mo, Cd, Cs, Ba and U, CRM recovery was 109, 73, 95, 118, 109, 106, 98, 92, 92, 88, 99, 129, 71, 85, 89, 95, 141, 139, 100, 105, 89, 102, 76, 101 and 49 % respectively.

6.3.3 Magnesium response experiment: effects of exogenous magnesium concentration on leaf mineral composition and photosynthetic activity

6.3.3.1 Growth of plant material using Nutrient Film Technique

Plant growth took place in a glasshouse at the Sutton Bonington campus of the University of Nottingham (52°49'58.9" N, 1°14'59.2" W) between 12th January and 15th February 2018. The glasshouse was heated to 20°C (venting at 22°C) during the day, and 18°C (venting at 20°C) at night. Artificial lighting was used to maintain day lengths of 18 hours. Supplementary heating and lighting was controlled by a Priva Integro control system (Hortech Systems Ltd., Spalding, UK). Backcrossed F_5 (BC_1F_5; Figure 6.1) seeds of Bra.MgA, SegWT and R-o-18 lines were sown into 25 x 25 x 40 mm rockwool plugs (Grodan, Roermond, The Netherlands) soaked with half strength Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture; Sigma-Aldrich, Darmstadt, Germany) buffered to pH 6 with NaOH. Rockwool plugs were kept moist with deionised water throughout 13 days of plant growth.

Following plant establishment, the rockwool plugs were transferred to a hydroponic system based on the nutrient film technique (NFT) as described previously (Broadley et al., 2003). The system comprised eight individual gullies, each custom built from flat-bottomed, 80 - 120 mm wide, trapezoid, PVC guttering (John A Stephens Ltd., Nottingham UK) covered with flat, PVC strips. Each gully was 4 m long, with 10, equally spaced holes cut into the top 3 m of the PVC strip covers. Each gully was fed by an individual, 84 L water tank (Really Useful Box; Key, Verwood, UK), with nutrient solutions pumped to the upper end of the gully by an Aquarius Universal Classic 1500 Pump (Hortech Systems Ltd.). All fixtures and fittings for pipe work were supplied by Hortech Systems Ltd. Gullies were laid horizontally at a slight incline (~2°), such that nutrient solutions would trickle down to the lower end of the gully under the influence of gravity to be collected back into the gully-specific water tank and recycled. Solution flow rate was maintained at approximately 3 L per minute, which ensured a thin layer of nutrient solution at the bottom of each gully. Each gully's water tank was filled with 30 L deionised water into which mineral nutrient salts were added. Each solution contained 2 mM $Ca(NO_3)_2$, 2 mM NH_4NO_3, 0.75 mM H_2SO_4, 0.5

mM KOH, 0.25 mM KH_2PO_4, 0.1 mM FeNaEDTA, 30 µM H_3BO_3, 10 µM $MnSO_4$, 3 µM $CuSO_4$, 1 µM $ZnSO_4$ and 0.5 µM Na_2MoO_4. Each water tank also contained varying concentrations of $MgCl_2$, which formed 8 Mg treatments. These were 0.0075 mM, 0.0375 mM, 0.075 mM, 0.375 mM, 0.75 mM, 1.125 mM, 1.5 mM and 3 mM. Each nutrient solution was buffered to pH 6 with NaOH. The position of each of the eight Mg treatments was randomly distributed within the eight gullies (Supplementary Table 6.2). Within each gully, three plants per genotype were allocated based on a one-way, blocked design generated in GenStat (17th Edition, VSN International, Hemel Hempsted, UK; Supplementary Table 6.2), such that each genotype was represented three times per Mg treatment, and once per block within each gully. After 12 days of growth, nutrient solutions in each water tank were replaced with freshly made solutions.

6.3.3.2 Leaf gas exchange measurements

After 19 days of growth under NFT conditions, photosynthetic carbon assimilation and stomatal conductance to water were measured on fully expanded 5th or 6th leaves of all plants using infrared gas analysis (LI-6400 Portable Photosynthesis System; LI-COR, Lincoln, NE, USA). Photosynthetic Photon Flux Density was set to 2000 µmol m^{-2} s^{-1}, provided by an integrated LED light source. Leaf chamber temperature was set to 22°C and CO_2 concentration was maintained at 400 µmol mol^{-1} throughout measurements. Leaves were equilibrated within the chamber for 2 minutes prior to single, spot measurements being taken for each leaf. All measurements were taken between 11:00 and 13:00 to reduce circadian effects on photosynthesis.

6.3.3.3 Sampling, digestion and mineral analysis of leaf samples

After 21 days of growth under NFT conditions, whole 3rd and 4th leaves were sampled from each plant for ICP-MS mineral analysis. Leaves from each plant were placed into individual paper bags, and dried in an incubator (Gallenkamp Economy Incubator with Fan, Size 2) for five days. They were then digested in a Multiwave 3000 platform with a 48-vessel MF50 rotor, and analysed by ICP-MS as described above. The elements Li, Be, V, Cr, Co, Ni, As, Se, Rb, Ag, Cd, Cs, Ba, Tl, Pb and U had mean leaf

concentrations below element-specific LODs, and hence were disregarded from subsequent analyses, as were any values below LODs for each element. It is worthy of note that the low concentrations measured for these elements is reflective of the method of plant growth in this experiment, which was highly selective over what elements were available for plant uptake. CRM recovery for the elements B, Na, Mg, P, S, K, Ca, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd, Cs, Ba and U, was 91, 70, 81, 100, 97, 92, 93, 73, 88, 117, 95, 89, 93, 99, 85, 90, 110, 114, 94, 98, 87, 95, 92, 94 and 70 % respectively.

6.3.4 Spatial-distribution of minerals at the tissue and cellular level

6.3.4.1 Growth of plant material for all spatial-distribution experiments

Seeds of the Bra.MgA, SegWT and R-o-18 lines were sown directly onto homogenised, high nutrient compost (Levington M3) in 130 x 100 mm (750 mL) pots. Sixteen seeds were sown per genotype, leading to a total of 48 plants. These were grown in a glasshouse at the Sutton Bonington campus of the University of Nottingham (52°49'58.9" N, 1°14'59.2" W) from 17th November to 10th December 2017 with 18 hour supplementary lighting and heating set to 20°C during the day (venting at 22°C) and 18°C at night (venting at 20°C). Watering was daily by hand as required. All plants were arranged in a one-way randomised design generated in GenStat (Supplementary Table 6.3).

6.3.4.2 Whole plant sampling and shipment for further preparation

After 23 days of growth (typically four true-leaves showing per plant), 10 plants of each of the Bra.MgA, SegWT and R-o-18 lines were sampled for spatial distribution analysis. Plants were removed from pots, and compost was gently washed off into a water filled bucket by hand. Roots were wrapped in moistened, 2-ply, blue tissue roll (Kimberly-Clark Kimwipes Blue Barrell Roll; Scientific Laboratory Supplies, Nottingham, UK) and whole plants were placed into 150 x 230 mm grip seal bags (Appleton Woods Ltd, Birmingham, UK) and sealed. Bagged plant samples were placed into a protected box and sent with a 24 hour air service (DHL EXPRESS 12:00; DHL, Colnbrook, UK) to the University of Ljubljana, Biotechnical Faculty (46°03'04.5"N 14°28'13.1"E) for further sample preparation.

6.3.4.3 Plant leaf dissection, freeze drying, and sectioning

On arrival at the University of Ljubljana, samples were prepared immediately for freezing. The first or second true-leaf of five plants of each of the Bra.MgA, SegWT and R-o-18 lines was taken, and three small pieces (approx. 3 x 5 mm), 1 – 2 cm in from the tip of the leaf and including the midrib were cut using a scalpel (Figure 6.2A). These were folded slightly by hand along the midrib, taking care to avoid snapping the leaf sample, and placed into 2 mm stainless steel needles, with the leaf partially protruding. Needles and protruding leaf samples were dipped into Jung Tissue Freezing Medium (Leica Microsystems Nussloch GmbH, Germany) to support the tissue during sectioning. Samples were then rapidly frozen in liquid propane cooled by liquid nitrogen to prevent elemental losses or redistribution, and then left in a frozen state in liquid nitrogen. Prior to sectioning, frozen samples were placed into a Leica CM3050 Cryostat microtome (Leica, Bensheim, Germany) with chamber and cutting head temperature set to -20°C and allowed to warm to this temperature for at least two hours. Sections were then cut transversely (Figure 6.2B) from protruding leaf samples at 60 and 25 µm thicknesses for micro-proton induced x-ray emission (micro-PIXE) and micro-X-ray fluorescence (micro-XRF) spectrometry respectively. One leaf sample per plant was used, the remaining prepared samples kept as reserves. Typically 10 sections were cut per thickness per used sample. Sections were placed into custom aluminium pots lined with filter paper and weighed down with stainless steel lids. They were then freeze-dried in a Christ LDC-1M Alpha 2-4 freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at -25°C and a pressure of 0.08 mbar for three days.

When dry, 60 µm sections were mounted onto 14 x 20 mm aluminium holders (Jožef Stefan Institute, Ljubljana, Slovenia), with sections sandwiched between two layers of Pioloform foil (approx. 400 nm thickness; SPI supplies, West Chester, PA, USA). All sections were inspected by light microscopy using an Axioskop 2 MOT microscope to select suitable regions for micro-PIXE analysis. Micrographic images of sections were taken with an attached Axiocam MRc colour digital camera (Carl Zeiss, Goettingen, Germany) and processed with AxioVision (version 4.1) software. The 25 µm sections

were mounted onto custom, 35 mm diameter, circular, aluminium holders between two layers of Pioloform foil. Each holder had four, two mm diameter sample spaces, and were prepared such that each of three holders contained samples from only one of the Bra.MgA, SegWT or R-o-18 genotypes.

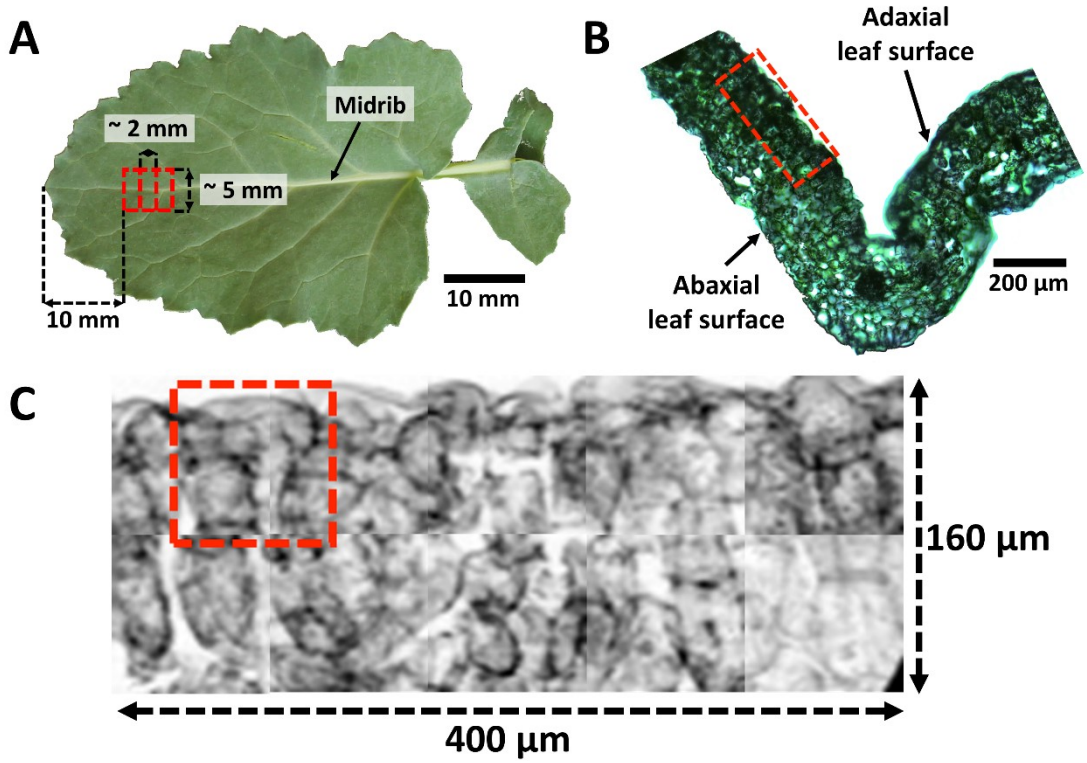


Figure 6.2. Leaf preparation work-flow for micro-proton induced X-ray emission (micro-PIXE) and micro-X-ray fluorescence (micro-XRF) analyses. A: First / second leaf removed from plant and three segments cut starting approximately 10 mm in from the tip of the leaf. Segments were typically 2 x 5 mm, and included the midrib of the leaf (segment locations represented by dashed red lines). B: cross-sections of freeze-dried segments cut by cryo-tomography. Element distributions of whole cross-sections were mapped using micro-PIXE analyses. Adaxial leaf lamina tissue (within region denoted by red dashed line) mapped using micro-XRF analyses. C: Cellular structure elucidated using X-ray imaging prior to micro-XRF analysis and 80 x 80 µm region comprising upper epidermal and palisade mesophyll cells (typical example marked by dashed red lines in C) selected for spatial distribution mapping. Scale bars in each panel shown to indicate typical size of leaf samples and cross-sections.

6.3.4.4 Micro-proton induced X-ray emission spatial distribution analysis

Localisation of elements within 60 µm leaf cross sections of Bra.MgA, SegWT and R-o-18 plants was assessed at the tissue level by micro-PIXE spectrometry as described previously (Pongrac et al., 2013). This was performed at the nuclear microprobe in the tandetron accelerator laboratory at the Jožef Stefan Institute (Ljubljana, Slovenia; Simičič et al., 2002). The sample area investigated ranged from 800 x 800 to 1200 x 1200 µm (Figure 6.2B), at approximately 4 µm resolution, and included relevant and easily identifiable tissue types including upper and lower epidermis, palisade and spongy mesophyll, and, where possible, major vasculature. Samples were scanned in a vacuum (10^{-6} mbar). The micro-PIXE analysis made use of an H^- beam extracted from hydrogen plasma created in a duoplasmatron ion source, with protons formed in a stripping process within a tandem accelerator. The accelerated proton beam was subsequently focussed onto the target. X-rays were emitted from the sample at element-specific energies of 1 – 25 keV. The elements Na, Mg, P, S, Cl, K and Ca were detected by a silicon drift detector, and the Mn, Fe, Cu and Zn were detected by a high-purity germanium detector. Samples were irradiated with low-energy electrons during analyses to avoid sample charging. Proton dose was measured using a rotating, in-beam chopper as described previously (Vogel-Mikuš et al., 2009) in order to enable element quantification. Backscattered spectra from the chopper were recorded in parallel with the PIXE spectra. Simultaneously, an on – off axis scanning transmission ion microscopy was performed to determine beam exit energy from the sample, related to the sample local tissue density (Pallon et al., 2004; Vavpetič et al., 2013).

The micro-PIXE spectra were analysed and numeric concentration matrices (pixel-by-pixel concentration matrices) were generated using GeoPIXE II software package (Ryan, 2000). Spatial distribution maps were generated using numeric concentration matrices in PyMCA (version 5.2.2; Solé et al., 2007). The micro-PIXE output files were first converted to .edf file format using an in-program converter, and then distribution maps were saved using the Matplotlib function, with bilinear pixel interpolation. Mean element concentrations within whole leaf tissue, upper

epidermis, lower epidermis and palisade mesophyll tissues were generated for each cross-section from numeric concentration matrices. Concentrations were extracted by manually encircling regions of interest in ImageJ (version 1.47; Schneider et al., 2012) in the element distribution maps.

6.3.4.5 TwinMic low energy X-ray fluorescence spatial distribution analysis

Cellular level spatial distribution of elements within 25 µm cross-sections was determined using the TwinMic beamline at the Elettra Synchrotron (Trieste, Italy; Gianoncelli et al., 2016). Synchrotron storage ring energy was 2.0 GeV. Operational modes were scanning transmission X-ray microscopy (STXM) and low energy X-ray fluorescence (LEXRF), with beam energy set to 2319 eV, and a max photon flux of $8x10^{10}$ photons s^{-1} mA^{-1}. Samples were scanned in a vacuum (10^{-6} mbar). For each sample, an 80 x 80 µm region of the cross-section comprising upper epidermal and palisade mesophyll cells was analysed (Figure 6.2C). A resolution of 1.2 µm was achieved by focussing the X-ray beam using a scanning X-ray microscope zone plate. Structural makeup of leaf samples was determined by X-ray absorption and phase contrast imaging. Elements mapped with LEXRF were O, F, Na, Mg, Al, Si, P, Fe and Ni. Spatial distribution maps were generated from TwinMic output .edf files in PyMCA. Images were saved using the Matplotlib function, with bilinear pixel interpolation. Co-localisation images were generated using RGB Correlator tools included within PyMCA.

6.3.4.6 Tissue sampling for leaf mineral analysis

Of the remaining six plants per genotype grown at the University of Nottingham, four were sampled for mineral analysis by ICP-MS, in order to confirm consistent phenotypes. All four true leaves were cut at the stem, including petiole, and placed into paper bags. These were dried in an incubator (Gallenkamp Economy Incubator with Fan, Size 2) set to 50°C for two days, and then homogenised by hand. Samples were then microwave digested and analysed by ICP-MS as described above.

6.3.5 Bulked segregant analysis and fine SNP mapping

Sequencing of bulked segregant DNA samples extracted from Bra.MgA, SegWT and R-o-18 genotypes was previously carried out (Thomas, 2017). Briefly, the 5th leaf from 12 plants of each genotype was collected and snap frozen in liquid nitrogen. Samples were then homogenised in a mortar and pestle and DNA was extracted from each sample according to the protocol of the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were pooled within each genotype, and sequenced by TGAC (The Genome Analysis Centre, now Earlham Institute, Norwich, UK) using an Illumina HiSeq2500 (Illumina, San Diego, CA, USA) with 125 bp paired end read metric.

Raw FastQ files were mapped to an unpublished, R-o-18 reference sequence (R-o-18 version 1.2; King GJ, Southern Cross University, NSW, Australia, personal communication) using the Burrows-Wheeler Aligner (BWA) software package (Li and Durbin, 2009) implemented in the Galaxy software platform (Afgan et al., 2018) as Map with BWA for Illumina (version 1.2.3). Single nucleotide polymorphisms (SNPs) between the three genotypes and the R-o-18 reference sequence were called using BCFtools (http://samtools.github.io/bcftools/bcftools.html). Resulting .vcf files were then filtered based on read quality, such that SNPs with combined read depth across sample and phred-scaled genotype posterior probability scores below 50 were removed from subsequent analysis. Conserved SNP calls between SegWT and R-o-18 genotypes and Bra.MgA, were disregarded in order to isolate SNPs unique to Bra.MgA. Remaining SNPs were binned into 20 kbp regions using COUNTIF functions in Excel 2013 (Microsoft Corporation, Washington, USA) in order to determine genomic region speficic SNP density. The R-o-18 reference sequence was previously mapped to the annotated *B. rapa* var. chiifu (Wang et al., 2011) sequence (King GJ, personal communication). All SNPs that fell within coding DNA sequences in Bra.MgA were thus determined. Gene function was inferred from *Arabidopsis thalina* orthologue data obtained from The Arabidopsis Information Resource (TAIR; Huala et al., 2001) in order to identify candidate genes, allelic variation in which may have led to the observed high magnesium trait in the Bra.MgA genotype.

6.3.6 Statistical analysis and graph generation

All statistical analyses comparing mineral concentration and photosynthetic trait data between Bra.MgA and SegWT genotypes was carried out in GenStat (17th edition; VSN International, Hemel Hempstead, UK). Mineral concentration data from ICP-MS analysis of BC_1F_4 and BC_1F_5 plants was analysed by one-way ANOVA with genotype included as the treatment. Mineral concentration and photosynthetic trait data from the Mg response experiments were analysed by two-way ANOVA with genotype and exogenous Mg concentration included as treatments. Trends between treatments and genotype mineral concentrations were calculated using regression functions. Tissue specific mean concentration data derived from micro-PIXE analysis was analysed by two-way ANOVA with genotype and tissue type included as treatments. Individual elements were handled in separate analyses in each experiment. Means of Bra.MgA and SegWT genotypes were compared in each case using Least Significant Difference (LSD) functions in GenStat at $P < 0.05$, $P < 0.01$ and $P < 0.001$ levels. All results figures excluding spatial distribution maps were generated in SigmaPlot (13th edition; Systat Software Inc., San Jose, CA, USA).

6.4 Results

6.4.1 Elevated leaf Mg concentration in the *Brassica rapa* Mg accumulator is accompanied by perturbations in leaf Na, P, S, K and Ca concentrations

Of the essential / beneficial plant macro-nutrients (typically present at 1000– > 10,000 mg kg^{-1} leaf dry weight, DW), 3rd / 4th leaf concentrations of Na, Mg, P, S, K and Ca were significantly perturbed in the BC_1F_4 (Figure 6.1) generation of the *Brassica rapa* Mg accumulator (Bra.MgA) genotype compared to wild-control plants (Figure 6.3). In Bra.MgA, mean leaf Na, Mg, P, S, K and Ca concentrations were 3121, 34293, 8309, 20770, 31980 and 24913 mg kg^{-1} DW, respectively (replicate number [n] = 20). In out-segregating wild-type plants (SegWT) mean leaf Na, Mg, P, S, K and Ca concentrations were 2469, 18919, 7399, 18638, 50639 and 27792 mg kg^{-1} DW, respectively (n = 20). In un-mutagenised wild-type (R-o-18) plants, mean leaf Na, Mg, P, S, K and Ca concentrations were 2908, 16341, 6720, 18635, 60254 and 26774 mg kg^{-1} DW, respectively (n = 19). This corresponds to an increase of 26 ($p < 0.05$), 81 ($p < 0.001$), 12 ($p < 0.05$) and 11 ($p < 0.01$) % in the concentrations of Na, Mg, P and S, respectively, and a decrease of 37 ($p < 0.001$) and 10 % ($p < 0.05$) in the concentrations of K and Ca, respectively in Bra.MgA compared to SegWT plants (Figure 6.3; Supplementary Table 6.4).

Perturbations were also observed in the concentrations of a number of other minerals in Bra.MgA, in both plant essential micro-nutrients and non-essential or toxic elements. This includes Ni, Zn, As and Se which were present in greater concentrations in leaves of Bra.MgA compared to SegWT ($p < 0.001$, < 0.01, < 0.001 and < 0.001, respectively) and Mn, Fe, Cu, Rb and Cd, which were present in lower concentrations in leaves of Bra.MgA compared to SegWT ($p < 0.001$, < 0.05, < 0.001, < 0.001 and < 0.001, respectively; Supplementary Figure 6.1, Supplementary Table 6.4).

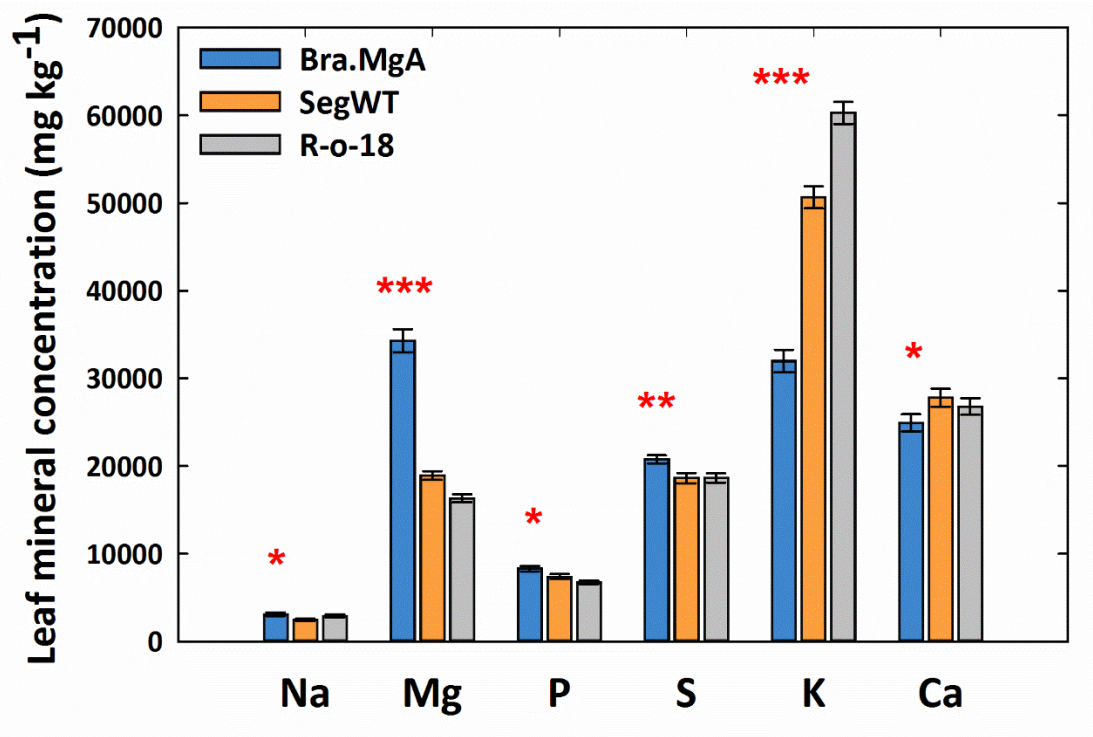


Figure 6.3. Concentrations of macro-mineral elements (present at greater than 1,000 parts per million; Na, Mg, P, S, K and Ca) in leaves of 45 day old (post seed sowing) BC_1F_4 *generation* Brassica rapa *magnesium accumulator (Bra.MgA; n = 20), out-segregating wild-type (SegWT; n = 20) and un-mutagenised wild-type (R-o-18; n = 19) plants. Where possible, 6th leaf from base taken for analysis, otherwise next available leaf taken (e.g. 7th leaf). Error bars represent standard error of the mean. Stars above groups represent significant differences in mineral concentrations between Bra.MgA and SegWT plants at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).*

6.4.2 The *Brassica rapa* Mg accumulator responds to exogenous Mg supply by accumulating greater leaf Mg concentrations than wild-type plants

Trends between exogenous Mg concentration and 3rd / 4th leaf Mg concentrations were observed in Bra.MgA (r^2 = 0.934, p < 0.001), R-o-18 (r^2 = 0.840, p < 0.001) and SegWT (r^2 = 0.557, p < 0.001). Mean leaf Mg concentrations observed in Bra.MgA under 0.0075, 0.0375, 0.075, 0.375, 0.75, 1.125, 1.5 and 3 mM exogenous Mg concentration treatments were 1783, 1888, 3518, 10747, 15067, 20915, 20540 and 37595 mg kg^{-1}, respectively. In SegWT, mean leaf Mg concentrations were 1228, 1670, 1457, 4581, 7179, 6503, 4834 and 11911, respectively. In R-o-18, mean leaf Mg concentrations were 2398, 2262, 2308, 3305, 7687, 7653, 8235, 13399, respectively (Figure 6.4; Supplementary Table 6.5). This corresponded to greater mean leaf Mg concentrations in Bra.MgA compared to SegWT of 135 % at 0.375 mM Mg (p < 0.05), 110 % at 0.75 mM (p < 0.01), 222 % at 1.125 mM Mg (p < 0.01), and 216 % at 3 mM Mg (p < 0.01; Figure 6.4). For each genotype at each treatment, n = 3, excluding Bra.MgA at 1.5 mM Mg; n = 2.

In 3rd and 4th leaf tissue of Bra.MgA, there were also 205, 270, 163, 55, 223 and 85 % greater mean concentrations of Na compared to SegWT at exogenous Mg concentrations of 0.0075 mM (p < 0.05), 0.075 mM (p < 0.01), 0.375 mM (p < 0.01), 0.75 mM (p < 0.05), 1.5 mM (p < 0.05) and 3 mM (p < 0.01), respectively; an 85 % greater concentration of P compared to SegWT at 3 mM Mg (p < 0.05); 185, 256, 51 and 86 % greater concentrations of S compared to SegWT at 0.0075 mM Mg (p < 0.05), 0.375 mM Mg (p < 0.05), 0.75 mM Mg (p < 0.001), and 3 mM Mg (p < 0.05), respectively; 73, 78 and 85 % lower concentrations of K observed at 0.375 mM Mg (p < 0.05), 0.75 mM Mg (p < 0.01) and 3 mM Mg (p < 0.05), respectively; and 255, 153 and 38 % greater concentrations of Ca compared to SegWT at 0.075 mM Mg (p < 0.01), 0.375 mM Mg (p < 0.05) and 0.75 mM Mg (p < 0.05), respectively (Supplementary Figure 6.2; Supplementary Table 6.5). There were no trends between concentrations of Na, P, S, K or Ca and exogenous Mg supply in all genotypes, excluding weak, negative correlations between exogenous Mg supply and leaf K (r^2 = 0.211, p < 0.05) and Ca (r^2 = 0.213, p < 0.05) concentration observed in Bra.MgA.

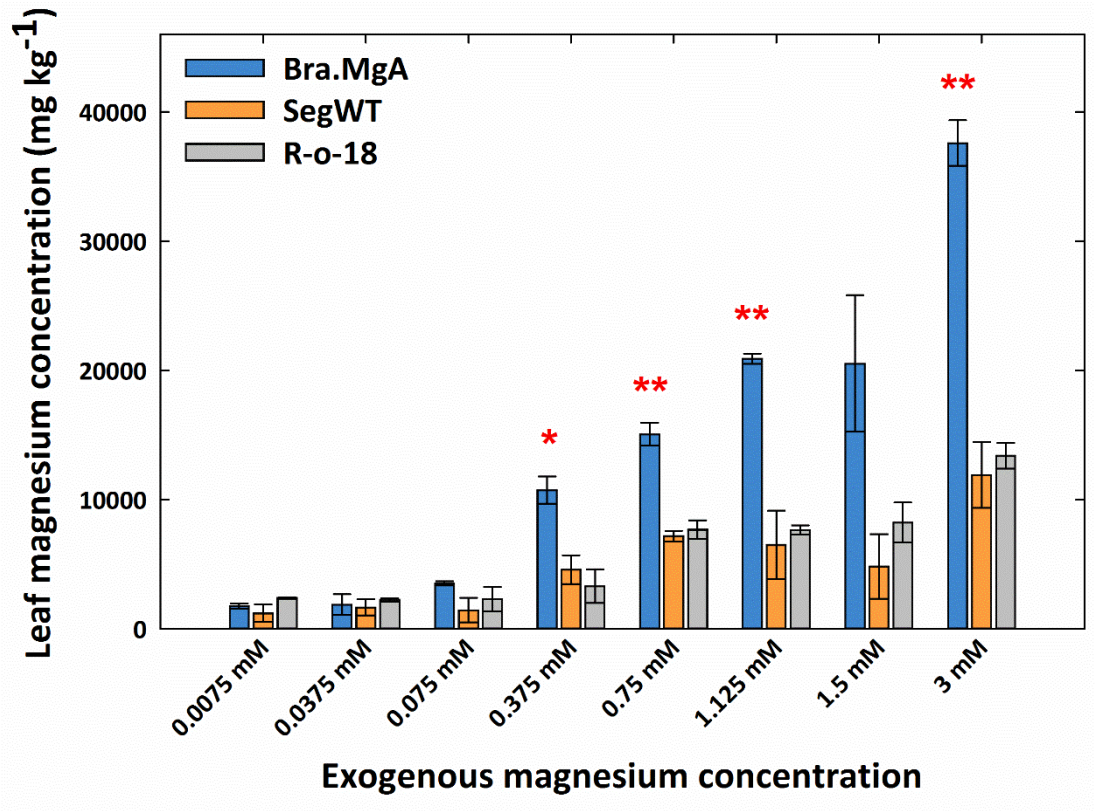


Figure 6.4. Concentrations of Mg in leaves of 34 day old (post seed sowing, including 21 days under varying Mg treatments) BC_1F_5 *generation* Brassica rapa *magnesium accumulator (Bra.MgA), out-segregating wild-type (SegWT) and un-mutagenised wild-type (R-o-18) plants at eight different exogenous Mg concentrations (n = 3 for all genotypes at all treatments except Bra.MgA at 1.5 mM exogenous Mg, n = 2). Error bars represent standard error of the mean. Stars above groups represent significant differences in Mg concentrations between Bra.MgA and SegWT plants at p < 0.05 (*) and p < 0.01 (**).*

6.4.3 The Brassica rapa Mg accumulator has a lower photosynthetic rate than wild-type plants that is not dependent on exogenous Mg supply

Photosynthetic rate, measured as mean rate of photosynthetic carbon assimilation, in 5th / 6th leaves of Bra.MgA at exogenous Mg concentrations of 0.0075, 0.0375, 0.075, 0.375, 0.75, 1.125, 1.5 and 3 mM was 17.5, 16.06, 15.32, 13.86, 17.84, 13.1, 15.4 and 15.89 µmol CO_2 m^{-2} s^{-1}, respectively. In SegWT, mean photosynthetic carbon assimilation rates were 24.89, 16.17, 23.24, 24.07, 29.08, 19.82, 19.31 and 25.46 µmol CO_2 m^{-2} s^{-1}, respectively, and in R-o-18, they were 22.97, 25.02, 23.38, 16.34, 30.65, 22.99, 14.02 and 28.82 µmol CO_2 m^{-2} s^{-1}, respectively (Figure 6.5; Supplementary Table 6.6). Lower photosynthetic rates were observed in Bra.MgA compared to SegWT at exogenous Mg concentrations of 0.375 mM ($p < 0.05$), 0.75 mM ($p < 0.01$) and 3 mM ($p < 0.05$; Figure 6.5). Comparisons are based on three replicate plant samples for each genotype and treatment, excluding Bra.MgA at 1.5 mM Mg; n = 2. No trends were observed between exogenous Mg concentration and photosynthetic carbon assimilation rate in any of the genotypes.

Of the other traits related to photosynthesis analysed, comprising stomatal conductance to water, intercellular CO_2 concentration, and transpiration rate, there were no differences between trait values in Bra.MgA compared to SegWT, except for a 30 % greater mean intercellular CO_2 concentration observed in Bra.MgA at an exogenous Mg concentration of 1.125 mM ($p < 0.001$; Supplementary Figure 6.3; Supplementary Table 6.6).

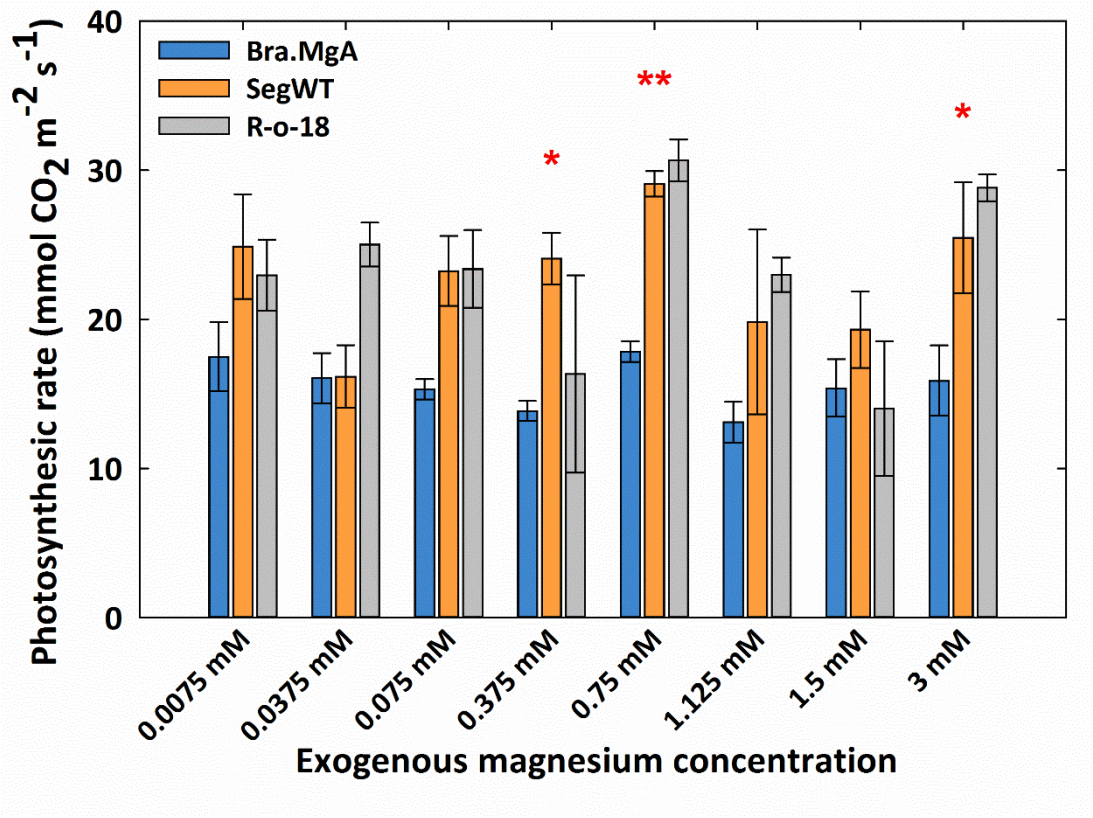


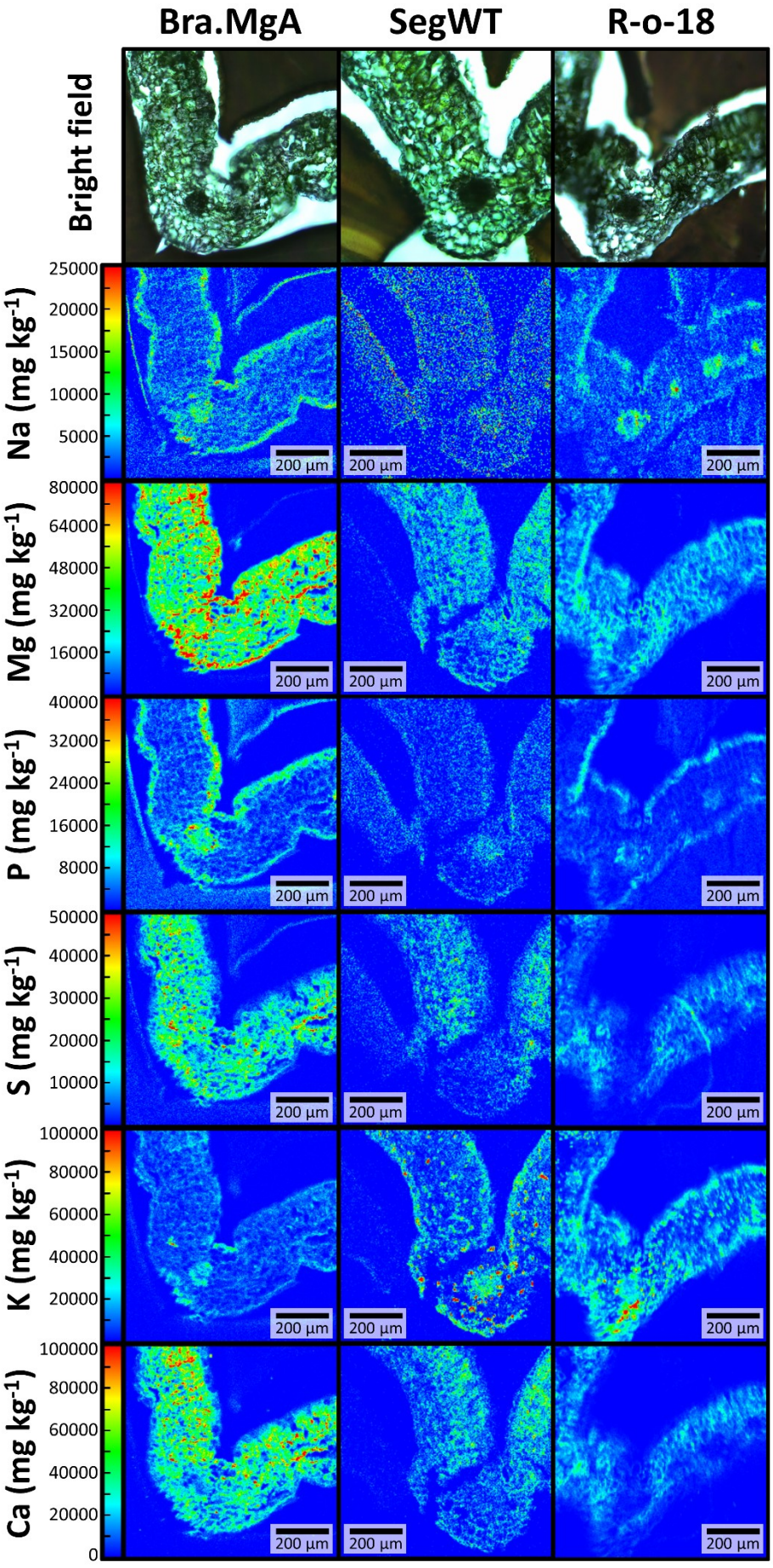
Figure 6.5. Photosynthetic rate measured as rate of photosynthetic carbon assimilation of leaves of 34 day old (post seed sowing, including 21 days under varying exogenous Mg treatments) BC_1F_5 *generation* Brassica rapa *magnesium accumulator (Bra.MgA), out-segregating wild-type (SegWT) and un-mutagenised wild-type (R-o-18) plants at eight different exogenous Mg concentrations (n = 3 for all genotypes at all treatments except Bra.MgA in 1.5 mM exogenous Mg, n = 2). Error bars represent standard error of the mean. Stars above groups represent significant differences in mineral concentrations between Bra.MgA and SegWT plants at p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).*

6.4.4 Unique, tissue specific Na, Mg, P, S and Ca distributions revealed in *Brassica rapa* Mg accumulator plants by micro-PIXE analysis

Following plant sampling for spatial distribution analysis, remaining plants were sampled for bulked leaf ICP-MS analysis to confirm consistent leaf phenotype. Trends in leaf macro-nutrient concentrations were largely identical to those measured previously, with elevated concentrations of Na, Mg, P and S in Bra.MgA compared to wild-type controls, and lower concentrations of K (Supplementary Figure 6.4).

Micro-PIXE spatial distribution mapping and analysis led to the development of quantified numeric concentration matrices. These are represented visually in Figure 6.6, in which each panel represents an 800 x 800 µm segment of a leaf cross-section from either Bra.MgA, SegWT or R-o-18, comprising leaf midrib / major vasculature, and immediately adjacent leaf lamina tissue. A bright field microscopy image of each sample is included for determination of leaf tissue type. Spatial distribution maps reveal perturbations in leaf mineral element distribution in Bra.MgA compared to wild-type controls. Concentrations of Na and P in Bra.MgA appear to be particularly elevated in the upper and lower leaf epidermis, along with elevated concentrations in vascular tissue. Leaf Mg also appears to be elevated in the upper and lower leaf epidermis in Bra.MgA, though there is also an increase in Mg concentration in all leaf tissue types. Likewise, concentrations of S and Ca appear to be greater in all tissue types in Bra.MgA compared to both wild-type controls. Leaf K concentrations appear to be lower in all tissue types in Bra.MgA, though concentrations were particularly lower in the upper and lower epidermis of leaf lamina tissue (Figure 6.6).

[next page] Figure 6.6. Bright field microscopy structure and tissue-level distribution of sodium (Na), magnesium (Mg), phosphorus (P), sulphur (S), potassium (K) and calcium (Ca) in leaf cross sections of 23 day old (post seed-sowing) Brassica rapa *magnesium accumulator (Bra.MgA), out-segregating wild-type (SegWT) and un-mutagenised wild-type (R-o-18) plants as measured through micro-proton induced X-ray emission (micro-PIXE) spectrometry. Images represent 800 µm^2 per cross section.*


Bra.MgA
SegWT
R-o-18
Bright field
Na (mg kg-1)
25000
20000
15000
10000
5000
Mg (mg kg-1)
80000
64000
48000
32000
16000
P (mg kg-1)
40000
32000
24000
16000
8000
S (mg kg-1)
50000
40000
30000
20000
10000
K (mg kg-1)
100000
80000
60000
40000
20000
Ca (mg kg-1)
100000
80000
60000
40000
20000
0
200 µm

Quantified numeric concentration matrices, derived from micro-PIXE analysis of spatial distribution of mineral elements, enabled characterisation of tissue specific mean concentration values. Across all tissue quantified, Na, Mg, P, S, K and Ca were present at mean concentrations of 4310, 39038, 9775, 16442, 15557 and 37129 mg kg^{-1} DW, respectively, in Bra.MgA (n = 3). In SegWT, these were present at mean concentrations of 3064, 10667, 2523, 5197, 20182 and 10769 mg kg^{-1} DW, respectively (n = 2). In R-o-18, these were present at mean concentrations of 3204, 12518, 3233, 5350, 30428 and 12086 mg kg^{-1} DW, respectively (n = 2; Figure 6.7; Supplementary Table 6.7). This corresponds to 266, 287, 216 and 255 % greater mean concentrations of Mg ($p < 0.01$), P ($p < 0.05$), S ($p < 0.001$) and Ca ($p < 0.05$) in Bra.MgA compared to SegWT across all tissue types (Figure 6.7).

Tissue specific effects of genotype were revealed when numeric concentration matrices were split into specific tissue types; upper epidermis, lower epidermis, and mesophyll. Mean Na concentrations were 67 and 71 % greater in Bra.MgA leaf upper ($p < 0.01$) and lower epidermis ($p < 0.01$) tissue, respectively, than in SegWT, though no differences were detected between Na concentrations in leaf mesophyll cells. Mean Mg concentrations were 338, 338 and 230 % greater in Bra.MgA leaf upper ($p < 0.001$) and lower epidermis ($p < 0.01$), and mesophyll ($p < 0.01$) tissue, respectively, than in SegWT. Mean P concentrations were 386 and 352 % greater in Bra.MgA leaf upper ($p < 0.01$) and lower epidermis ($p < 0.05$) tissue, than in SegWT, with no differences detected between P concentrations in leaf mesophyll cells. Mean S concentrations were 258, 252 and 195 % greater in Bra.MgA leaf upper ($p < 0.05$) and lower epidermis ($p < 0.05$), and mesophyll ($p < 0.01$) tissue, respectively, than in SegWT. Mean Ca concentrations were 232, 275 and 242 % greater in Bra.MgA leaf upper ($p < 0.05$) and lower epidermis ($p < 0.05$), and mesophyll ($p < 0.01$) tissue, respectively, than in SegWT. No significant differences were detected in tissue specific K concentrations between genotypes.

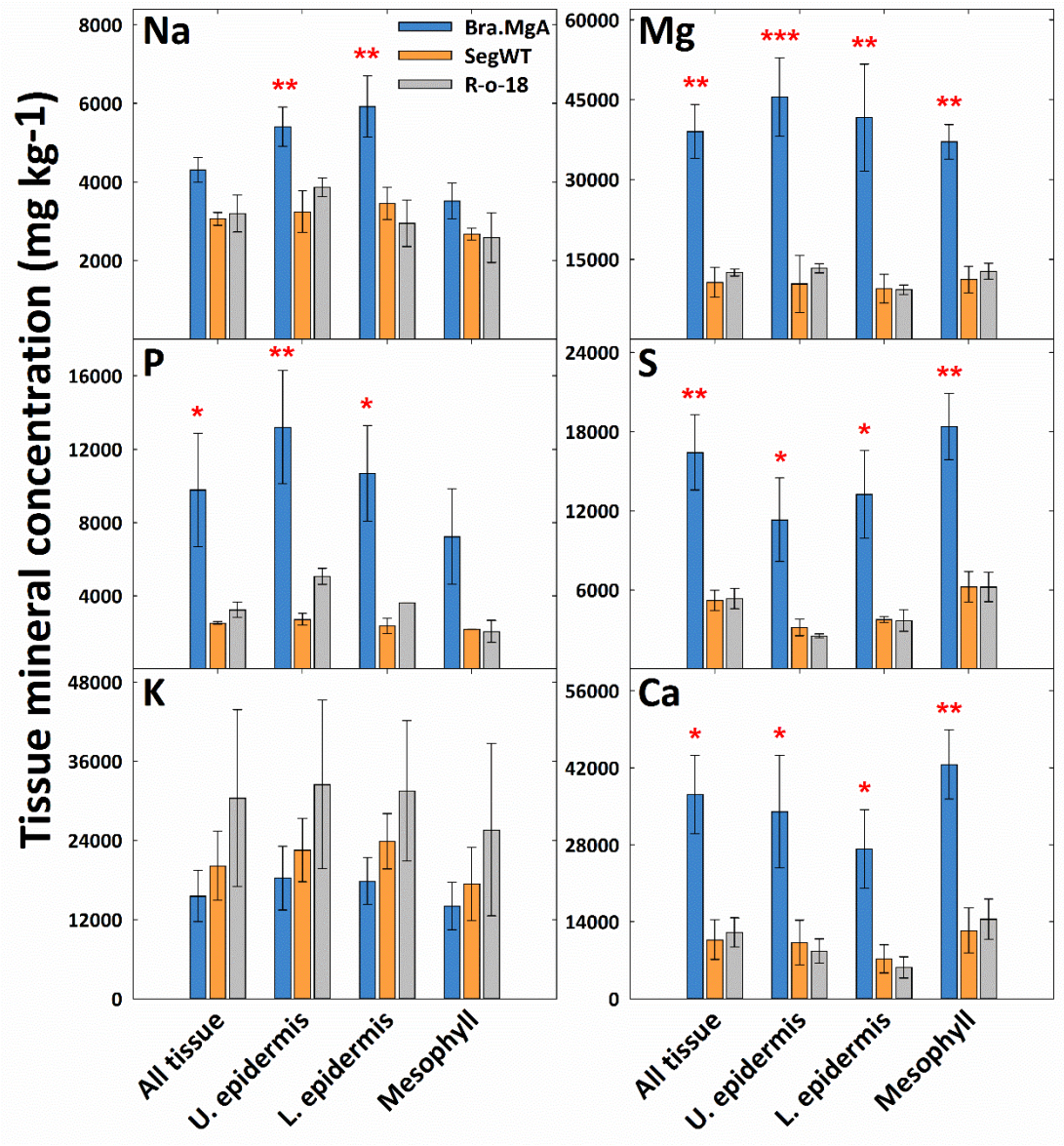


Figure 6.7. Concentrations of sodium (Na), magnesium (Mg), phosphorus (P), sulphur (S), potassium (K) and calcium (Ca) in all tissue, upper epidermis, lower epidermis and mesophyll (excluding vasculature) tissue of leaf cross sections of 23 day old (post seed-sowing) Brassica rapa *magnesium accumulator (Bra.MgA), out-segregating wild-type (SegWT) and un-mutagenised wild-type (R-o-18) plants (n = 3 for each genotype). Measurements performed using micro-proton induced X-ray emission (micro-PIXE) spectrometry, with tissue average concentrations calculated in ImageJ. Error bars represent standard error of the mean. Stars above groups represent significant differences in mineral concentrations between Bra.MgA and SegWT plants at p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).*

6.4.5 Intra- and inter-cell perturbations in Bra.MgA Na, Mg and P spatial distribution revealed through micro-XRF analysis

Semi-quantitative distribution of elements at the cellular level was determined by micro-XRF analysis. Representative maps showing the spatial distribution of Na, Mg and P in Bra.MgA, SegWT and R-o-18 genotypes are presented in Figure 6.8. Each panel represents an 80 x 80 µm segment of a leaf cross-section. For each sample, an X-ray image generated by X-ray absorption and phase contrast imaging is included for cell type identification. Each image features an edge, and is oriented such that this edge and upper epidermal cells are at located towards the top of each panel, with palisade mesophyll cells adjoining below.

In wild-type samples (SegWT, R-o-18), leaf Na was present at largest concentrations in apoplastic regions of both upper epidermal and mesophyll cells. Moderate concentrations of Na were also detected within the cytoplasm of cells. In contrast, much of the Mg in wild-type samples appeared to localise within the cytoplasm of cells in a 'speckled' manner, with lower concentrations observed in apoplastic regions. Concentrations of P in wild-type samples were greatest in the apoplastic regions of cells. Elevated concentrations of Na, Mg and P were observed in Bra.MgA samples compared to both wild-type controls. In Bra.MgA, each of these elements were particularly localised to upper epidermal cells. Greater concentrations of Na, Mg and P were also evident in mesophyll cells of Bra.MgA than in SegWT and R-o-18. However, the majority of this appeared to be within apoplastic regions of mesophyll cells. Within the cytoplasm of mesophyll cells, concentrations of Na, Mg and P were only marginally greater in Bra.MgA than in SegWT and R-o-18 (Figure 6.8).

In SegWT and R-o-18 lines, Mg and P (shown in red and green, respectively; Figure 6.9) generally localised separately in upper epidermal and mesophyll cells. A greater co-localisation of Mg and P was observed in Bra.MgA, as shown in yellow. This was particularly apparent within epidermal cells, and within apoplastic regions of mesophyll cells. Within the cytoplasm of mesophyll cells of Bra.MgA, a lower co-localisation of Mg and P was indicated (Figure 6.9).

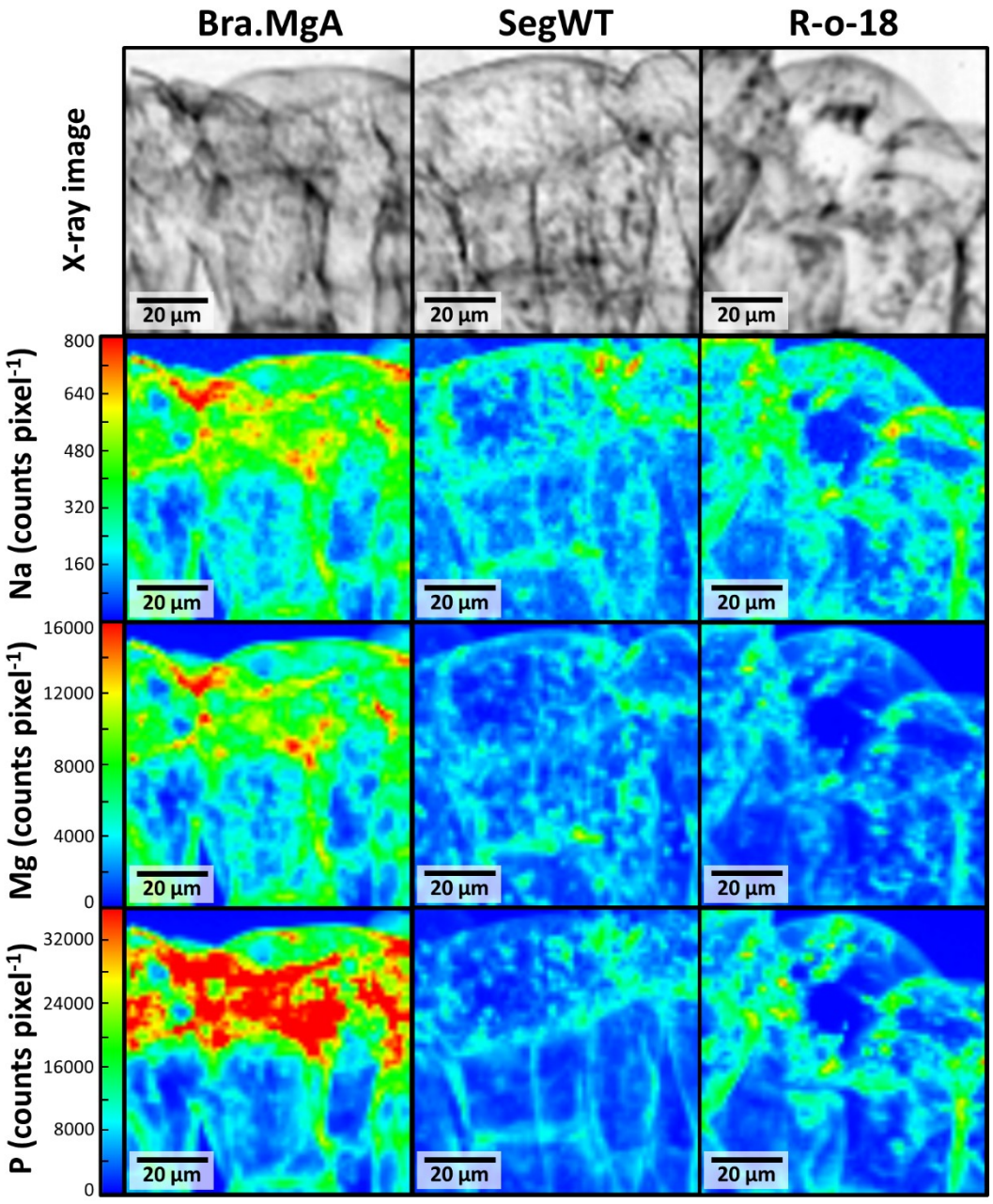


Figure 6.8. X-ray structure and cellular-level distribution of sodium (Na), magnesium (Mg) and phosphorus (P) in leaf cross sections of 23 day old (post seed-sowing) Brassica rapa *magnesium accumulator (Bra.MgA), out-segregating wild-type (SegWT) and un-mutagenised wild-type (R-o-18) plants as measured through micro-X-ray fluorescence (micro-XRF) spectrometry. Images represent 80 µm^2 mapped at 1.2 µm resolution, with the field of view focussed on upper epidermal and palisade mesophyll cells.*

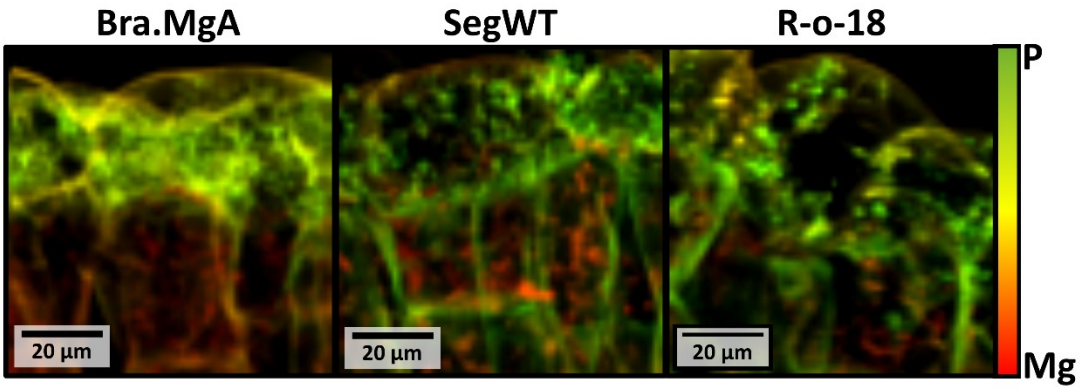


Figure 6.9. Co-localisation of magnesium (Mg) and phosphorus (P) in leaf cross sections of 23 day old (post seed-sowing) Brassica rapa *magnesium accumulator (Bra.MgA), out-segregating wild-type (SegWT) and un-mutagenised wild-type (R-o-18) plants as measured through micro-X-ray fluorescence spectrometry. Images represent 80 µm^2 mapped at 1.2 µm resolution, with the focus on upper epidermal and palisade mesophyll cells. P represented in green colouration, Mg in red, and co-localisation in yellow. Image created using RGB correlator tool in PyMCA.*

6.4.6 Bulked segregant analysis reveals candidate SNPs and genes that may be responsible for elevated leaf Mg concentration trait

Mapping of FastQ files generated through Illumina sequencing revealed 33539 SNPs between Bra.MgA and the R-o-18 reference genome (R-o-18 version 1.2; King GC, personal communication) with combined read depth across samples and phred-scaled genotype posterior probabilities above 50. There were also 43160 SNPs detected between SegWT and the reference genome, and 34375 SNPs detected between R-o-18 sequences generated here and the reference genome. Of these, 5532 SNPs were unique to Bra.MgA, of which 4764 mapped to physical locations on *B. rapa* chromosomes. Particularly high SNP density was observed in distinct regions of the Bra.MgA genome. In a region of chromosome A2 between chromosome positions (measured in nucleotide base pairs) 21080000 & 21100000, 21 SNPs were detected. Thirteen SNPs were also detected between positions 13220000 & 13240000. On chromosome A3, 12 and 15 SNPs were detected between positions 13840000 & 13860000, and 23740000 & 23760000, respectively. On chromosome A5, the three regions between positions 2040000 & 2060000, 13540000 & 13560000 and 25280000 & 25300000 had SNP counts of 15, 15 and 17, respectively. The regions of chromosome A9 between positions 14640000 & 14660000 and 31520000 & 31540000 had SNP counts of 12 and 15, respectively. Finally, on chromosome A10, 13 SNPs were detected between positions 620000 & 640000, and a total of 59 SNPs were detected within a larger, high SNP-density region between positions 7580000 & 8740000 (Figure 6.10).

A total of 891 unique SNPs mapped to coding DNA sequences of genes, which corresponds to 2234 locations in the published, annotated *B. rapa* genome (Chiifu-401-42; (Wang et al., 2011) due to sequence homology between paralogous genes. By investigating gene function annotations of *Arabidopsis thaliana* orthologues, 14 candidate genes were identified, spanning nine *B. rapa* chromosomes (Table 6.1). These include a number of known nutrient transporter genes, as well as transcription factors that may be influencing the observed high Mg trait through altering expression of downstream genes.

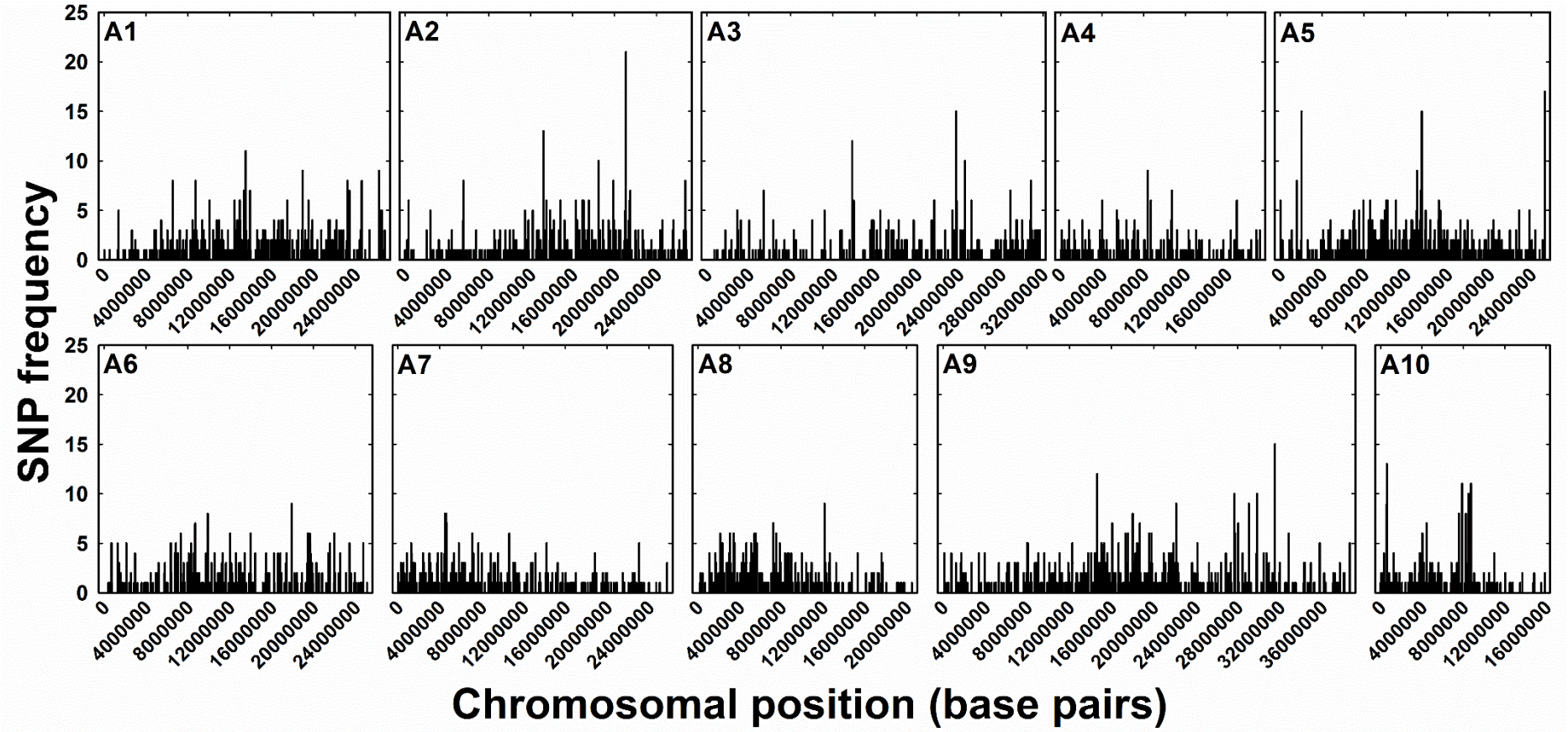


Figure 6.10. Distribution of SNPs unique to the Brassica rapa *magnesium accumulator based on frequency per 20,000 nucleotide base pairs. Panels represent individual chromosomes, scaled according to chromosome size in base pairs (Wang et al., 2011).*

Table 6.1. List of candidate genes for high leaf Mg concentration trait identified through bulked segregant analysis. All candidate genes have a least one SNP within coding sequences in Bra.MgA.

Position on R-o-18 genome	***Arabidopsis* ID**	***Brassica rapa* (chiifu) orthologue gene ID**	**Description**
A01: 22847369-22851907	AT3G15990	Bra027197,Bra021152,Bra019289,Bra001617	SULTR3;4 sulfate transporter
A02: 21098330-21098554	N/A	Bra033137	Paralogue of Bra030355 (AT5G39210) CHLORORESPIRATORY REDUCTION 7
A02: 25600727-25602199	AT5G62160	Bra029282	ZIP12 cation transporter
A03: 6601560-6614625	AT5G51870	Bra029154,Bra028283,Bra029155,Bra028282	MADS-box protein (AGL72)
A03: 11808986-11821408	AT2G24690	Bra000563	DNA binding / transcription factor
A04: 7444040-7451920	AT5G39460	Bra028250,Bra028249,Bra028247,Bra028246	F-box family protein
A06: 17936889-17942210	AT5G24810	Bra020472,Bra009794	ABC1 family protein
A07: 7136193-7138497	AT1G33720	Bra039329,Bra039328,Bra014953,Bra014462,Bra014461	Cytochrome P450 family 76
A07: 22033849-22051913	AT1G70300	Bra023490,Bra016225	KUP6 potassium transporter
A08: 12212728-12228190	AT4G32730	Bra037045,Bra034531,Bra011376	MYB3R1 transcription factor
A08: 19264329-19278740	AT1G11950	Bra034494,Bra027055,Bra016801	Transcription factor
A09: 25890237-25897761	AT1G22550	Bra008009,Bra003898,Bra024520,Bra016375,Bra012334, Bra011003	NPF5.16 nitrogen transporter
A10: 1456393-1467923	AT2G32270	Bra021776,Bra015415,Bra005579	ZIP3 zinc transporter
scaffold2313432: 156022-166836	AT3G13080	Bra039348,Bra039369,Bra039368,Bra034706,Bra001490, Bra001488	ATMRP3 chlorophyll catabolite transporter

6.5 Discussion

6.5.1 Leaf mineral concentrations measured here are consistent with those observed in previous generations of the Bra.MgA genotype

Brassica rapa Mg accumulator (Bra.MgA) plants of the BC_1F_4 generation (Figure 6.1) had elevated leaf concentrations of Na, Mg, P and S compared to out-segregating wild-type (SegWT) and un-mutagenised wild-type (R-o-18) lines. The largest increase was observed for leaf Mg, which were present at 81 % higher concentrations in Bra.MgA than in SegWT. Of the plant macro-nutrients, concentrations of K and Ca were also lower in Bra.MgA compared to both wild-type lines. In previously analysed plants of the BC_1F_3 generation, similar perturbations in leaf Na, Mg, P, S and K concentrations were observed (Thomas, 2017). However, the percent elevation in leaf Na, Mg, P and S concentration in Bra.MgA compared to SegWT observed here was lower than that measured previously. As well as this, the percent difference in leaf K concentration between Bra.MgA and SegWT was greater in BC_1F_4 plants measured here. Intriguingly, there was a different effect observed for leaf Ca concentration, which was lower in Bra.MgA compared to SegWT in the present study, but greater in BC_1F_3 plants measured previously (Thomas, 2017). The discrepancies in leaf mineral concentrations apparent between BC_1F_4 plants characterised here and BC_1F_3 plants measured previously are likely due to developmental stage of the tissue sampled for analysis. Tissue taken in this study was from sixth leaves of 45 day old plants, whereas those analysed previously were of younger, fourth leaves. First and eighth leaves of BC_1F_3 plants were also characterised in the study of Thomas (2017), and the percent difference in Mg concentration between Bra.MgA and SegWT plants was far greater in the former leaves. It is thus evident that the high Mg trait of Bra.MgA is more prevalent earlier in plant development. Despite this, is remains throughout developmental stages, and has been shown to be consistent between generations.

The mean leaf Mg concentration of BC_1F_4 generation plants measured here was 34,293 mg kg^{-1}, which corresponds to over 3.4 % leaf matter on a dry weight basis.

Whilst higher than concentrations observed in wild-type plants of the same ancestry, this is also far higher than Mg concentrations observed previously in a wide range of crop types, as well as within large populations of more 'natural' *Brassica* germplasm. For instance, the highest mean shoot Mg concentration of 117 angiosperm species representing 24 orders grown hydroponically was only 0.92 % DM (Broadley et al., 2004). Similarly, in a study that investigated the phylogenetic effects on shoot Mg concentration specifically, the highest shoot Mg concentration measured was 1.95 % DW, observed in *Hereroa ordorata*, of the order Caryophyllales (White et al., 2015). In a diversity population of 383 genotypes of *B. napus* grown in compost, the highest leaf Mg concentration observed was 13,415 mg kg^{-1} DW, or around 1.3 % of dry leaf matter (Thomas et al., 2016; chapter 2). In a population of 429 *B. oleracea* genotypes grown in compost, the highest shoot magnesium concentration observed between varieties was 0.80 % DW (Broadley et al., 2008). Hence, it is clear that the > 3.4 % DM Mg concentration observed in the *B. rapa* genotype analysed here (Bra.MgA) is incredibly large compared to other species and *Brassica* varieties. It is also well above the 50 % target increase in Mg concentration reported to be required to bring 2 million people in the UK alone above the LRNI (Broadley and White, 2010). This indicates great potential for this trait to be made use of in genetic biofortification strategies to reduce widespread human Mg deficiency risks, providing it can be bred into suitable crop cultivars.

A potential disadvantage of the elevated leaf Mg concentration observed in Bra.MgA plants is an associated decrease in leaf concentrations of K. Interestingly, previous analysis of mineral concentration in BC_1F_3 generation plants indicated elevated concentrations of K in roots of Bra.MgA compared to wild-type plants (Thomas, 2017). A negative relationship between greater exogenous K concentration and Mg uptake efficiency have been previously reported in rice (Ding et al., 2006) and maize (Jakobsen, 1993). Similarly, increasing exogenous Mg concentration was previously shown to have antagonistic effects on the uptake of both K and Ca in groundnut (Fageria, 1974). These effects may be linked to competition between K^+ and Mg^{2+} for active sites in plasma membrane bound transport proteins. Whilst it may be expected that this effect is most prevalent under unbalanced ratios of K to Mg in the soil, it

may be reasonable to assume that an increased selection for Mg translocation into shoot tissue could negatively impact on K translocation. However, further work will be required to elucidate the mechanisms controlling this.

6.5.2 Response to exogenous Mg supply suggests Bra.MgA may be amenable to agronomic biofortification strategies

Agronomic biofortification strategies aim to increase concentrations of minerals available for plant uptake through fertilisation or improvement of solubilisation and mobilisation of soil minerals (White and Broadley, 2009). However, such strategies would benefit from selection of crops that respond well to application of the mineral being applied, such that they uptake greater quantities as exogenous supply increases and mobilise it to the target tissue effectively. In the Mg response experiment performed here, all genotypes responded to increased exogenous Mg supply with increased shoot Mg accumulation. This is consistent with effects observed previously in wild-type *B. rapa* plants grown under varying exogenous Ca and Mg supply (Rios et al., 2012). However, a particularly large response was observed in Bra.MgA plants, which accumulated 216 % more leaf Mg than Seg-WT plants at the highest exogenous Mg concentration treatment (3 mM). Therefore, this trait may prove useful in a combination of genetic and agronomic biofortification strategies; crossing the trait into suitable field cultivars will provide appropriate germplasm for increasing Mg supplies, and applying Mg mineral fertilisers will allow even greater concentrations to be effectively taken up into edible crop tissue. The negative correlation between exogenous Mg supply and leaf K and Ca concentration in Bra.MgA is consistent with the antagonistic effects between these mineral nutrients discussed above. However, the correlations were only very weak, and thus potential application of Mg fertiliser on crop plants with this trait are unlikely to exhibit negative impacts on productivity or nutrition.

6.5.3 Reduced photosynthetic rate of Bra.MgA indicates poor use efficiency of increased leaf Mg supplies

Relative chlorophyll content of Bra.MgA and wild-type plants was previously analysed in BC_1F_3 plants through spectral absorbance using a SPAD-502Plus meter (Konica

Minolta, Tokyo, Japan; Thomas, 2017). There was approximately 20 % less chlorophyll in leaves of Bra.MgA compared to SegWT. The SPAD meter gives only an indirect measure of relative chlorophyll content, based on the amount of light transmitted by the leaf in two wavelength regions in which the absorbance of chlorophyll is different. Because of this observation, it was decided to investigate photosynthetic ability of Bra.MgA plants further in this study through infrared gas analysis. Photosynthetic rate, measured as rate of photosynthetic carbon assimilation, was lower in Bra.MgA than wild-type plants under all exogenous Mg conditions studied. Whilst significant differences in photosynthetic carbon assimilation between genotypes were only observed at three exogenous Mg concentrations (0.375, 0.75 and 3 mM), only three replicate samples were typically included per genotype per treatment, and it is likely that a greater number of replicates would have revealed an even greater genotypic effect. Magnesium plays a key role in photosynthesis in plants. It forms the central atom of chlorophyll, and around 10 % of the total Mg content of mature leaves can be found here (Wilkinson et al., 1990). Thus, it might be expected that a greater leaf concentration of Mg would indicate an overall greater number of chlorophyll molecules and therefore a greater photosynthetic rate. That a lower photosynthetic rate was observed in Bra.MgA than wild-type plants despite much greater leaf Mg concentrations in Bra.MgA, suggests that the elevated Mg supply is not being used effectively by the plants. At the very least, it appears that Bra.MgA plants produce less chlorophyll, and work may be required to uncouple the high leaf Mg trait from this in order for it to be amenable for the production of high yielding, high shoot Mg crop cultivars.

6.5.4 Spatial distribution analysis reveals trends in tissue-specific mineral localisation in Bra.MgA

The spatial distribution of minerals between different tissue types revealed trends in mineral localisation in both wild-type and Bra.MgA plants. Based on quantified concentration data from micro-PIXE analysis, concentrations of Na in wild-type plants were consistent between epidermal and mesophyll cells, though spatial distribution maps (Figure 6.6) suggest a potential preference for Na localisation in vasculature tissue. However, a different distribution was observed in Bra.MgA plants, in which

greater concentrations of Na were observed in epidermal tissue than in the mesophyll. Interestingly, some halophytic species appear to preferentially accumulate Na in epidermal cells (Pongrac et al., 2013). Salt stress is a major factor affecting plant productivity by disturbing cellular osmotic balance and preventing water uptake (Türkan and Demiral, 2009). Exclusion of Na from photosynthetically active tissues, particularly the mesophyll, appears to be a common mechanism of salt tolerance in plants (Pongrac et al., 2013). Bra.MgA plants accumulate greater leaf concentrations of Na than wild-type plants, as evident from initial bulked sample mineral analysis. It is hence likely that the elevated Na concentration observed in epidermal cells is caused by the plant preferentially avoiding mesophyll accumulation.

Whilst there was a huge increase in leaf Mg concentration in Bra.MgA compared to both SegWT and R-o-18 plants, distribution appeared not to be particularly affected at the tissue level, with similar concentrations observed between mesophyll and epidermal cell types in all three genotypes. Previous analysis of the distribution of Ca and Mg between epidermal and mesophyll cell types in *B. rapa* indicated a similar distribution of Mg between cell types in plants grown under a relatively low exogenous Mg supply (Rios et al., 2012). However, at higher exogenous Mg supply, an overall increase in leaf Mg concentration was associated with particularly elevated concentrations in mesophyll cell types. This is in contrast to results observed here, in which the elevated leaf Mg concentration observed in Bra.MgA plants appears to be evenly distributed between cell types.

Concentrations of P, S and Ca appear to be elevated in Bra.MgA compared to wild-type plants based on micro-PIXE quantified concentration data. The differences observed here are much greater than those measured through ICP-MS analysis of bulked leaf samples of BC_1F_4 generation plants, and of BC_1F_5 plants grown in parallel to those sampled for spatial distribution analysis (Supplementary Figure 6.4). This could be due to an artefact of the micro-PIXE method, in which quantified data is inferred from sample thickness and proton dose, rather than more direct measurements of mineral concentration obtained from ICP-MS analysis.

Alternatively, ICP-MS analysis of bulked leaf samples may have masked differences specific to leaf apex tissue, where samples for spatial distribution analysis were taken from. Despite this discrepancy, further trends in mineral distribution between tissue types can be deduced. The distribution of both S and Ca appear to be similar those observed in wild-type leaves. However, based on spatial distribution maps, there appear to be particularly elevated concentrations of P in upper and lower epidermal tissue, as well as in the vascular tissue. This effect can also be observed, to a lesser degree, in R-o-18 plants, and is consistent with the greater P concentrations typically observed in epidermal cells of dicot plants grown under P sufficient conditions (Guilherme Pereira et al., 2018). The mechanism controlling the greater effect of genotype on P distribution in Bra.MgA, and the apparent co-localisation of P with Na is as of yet unknown.

Mean concentrations of K were lower in leaves of Bra.MgA than wild-type plants in all tissue types measured, though extensive variation within genotypes masked any statistically significant differences. However, based on spatial distribution maps, a perturbation in K distribution in Bra.MgA can be discerned. Wild-type plants appear to preferentially accumulate K in epidermal cells and vascular tissue. However, whilst K concentrations appear lower in all tissue types in Bra.MgA plants, particularly lower concentrations can be observed in upper and to a lesser degree lower epidermal cells. This is an opposite effect to that observed for Na and P distribution in leaves of Bra.MgA, and may suggest potential interactions between these elements in this genotype.

The use of micro-PIXE analysis to map and quantify the distribution of mineral elements between plant tissues here has revealed new patterns unique to Bra.MgA. However, further work will be required to dissect the molecular and genetic bases of the perturbations in leaf mineral distribution described above. This method has proven to be particularly efficacious in discerning differences in mineral concentrations which are only present between specific tissue types that may be masked in bulked leaf sample analyses. Micro-PIXE was especially useful for identifying differences in Na concentration between genotypes, for which only very

small differences were apparent between bulked leaf samples, whereas more notable differences were observed when particular tissue types were studied.

6.5.5 Intra- and inter-cellular distribution of Na, Mg and P indicates potential physiological basis of observed perturbations in leaf mineral concentrations

Cellular level spatial distribution analysis indicated that the elevated concentrations of Mg in Bra.MgA compared to wild-type plants were largely localised to upper epidermal cells. Interestingly, the symplast of Bra.MgA mesophyll cells did not appear to have particularly greater concentrations of Mg than wild-type plants. This contradicts previous work in *Arabidopsis thaliana* in which it was shown that excess Mg is stored in vacuoles of mesophyll cells, especially under greater exogenous supply (Conn et al., 2011). This also appears to contradict results from tissue level spatial distribution analysis, which suggested a fairly even distribution of Mg across leaf tissues. However, this may instead reveal new information regarding distribution patterns in Bra.MgA. Specifically, is seems apparent that whilst Mg is localised across all tissue types in leaves of Bra.MgA, in mesophyll regions, the excess Mg supply either lies in inter-cellular spaces, or within apoplastic regions of the cell, rather than entering the cytoplasm. This was reflected by micro-XRF analysis, which indicated greater concentrations of Mg around cell wall regions of mesophyll cells in Bra.MgA plants. Similar patterns were also observed in the distribution of Na and P, which were also present at elevated concentrations in epidermal cells of Bra.MgA than wild-type plants. This was consistent with results from micro-PIXE analysis. There was also a particularly apparent co-localisation of Mg with P in Bra.MgA plants. This could be indicative of elevated concentrations of compounds comprising both Mg and P such as phytate, which can form stable complexes with mineral cations including Mg (Raboy, 2001). However, further work will be required to elucidate the forms in which these elements coexist.

That the elevated Mg concentration in leaves of Bra.MgA is not found within the cytoplasm of mesophyll cells suggests a potential mode of action for the observed perturbations in mineral concentrations. It is possible that the EMS dose applied to ancestral plants led to a faulty, plasma-membrane bound Mg transporter that is thus

preventing efficient uptake of Mg into mesophyll cells. This would also explain the lower photosynthetic rate measured in Bra.MgA plants compared to wild-types; if less Mg can efficiently enter the mesophyll cells, it stands to reason that less chlorophyll can be produced, and photosynthesis will be affected. Control of plant magnesium transport is dominated by members of the MAGNESIUM TRANSPORTER (MGT) family of proteins, some of which, including MGT1 and MGT3, are localised to the plasma membrane (Waters, 2011). If a protein controlling Mg import into mesophyll cells is faulty, the resulting lower cellular concentration of Mg may lead to an apparent deficiency response, whereby processes are initiated within the plant to import greater concentrations of Mg from the soil and translocate it to leaf tissue. When in the leaf tissue, it is then reasonable to assume that the elevated concentrations of Mg will localise to tissue that they can occupy, including epidermal cells and inter-cellular spaces. This could also theoretically lead to an increase in the concentrations of numerous other nutrients in shoot tissue through mass flow of nutrients through the xylem (White, 2012). A change in the cation balance caused by elevated concentrations of Mg could also affect the distribution of elements within leaf tissue, as observed in experiments performed here.

6.5.6 Progress made towards dissecting the genetic basis of the high Mg trait through fine mapping of bulked segregant DNA sequences

Bulked segregant analysis works on the principle that DNA samples extracted from tissue from a number of separate organisms, in this case individual plants of Bra.MgA, SegWT and R-o-18, contain various polymorphisms between their genomes. It also assumes that the genetic basis of a heritable trait exhibited by all in one group will be conserved between all in that group. By selecting two or more groups with opposing, heritable traits, and pooling DNA of all individuals into the individual groups prior to marker analysis, any polymorphisms that are not associated with the trait of interest will likely differ between individuals within a group, and hence have increased heterozygosity at that loci. This, therefore, should allow a massive reduction in the number of candidate polymorphisms for a given trait following marker analysis, through filtering of polymorphisms with a greater heterozygosity index (Zou et al., 2016).

Between bulked DNA samples from Bra.MgA and the R-o-18 reference genome, after filtering based on combined read depth (DP) across samples and phred-scaled genotype posterior probabilities (GP), there were 33539 SNPs. This was far greater than the number of SNPs expected between this genotype and the reference genome. Previous analysis of SNP density in the *B. rapa* TILLING population from which Bra.MgA is derived estimated that each genotype should contain approximately 10,000 point mutations in the M_2 generation (Stephenson et al., 2010). Since Bra.MgA has been through a backcross with the wild-type background, even fewer than this number of SNPs should be expected in the generation that was sequenced, even taking into account variation between segregants in each bulked group. That there was a far greater number of SNPs identified in this genotype, even following filtering based on DP and GP is indicative of a potential issue with the original BAM/SAM files obtained following sequencing, or in the manner that they were handled subsequently. Indeed, there were also 34,375 SNPs called between R-o-18 DNA samples sequenced as part of this study and the R-o-18 reference. Whilst the R-o-18 accession utilised in this study has likely diverged to some degree from the reference accession by genetic drift, this identification of this many SNPs between the two is largely unreasonable.

Further mapping work will be required in order to elucidate the specific, likely processing issue leading to the overly high number of SNP calls mentioned above. Further quality checking of initial sequence files (BAM/SAM) will be the first necessary step, after which proper mapping should be possible. However, despite the further work required in order to fully analyse the bulk segregant DNA sequence data, progress has been made towards identifying candidate loci for controlling the high shoot Mg trait. For instance, after accounting for SNPs that were not unique to Bra.MgA, the number of SNPs called that could possibly control the trait was reduced to only 5532 of which 4764 mapped to physical locations on *B. rapa* chromosomes. Whilst still a considerable number of candidate SNPs remain, this amounts to significantly fewer than the ~270,000 SNPs identified previously (Thomas, 2017) prior to filtering based on DP and GP. There were also several regions of high SNP

frequency identified. Due to recombination patterns, it is likely that the presumed, causative SNP will fall within a highly SNP dense region, as further SNPs that remain from the initial EMS dose within linkage disequilibrium of the causative SNP would be inherited with it. Hence, these regions are of particular interest as candidate loci for control of the high Mg trait.

By considering genes containing SNPs unique to Bra.MgA, a total of 14 candidate genes were identified. There are limitations to this method, largely based around the assumption that the causative SNP lies within a coding sequence of a gene. Alternatively, it could lie within a promotor sequence of a causative gene, which is responsible for a change in gene expression. Further work will be required to look into this possibility. However, a number of convincing candidates were identified from this initial attempt to identify the causative gene. Perhaps the most convincing candidate is the sulphate transporter SULTR3;4. There were three SNPs detected in this gene that were unique to Bra.MgA. These map to four separate, paralogous genes on the published *B. rapa* genome (Wang et al., 2011), due to significant sequence homology. However, they all share a common orthologue in *A. thaliana* (AT3G15990). Mutants in this gene, based on *in silico* analysis of data obtained from the Ionomics Hub (Baxter et al., 2007), have perturbed ionomic profiles, and these are not dissimilar to the perturbations in Bra.MgA observed here. Specifically, one knockout line (SALK_068355_homo) had elevated shoot concentrations of Na and Mg compared to the background line, along with lower concentrations of K. Data at ePlant (Waese et al., 2017) indicates that this gene is also plasma membrane expressed, and is expressed in a range of tissue types in both the root and shoot. Hence, this is a highly convincing candidate for the control of the high Mg trait characterised here.

A number of other nutrient transport protein encoding genes were also identified as potential candidates, including two zinc transporters, a potassium transporter, and a nitrogen transporter. These are also worthy of further consideration. Several transcription factors were also identified; these may be responsible for downstream regulation of expression of further genes that are responsible for the high Mg trait.

Most of these are currently poorly characterised, and as such, their function within plants remains elusive. An exception is MYB3R1, which has shown to be critical for proper root and shoot development through cell cycle control (Wang et al., 2018). Whilst it is unclear how this might link to perturbations in mineral concentrations specifically, this transcription factor is clearly of high importance to plant development. Since it contains a unique SNP in Bra.MgA, it therefore remains of interest. A further candidate is Bra033137. This particular gene doesn't have an obvious orthologue in *A. thaliana*, or indeed any species outside of *Brassica*, and hence inference of possible function is difficult. Interestingly though, a paralogous gene within *Brassica rapa* is orthologous to Arabidopsis thaliana AT5G39210, which is required for assembly chloroplast NDH, and hence may be involved in chlororespiration (Kamruzzaman Munshi et al., 2005). Photosynthetic gas exchange measurements taken here indicated a lower photosynthetic rate in Bra.MgA compared to wild-type plants. It is therefore possible that a SNP in Bra033137 is leading to this difference. This contrasts with the mode of action hypothesised above, in which the cause of the lower photosynthetic rate was suggested to be a lower intracellular Mg concentration which prevented sufficient chlorophyll production. If correct, this new hypothesis could be potentially beneficial, as it may suggest that it is possible to uncouple the high leaf Mg trait from a lower photosynthetic rate. However, whilst it is currently unclear as to which of the above hypotheses is most accurate, the former hypothesis seems most likely at this time.

Characterisation of allelic variants of the candidate genes described above will be required to confirm gene function in *B. rapa*, and to elucidate their possible role in the high Mg trait. Currently, there are a reasonable number of candidate genes, but a much larger number of genes containing unique SNPs in Bra.MgA. Hence, this may not be the most efficient next step. Ancestral plants of Bra.MgA were subjected to very high levels of EMS (Stephenson et al., 2010) and this led to a high background mutation load. Only single backcross to un-mutagenised *B. rapa* plants has been performed so far, and hence there has been little opportunity for this mutation load to be reduced. Further backcrosses, followed by several generations of self-fertilisation to induce widespread genomic homozygosity, will likely lead to the

development of plants for which the bulked segregant approach is more suited. This will take time, but will likely be worth it in the long run, as it should allow more accurate identification of the genetic basis of this highly interesting and potentially very useful trait.

6.5.7 Next steps: breeding viable, high magnesium *Brassica* cultivars

A crucial next step towards developing novel, high Mg germplasm suitable for cultivation is the initiation of a programme to introgress the high magnesium trait of Bra.MgA into suitable, high performing, *Brassica* field varieties. Particular focus should be on the generation of vegetable crops, which are likely to be excellent vehicles for increasing human dietary magnesium intakes. Within the *B. rapa* species are numerous vegetable crop types ranging from leafy vegetables including pak choi, napa cabbage, and rapini, to root vegetables including turnip. Elevated Mg concentrations have been observed in both shoot and root tissue of Bra.MgA plants, as shown here and previously (Thomas, 2017). Hence, the development of both root and leafy vegetable types with elevated Mg concentrations should be possible. There is also the opportunity to generate novel germplasm of closely related *B. napus*, which contains a full set of diploid chromosomes from a *B. rapa* ancestor, as well as from *B. oleracea* (Chalhoub et al., 2014). *Brassica napus* comprises the two major crop types oilseed rape and swede. Oilseed rape is the most widely grown oil crop in Europe (Eurostat, 2017), and is the third largest source of vegetable oil globally (USDA, 2018). Whilst further work is required to deduce possible elevated Mg concentrations in seed oil, this is a further potential application of the high Mg trait, which could lead to a higher value oil crop. Swede has a further application; whilst root portions of the crop are prepared for human consumption, the remaining plant tissue is commonly used for livestock feed. Magnesium deficiencies are common in grazing livestock (Grunes and Welch, 1989). This can lead to a condition known as ‘grass tetany’, which can progress to violent convulsions, coma and death if not treated rapidly (McCaughan, 1992). Introgression of the Mg hyper-accumulator trait into swede could hence benefit both human and animal nutrition through increasing dietary Mg intakes.

It is likely that a better understanding of the genetic basis of the high Mg trait could lead to faster generation of suitable, high Mg crop cultivars. However, the Mg concentrations observed in all homozygous and segregating Bra.MgA generations to date have been sufficiently higher than in wild-type plants to make correct identification of successful introgressions easy. Hence, introgression of the high Mg trait should be possible in the absence of molecular markers. Perhaps of greater importance, is understanding the basis of the potentially negative effects associated with the high Mg trait, including lower shoot K concentration, and lower photosynthetic rate. Identifying gene/s controlling all traits identified in Bra.MgA to date may allow the uncoupling of positive from negative traits, and eventually lead to the development of crop cultivars with even better potential to reduce Mg deficiencies in human and animal populations.

6.6 Summary and perspective

Work performed here has made significant progress towards understanding the physiological basis of a mutation conferring up to ~200 % greater shoot Mg concentrations in *Brassica rapa*. Of particular importance are the insights into genotypic effects on element specific partitioning identified through spatial distribution mapping. This has led to the development of novel hypotheses regarding the mode of action of a number of perturbed traits observed in the *B. rapa* Mg accumulator. Specifically, it seems likely that the causative mutation has led to a faulty, plasma membrane bound transporter that is negatively affecting Mg import into mesophyll cells. Progress has also been made towards dissecting the genetic basis of this trait, including the identification of SNPs that fall within genes. Among these genes are several that encode plasma membrane bound transporters which are worthy of further consideration. The perturbations observed in Bra.MgA offer a fantastic opportunity to study mechanisms controlling Mg uptake and distribution in plants on a fundamental level. Despite the importance of Mg in plants, knowledge regarding its homeostasis lags behind that of other mineral elements (Waters, 2011). Work performed here has also brought us closer to the realisation of crop cultivars with elevated concentrations of Mg in edible tissue. Developing such cultivars has the potential to reduce widespread human and animal Mg undernutrition, and to reduce deficiency risks. Hence, from here, efforts to introgress the high Mg trait into suitable crop cultivars should be made a priority.

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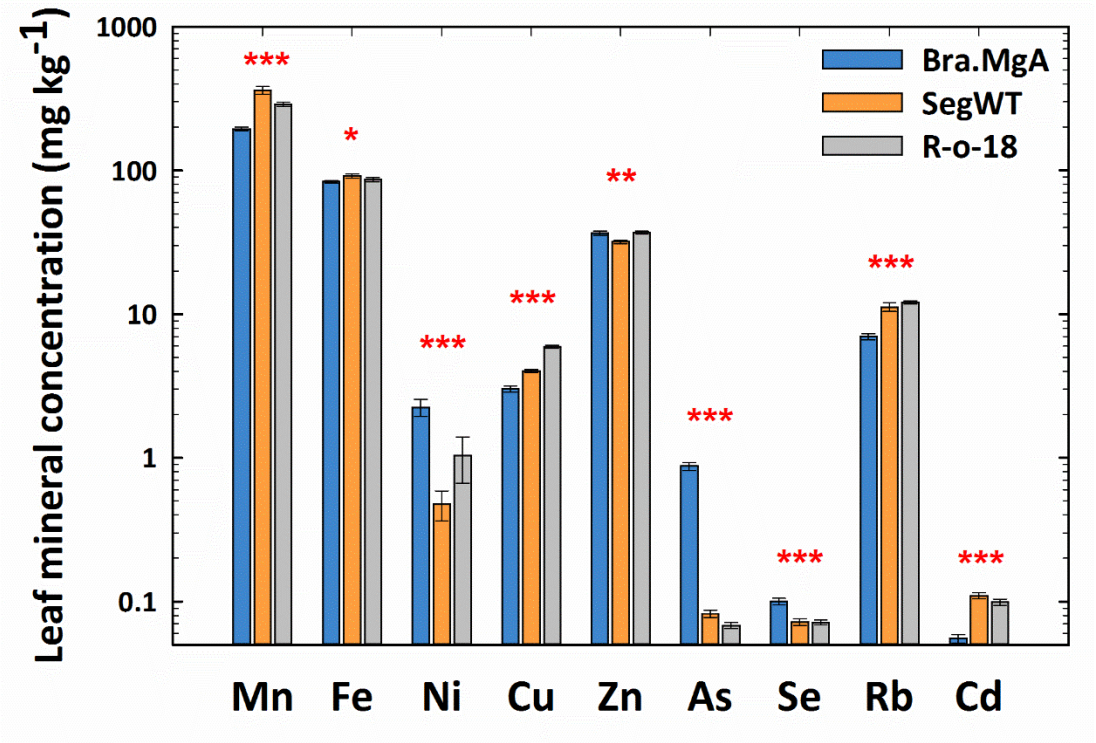
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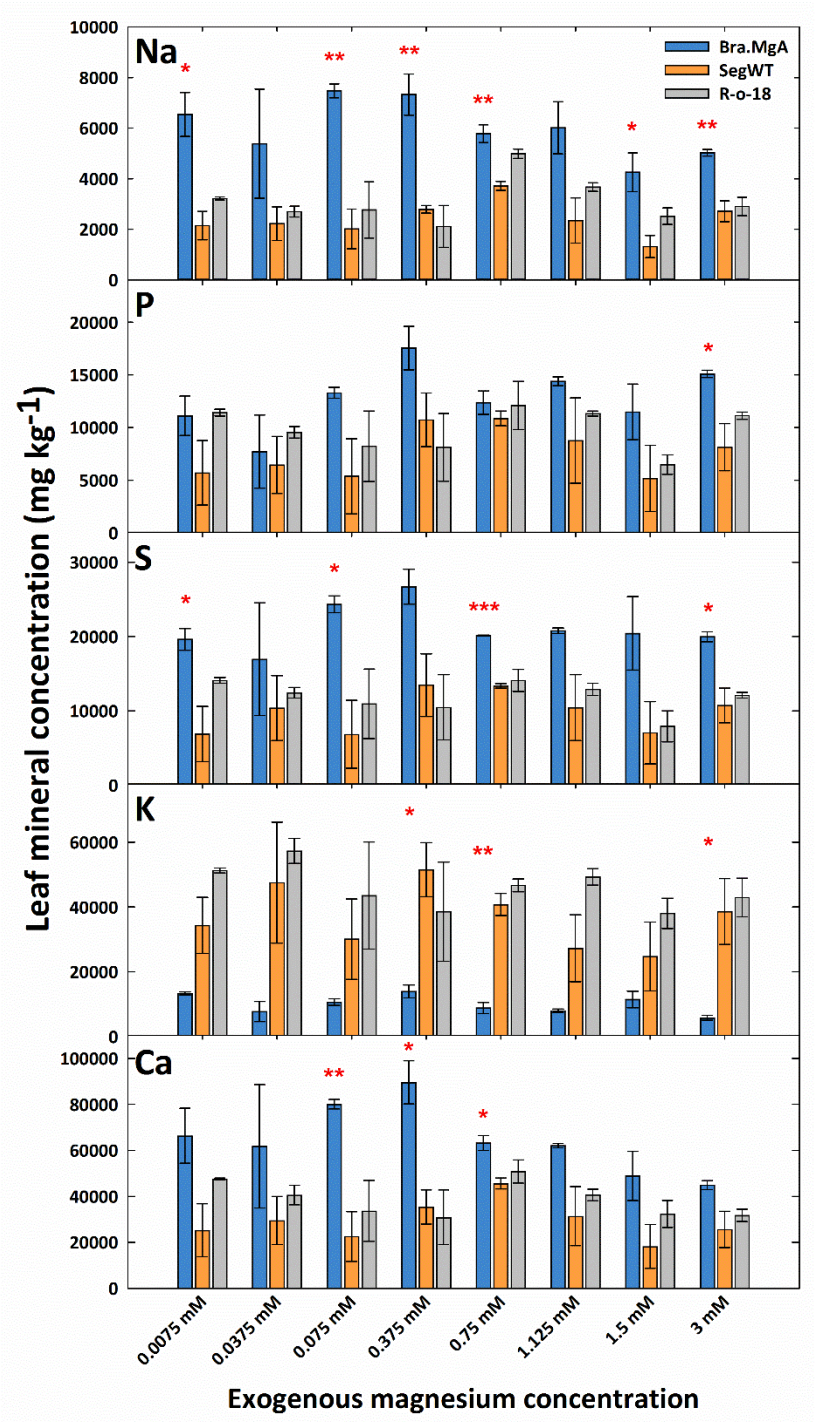
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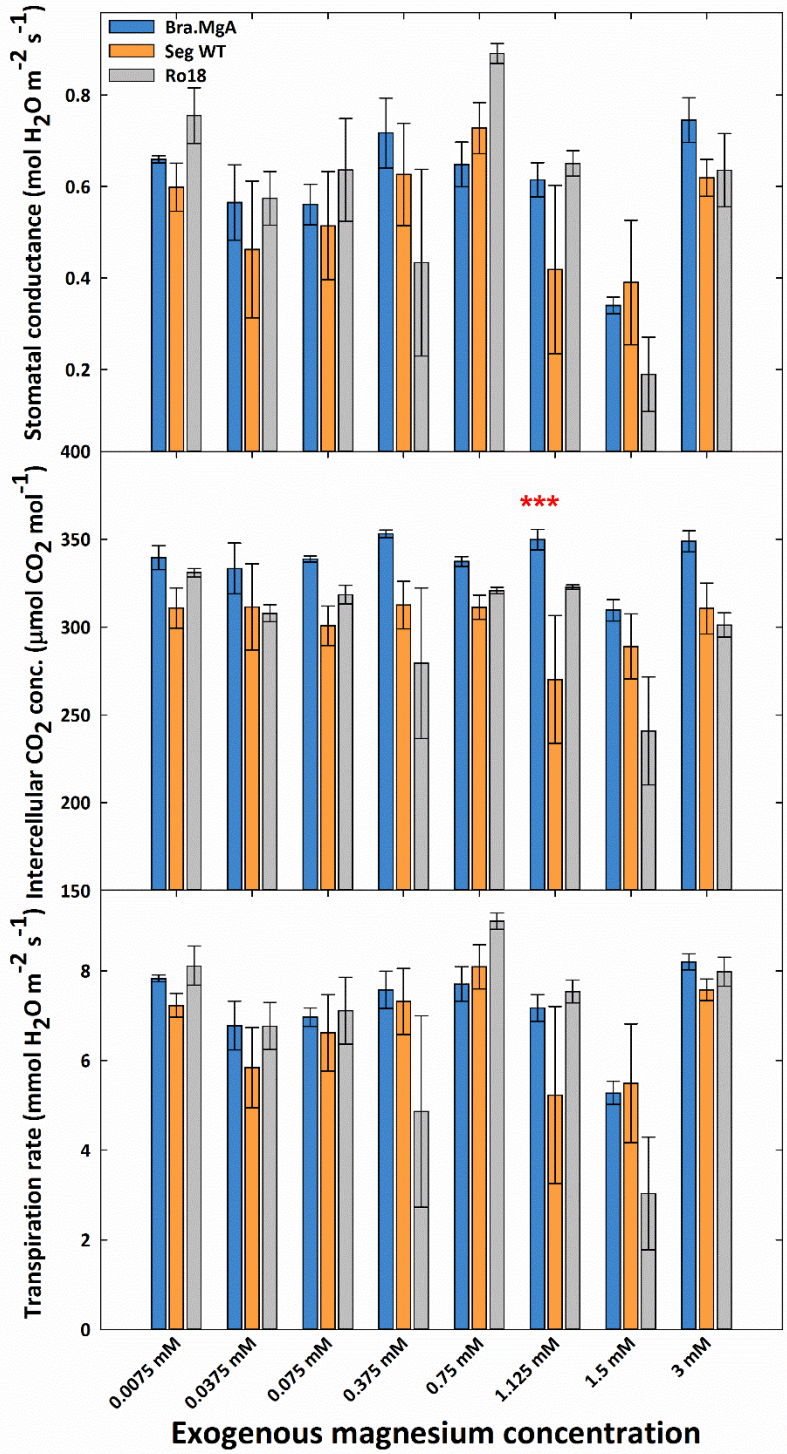
6.8 Supplementary Figures



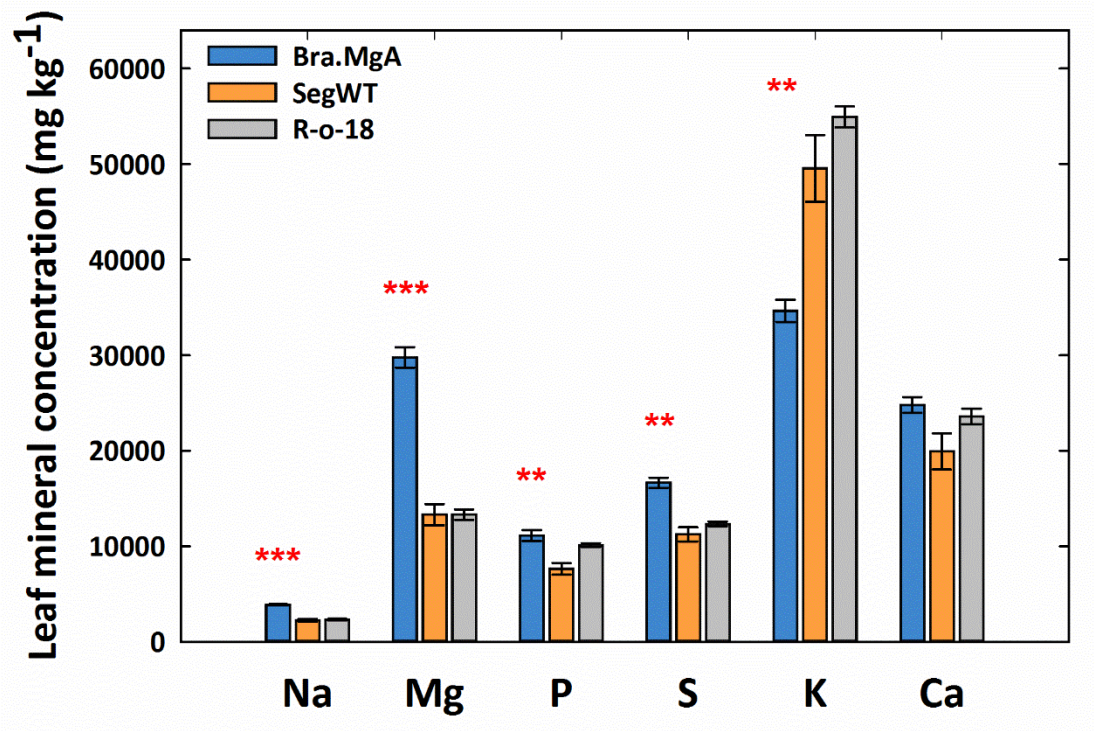
Supplementary Figure 6.1. Concentrations of elements present at less than 1,000 parts per million (Mn, Fe, Ni, Cu, Zn, As, Se, Rb and Cd) in leaves of 45 day old (post seed sowing) BC_1F_4 *generation* Brassica rapa *magnesium accumulator (Bra.MgA; n = 20), out-segregating wild-type (SegWT; n = 20) and R-o-18 (n = 19) plants. Where possible, 6th leaf from base taken for analysis, otherwise next available leaf taken (e.g. 7th leaf) Mineral concentration shown in* log_{10} *scale for ease of visualisation. Error bars represent standard error of the mean. Stars above groups represent significant differences in mineral concentrations between Bra.MgA and SegWT plants at* $p < 0.05$ *(*),* $p < 0.01$ *(**), and* $p < 0.001$ *(***).*



Supplementary Figure 6.2. Concentrations of Na, P, S, K and Ca in leaves of 34 day old (post seed sowing, including 21 days under varying Mg treatments) BC_1F_5 *generation Bra.MgA), SegWT, and R-o-18 plants at eight different exogenous Mg concentrations (n = 3 for all genotypes at all treatments except MgHA in 1.5 mM exogenous Mg, n = 2). Error bars represent standard error of the mean. Stars above groups represent significant differences in mineral concentrations between Bra.MgA and SegWT plants at p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).*



Supplementary Figure 6.3. Photosynthetic traits of leaves of 34 day old (post seed sowing, including 21 days under varying exogenous Mg treatments) BC_1F_5 *generation Bra.MgA, SegWT, and R-o-18 plants at eight different exogenous Mg concentrations (n = 3 for all genotypes at all treatments except MgHA in 1.5 mM exogenous Mg, n = 2). Error bars represent standard error of the mean. Stars above groups represent significant differences in mineral concentrations between Bra.MgA and SegWT plants at p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).*



Supplementary Figure 6.4. Concentrations of sodium (Na), magnesium (Mg), phosphorus (P), sulphur (S), potassium (K) and calcium (Ca) in leaves of 21 day old (post-germination) BC_1F_5 *generation Bra.MgA (n = 4), SegWT (n = 4) and R-o-18 (n = 4) plants. Pooled 3rd and 4th leaves used for analyses. Error bars represent standard error of the mean. Stars above groups represent significant differences in mineral concentrations between Bra.MgA and SegWT plants at p < 0.01 (**), and p < 0.001 (***).*

Chapter 7. General Discussion

7.1 Variation in leaf and seed ionomic traits in the RIPR diversity panel indicates scope for breeding for increased nutrient uptake and use efficiency using naturally available germplasm

In order to assess variation in mineral uptake and distribution, this study characterised root architecture and leaf and seed ionomic traits across a population of 383 genotypes of *Brassica napus*. *Brassica napus* is likely to have arisen through multiple, independent crosses between *B. rapa* and *B. oleracea*. It is an amphidiploid species and, as such, its genome comprises a full set of diploid chromosomes from each of the two parent species (Chalhoub et al., 2014). Studying variation in traits between multiple genotypes of *B. napus* and identifying genetic loci under their control is thus likely to be indicative of variation across all three, closely related species. The population used here is referred to here as the RIPR population, after the project that led to its creation (Biotechnology and Biological Sciences Research Council [BBSRC] Renewable Industrial Products from Rapeseed [RIPR] Programme Grant BB/L002124/1).

Large amounts of variation were observed across all traits measured in the RIPR population. This included 2.6- to 4.3-fold variation across root architecture traits, and 1.7- to 14.1-fold (but typically 2- to 4-fold) variation in leaf and seed mineral concentration traits (Thomas et al., 2016; Chapter 2), as well as 8.3-fold variation in leaf nitrate (NO_3^-) concentration (Chapter 3). Variance components analysis also revealed that much of the variation observed within leaf and seed ionomic traits could be explained by genotype, indicating that many of these traits are highly heritable. For instance, genotype accounted for 36 % of the variation observed in leaf magnesium (Mg), 35 % of the variation in leaf potassium (K), 26 % of the variation in leaf calcium (Ca), and 21 % of the variation in leaf phosphorus (P) concentration. Genotype also accounted for 39 % of the variation in leaf NO_3^- concentration. Genotype generally accounted for less variation in seed mineral traits, but still explained 21 % of the variation in seed K, 16 % of the variation in seed Ca, 12 % of the variation in leaf P and 6 % of the variation in leaf Mg concentration. The large amounts of phenotypic variation observed in the RIPR population, and the apparent

high heritability of such traits, indicates that there is significant scope for breeding for increased nutrient accumulation and use efficiency in *B. napus* whist only using currently available germplasm. Also, whilst there were differences in leaf and seed ionomic traits observed between plants of different crop habits (winter, semi-winter and spring oilseed rape, fodder, swede types), habit in general accounted for less variation than genotype within habit groups. Therefore, there is also likely to be beneficial allelic variation within all crop habits that could be made use of for crop improvement. This could be realised through traditional breeding approaches, involving crosses between genotypes with previously under-utilised, desirable traits and modern, high-yielding cultivars. The RIPR population likely reflects the majority of within-species variation in *B. napus*. Hence, the data produced here forms a highly useful resource, which may prove beneficial for identifying genotypes suitable for trait introgression for years to come.

Interestingly, root architecture traits largely did not correlate with leaf or seed nutrient concentration. This is perhaps surprising, given that variation in plant nutrient uptake is largely thought to depend on variation in root traits. For instance, root architecture (Lynch, 1995; Liao et al., 2001; Miller et al., 2003) and more specifically root hair length (Bates and Lynch, 2000) and total root length (Ma et al., 2003) have been shown to influence P uptake in a range of environments. Further root traits, including smaller root diameter, have also been linked to increased access to water and nutrients in the soil through more efficient access to larger soil volumes (Hufnagel et al., 2014). The root architecture measurements characterised in Thomas et al. (2016; Chapter 2) were of plants grown on absorbent germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA) saturated in 25 % strength Hoagland's solution (No. 2 Basal Salt Mixture, Sigma Aldrich, Dorset, UK). Excluding the occurrence of nutrient depletion zones surrounding roots, it may be expected that all nutrients required for growth were readily available to root tissue, and hence any responses to nutrient unavailability in the form of increased root length, lateral root density, etc. are likely to have been masked. Similarly, plants grown for leaf nutrient concentration analyses were cultivated on Levington C2 compost (Scotts Professional, Ipswich, UK), which is likely to have much higher available nutrient

concentrations than those typically experienced in the soil. It is possible that greater levels of variation would be observed in both root architecture and leaf and seed ionomic traits between genotypes of the RIPR population if plants are grown in more nutrient-devoid conditions. In particular, this may reflect differences in nutrient uptake efficiency at low exogenous supply, which may be expected to be more dependent on root architecture traits. Despite this, the 2.6- to 4.3-fold variation observed in all root traits measured here still indicates sufficient, heritable variation in between *B. napus* genotypes. This could be made use of in breeding strategies for increasing the efficiency of soil nutrient uptake and subsequent allocation to leaf and seed tissue.

Root hair length and density traits were not measured as part of this study. If performed in future, this would likely reveal further amenable variation between genotypes of the RIPR population. Root hairs are an effective strategy for increasing plant root soil contact, expanding the area from which nutrients can be absorbed from and providing a greater surface area on which to absorb them (Gilroy and Jones, 2000). Characterising variation in root hair traits between genotypes of the RIPR population may indicate suitable germplasm for use in crop breeding strategies to increase root hair length or density. There are obvious difficulties associated with measuring root hair traits on plants grown either in soil in a field or in compost, and so such measurements may have to take place in a more artificial environment. This may mask some genotypic effects, however should give a preliminary insight into the variation available within the species. Investigation into potential correlations between root hair traits and plant nutrient acquisition will also reveal the efficacy of such a physiological marker for assessing nutrient status in *B. napus*.

7.2 Quantitative trait loci identified through Associative Transcriptomics form genetic markers amenable for use in marker-assisted breeding strategies

As revealed through variance components analysis on leaf mineral concentration traits described above, variation in leaf NO_3^-, P, K, Ca and Mg concentration could be explained to a relatively large degree by genotype. Thus, it is likely that these traits

are under strong, heritable, genetic control. For this reason, coupled with the importance of these elements for plant productivity and human and animal nutrition, these were selected for Associative Transcriptomics analysis (Harper et al., 2012; Havlickova et al., 2018). The Associative Transcriptomics approach makes use of both single nucleotide polymorphisms (SNPs) and gene expression markers (GEMs), generated through RNA-sequencing, to associate genomic loci with variation in quantifiable traits. The use of coding-sequence-specific molecular markers reduces the genetic complexity observed within the polyploid *B. napus* genome (Trick et al., 2009; Bancroft et al., 2011; Harper et al., 2012; He et al., 2016). The generation of GEMs through quantification of transcript abundance also allows the consideration of gene expression in trait control, which may be particularly important in polyploid organisms in which gene duplication may have led to unequal expression (Adams et al., 2003).

Significantly associated SNP markers were detected for leaf NO_3^-, Ca, and Mg concentration. Significantly associated GEMs were also detected for leaf K, Ca and Mg concentration. Many of these fell within distinct, closely linked, association peaks. Trait specific peaks along with their locations and potential importance and amenability for crop breeding are discussed within previous Chapters (Chapters 3 and 4 for leaf NO_3^-, P and K concentration, & Ca and Mg concentration, respectively). However, worthy of note are association peaks that co-localised for more than one trait. Numerous association peaks co-localised for leaf Ca and Mg concentration, including loci on chromosomes A2, A3, A7, A10, C2, C3 and C9. The association peak observed on A10 is of particular interest. This comprised many of the most highly associated markers for Ca and Mg concentration. It also co-localised with an association peak for leaf NO_3^- concentration. Whilst not as closely associated with the latter trait, this locus forms a highly promising target for crop improvement, which may be suitable for influencing the accumulation of multiple, essential plant nutrients.

No SNPs or GEMs were significantly associated with leaf P concentration, and only few GEMs were associated with leaf K concentration. Given the apparently high

heritability of these traits, as discussed above, this was a surprising result. It is possible that these traits are influenced by a larger number of genes than other traits in *B. napus*, and this may have led to excessive noise in the dissection of their genetic control. It is also possible that these traits are influenced to a greater degree by genetic loci not reflected by the markers used in the analyses. The SNP resource made use of here comprised 355,536 SNP markers, which is roughly equivalent to one SNP every 0.33 kb across mapped sequences in the *B. napus* pan-transcriptome (Havlickova et al., 2018). This forms a high density molecular mapping resource. However, since all SNP markers are based on coding DNA sequences, SNPs in other regions, such as gene promoter sequences, will not be reflected. As well as this, while gene expression differences between accessions are reflected through GEMs, the tissue sampled for RNA extraction and subsequent sequencing was from young leaves (He et al., 2016). Thus, only differences in transcript abundance of genes expressed in this tissue will be reflected in the analyses performed here. Perhaps of greater importance to the control of leaf nutrient concentration are genes controlling the uptake of nutrients from the soil solution, or genes controlling allocation to shoot tissue. If root transcript abundance were quantified in the RIPR population, it may allow the elucidation of further genetic loci controlling nutrient uptake, including loci controlling leaf concentrations of both P and K.

A similar restriction of using leaf transcript abundance as a marker for trait variation is manifested when variation in gene expression throughout plant development, and under varying environmental conditions, is considered. Tissue taken from all plants for RNA sequencing was at a single growth stage, and under specific, controlled, environmental conditions (He et al., 2016). Variation in genes only expressed under different environmental conditions, or during different developmental stages, is thus also unlikely to be represented among GEMs used here. This becomes particularly apparent when it is considered that significant expression was only quantified for 53,889 out of 116,098 gene models (Havlickova et al., 2018). A plethora of further genetic information and potential links to the control of phenotypic variation could be revealed if further samples were taken from genotypes of the RIPR population for RNA sequencing. This could include samples taken at multiple growth stages, from

multiple tissue types, and from plants grown in a range of environmental conditions. There are obvious difficulties with this approach. With 383 genotypes in the RIPR population, sample numbers could very quickly reach many thousands, which is likely to be cost-prohibitive. Despite this, quantifying root transcript abundance offers clear potential for elucidating trait control mechanisms in *B. napus*. This forms a suitable direction for further work with the RIPR population. It is also likely to be relatively cost effective, so long as a suitable growth stage and environment can be selected for tissue sampling, maximising measureable genetic variation, whilst minimising any artefacts associated with methodology.

Also important to note once again is that plants grown for the initial leaf mineral concentration screen were all grown on Levington C2 compost. This growth medium comprises nutrient concentrations deemed sufficient for growth. Hence, it is unlikely that any of the plants grown in this environment were suffering from any nutrient stresses. Due to the vast variation in leaf mineral concentration traits in varieties of the RIPR population observed under sufficient nutrient conditions, it is reasonable to assume that there are similarly large differences between genotypes in their ability to uptake nutrients under stressed conditions. Such potential variation would be highly interesting to explore, as it may indicate genotypes suitable for breeding for improved growth on nutrient impoverished soils. However, perhaps of greater impact would be the ability to identify genetic markers associated with variation in leaf mineral status under nutrient stressed conditions. It has previously been shown that starvation of various nutrients, such as P, leads to various changes in gene expression in plants (Hammond et al., 2003). Thus, it is likely that utilising leaf mineral concentration data from nutrient stressed plants in Associative Transcriptomics analyses would lead to the association of further molecular markers additional to those identified in this study. Crucially, it could lead to the identification of a number of molecular markers associated with leaf P and K concentration, which are currently limited in number.

In order for this data to be made available, it would be necessary to grow each genotype of the RIPR population under lower nutrient conditions in an additional

designed experiment. There are a number of potential constraints to this, not least the associated costs and workload. Important to decide are the nutrient concentration to be made available in the new growth media. If made too high, differences in nutrient concentration traits between this and the initial experiment may not significantly differ. If made too low, there is a chance that some plants would be too stressed for data to be reliably gathered. A suitable first step would be selection of a small but diverse subset of genotypes of the RIPR population. These could be grown in a range of exogenous nutrient supply conditions, tissue taken from each plant, and samples analysed for variation in mineral concentration traits. This data could then be used to inform the likely efficacy of performing a larger experiment, comprising all genotypes of the RIPR population, in order to identify novel genetic loci associated with variation in leaf mineral concentration traits under low nutrient conditions. This would ideally be coupled with repeating extraction and sequencing of RNA samples from plants under nutrient stressed conditions, which may allow further elucidation of genetic loci required for nutrient uptake under lower exogenous supply, as discussed above.

Despite the limitations discussed here, and the lack of significantly associated markers for leaf P and K concentration, potentially useful allelic variation was characterised between genotypes of the RIPR population. For instance, at lead-marker loci within association peaks, individual SNPs were associated with up to around 2-fold variation in leaf NO_3^- concentration (Chapter 3). It is important to note that the SNPs compared between genotypes of the RIPR population that associate with trait variation are unlikely to be directly involved in trait control. Far more likely, is that they are in close linkage disequilibrium with genes that do directly control trait variation. Nevertheless, these form suitable markers for trait values, which may prove useful in marker-assisted selection strategies to rapidly breed for increased nutrient uptake and use efficient crop cultivars. Around 1.2-fold variation in leaf P concentration was also detected between allelic variants at lead-marker positions (Chapter 3). Whilst less phenotypic variation was associated with specific SNPs for leaf P than NO_3^- concentration, the identification of allelic variants conferring significant variation in leaf P concentration indicates further breeding potential.

Transcript abundance of several genes also significantly correlated with leaf K concentration. Whilst the effects were still relatively low, the genes described here (Chapter 3) are more likely to play a more direct role in trait variation. Hence, their expression levels may prove useful both as a marker for leaf K concentration, and as a potential avenue for more directed crop improvement.

7.3 Generation of novel alleles in candidate genes may lead to the development of greater nutrient accumulation or use efficiency traits than those available in current germplasm

Associative Transcriptomics based on a diverse population of a large number of genotypes is able to detect loci controlling heritable traits at high resolution. Typically > 100 genes are likely to be within linkage disequilibrium (LD) decay of associated molecular markers. However, this is still much higher resolution than that possible through more traditional QTL mapping, due to further genetic divergence between members of a diversity population than a bi-parental mapping population (Brachi et al., 2011). In Chapters 3 and 4, this technique was used to identify genes controlling variation in leaf NO_3^-, P and K concentration, & leaf Ca and Mg concentration, respectively. Due to the high resolution achievable through Associative Transcriptomics, this enabled not only identification of molecular markers for a given trait, as discussed above, but also inference of candidate genes that may directly play a role in trait control. This was largely achieved by making use of annotated, gene function data available for orthologous genes in *Arabidopsis thaliana*, a model plant species closely related to *B. napus*. This reduced all potentially trait influencing genes within LD of associated markers to a more manageable number of candidate genes that are more amenable to functional analysis.

Specific candidate genes, their likely function, and potential significance in crop improvement through increasing productivity or the accumulation of essential human nutrients, are discussed in previous Chapters (Chapters 3 and 4 for leaf NO_3^-, P and K & Ca and Mg candidates, respectively). However, some candidate genes are worthy of additional emphasis here. These include three genes that are orthologous

to distinct *A. thaliana* ABC transporter encoding genes G2, G6, and G20. Each of these have proven to be important for the control of root suberin synthesis (Yadav et al., 2014), and in fact are the only three ABC transporter genes that have been directly linked to such a function. Throughout the *B. napus* genome, due to historic hybridisation events, there are two copies of ABC G2, two copies of ABC G6, and four copies of ABC G20. Of these eight genes, three were found within LD of SNP markers associated with leaf NO_3^- concentration, one of which was orthologous to *A. thaliana* ABC G2 and two of which were orthologous to *A. thaliana* G20. This enrichment of ABC transporter genes with such a function close to such peaks indicates that they are highly promising candidates for controlling leaf NO_3^- concentration. A further orthologue of *A. thaliana* ABC G6 was also identified near an association peak for leaf P concentration, indicating a role of root suberin synthesis in P uptake as well. Root suberin is a major component of the Casparian strip. This forms a chemical barrier restricting extracellular transport of water and dissolved nutrients to the vascular tissue (Baxter et al., 2009). It is clear that this is an important mechanism for controlling the movement of numerous nutrients from root to shoot tissue. The existence of genes likely to be involved in its formation within LD of markers for leaf NO_3^- and P concentration thus suggests that there is variation within Casparian strip physiology in *B. napus* that is capable of influencing shoot nutrient concentrations.

Several genes orthologous to members of the *A. thaliana* nitrate transporter family NRT2 were also identified close to markers highly associated with leaf NO_3^- concentration. Similarly to the ABC transporter genes described above, these were also highly enriched, with a total of 7 distinct orthologues of the NRT2 family found close to associated markers, out of the 27 orthologues which are present in *B. napus*. Particularly enriched were orthologues of the gene family members NRT2.3 and NRT2.4. Of the seven copies present throughout the *B. napus* genome, five were localised within association peaks for leaf NO_3^- concentration. This indicates that these are highly convincing candidate genes, allelic variation in which may be amenable for use in breeding strategies for increasing NO_3^- use efficiency.

A further notable candidate gene was associated with leaf K concentration and is orthologous to *A. thaliana* AT2G04850. Whilst this gene has not been specifically characterised in detail, annotation data at The Arabidopsis Information Resource (TAIR; Columbus, OH, USA) derived from The Institute for Genomic Research (Rockville, MD, USA) indicates that it is auxin responsive. This data is based on sequence similarity with a number of genes described in an early study which isolated mRNAs that accumulated during auxin-induced lateral root formation (Neuteboom et al., 1999). Proteins encoded by the genes described in this study started to accumulate within 4 hours of the addition of active auxin, suggesting that they may have a role in lateral root formation. Since nutrient uptake is considered to be highly dependent on root architecture traits, the association of a gene relating to lateral root formation with leaf K concentration would perhaps be unsurprising. However, given the lack of work performed on the specific auxin responsive gene that was associated, this gene may indicate a novel target for consideration in identification of favourable alleles for improved root architecture for nutrient acquisition.

Similarly notable candidate genes include orthologues of *A. thaliana ACA8* and *MGT7* which were identified within LD of markers for both leaf Ca and Mg concentration. These encode a Ca^{2+}-transporting ATPase (Bonza et al., 2000) and a membrane-bound Mg transporter (Mao et al., 2008; Gebert et al., 2009), respectively. These form excellent candidates for the control of leaf Ca and Mg concentration in *B. napus*.

In this thesis, the role of a number of candidate genes in controlling shoot Ca and Mg concentration were analysed using T-DNA mutant genotypes of *A. thaliana* (Alcock et al., 2017; Chapter 4). This work indicated such a role for several candidates, as reflected through perturbed mineral concentrations observed in respective T-DNA mutants. Further work was performed using mutated *B. rapa* germplasm from an ethyl methanesulfonate (EMS) mutagenesis background (Chapter 5). This latter work focussed on a genotype comprising a splice donor mutation in an orthologue of *A. thaliana MGT7*, which formed an effective knockout mutation through truncation of the *B. rapa* MGT7 protein. The mutated *B. rapa* genotype was found to accumulate greater leaf concentrations of Mg than out-segregating wild-type plants under

relatively low exogenous Mg supply. The direction of the effect of the mutation was opposite to that expected. Despite this, the altered leaf Mg concentration observed in *B. rapa mgt7* mutant plants provided further evidence for the role of this gene in Mg uptake and / or distribution in *Brassica* spp.

All of the mutated genotypes used to examine gene function in this thesis were effective knock-out mutations, which are likely to have led to loss of function of encoded proteins. Whilst characterising the effects of loss-of-function mutations is a highly useful approach to determine gene function (Thorneycroft et al., 2001), perhaps a better approach towards identifying traits amenable to crop improvement is characterising trait variation in allelic variants of candidate genes. This offers the potential of identifying beneficial variants, which could be made use of in order to improve crop traits. Beneficial allelic variants could be identified through screening sequence polymorphisms in naturally available germplasm. Novel variants could also be generated through genetic manipulation techniques, such as through the use of EMS mutagenesis to induce point mutations across a plant's genome (Kim et al., 2006). Subsequent screening for mutations in target genes could reveal a multitude of allelic variants, some of which could confer beneficial traits. This approach has previously been used in *B. rapa* (Graham et al., 2014). Significant phenotypes compared to out-segregating wild-type plants were observed for three independent mutant genotypes in the *CAX1* gene, each containing a different point mutation. The *CAX1* gene encodes a Ca^{2+} / H^{+} antiporter of the calcium exchanger (CAX) protein family. Intriguingly, plants of one of the allelic variants characterised had around 64 % greater shoot Ca concentrations than those observed in the respective, out-segregating wild-type line, as well as 49 % greater leaf Mg concentrations. Whilst further work is required to elucidate the mechanism controlling this, the relative increases in both leaf Ca and Mg concentration in this genotype offer the potential for this novel variation to be bred into commercially viable *Brassica* crop cultivars. This could lead to greater dietary Ca and Mg intakes in humans and animals, which could contribute to reducing widespread undernutrition. By using this approach within further investigation of the candidate genes identified as a part of this thesis, further, beneficial allelic variants could be generated. This could additionally

contribute to the generation of novel crop cultivars with increases in crop productivity, nutrient use efficiency, and elevated accumulation of essential human and animal nutrients in edible crop tissue.

7.4 New opportunities towards breeding novel, high magnesium crop cultivars, and dissecting the physiological and genetic bases of Mg homeostasis in plants

In Chapter 6, an EMS mutagenised genotype of *B. rapa* that accumulates up to ~200 and 60 % greater shoot and root concentrations of Mg than wild-type plants, respectively, was characterised. This genotype has huge potential for alleviating widespread human and animal Mg deficiencies (Broadley and White, 2010), as long as the beneficial traits can be bred into commercial viable crop cultivars. However, to date, the causative genetic variant responsible for this huge increase in shoot Mg is not known. The EMS concentrations applied to ancestral plants formed a high dose, and it is likely that M_3 plants of this population contain around 10,000 mutations (Stephenson et al., 2010). Despite a backcross to un-mutagenised background plants having been performed with the *B. rapa* Mg accumulator (Bra.MgA), followed by multiple rounds of self-fertilisation, it is probable that a large proportion of these remain. Segregation patterns within offspring of heterozygous parents suggest that only a single mutation is leading to the observed high Mg concentrations. This may reduce the complexity of unravelling the genetic basis of the traits. However, even with efforts to detect sequence differences between Bra.MgA and wild-type plants having taken place as a part of this thesis, the exact, causative mutation remains elusive.

Nevertheless, progress towards understanding both the genetic and physiological basis of the observed traits has been made. Work performed in this thesis focussed on elucidating perturbations in a range of traits including bulk leaf mineral concentrations, photosynthetic activity and leaf tissue and cellular level spatial distribution of Mg and other elements in Bra.MgA. Notably, this genotype also has higher leaf concentrations of sodium (Na), P and sulphur (S) than wild-type plants. Varying effects were also observed for leaf Ca concentrations, which were also

elevated in Bra.MgA under certain but not all tissues and generations. Despite an increase in leaf Mg concentration, it is apparent that Bra.MgA has lower photosynthetic activity. Due to the central role of Mg in the formation of chlorophyll (Wilkinson et al. 1990), this was surprising, and indicates a low use-efficiency of increased leaf Mg supplies. Profound differences in the spatial distribution of Mg, Na, P and K were also observed in Bra.MgA compared to wild-type plants. Particularly large reserves of Mg and P localised to upper epidermal cells, and in the cell wall regions of palisade mesophyll cells. Similar effects were also observed for Na. An almost opposite effect was observed for K, with lower leaf epidermis concentrations in Bra.MgA than in wild-type plants. Concentrations of Mg within mesophyll cells were not obviously higher than in wild-type plants, despite clearly greater Mg concentrations in bulked leaf samples. This provides an explanation for the unexpected effect on photosynthetic activity; if the excess Mg is not found within the bounds of the cell membrane, it cannot be used for the production of chlorophyll.

This new information goes some way towards uncovering the physiological mechanisms controlling perturbed traits in Bra.MgA. It is possible that the EMS dose applied to ancestral plants has led to a faulty, plasma-membrane bound Mg transporter in Bra.MgA. If this results in lower cellular concentrations of Mg, despite sufficient concentrations being locally present, it may lead to an apparent deficiency response, whereby processes are initiated within the plant to import greater concentrations of Mg from the soil and translocate it to leaf tissue. The elevated leaf concentrations of Mg that result will then localise to tissue that they can occupy, including epidermal cells and inter-cellular spaces. This could also explain the increase in concentrations of other nutrients in shoot tissue, which may have accumulated in excess due to mass flow of nutrients through the xylem (White, 2012).

Bulked segregant analysis performed as part of this thesis indicated genetic loci that are at increased likelihood of containing the causative, presumed SNP. It also allowed the identification of several candidate genes. However, further work will be required to elucidate fully the genetic basis of the high Mg trait. DNA was extracted for

sequencing from plants that had only undergone a single back-cross to wild-type background plants. It is likely that further back-crosses, followed by selection of plants that maintain the high Mg trait, will significantly reduce background mutation load, and reduce noise in any future bulked segregant analysis efforts. Providing sufficient resources can be obtained, this is a useful next step to take. It is also worth investigating any downstream effects of the mutation. To this end, transcriptomic analysis of root and shoot samples should be performed in future through RNA sequencing. This will assess whether there are changes in expression of genes associated with nutrient uptake in Bra.MgA, which may contribute to the observed perturbed traits. It is currently unknown whether the causative SNP is directly leading to the observed high shoot magnesium trait in Bra.MgA. Alternatively, it may be altering the expression of genes and pathways that control Mg accumulation, as discussed above. Studying transcript abundance at multiple exogenous Mg concentrations may also provide additional insights into the control of Mg uptake in response to environmental conditions. Following the identification of candidate SNPs and genes contributing to high shoot Mg concentration, germplasm comprising allelic variation in relevant genomic loci should be acquired and / or generated. Characterisation of this germplasm may then confirm gene function and contribution to the observed increase in shoot Mg concentration in Bra.MgA.

Whilst elucidation of the genetic control of the high Mg trait will provide suitable molecular markers for breeding for improved crop cultivars for human and animal nutrition, it is also possible for breeding programmes to be initiated in their absence. Up to now, only the elevated shoot Mg concentration has been required to follow the high Mg trait through multiple generations. Thus, it should be possible to begin introgression of the high magnesium trait into suitable, commercially viable *Brassica* crop cultivars, in order to generate novel germplasm suitable for cultivation. Particular focus should be on the generation of vegetable crops, which are likely to be excellent vehicles for increasing human dietary magnesium intakes. Within the *B. rapa* species are numerous vegetable crop types ranging from leafy vegetables, including pak choi, napa cabbage and rapini, to root vegetables, including turnip. Since elevated Mg concentrations have been observed in both shoot and root tissue

of Bra.MgA plants, it should be possible to generate high Mg varieties of a multitude of crop types.

Despite the importance of Mg in plants, knowledge regarding its uptake, distribution and homeostasis lags behind that of other mineral elements (Waters, 2011). For this reason, the perturbed traits in Bra.MgA also offer an additional opportunity. Elucidating variation in gene sequences and expression leading to the observed differences in leaf Mg concentration and distribution between tissue types and at a cellular levels offers a fantastic opportunity to study mechanisms controlling Mg homeostasis in plants on a fundamental level. Such insights could be generated alongside efforts to breed for higher Mg concentrations in edible crop tissue. This will provide a duality of beneficial outputs that will contribute to reducing widespread human and animal undernutrition whilst maintaining relevance to the wider research community for years to come.

7.5 The road is long, with many a finding

A particular focus throughout this research has been on the potential applications of an increased understanding of the genetic control of nutrient accumulation in *Brassica* spp. Elucidating naturally available variation within *B. napus*, as described in Chapter 2, has the obvious application of indicating suitable genotypes for use in crop breeding strategies. Subsequent work, which focussed on dissecting the genetic basis of shoot nutrient accumulation, has also led to a number of amenable genetic markers, which may prove useful for marker-assisted selection strategies towards the generation of novel crop cultivars. Introgression of beneficial traits may lead to the generation of more productive cultivars than available through current, high-yielding germplasm. It could also lead to the development of commercially viable cultivars that are able to accumulate greater concentrations of Ca and Mg in edible crop tissue. Both Ca and Mg are important elements in human and animal nutrition, and risks of deficiency in these nutrients are likely to be widespread. Increasing concentrations of these elements in edible horticultural produce is likely to be an effective strategy to reduce deficiency risks in human populations (Broadley and White, 2010). Such an end could be met particularly effectively by making use of the highly beneficial traits

characterised in the *B. rapa* Mg accumulator described in Chapter 6. Since a genotype that already accumulates far greater shoot Mg concentrations than naturally available germplasm has been identified, the development of high Mg crop cultivars could be realised with relatively little effort. More challenging will be efforts required to make such cultivars commercially viable, which will likely require further testing of product quality, along with discussions with seed producers, farmers and supermarkets. Despite further work being required to fully understand the genetic basis of this trait, as well as the amenability of allelic variants and candidate genes for improved N, P and K uptake and use efficiency, the work recorded in this thesis has made significant progress towards understanding nutrient accumulation in *Brassica* spp., which may prove useful to future research for many years.

7.6 References

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