Title
Biofortifying *Brassica* with calcium (Ca) and magnesium (Mg)

Authors
Broadley, Martin R
Hammond, John P
King, Graham J
et al.

Publication Date
2009-09-15

Peer reviewed

Introduction

Humans require more than 22 mineral elements, all of which can be supplied by an appropriate diet. However, over half of the world's population is deficient in one or more of calcium (Ca), iron (Fe), iodine (I), magnesium (Mg), selenium (Se) and zinc (Zn) (Graham et al., 2007; White & Broadley 2009). Most mineral deficiencies are caused by the consumption of plants, directly or via livestock, containing insufficient minerals. Agronomic (fertilisers) and genetic (crop improvement) biofortification strategies can increase the delivery of bioavailable minerals to humans and livestock (Grusak & DellaPenna, 1999; Morris et al., 2008; White & Broadley, 2009). Here, we describe the potential for biofortifying vegetable *Brassica* with Ca and Mg by exploiting natural genetic variation within its genepool.

Calcium and Mg are the most abundant Group II minerals in humans. Most Ca (99% w/w; >1 kg) is associated with bones and teeth. The remainder has signalling and metabolic functions including involvement in vitamin D metabolism. Under Ca deficiency, soft tissue Ca is maintained, reducing bone strength and increasing fracture-risks and osteoporosis (Francis 2008). Calcium deficiency causes rickets in rural populations in developing countries (Thacher et al., 2006). Adults contain ~25 g Mg, 60% in hard tissues, 40% in muscle/soft tissue, and 1% in extracellular fluids. Magnesium is involved in energy, protein, and fatty acid metabolism and in cellular ionic balance (Gums, 2004). Magnesium deficiency is more widespread than Ca due to relatively higher soft-tissue requirements and is linked to hypertension, cardiovascular disease, and preeclampsia (Bo & Pisu, 2008). Although billions are at risk globally from Ca and Mg deficiency, reliable biomarkers are lacking. Risk assessment therefore relies on dietary surveys. In the UK, Ca and Mg deficiency affects at least 5-10% of the population according to Reference Nutrient Intake (RNI) criteria (NDNS, 2003). The RNI is a population mean intake which provides adequacy for ~97.5% of individuals. The UK RNI for Ca is 700 mg d^{-1} (19-64 yrs); the RNI for Mg is 270 (♀) or 300 (♂) mg d^{-1}. An individual intake below the Lower RNI (LRNI), i.e. *c.* two standard deviations below the RNI, is considered deficient. LRNIs are 400 mg Ca d^{-1} for all adults, and 150 and 190 mg Mg d^{-1} for women and men, respectively. Approximately 4% of UK adults (~2 million) have intakes below the LRNI for Ca and 11% (~5 million) for Mg. Crop plants supply 90-95% of total Ca and Mg intake either directly, or via animal feed-chains with the remainder coming from drinking water. In the UK, 50% and 25% of total Ca and Mg intake, respectively, is derived from dairy, meat and eggs (NDNS, 2003).

Leafy vegetable crops such as *Brassica* are appropriate targets for genetic biofortification, even where vegetable consumption is relatively low. For example, in the UK, vegetables (excluding potatoes) contribute 5% of Ca (44.4 mg Ca d^{-1}) and 8% of Mg (21.4 mg Mg d^{-1}) intake among adults (NDNS, 2003). UK food composition tables report values of 54 mg Ca 100 g^{-1} FW and 7 mg Mg 100 g^{-1} FW for Chinese cabbage (*B. rapa*), and similar in white, red, and Savoy cabbage (*B. oleracea*; FSA, 2002). We routinely observe >500 mg Ca 100 g^{-1} FW and >120 mg Mg 100 g^{-1} FW in edible leaves of *B. oleracea* (Broadley et al., 2008). A target of >150 mg Ca 100 g^{-1} FW and >50 mg Mg 100 g^{-1} FW thus appears feasible through breeding and/or agronomy, and a single 100 g FW portion of Ca/Mg-biofortified leaf material could significantly improve intakes.

Here, we dissected genetic variation in shoot or leaf Ca and Mg concentration (shoot/leaf-Ca/Mg) in the genepools of: (1) *Brassica oleracea* L. (C-genome; 1n=9; cabbage, calabrese, cauliflower etc.); (2) *Brassica rapa* L. (A-genome; 1n=10; Chinese cabbage, pak choi, turnip

etc.); (3) *Brassica napus* L. (amphidiploid AC-genome; 1n=19; canola/colza/oilseed rape (OSR), rutabaga/swede etc.). Phenotypic variation in shoot/leaf-Ca and Mg is marked, with a strong genetic component (heritability). Allelic recombination among the progeny of homozygous lines of both *B. oleracea* and *B. rapa* causes variation in shoot/leaf-Ca and Mg comparable to that encountered among the wider genepool. Quantitative trait loci (QTL) for shoot/leaf-Ca and Mg occur in potentially paralogous regions in *B. oleracea* and *B. rapa*. Efforts to identify genes underlying these loci, and their regulation, are ongoing through high resolution recurrent backcrossing.

Materials and Methods

Plant Material

Four *Brassica* populations were studied for their shoot/leaf-Ca/Mg. (1) A *B. oleracea* Diversity Foundation Set (BolDFS): a structured sample of 376 accessions plus a set of 74 varieties, mostly F_1s, capturing the major morphotypes under cultivation in N. Europe, described previously (Broadley et al., 2008). The DFS is likely to represent most of the common allelic variation in the species. (2) A *B. oleracea* mapping population (BolAGDH): 90 doubled-haploid (DH) lines from a segregating population of 206 lines generated through anther culture of the F_1 of a cross between a DH rapid-cycling accession *B. oleracea* var. *alboglabra* ('A12DHd', ♀) and a DH accession derived from an F_1 hybrid calabrese cultivar, 'Green Duke', *B. oleracea* var. *italica* ('GDDH33', ♂). A linkage map of 906 cM was used, with a mean distance between marker loci of 1.92 ± 3.49 cM of a marker (Broadley *et al*., 2008). (3) A *B. napus* DFS (BnaDFS): a set of 130 *B. napus* lines representing a structured sampling of the genetic diversity across the global *B. napu*s genepool, and encompassing winter and spring OSR, swedes and fodder, forage and salad kales (www.oregin.info/resources/plantdiversity.php). (4) A *B. rapa* mapping population (BraIRRI): a subset of 81 lines from a larger population of 150 lines (S_8s), derived from a cross between IMB211 rapid cycling Chinese cabbage (ssp. *pekinensis*) from Japan (♀) and R500 Yellow Sarson seed oil genotype (ssp. *tricolaris*) from USA (♂) (Dechaine et al., 2007). A linkage map of 1125 cM and a mean distance between marker loci of 5.7 cM was used (www.ars.usda.gov/research/publications/Publications.htm?seq_no_115=207998).

Growth conditions and data analysis

Plants were grown under glasshouse conditions at low and high P-supply to induce a growth response (see Broadley et al., 2008; Hammond et al., 2009). Phosphorus was added as single superphosphate (SSP, 7% P) to 1-L pots containing a 25% sand:75% (v/v) compost mix (Shamrock medium grade sphagnum peat; Scotts, Ipswich, UK) at 5.25 mg L^{-1} (low $[P]_{ext}$) or 15.75 mg L^{-1} (high $[P]_{ext}$). Other nutrients were incorporated as appropriate. The *B. oleracea* experiments represent a complete experimental design (Broadley et al., 2008). The *B. rapa* and *B. napus* experiments are three experimental runs per population, but are not a complete design. Shoots (*B. oleracea* and *B. napus*) or leaves (*B. rapa*) were sampled during early vegetative growth, typically 5-to-6 weeks growth or sooner. The fresh weight (FW) and dry weight (DW; dried at 80 ºC for 72 h) of material was determined. Shoot/leaf-Ca and Mg was determined by inductively coupled plasma emission spectrometry (ICP-ES). Variance components analyses and accession means were estimated using residual maximum likelihood (REML) procedures, and a simplified model ([run+(treatment*accession)]) to that described in Broadley et al. (2008) in GenStat (Version 11.1.0.1575, VSN International, Oxford, UK). QTL mapping was performed using composite interval mapping (CIM) in QTL Cartographer 2.0 (Wang et al., 2001-2004).

Results and Discussion

Wide variation in shoot/leaf Ca and Mg concentration among three species of Brassica

Shoot/leaf-Ca and Mg varied widely among accessions in each population (Fig. 1). *Brassica napus* had a lower average shoot-Ca (1.1-2.3%, DW) but similar shoot-Mg (0.34-0.66%) compared to *B. oleracea* (Ca, 1.7-3.3%; Mg, 0.35-0.80%). The BolAGDH population (shoot-Ca, 1.6-2.7%; Mg, 0.47-0.76%) captured a substantial proportion (>70%) of the species-wide variation of *B. oleracea*; the two parents were significantly different (♀>♂). The two parents of the *B. rapa* BraIRRI population (leaf-Ca, 1.6-2.7%; Mg, 0.47-0.76%) also differed (♀>♂), but there was less variation among the population than in the BolAGDH. Transgressive segregation was evident among progeny in both the BolAGDH and BraIRRI populations. We conclude that sufficient variation in shoot/leaf-Ca and Mg is evident across all four populations to imply that breeding for these traits will be feasible, if suitable allelic combinations are identified and are heritable.

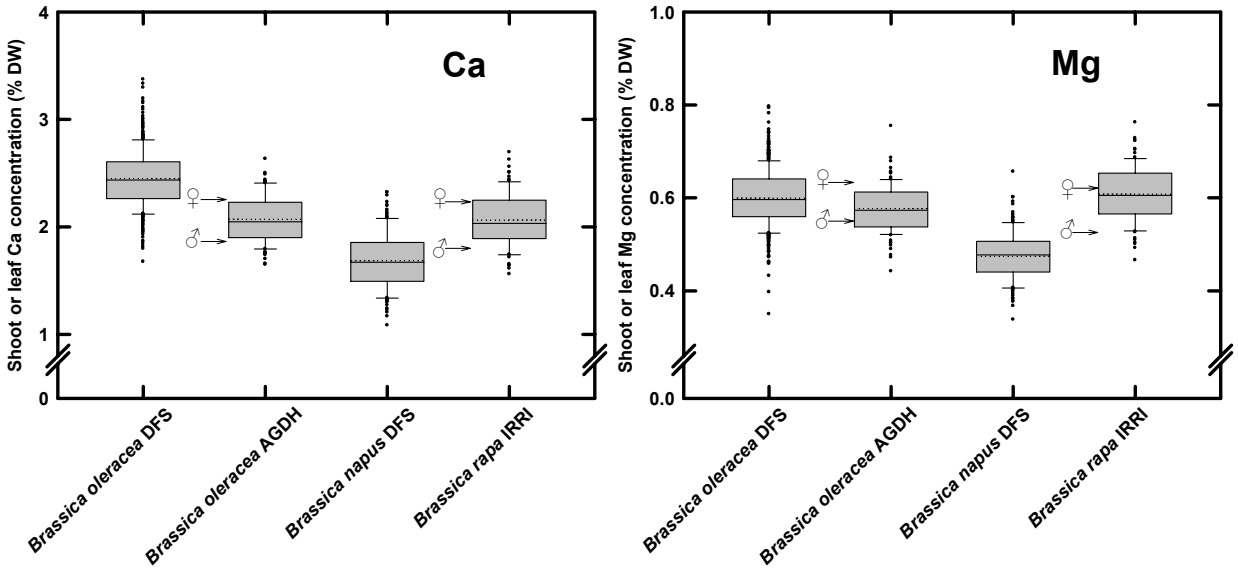

Figure 1. **A.** The Ca and Mg concentration of *Brassica oleracea* and *B. napus* shoots and *B. rapa* leaves. The boundaries of the box proximal and distal to zero are 25th and 75th percentiles, respectively. Solid and dotted lines within the box indicate the median and mean, respectively. Error bars indicate the 10th and 90th percentiles. Circles indicate outliers.

Shoot/leaf Ca and Mg concentration is significantly and highly heritable

Plant genotype contributed a significant proportion of the total variation in shoot/leaf-Ca and Mg in the four populations of *Brassica* (Table 1). Within the *B. napus* and *B. oleracea* DFSs up to 28% of the variation was due to genotype. Within the BolAGDH, genotype contributed 37.5% and 39.6% of the variation for shoot-Ca and Mg. Within the BraIRRI population, there was a highly significant genotypic contribution of c. 10% for leaf-Ca and Mg. Plant growth effects on shoot/leaf-Ca and Mg induced by P-fertilisation were minimal. Although further experiments are needed for BnaDFS and BraIRRI, these data indicate that breeding for altered shoot/leaf-Ca and Mg is feasible.

Table 1. Variance components analysis of shoot/leaf Ca and Mg concentration for four *Brassica* populations. Line variance approximates heritability.

Population	Variance component	Shoot/leaf Ca concentration			Shoot/leaf Mg concentration		
		% variation	*P*	d.f.	% variation	*P*	d.f.
Brassica oleracea DFS	Line	18.1	<0.001	429	15.7	<0.001	429
	Treatment	1.1	<0.001	1	1.7	<0.001	1
	Line*Treatment	0.0	0.962	425	0.0	0.773	425
Brassica napus DFS	Line	28.0	<0.001	129	17.2	0.036	129
	Treatment	0.1	0.345	1	2.1	0.019	1
	Line*Treatment	0.0	0.983	126	0.0	0.988	126
Brassica oleracea AGDH mapping population	Line	37.5	<0.001	99	39.6	<0.001	99
	Treatment	0.2	0.039	1	4.2	<0.001	1
	Line*Treatment	0.0	0.947	99	0.0	0.934	99
Brassica rapa IRRI mapping population	Line	10.1	<0.001	82	10.6	<0.001	82
	Treatment	4.4	<0.001	1	1.8	<0.001	1
	Line*Treatment	0.0	0.939	81	0.2	0.524	81

***Quantitative trait loci (QTL) affecting shoot/leaf Ca and Mg concentration occur in potentially paralogous regions of* Brassica oleracea *and* B. rapa**

We identified regions of the *B. oleracea* genome having a substantial effect on shoot-Ca and Mg. QTL for shoot-Ca and Mg co-localised on C6, C8 and C9 (Broadley et al., 2008; Fig. 2). Significant QTL also occurred on C7 for shoot-Ca, and on C2 for shoot-Mg. The most significant QTL occurred on C9, accounting for 31% (LOD=8.1) and 14% (LOD=3.4) of the population-wide additive genetic variation (V_A) for shoot-Ca and Mg, respectively. QTL on C9 were driven by positive effect of the female parental allele. Analyses of recurrent backcross substitution lines (AGSLs), in which segments of the male GDDH33 line had been introgressed into the female A12DHd background located a shoot-Ca QTL to a 4 cM map interval (43-47 cM) on C9, and supported a further shoot-Ca and Mg QTL distal to this on C9 (Broadley et al., 2008). Comparative alignment of *Brassica* and *Arabidopsis* (Parkin et al., 2005) indicates that 43-47 cM on C9 corresponds to a 0.41 Mb region on *Arabidopsis* Chromosome 5 and further backcrosses are required to resolve these loci. Preliminary analysis of the *B. rapa* A-genome reveals a significant QTL for leaf-Ca on Chromosome A8 (18.7 cM; LOD 3.7; Fig. 2) and a suggestive QTL for leaf-Ca on Chromosome A9 (53.7 cM; LOD 2.2). Chromosomes A8 and A9 contain regions collinear with C-genome regions on chromosomes C7, C8 and C9, which contain QTL. If such loci are confirmed to be paralogous, they are potentially useful in biofortification breeding programmes in vegetable *Brassica*.

The BraIRRI population is a good model for resolving potentially paralogous loci in segmentally duplicated diploid A- and C-genomes of *Brassica,* for a number of reasons. The IMB211 maternal line has a short generation time (8 weeks) and is self-compatible. Moreover, a large proportion of the A-genome sequence is now complete (www.brassica.info/resource/sequencing/status.php), with complete coverage anticipated during

2009 from new-generation sequencing approaches. In conjunction with high density transcriptome (Trick et al., 2009), this is facilitating the navigation between genetic and physical maps and sequence, and *Arabidopsis* gene models. Finally, the recent release of high mutant-load TILLING (Targeting Induced Local Lesions IN Genomes) populations in A-genome *Brassica* (www.brassica.info/research/activities/tilling.php) will facilitate the rapid functional analysis of candidate genes and, ultimately, to understand the regulation and function of these genes in controlling Ca and Mg dynamics in plants.

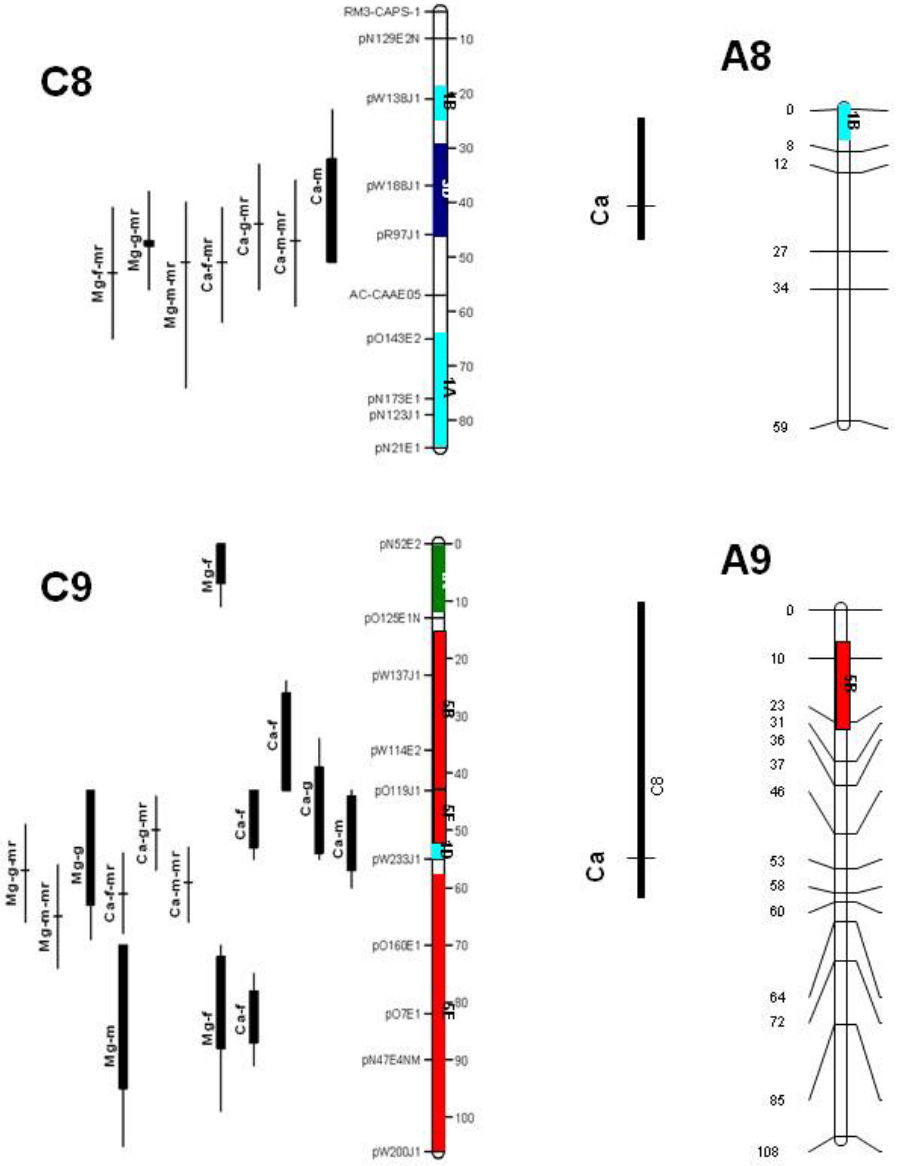

Fig. 2. Quantitative trait loci (QTL) for shoot/leaf Ca and Mg concentration on chromosomes C8, C9 (*B. oleracea*), A8 and A9 (*B. rapa*). Details of trait codes for the C-genome follow those in Broadley et al. (2008). A-genome QTL are given as position (cM, cross-hatch) +/- 1 LOD support (black vertical bar). Chromosome segments collinear with *Arabidopsis* are *sensu* Parkin et al. (2005).

Acknowledgements
The authors acknowledge financial support from the UK Biotechnology & Biological Sciences Research Council, the UK Department for Environment, Food, & Rural Affairs, and the Scottish Government Rural & Environmental Research & Analysis Directorate.

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