Shoot Calcium and Magnesium Concentrations Differ between Subtaxa, Are Highly Heritable, and Associate with Potentially Pleiotropic Loci in *Brassica oleracea*[1][W][OA]

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Calcium (Ca) and magnesium (Mg) are the most abundant group II elements in both plants and animals. Genetic variation in shoot Ca and shoot Mg concentration (shoot Ca and Mg) in plants can be exploited to biofortify food crops and thereby increase dietary Ca and Mg intake for humans and livestock. We present a comprehensive analysis of within-species genetic variation for shoot Ca and Mg, demonstrating that shoot mineral concentration differs significantly between subtaxa (varietas). We established a structured diversity foundation set of 376 accessions to capture a high proportion of species-wide allelic diversity within domesticated *Brassica oleracea*, including representation of wild relatives (C genome, $1n = 9$) from natural populations. These accessions and 74 modern F_1 hybrid cultivars were grown in glasshouse and field environments. Shoot Ca and Mg varied 2- and 2.3-fold, respectively, and was typically not inversely correlated with shoot biomass, within most subtaxa. The closely related *capitata* (cabbage) and *sabauda* (Savoy cabbage) subtaxa consistently had the highest mean shoot Ca and Mg. Shoot Ca and Mg in glasshouse-grown plants was highly correlated with data from the field. To understand and dissect the genetic basis of variation in shoot Ca and Mg, we studied homozygous lines from a segregating *B. oleracea* mapping population. Shoot Ca and Mg was highly heritable (up to 40%). Quantitative trait loci (QTL) for shoot Ca and Mg were detected on chromosomes C2, C6, C7, C8, and, in particular, C9, where QTL accounted for 14% to 55% of the total genetic variance. The presence of QTL on C9 was substantiated by scoring recurrent backcross substitution lines, derived from the same parents. This also greatly increased the map resolution, with strong evidence that a 4-cM region on C9 influences shoot Ca. This region corresponds to a 0.41-Mb region on Arabidopsis (*Arabidopsis thaliana*) chromosome 5 that includes 106 genes. There is also evidence that pleiotropic loci on C8 and C9 affect shoot Ca and Mg. Map-based cloning of these loci will reveal how shoot-level phenotypes relate to Ca^{2+} and Mg^{2+} uptake and homeostasis at the molecular level.

An appropriate plant-based diet can supply sufficient minerals to satisfy human dietary requirements. 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), or zinc (Zn; Welch and Graham, 2004). Ca and Mg are the most abundant group II elements in both plants and animals. In adult humans, 99% (w/w) of Ca (>1 kg) is associated with bones and teeth (National Research Council, 1989). The remainder is involved in signaling, maintenance of cell structure, and other metabolic functions. Under Ca deficiency, soft tissue Ca is maintained, thereby reducing bone strength and increasing the risk of Ca-related clinical symptoms including osteoporosis (National Research Council, 1989). Dietary Ca deficiency is the major cause of nutritional rickets affecting rural populations (Thacher et al., 2006). The increased prevalence of nutritional rickets has been attributed to a change from bean-rich to cereal-rich diets in areas where dairy and meat consumption is low (Welch and Graham, 2004). Adult humans typically contain 25 g Mg, about 60% of which is in hard tissues, 40% in muscles and soft tissues, and 1% in extracellular fluids, where it is under tight homeostatic control (National

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Research Council, 1989). Magnesium is a component of numerous enzymatic reactions, including energy, protein, and fatty acid metabolism, and in maintenance of cell ionic balance (Gums, 2004). Human Mg deficiency is linked to numerous conditions, including hypertension, heart dysfunction, diabetes, and preeclampsia. Ultimately, all dietary mineral deficiencies are caused by the consumption of plants—either directly or indirectly via the livestock food chain—which contain insufficient minerals. Agronomic biofortification using fertilizers can increase the delivery of dietary minerals to humans and livestock (Broadley et al., 2006b). Genetic biofortification, i.e. breeding crops with increased mineral content, is an attractive complementary strategy (Grusak and DellaPenna, 1999; Welch and Graham, 2004; White and Broadley, 2005a; Morris et al., 2008).

Typically, shoot Ca and shoot Mg concentration (shoot Ca and Mg) is in the low percentage (w/w) dry weight (DW) range in plants, although very wide ranges (Ca, 0.02%–13.1%; Mg, 0.03%–3.2%) are reported in surveys of terrestrial plant species (Broadley et al., 2003, 2004; Watanabe et al., 2007). The divalent cation Ca^{2+} has structural roles in plant cell walls and membranes, is a counter cation for inorganic and organic anions in the vacuole, and is an intracellular messenger in the cytosol (White and Broadley, 2003). Calcium uptake is mediated by both symplastic and apoplastic fluxes in the root, while entry and exit of Ca to the symplast is exquisitely controlled by plasma membrane-localized Ca^{2+}-permeable ion channels and Ca^{2+}-ATPases, respectively. Magnesium has a critical role in the activation of a wide range of enzymes in plants, and is a key structural component of the chlorophyll molecule and ribosomal aggregates (Shaul, 2002). Calcium is relatively immobile in the phloem of plants, and source-to-sink transfer rates are invariably low. Cereal grains, fruits, and root storage organs typically have low Ca concentrations (White and Broadley, 2005a). In general, Mg has higher phloem mobility than Ca. Despite their unique physiological and biochemical roles in planta, Ca^{2+} and Mg^{2+} homeostasis is likely to be controlled in part by common regulatory networks, as in yeast (*Saccharomyces cerevisiae*; Wiesenberger et al., 2007), at the uptake, transport, and tissue localization levels due to the chemical similarity of Ca^{2+} and Mg^{2+} ions.

The genetics of shoot Ca and Mg have not yet been characterized or dissected within any plant species. However, shoot Ca and Mg are likely to be under strong genetic control and we have shown previously that shoot Ca and Mg varies between species of different plant families, either due to past selection pressures or trait canalization. For example, shoot Ca and Mg are typically much lower in species of commelinid monocotyledon families, which include the Poaceae and Cyperaceae, than in species from other dicot and monocot families of angiosperms (Broadley et al., 2003, 2004). Differences in cell wall chemistry and cation-exchange capacity may correspond with family level differences in shoot Ca (White and Broadley, 2003; White, 2005). Furthermore, species from families within the order Caryophyllales (which includes the families Amaranthaceae and Caryophyllaceae) tend toward higher shoot Mg, and thereby lower shoot Ca to shoot Mg ratios, than species of many terrestrial plant families (Broadley et al., 2004; Watanabe et al., 2007).

We have conducted a species-wide dissection of variation in shoot Ca and Mg in the domesticated genepool of *Brassica oleracea*. *B. oleracea* (C genome; $1n = 9$) is a widely consumed leafy-vegetable crop, well suited to genetical analyses due to the availability of well-structured genetic variation defined by ecogeographic regions and crop type, as well as the tractability of resolving genetic loci. The genus *Brassica* contains the closest crop relatives of Arabidopsis (*Arabidopsis thaliana*) and the duplicated genomic structure and relationships within the Brassicaceae are becoming well characterized (Lysak et al., 2005; Parkin et al., 2005; Kim et al., 2006). The C genome of *B. oleracea* is also a component of the widely grown amphidiploid species *Brassica napus* (AC genome; canola/oilseed rape/colza/rutabaga/swede), as well as *Brassica carinata* (BC genome; Ethiopian mustard). We used variance components analyses to assign variation in shoot Ca and Mg to genetic and environmental components. We then tested whether genetic regulation of shoot Ca and Mg was likely to be under strong selection pressure by assessing if recombination of alleles present within two homozygous lines could contribute to a range of variation comparable with that encountered in the wider genepool. We also tested whether physiological attributes of these homozygous lines corresponded with shoot Ca and Mg. We were able to detect quantitative trait (QTL) for shoot Ca and Mg, and then confirm and resolve these loci to much shorter mapping intervals, using a backcrossed substitution line population. Map-based cloning of QTL ultimately offers the prospect of relating shoot Ca and Mg phenotypes to underlying molecular mechanisms of Ca^{2+} and Mg^{2+} uptake and homeostasis.

RESULTS

Shoot Ca and Mg Varies Widely in *B. oleracea* and Is Not Correlated with Shoot Biomass

We have defined a diversity fixed foundation set (DFFS) as an informative set of genetically fixed lines representing a structured sampling of diversity across a genepool (Supplemental Table S1). A diversity foundation set (DFS) consists of founder lines that may include landrace, open-pollinated, and more uniform F_1 or inbred lines. There was considerable variation in shoot Ca and Mg among 355 DFS accessions of the *Brassica* C genome and 74 F_1 cultivars in the DFS. Shoot Ca varied 2-fold (1.7%–3.3%) and shoot Mg 2.3-fold (0.35%–0.80%; Fig. 1; Supplemental Table S1) across all treatment factors. The treatment factors included dif-

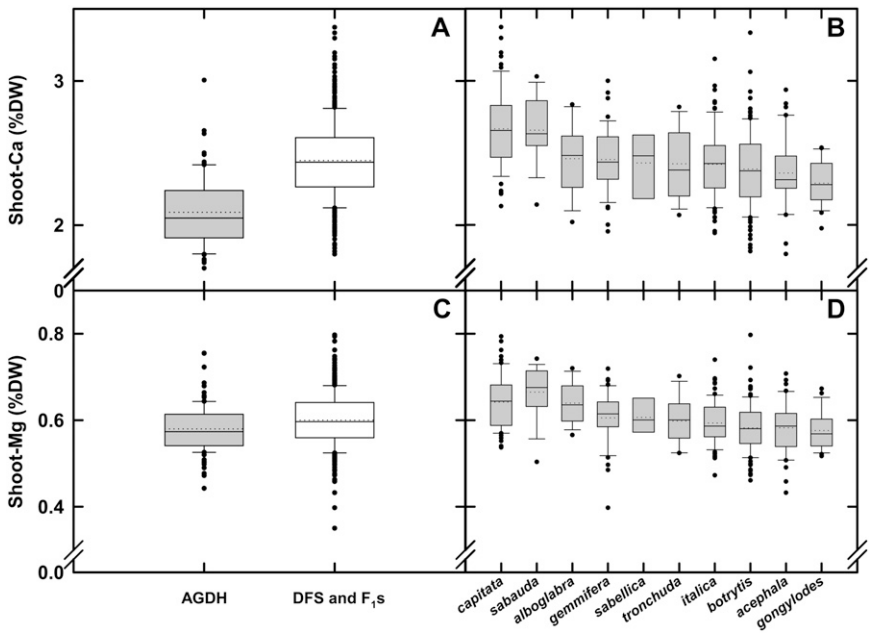

Figure 1. Shoot Ca (A and B) and Mg (C and D) concentration of *Brassica* C-genome accessions. A and C, Data are REML-estimated means, averaged across all treatment factors, from species-wide (DFS and F_1s, compare with experiment *GE1*) and mapping population (AGDH, compare with experiment *GE2*) experiments. B and D, Subtaxa (varietas) rankings of mean leaf Ca and Mg in *GE1*. The boundaries of the box closest and farthest to zero indicate the 25th and 75th percentiles, respectively. The solid and dotted lines within the box indicate the median and mean, respectively. Error bars indicate the 10th and 90th percentiles. Circles indicate outliers.

ferent phosphate conditions ($[P]_{ext}$). These $[P]_{ext}$ conditions were chosen based on previous growth response studies (Greenwood et al., 2005, 2006) in which high $[P]_{ext}$-grown plants had higher shoot fresh weight (FW) and DW than plants grown at low $[P]_{ext}$ (Supplemental Table S1). Young glasshouse-grown plants were sampled before visible morphological differentiation occurred between subtaxa (varietas), although some of the rapid cycling *alboglabra* accessions bolted on occasion. To remove any potential confounding effects of morphology, correlation analyses were conducted within each subtaxa. Within most subtaxa, there was no correlation between shoot Ca and Mg and shoot FW or DW. However, within two of the 10 subtaxa with the smallest representation, there was a weak negative correlation between shoot DW and shoot Ca (*gongylodes*, kohlrabi, $r = 0.43$, $P = 0.042$, degrees of freedom [df] = 21; *sabauda*, Savoy cabbage, $r = 0.53$, $P = 0.041$, df = 13). There were also weak negative correlations between shoot DW and shoot Mg within *acephala* (kale, $r = 0.41$, $P = 0.009$, df = 38) and within *gongylodes* ($r = 0.49$; $P = 0.017$, df = 21).

Shoot Ca and Mg differed significantly ($P < 0.001$) between different subtaxa, with the closely related *capitata* (cabbage) and *sabauda* having highest mean shoot Ca and Mg, and *gongylodes* having lowest mean shoot Ca and Mg (Fig. 1). Other subtaxa had intermediate shoot Ca and Mg; subtaxa-level mean shoot Ca and Mg correlated significantly across the 10 subtaxa ($r = 0.97$; Student's t test approximation = 11.84; $P < 0.001$; df = 8). Shoot Ca and Mg also correlated within all 10 subtaxa (data not shown). While the subtaxa term explained approximately 20% of the genetic variance component (data not shown), wide genetic variation in shoot Ca and Mg still occurred within each subtaxa (Fig. 1). We also tested whether selection pressures have resulted in reductions in shoot Ca or Mg within subtaxa. There was no overall difference in shoot Ca or Mg between likely landrace and nonlandrace accessions within any of the subtaxa except for *italica* (broccoli). Within *italica*, shoot Ca and Mg ($\bar{x} = 2.47$ and 0.60%, respectively, $n = 59$) were slightly higher ($P < 0.05$) in likely landrace accessions than in nonlandrace accessions ($\bar{x} = 2.34$ and 0.58%, respectively, $n = 29$).

Shoot Ca and Mg in *B. oleracea* Is Highly Consistent between Glasshouse and Field Environments

Variation in shoot Ca and Mg among commercial F_1 hybrid cultivars represented >80% of the species-wide variation in shoot Ca and Mg in the DFS (Supplemental Table S1). Among these genetically uniform F_1s, we tested if shoot Ca and Mg correlated between glasshouse and field environments. The field experiment included four different $[P]_{ext}$ conditions, a nutritional axis based on previous growth-response studies (Greenwood et al., 2005; Supplemental Table S1). Across all treatment factors, shoot Ca and Mg were highly positively correlated between glasshouse and field environments (Fig. 2). These data show that glasshouse conditions can be used to represent variation in shoot Ca and Mg in field situations, and that shoot Ca and Mg is likely to be highly heritable. Indeed, genetic variance components for accessions accounted for 15.5% and 13.9% (glasshouse conditions), and 27.9% and 11.3% (field conditions) of the total variation in shoot Ca and Mg, respectively (Table I). There was little or no significant effect of $[P]_{ext}$ on shoot Ca or Mg and no G × E interaction (Table I). Unsurprisingly, a large fraction of the variation in shoot FW and DW in glasshouse conditions was due to

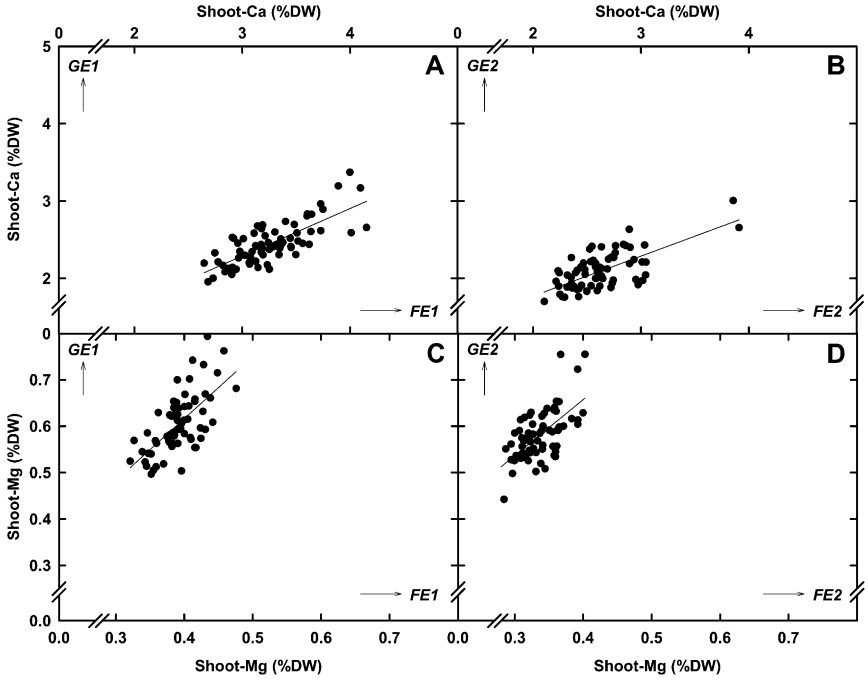

Figure 2. Shoot Ca (A and B) and Mg (C and D) concentration of *B. oleracea* genotypes grown under both field (*x* axes data, *FE1*, *FE2*) and glasshouse (*y* axes data, *GE1*, *GE2*) conditions. Data are REML-estimated means, averaged across all treatment factors, for 69 F_1s (A and C) and for 61 informative DH accessions, plus the two parental AGDH accessions, and eight reference F_1s (B and D). Correlation coefficients are (A, $r = 0.76$; B, $r = 0.67$; C, $r = 0.65$; D, $r = 0.64$; all correlations $P < 0.0001$ [A and C = 67 df, B and D = 69 df]). Fitted lines represent linear regressions.

$[P]_{ext}$, with this treatment factor accounting for >40% of total variation in shoot FW and DW (Table I).

Shoot Ca and Mg Is Highly Heritable

The heritability of shoot Ca and Mg was determined using a previously well-characterized mapping population of doubled-haploid (DH) segregants (the AGDH population; Fig. 3). Shoot Ca and Mg were highly heritable, with genetic variance components for shoot Ca and Mg of 36.0% and 37.7%, respectively, under glasshouse conditions (*GE2*), and 41.5% and 25.3%, respectively, under field conditions (*FE2*; Table I). These genetic variance components approximate the population-wide additive genetic variation (V_A), or narrow-sense heritability. Consistent with our findings for F_1 accessions, shoot Ca and Mg correlated between glasshouse and field conditions in the AGDH population (Fig. 2, B and D; Supplemental Table S1). Also, consistent with most within-subtaxa comparisons, there was no correlation between shoot Ca and Mg and shoot FW or DW. Among the AGDH population, transgressive segregation in shoot Ca and Mg was evident (Fig. 3). In glasshouse-grown AGDH plants, shoot Ca varied from 1.7% to 2.7%, and shoot Mg from 0.44% to 0.79%. This segregation, resulting from recombination of only two sets of homozygous parental alleles, surprisingly corresponded to a major proportion (75%–80%) of the species-wide variation in shoot Ca and Mg (Fig. 1, A and C).

Shoot Ca and Mg among AGDH Lines Correlates with Physiological Traits

We tested hypotheses that variation in shoot Ca and Mg is associated with root and/or shoot physiological traits. These traits included root and shoot length (measured after 15 d growth), biomass production rates, root development, seed nutritional status, germination time, and germination response to heat and water stress, scored previously in the AGDH population (Bettey et al., 2000; Cogan et al., 2002, 2004). There were 41 AGDH lines common to all studies (39 df for Spearman's rank correlation analysis). Shoot Ca and Mg correlated with two physiological parameters. A weak positive correlation was observed between shoot length and both shoot Ca ($r = 0.36$, $P = 0.019$) and Mg ($r = 0.33$, $P = 0.033$) in glasshouse-grown plants, as well as shoot length and shoot Ca ($r = 0.40$, $P = 0.010$) in field-grown plants. There was also a weak negative correlation between root length growth rate and shoot Ca in glasshouse-grown plants ($r = -0.34$, $P = 0.032$).

QTL for Shoot Ca and Mg Map to Linkage Groups C2, C6, C7, C8, and C9

We detected several regions of the *B. oleracea* genome having a substantial effect on shoot Ca and Mg (Fig. 4). QTL for shoot Ca and Mg potentially colocalized on C6, C8, and C9 (Fig. 4). Thus, several QTL may be pleiotropic for both shoot Ca and Mg. Significant QTL were also detected on C7 for shoot Ca and on C2 for shoot Mg. The most significant QTL for shoot Ca and Mg occurred on C9. For shoot Ca, C9 QTL accounted for 31% (log of the odds [LOD] = 8.1) and 55% (LOD = 5.0 and 6.3) of V_A, under glasshouse (*GE2*) and field (*FE2*) conditions, respectively. For shoot Mg, C9 QTL accounted for 14% (LOD = 3.4) and 33% (LOD = 5.1 and 4.4) of V_A, under glasshouse and field conditions, respectively. QTL on chromosomes C2, C8, and C9 were driven by a positive additive effect of the female

Table I. *Variance components analyses of shoot FW and DW, percentage DW (%DW), and shoot Ca and Mg concentration of B. oleracea grown in glasshouse (GE1, GE2, GE3) and field (FE1, FE2) experiments*

		Shoot FW			Shoot DW			DW			Shoot Ca			Shoot Mg		
		g			*g*			%			%			%		
Glasshouse Experiment 1 (*GE1*)	Variance component (%)															
DFS and F_1 cultivars	Occasion	27.9			27.3			6.3			19.6			19.2		
	Occasion/bench	1.7			2.7			2.9			3.7			4.9		
	Occasion/bench/run	2.3			3.6			6.3			10.1			6.8		
	Accession	6.7			5.5			9.3			15.5			13.9		
	$[P]_{ext}$	43.8			40.4			1.1			1.2			1.7		
	$[P]_{ext}$/accession	2.1			3.6			0.0			0.4			0.0		
	Residual	15.5			16.8			74.2			49.5			53.4		
	Fixed term	W[a]	d.f.[b]	χ^2[c]	W	d.f.	χ^2	W	d.f.	χ^2	W	d.f.	χ^2	W	d.f.	χ^2
	Accession	1,960	429	<0.001	1,768	429	<0.001	786	429	<0.001	1,432	428	<0.001	1,290	428	<0.001
	$[P]_{ext}$	4,939	1	<0.001	4,335	1	<0.001	24	1	<0.001	41	1	<0.001	55	1	<0.001
	$[P]_{ext}$/accession	655	428	<0.001	766	428	<0.001	354	428	0.996	436	424	0.328	454	424	0.151
	Standard errors of differences of mean															
	Average:	1.86			0.19			1.28			0.24			0.06		
	Maximum:	3.32			0.35			2.29			0.42			0.10		
	Minimum:	1.06			0.11			0.73			0.13			0.03		
Field Experiment 1 (*FE1*)	Variance component (%)															
F_1 cultivars	Occasion	68.5			62.4			36.8			8.8			44.7		
	Occasion/replicate	1.4			3.7			3.8			0.0			0.0		
	Occasion/replicate/block	0.8			0.2			2.3			10.6			6.8		
	Occasion/replicate/block/main_plot	2.7			1.9			3.3			15.6			11.3		
	Accession	5.4			6.6			23.1			27.9			11.3		
	$[P]_{ext}$	1.0			0.5			2.5			0.0			2.3		
	$[P]_{ext}$/accession	0.0			0.0			0.0			0.0			0.0		
	Residual	20.1			24.6			28.2			37.0			23.6		
	Fixed term	W	d.f.	χ^2	W	d.f.	χ^2	W	d.f.	χ^2	W	d.f.	χ^2	W	d.f.	χ^2
	Accession	708	71	<0.001	722	71	<0.001	2,001	71	<0.001	1,826	71	<0.001	1,177	71	<0.001
	$[P]_{ext}$	22	3	<0.001	22	3	<0.001	25	3	<0.001	0	3	0.958	10	3	0.017
	$[P]_{ext}$/accession	190	213	0.870	190	213	0.873	142	213	1.000	175	213	0.974	189	213	0.883
	Standard errors of differences of mean															
	Average:	6.75			0.73			0.24			0.10			0.01		
	Maximum:	7.04			0.76			0.25			0.10			0.01		
	Minimum:	6.64			0.72			0.24			0.10			0.01		
Glasshouse Experiment 2 (*GE2*)	Variance component (%)															
AGDH accessions	Occasion	37.7			14.0			32.7			12.8			5.2		
	Occasion/replicate	0.8			4.6			14.8			6.6			2.0		
	Occasion/replicate/block	4.6			7.4			7.4			6.3			4.9		
	Occasion/replicate/block/plot	0.5			0.0			7.9			12.0			14.4		
	Accession (V_A)	10.4			14.2			13.2			36.0			37.7		
	$[P]_{ext}$	21.0			24.2			0.0			0.2			4.0		
	$[P]_{ext}$/accession	0.2			1.0			0.6			1.4			1.1		
	Residual	24.8			34.7			23.3			24.8			30.8		
	Fixed term	W	d.f.	χ^2	W	d.f.	χ^2	W	d.f.	χ^2	W	d.f.	χ^2	W	d.f.	χ^2
	Accession	771	99	<0.001	784	99	<0.001	670	99	<0.001	1,364	99	<0.001	1,188	99	<0.001
	$[P]_{ext}$	765	1	<0.001	633	1	<0.001	2	1	0.209	8	1	0.004	120	1	<0.001
	$[P]_{ext}$/accession	108	99	0.262	123	99	0.049	121	99	0.068	148	99	0.001	130	99	0.019
	Standard errors of differences of mean															
	Average:	0.90			0.09			0.44			0.09			0.02		
	Maximum:	0.95			0.10			0.46			0.10			0.02		
	Minimum:	0.88			0.09			0.43			0.09			0.02		
Field Experiment 2 (*FE2*)	Variance component (%)															
AGDH accessions	Replicate	0.0			0.4			11.2			12.3			12.0		
	Replicate/block	2.3			1.8			0.6			4.6			1.4		
	Replicate/block/main_plot	9.2			6.9			19.2			8.3			5.4		
	Accession (V_A)	44.2			48.8			8.3			41.5			25.3		
	$[P]_{ext}$	7.9			3.9			12.9			11.1			27.9		
	$[P]_{ext}$/accession	0.3			0.0			1.0			2.1			0.0		
	Residual	36.1			38.2			46.7			20.1			28.0		

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

		Shoot FW			Shoot DW			DW			Shoot Ca			Shoot Mg		
	Fixed term	*W*	d.f.	χ^2	*W*	d.f.	χ^2	*W*	d.f.	χ^2	*W*	d.f.	χ^2	*W*	d.f.	χ^2
	Accession	1,017	71	<0.001	1,068	71	<0.001	211	71	<0.001	1,726	71	<0.001	786	71	<0.001
	$[P]_{ext}$	21	3	<0.001	14	3	0.003	35	3	<0.001	20	3	<0.001	105	3	<0.001
	$[P]_{ext}$/accession	213	213	0.488	199	213	0.740	223	213	0.308	281	213	0.001	180	213	0.950
	Standard errors of differences of mean															
	Average:	7.34			0.75			0.55			0.09			0.01		
	Maximum:	7.79			0.80			0.58			0.10			0.01		
	Minimum:	7.18			0.74			0.53			0.09			0.01		
Glasshouse Experiment 3 (*GE3*)	Variance component (%)															
AGSL accessions	Accession	7.6			10.1			43.5			59.5			47.5		
	$[P]_{ext}$	63.0			57.4			2.8			8.2			4.3		
	$[P]_{ext}$/accession	4.5			4.2			0.0			6.8			17.4		
	Residual	24.9			28.2			53.8			25.6			30.8		
	Fixed term	*W*	d.f.	χ^2	*W*	d.f.	χ^2	*W*	d.f.	χ^2	*W*	d.f.	χ^2	*W*	d.f.	χ^2
	Accession	137	30	<0.001	143	30	<0.001	182	30	<0.001	536	30	<0.001	359	30	<0.001
	$[P]_{ext}$	259	1	<0.001	207	1	<0.001	6	1	0.014	41	1	<0.001	24	1	<0.001
	$[P]_{ext}$/accession	45	30	0.039	42	30	0.070	26	30	0.652	57	30	0.002	88	30	<0.001
	Standard errors of differences of mean															
	Average:	1.78			0.18			0.49			0.09			0.02		
	Maximum:	1.80			0.19			0.50			0.09			0.02		
	Minimum:	1.46			0.15			0.40			0.07			0.02		

[a]Wald test statistic. [b]Degrees of freedom. [c]Chi-squared function.

parental allele (from A12DHd var. *alboglabra*), whereas QTL on chromosomes C6 and C7 were driven by a positive additive effect from the male parental allele (GDDH33 var. *italica*).

Confirmation and Resolution of QTL for Shoot Ca and Mg

The presence of QTL for shoot Ca and Mg on C9 were confirmed and resolved further using recurrent backcross substitution lines (AGSLs), in which segments of the GDDH33 line are introgressed into the A12DHd background (Rae et al., 1999; Supplemental Table S3; Fig. 5). AGSLs were scored for shoot Ca and Mg in a glasshouse experiment (*GE3*), including five AGSLs containing GDDH33 allele substitutions on C9: AGSL121, AGSL122, AGSL129, AGSL141, and AGSL173 (Supplemental Table S3; Fig. 5). There was no significant difference in shoot Ca or Mg between A12DHd and AGSL121 (Fig. 5). AGSL122 and AGSL129 had lower shoot Ca than A12DHd ($P < 0.01$), but with no significant difference in shoot Mg. AGSL141 and AGSL173 had lower shoot Ca and Mg than A12DHd ($P < 0.001$). There was a negative allelic effect on shoot Ca and Mg in all AGSLs containing a GDDH33 allele on C9. Thus, these data are entirely consistent with data from the AGDH lines, although shoot Ca and Mg was not significantly different to A12DHd in all cases (Fig. 5). These observations provisionally locate a shoot Ca QTL to a 4-cM map interval (between 43 and 47 cM) on C9, and provide evidence of a further shoot Ca and Mg QTL distal to this on C9. However, since all five of these AGSLs contain a small number of short chromosomal segments containing GDDH33 alleles elsewhere on the genome (Supplemental Table S3; Fig. 5), including on C2 and C8, further backcrosses are required to verify and resolve these loci.

DISCUSSION

We have carried out the first systematic dissection of genetic variation in shoot Ca and Mg in plants. A DFS was selected to provide an informative set of lines representing a structured sampling of diversity across the *B. oleracea* genepool, together with wild C-genome relatives that are interfertile with this species, including *Brassica villosa* Biv. and *Brassica hilarionis* Post., as well as the A-genome *Brassica rapa*. *Brassica* C-genome species have adapted to a range of environmental niches in the Mediterranean center of diversity and appear to

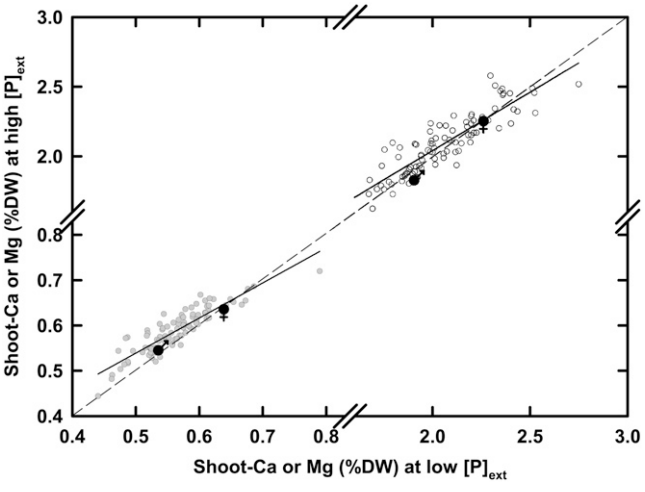

Figure 3. Shoot Ca (white circles) and Mg (gray circles) concentration of 90 informative DH accessions, plus two parental accessions of *B. oleracea* AGDH mapping population grown under contrasting $[P]_{ext}$ in glasshouse experiment *GE2*. Correlations of shoot Ca and Mg are both $r = 0.89$, $P < 0.0001$ (90 df). Trait values of the female (A12DHd) and male (GDDH33) DH parent lines are indicated. Dashed line represents unity.

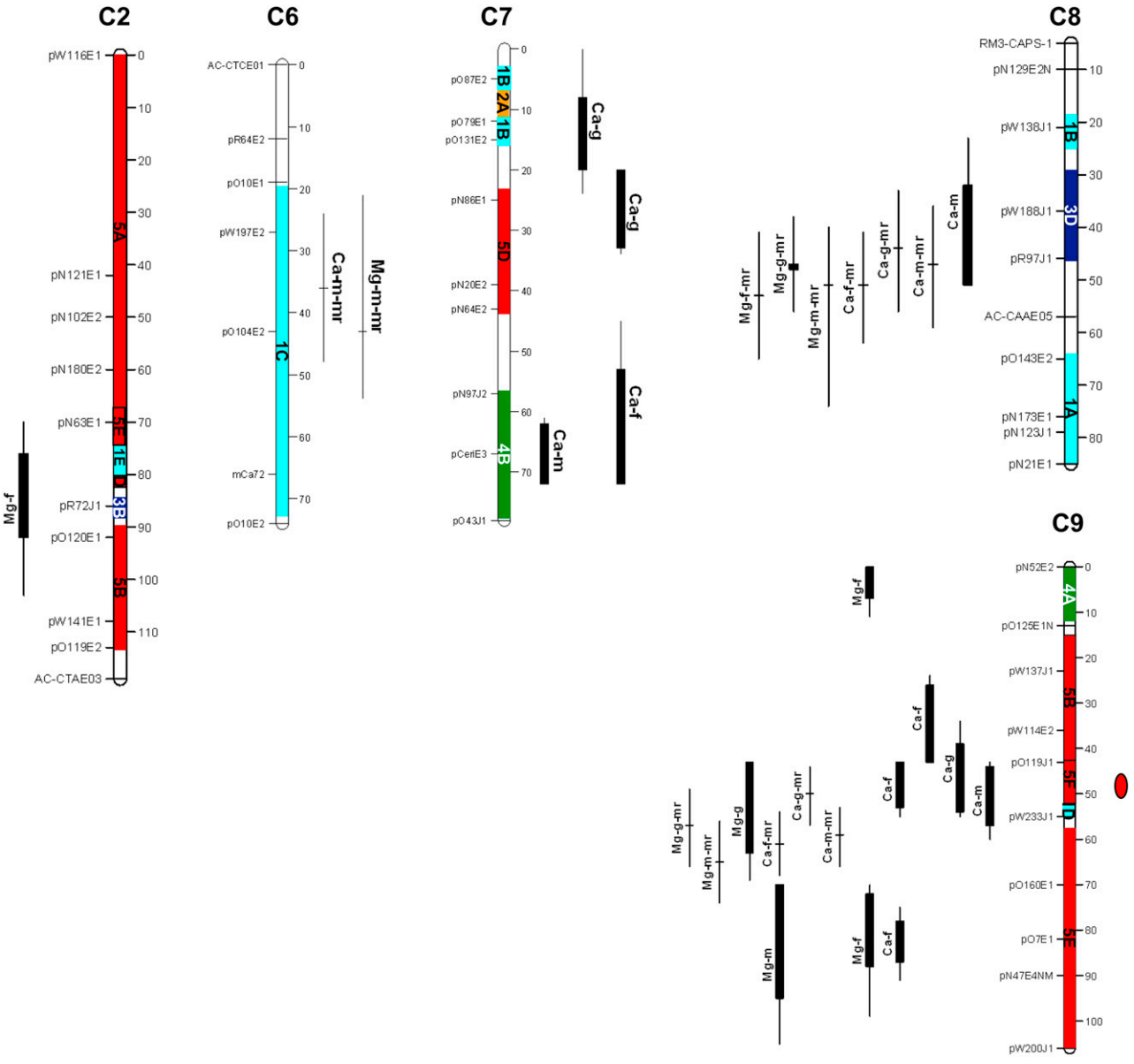

Figure 4. QTL for shoot Ca and Mg concentration (shoot Ca, Mg) on *B. oleracea* chromosomes C2 and C6 to C9. QTL with positive additive effects arising from female parental (A12DHd) alleles are shown to the left of the chromosomes, while those with additive effects from the male parent (GDDH33) are shown to the right. Segments of chromosomes collinear with those in the Arabidopsis genome are indicated, with block nomenclature as defined by Parkin et al. (2005). The 4-cM section of C9 resolved using substitution lines is indicated by a red oval. QTL detected by CIM are indicated as follows: Ca-f: shoot Ca in field; Ca-g: shoot Ca in glasshouse; Ca-m: mean of shoot Ca in field and glasshouse. QTL detected by multiple marker regression, as above, have -mr suffix (e.g. Mg-m-mr). For CIM, the thick vertical line indicates the 1-LOD interval, and the thin whisker line the extent of the 2-LOD interval; for multiple marker regression, the midpoint of the simulated QTL is shown by a horizontal mark, with the 95% confidence interval shown by the vertical whisker line.

represent a wide range of genetic variation (Allender et al., 2007). In addition, 74 commercially grown F_1 hybrid cultivars were included, representing a morphologically diverse group of *B. oleracea* domesticated crop subtaxa, including the following varietas: *acephala* (kale/collards), *alboglabra* (oriental kale), *botrytis* (cauliflower), *capitata* (cabbage), *gemmifera* (Brussels sprout), *gongylodes* (kohlrabi), *italica* (broccoli/calabrese), *sabauda* (Savoy cabbage), *sabellica* (borecole/curly kale), and *tronchuda* (Portuguese cole). Theoretical studies (Lawrence et al., 1995a, 1995b) indicate that 400 homozygous accessions, collected from natural populations throughout the world, should contain 99% of the allelic polymorphism (i.e. for alleles with frequencies >2%) present in the species. While the DFS and F_1s selected for this study are likely to represent most of the common allelic variation within the species, canalization of alleles associated with domestication may

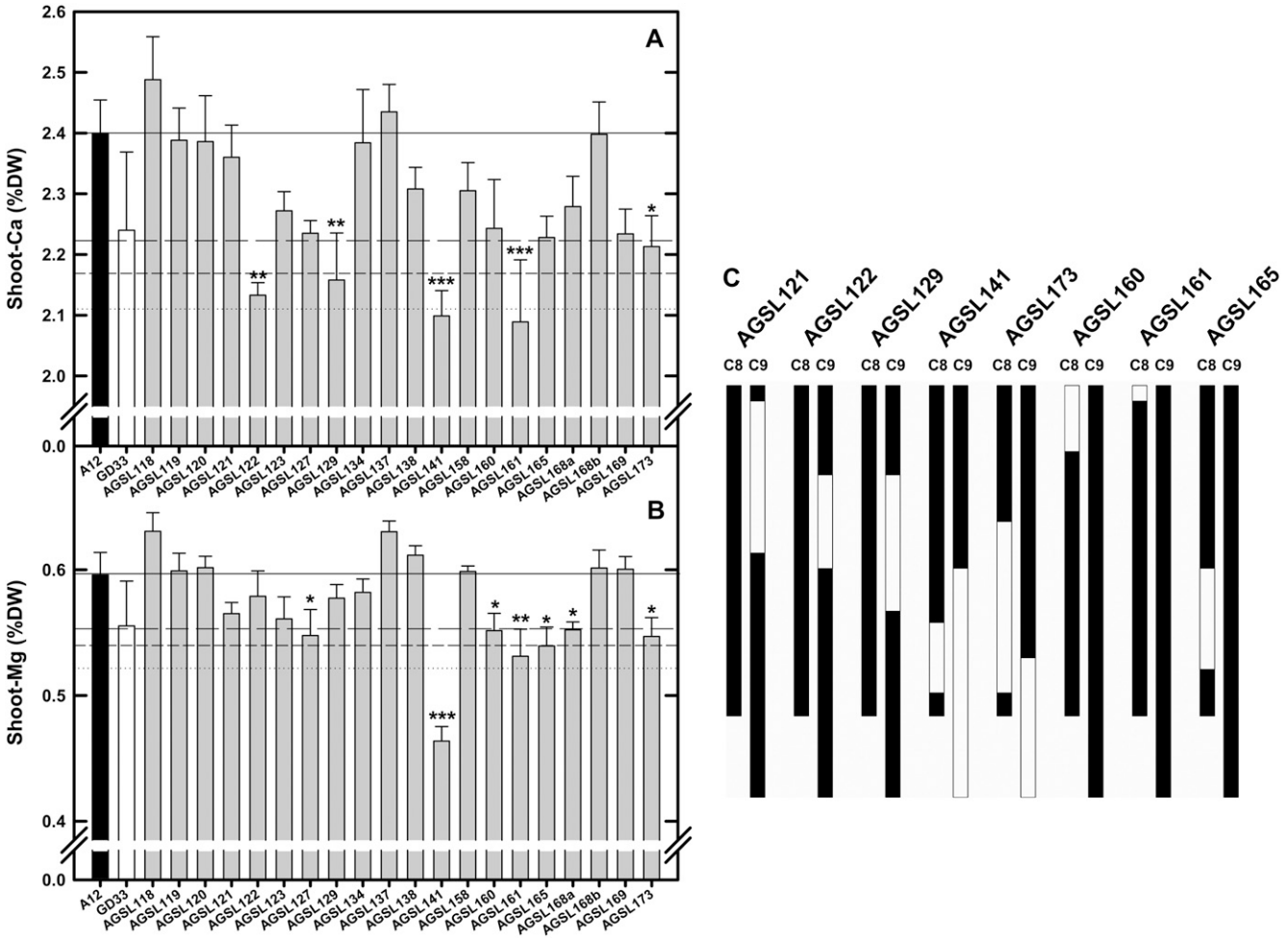

Figure 5. Shoot Ca (A) and Mg (B) concentration of 20 substitution lines derived from the *B. oleracea* AGDH mapping population (compare with glasshouse experiment *GE3*). Black bars, female (A12, A12DHd), and white bars, male (GD33, GDDH33) DH parent lines. Solid line is mean A12DHd trait value; long-dashed, short-dashed, and dotted lines are LSD thresholds at $P = 0.05$ (*), 0.01 (**), and 0.001 (***), respectively (one-way ANOVA). Error bars represent +1 SEM ($n = 6$). Introgressed regions of GDDH33 on chromosomes 8 and 9 of the AGSLs are indicated in white in C. All introgressed regions are provided in Supplemental Table S3.

lead to underrepresentation of some species-wide allelic variation, hence the inclusion of wild relatives (Allender et al., 2007). Here, based on initial genetic analysis of a small number of simple sequence repeat (SSR) markers ($n = 29$), scored on 95 of the DFS lines, there is support for monophyletic *capitata*, *gemmifera*, *tronchuda*, *gongylodes*, *botrytis*, and *acephala* groups, and polyphyletic *italica* and *acephala* groups (G.R. Teakle, unpublished data). These observations are consistent with considerable allelic canalization occurring within most subtaxa of this species. The ongoing production of a homozygous DFFS using DH techniques and/or single seed descent is expected to take several more years to complete.

Shoot Ca and Mg varied by >2-fold across a series of extensive experiments conducted in both glasshouse and field environments, with a high genetic contribution to total phenotypic variation. Extensive sets of Ca and Mg concentration data in shoots or leaves are not available in published form, nor have these previously been resolved into G × E variance components. Among 19 accessions of chickpea (*Cicer arietinum*), leaf Ca and Mg concentration varied by 1.5- and 1.8-fold, respectively (Ibrikci et al., 2003). Among 22 accessions of *B. oleracea* L. var. *acephala* (kale/collards), shoot Ca ranged from 1.2% to 3.1% and shoot Mg from 0.3% to 0.6%, with cultivar rankings consistent between years (Kopsell et al., 2004). In spinach (*Spinacia oleracea*), shoot Ca and Mg varied from 0.3% to 1.2% DW (w/w) and 0.5% to 1.5%, respectively, among 322 accessions (U.S. Department of Agriculture [USDA], Agricultural Research Service [ARS], National Genetic Resources Program, Germplasm Resources Information Network [online database], National Germplasm Resources Laboratory, Beltsville, MD [http://www.ars-grin.gov/cgi-bin/npgs/html/listdsc.pl?SPINACH]; M.A. Grusak, personal communication). Across all treatment factors, shoot Ca was greater and shoot Mg was

less in *B. oleracea* than in spinach (Fig. 6). Shoot Ca to Mg ratios in *B. oleracea* (4.09, SEM 0.014) are therefore much higher than in spinach (1.43, SEM 0.019). Since the spinach was irrigated with nutrients throughout their 4 to 5 weeks of growth (M.A. Grusak, personal communication), both shoot Ca and Mg should feasibly be greater in spinach than in *B. oleracea*, which was irrigated with deionized water. However, spinach is a member of the Amaranthaceae family and species from this and other families in the order Caryophyllales typically have very high shoot concentrations of essential and nonessential elements, including Mg, compared to other terrestrial plant families (Fig. 6; Broadley et al., 2004; Watanabe et al., 2007). The hypothesis that species of Caryophyllales have altered shoot mineral composition warrants testing using a focused sampling strategy.

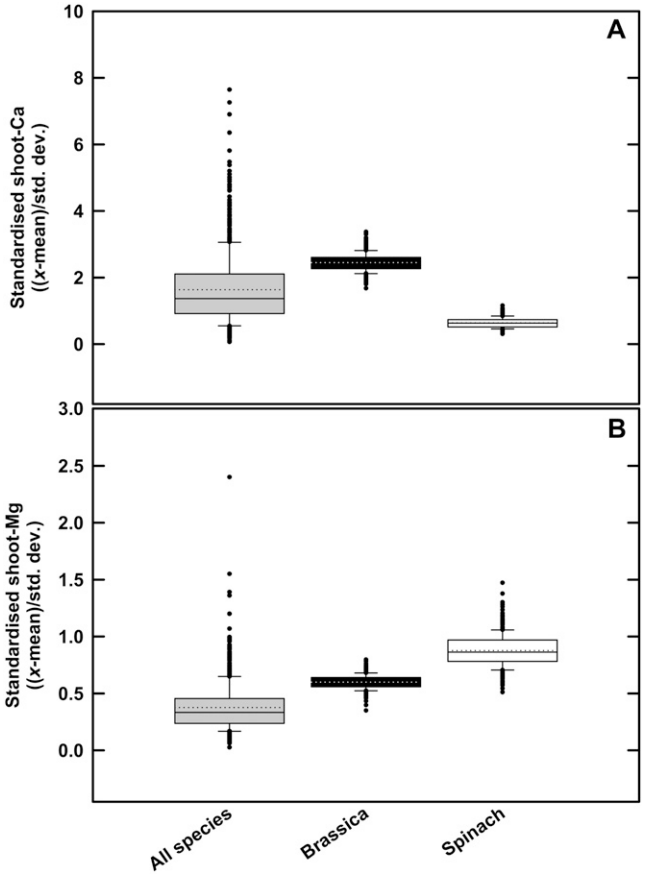

Figure 6. Shoot Ca (A) and Mg (B) concentration in higher plants (n = 670; gray-filled bars), *B. oleracea* (n = 429; black-filled bars), and spinach (n = 325; white-filled bars). Higher plant data are from Watanabe et al. (2007). *B. oleracea* data are averages across treatment factors, from species-wide experiments (compare with experiment *GE1*). Spinach data are from USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network (online database, National Germplasm Resources Laboratory, Beltsville, MD, http://www.ars-grin.gov/cgi-bin/npgs/html/listdsc.pl?SPINACH, February 19, 2007). Data were standardized to the same scale by subtracting the global trait mean and dividing by the global SD for all data points. The boundaries of the box closest and farthest to zero indicate the 25th and 75th percentiles, respectively. The solid and dotted lines within the box indicate the median and mean, respectively. Error bars indicate the 10th and 90th percentiles. Circles indicate outliers.

Our shoot Ca and Mg data are consistent with substantial genetic variation in Ca and Mg concentrations in edible tissues of several crop species; Ca and Mg are clearly, therefore, attractive targets for genetic biofortification (White and Broadley, 2005a). For example, grain Ca varied 3-fold among 132 bread wheat (*Triticum aestivum*) accessions (Graham et al., 1999), seed Ca varied 9-fold among 70 wild and cultivated common bean (*Phaseolus vulgaris*) accessions, 9-fold among 120 segregating $F_{2,3}$s from a wide common bean cross (Guzmán-Maldonado et al., 2000, 2003), and 6-fold among 1,072 common bean accessions sampled from core collections (Islam et al., 2002). Among 481 accessions of pea (*Pisum sativum*), seed Ca and Mg ranged from 0.03% to 0.26% DW and 0.11% to 0.25% DW, respectively (Grusak and Cakmak, 2005; http://www.ars-grin.gov/cgi-bin/npgs/html/crop.pl?177). Genetic variation in inflorescence Ca and Mg has been observed in *B. oleracea* var. *italica* (broccoli/calabrese; Farnham et al., 2000; Rosa et al., 2002). In general, tissue Ca and Mg concentrations are correlated (White and Broadley, 2005a). In Arabidopsis seeds, where 30% to 40% of the Ca and Mg is in the seed coat, the significant positive correlation (r = 0.68, $P < 0.001$) between seed Ca and Mg among 25 natural accessions was not seen among a recombinant inbred population of 158 lines (Vreugdenhil et al., 2004), implying that seed Ca and Mg is controlled, at least in part, by independent processes.

For many crops, it has been hypothesized that increasing crop yields over time has led to a decline in crop mineral concentration as a consequence of a dilution effect (Davis et al., 2004; Davis, 2006). Indeed, experimental studies have shown that, when grown under identical conditions, concentrations of some mineral elements are lower in genotypes yielding more edible biomass than in older, lower-yielding genotypes. For example, among 26 bread wheat cultivars, there was a negative correlation between yield and grain Fe, Zn, and phosphorus concentrations (Monasterio and Graham, 2000). Among 14 U.S. hard red winter wheat varieties, there were negative relationships between seed Fe, Zn, and Se concentrations and grain yields (Garvin et al., 2006). For most horticultural species, evidence for a dilution in mineral concentration over time has been restricted to literature-based analyses and remains unconvincing due to the paucity of replication within the original studies (Davis et al., 2004; White and Broadley, 2005b; Broadley et al., 2006a; Davis, 2006). Nevertheless, in *B. oleracea*, Farnham et al. (2000) found a strong negative relationship between inflorescence head weight and Ca and Mg concentrations among 27 broccoli genotypes. In this study, we found few inverse correlations between shoot Ca or Mg and shoot biomass within most subtaxa, nor any significant differences in shoot Ca or Mg between likely landrace and

nonlandrace accessions, except for *italica*. While this study is constrained by a major caveat that for many subtaxa (e.g. *botrytis*, *italica*, *gemmifera*, *gongylodes*), total shoot biomass does not correspond directly to an edible fraction, there was no evidence that higher shoot biomass yield was associated with lower shoot Ca or Mg in the cabbages and kales, i.e. subtaxa in which most of the shoot biomass is consumed directly.

We show for the first time (to our knowledge) that shoot mineral concentration differs significantly between subtaxa, with approximately 20% of genetic variation assigned to the subtaxa term. Thus, closely related cabbage and Savoy cabbage groups had the highest, and kohlrabi group the lowest, mean shoot Ca and Mg. Shoot morphological differences between subtaxa will undoubtedly account for some of the genetic variation assigned to the subtaxa term. However, there was still a strong correlation between glasshouse-grown plants (which were younger and morphologically more homogenous) and those grown in field environments ($n = 9$, df = 7), both in terms of shoot Ca ($r = 0.83$, Student's t test approximation = 3.99, $P = 0.005$) and Mg ($r = 0.75$, Student's t test approximation = 3.00, $P = 0.02$), at the subtaxa level. More importantly from the point of biofortification, since approximately 80% of the genetic variance in shoot Ca and Mg occurs within subtaxa, there is clearly scope for genetic improvement of shoot Ca and Mg through the introduction of alleles from the wider genepool into elite germplasm.

Among the DFS, alleles have been subjected to natural or artificial selection pressures. Thus, further genetical dissection was conducted using allelic recombinations from within an informative mapping population. Genomic regions associated with shoot Ca and Mg under glasshouse and field conditions were mapped, and selected loci were confirmed and resolved further using AGSLs (Rae et al., 1999). Shoot Ca and Mg is highly heritable, with V_A values of up to >40% even when all experimental variance components were considered simultaneously. This value is much greater than V_A values previously reported for cesium accumulation in Arabidopsis (<10%), an experiment conducted in vitro, yet analyzed using similar residual maximum likelihood (REML) variance components analyses (Payne et al., 2004). Rather surprisingly, 75% to 80% of the species-wide variation in shoot Ca and Mg was captured by the AGDH population, due to transgressive segregation beyond the parental lines GDDH33, consistently low in shoot Ca and Mg, and A12DHd, consistently high in shoot Ca and Mg. Since it is unlikely that the AGDH population contains most of the allelic variation that could potentially influence shoot Ca and Mg in *B. oleracea*, this study illustrates that positive natural selection for extreme shoot Ca and Mg has probably not occurred within the species, and that there is the breeding potential to alter shoot Ca and Mg substantially by artificially recombining alleles. Intriguingly, variation in shoot Ca and Mg among AGDH lines is weakly correlated to two physiological parameters. First, there is a weak positive correlation between shoot Ca and Mg and shoot length. Second, there is a weak negative correlation between root length growth rate and shoot Ca. Once genetic loci have been fine mapped, it will be necessary to establish more precisely the physiological and anatomical correlates of shoot Ca and Mg.

Using the AGDH population, significant QTL associated with shoot Ca and Mg in *B. oleracea* were identified on linkage groups C2, C6, C7, C8, and C9. On C2, C8, and C9, QTL were due to positive allelic effects of the A12DHd alleles (Fig. 4). In contrast, negative allelic effects of the A12DHd alleles were observed on C6 and C7. QTL on C8 and C9 potentially colocalized for shoot Ca and Mg. The existence of one or more pleiotropic loci affecting shoot Ca and Mg is supported by the observation that shoot Ca and Mg across all genotypes and environments is highly correlated ($P < 0.001$; Fig. 7). From analyses of AGSLs, one of the major QTL for shoot Ca was provisionally located to a 4-cM map interval (between 43 and 47 cM) on C9, with strong support for additional shoot Ca and Mg QTL distal to this region on C9. Based on comparative alignment of *Brassica* and Arabidopsis (Parkin et al., 2005) the region between 43 and 47 cM on C9 corresponds to a 0.41-Mb region on Arabidopsis chromosome 5 that includes 106 genes (TAIR6; Supplemental Table S5). Within this region are two divalent cation-permeable, plasma-membrane localized genes, At5g62160 (*AtZIP12*) and At5g62670 (a P-type ATPase). Neither gene is an obvious candidate for controlling Ca^{2+} (or Mg^{2+}) transport in plants (White and Broadley, 2003). This lack of obvious candidate genes constrains reverse genetic approaches to identifying genes con-

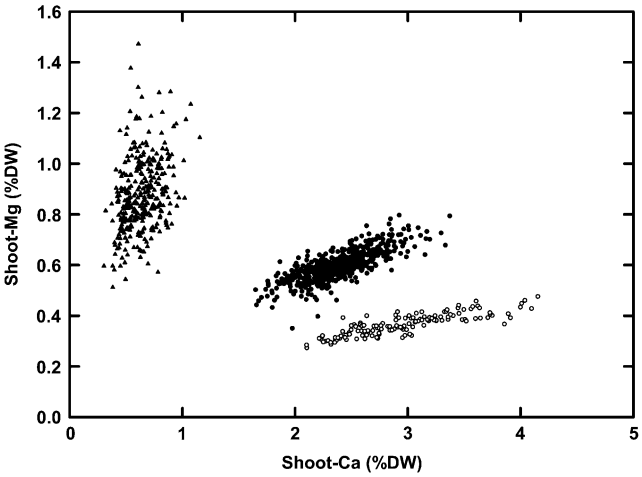

Figure 7. Shoot Ca and Mg concentration for all genotypes of *B. oleracea* (circles) and spinach (triangles). Black circles represent *B. oleracea* data from glasshouse experiments *GE1*, *GE2*, and *GE3*, and white circles represent field experiments *FE1* and *FE2*. Data are averages across all treatment factors. Spinach data are from USDA, ARS, National Genetic Resources Program, Germplasm Resources Information Network (online database, National Germplasm Resources Laboratory, Beltsville, MD, http://www.ars-grin.gov/cgi-bin/npgs/html/listdsc.pl?SPINACH, February 19, 2007).

trolling Ca^{2+} and Mg^{2+} uptake and homeostasis at the molecular level, and it indicates that map-based cloning of QTL for shoot Ca and Mg is an appropriate strategy for further dissection of these shoot-level traits. Nevertheless, overexpression of a plant Ca^{2+} transporter (CAX1) has recently been shown to increase bioavailable Ca levels in carrots (*Daucus carota*; Morris et al., 2008), and therefore genes with a putative Ca^{2+} transport function cannot be discounted. An important caveat to the interpretation of AGSL data is the occurrence of a small number of introgressions elsewhere in the genome (Supplemental Table S3). Thus, we have initiated a further backcrossing program to fine map genetic loci associated with shoot Ca and Mg. Further insights will also be gained from genes identified as affecting shoot Ca and Mg in very high-throughput screens of Arabidopsis fast-neutron mutants (Baxter et al., 2007).

There is high prevalence of horticultural brassicas in diets throughout the world, and it seems likely that greater quantities of *Brassica* leaf and nonreproductive shoot fractions are eaten directly by humans than from any other crop genus. For example, in 2005, 68.1 Mt $year^{-1}$ of cabbages and other brassicas, and a further 17.6 Mt $year^{-1}$ of cauliflower and broccoli, were consumed worldwide, compared to 61.4 Mt $year^{-1}$ of onions and shallots (*Allium cepa*; Food and Agriculture Organization, 2005, http://faostat.fao.org/). Thus, the genomic regions associated with shoot Ca and Mg could already be used to breed crops with higher shoot Ca and Mg and thereby improve human dietary intakes. Further, these genomic regions could be pyramided with other agronomically useful traits, including developmental and production traits of potential interest, and for which QTL have recently been identified in *B. oleracea*. Such traits include photosynthetic efficiency (Hall et al., 2005) and fatty acid profiles (Barker et al., 2007).

MATERIALS AND METHODS

Plant Material

From the >4,300 C-genome *Brassica oleracea* accessions in the Warwick HRI Genetic Resources Unit, a planned DFS was selected including the following subtaxa (varietas; F1–F376, Supplemental Table S1): *acephala* (n = 37), *alboglabra* (13), *botrytis* (100), *capitata* (51), *gemmifera* (38), *gongylodes* (14), *italica* (84), *sabauda* (6), and *tronchuda* (17). One accession each of *Brassica cretica*, *Brassica hilarionis*, *Brassica incana*, *Brassica insularis*, *Brassica macrocarpa*, *Brassica nigra*, *B. oleracea* var. *oleracea*, and *Brassica spinescens*, and four accessions each of *Brassica rapa* and *Brassica villosa* were included in the planned DFS (n = 376). Nine further accessions (six *italica*, and one each of *acephala*, *botrytis*, and *tronchuda*) were used in later runs to replace several nongerminating selections (Supplemental Table S1). Since theoretical studies (Lawrence et al., 1995a, 1995b) indicate that 400 homozygous accessions, collected from throughout the world, should contain 99% of the allelic polymorphism (i.e. for alleles with frequencies >2%) present in the species, this DFS is likely to represent most of the common allelic variation within the species. To assess existing genetic variation in current or recent cultivation in Northern Europe, a further set of genotypes—primarily F_1 hybrid cultivars—was included in the DFS, based on coverage of the major *B. oleracea* morphotypes and their availability from seed suppliers (C1–C77; Supplemental Table S1). This F_1 set comprised *acephala* (n = 6), *alboglabra* (1), *botrytis* (11 + 1 open pollinated [OP], cultivar), *capitata* (16), *gemmifera* (7), *gongylodes* (6 + 3 HRI-Genetic Resources Unit accessions), *italica* (9 + 1 OP), *sabauda* (10), and *sabellica* (5 + 1 OP; n = 77).

For the QTL mapping experiments, a subpopulation of 90 DH lines was selected from a larger segregating population of 206 lines representing the AGDH mapping population (Bohuon et al., 1996; Rae et al., 1999; Sebastian et al., 2000), based on the distribution of recombination break points covering all linkage groups. This subpopulation was therefore deemed to be maximally informative for resolution of map loci. The AGDH population was generated previously 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 for the AGDH mapping population was developed using markers developed from RFLP, amplified fragment length polymorphism (AFLP), and microsatellite (SSR) markers segregating in the 206 lines (Sebastian et al., 2000; G.R. Teakle, unpublished data; Supplemental Table S4). The mean distance between marker loci was 1.92 ± 3.49 cM, such that approximately 90% of the genome was within 5 cM of a marker.

To confirm and further resolve QTL in the AGDH population, 20 previously generated substitution lines (the AGSL population) were selected (Rae et al., 1999; Supplemental Table S3). These were designed to carry a single segment of a donor genotype in an otherwise pure genetic background. Briefly, DH backcross populations (B_1s) were generated using donor DH F_1s selected from the AGDH population (Rae et al., 1999; Sebastian et al., 2000). The A12DHd plant was the recurrent female parent and thus AGSLs contained the variety *alboglabra* cytoplasm. To minimize the potential for male GDDH33 donor segments to segregate independently and confound genetic analyses, selected AGSLs were backcrossed to generate a B_3. Individual B_3s were selfed on a further two or three occasions and genotyped to identify AGSLs with homozygous donor segments prior to seed multiplication. The substituted regions were mapped using 78 RFLP and 119 AFLP markers (Rae et al., 1999). A12DHd and GD33DH, and eight *B. oleracea* F_1s, used previously to develop appropriate growth conditions (Greenwood et al., 2005, 2006), were used as common reference cultivars in all AGDH and AGSL experiments: *B. oleracea* var. *alboglabra* ('Green Lance' hybrid), *botrytis* ('Fremont' hybrid), *capitata* ('Impuls' hybrid), *gemmifera* ('Maximus' hybrid), *gongylodes* ('Kolibri' hybrid), *italica* ('Marathon' hybrid), *sabauda* ('Midvoy' hybrid), and *sabellica* ('Reflex' hybrid).

Experimental Designs

Experiment 1 (*GE1*) was conducted to characterize species-wide genetic variation in shoot Ca and Mg using 376 DFS accessions and 74 F_1 hybrid cultivars grown in a compost-based medium, in pots, in the glasshouse. Two $[P]_{ext}$ were used to determine the effect of growth response on shoot Ca and Mg. Experiment 2 (*FE1*) was conducted to determine the consistency of species-wide variation in shoot Ca and Mg between glasshouse and field environments using 72 F_1 hybrid cultivars grown in the field at four $[P]_{ext}$. Experiment 3 (*GE2*) was conducted to identify QTL associated with variation in shoot Ca and Mg, using 90 of the most informative lines from the AGDH mapping population grown in a compost-based medium, in pots, in the glasshouse. Experiment 4 (*FE2*) was conducted to determine if QTL associated with shoot Ca and Mg were consistent between glasshouse and field environments, using 61 lines from the AGDH mapping population grown in the field. Experiment 5 (*GE3*) was conducted to determine if robust QTL associated with shoot Ca and Mg could be confirmed and resolved further using AGSLs in the glasshouse.

Experiment 1 (GE1)

GE1 was conducted between June 2003 and July 2004 in a 40-m^2 Cambridge-type glasshouse compartment at Wellesbourne, UK (latitude 52°12′31″ N, longitude 1°36′06″ W, 46 m above sea level). The compartment was set to maintain temperatures of 24°C by day and 15°C at night using automatic vents and supplementary heating. Daylight was supplemented by artificial lighting (Son-T 400-W Philips phi 0.85i) to maintain 16 h light d^{-1}. Suitable potting mixes and $[P]_{ext}$ were determined empirically in previous studies (Greenwood et al., 2005, 2006), based on the P responses of eight reference F_1 cultivars described above and four DH parental lines of DH mapping populations (Sebastian et al., 2000): var. *alboglabra* ('A12DHd'); var. *italica* ('GDDH33' and 'B line BI87053'); and var. *botrytis* ('N line CA25'). Briefly, the potting mix

comprised 25% sand and 75% (v/v) compost (Shamrock medium grade sphagnum peat; Scotts). Unfertilized, the potting mix had an Olsen extractable P (Olsen et al., 1954) of 8 mg L^{-1}. According to model fitting, plant-available P was equivalent to 3 mg L^{-1} added fertilizer P (Greenwood et al., 2005). Two $[P]_{ext}$ treatments of 6 and 18 mg L^{-1} added P were imposed by incorporating 0.075 and 0.225 g of sieved (500 μm) single superphosphate (7% P) per liter of compost. Other nutrients were incorporated in the potting mix in sufficient amounts to prevent deficiencies as follows: NH_4NO_3 (0.4 g L^{-1}), KNO_3 (0.75 g L^{-1}), ground limestone 2.25 g L^{-1}, magnesian limestone 2.25 g L^{-1}, and a fritted trace elements mixture containing boron, copper, Fe, manganese, molybdenum, and Zn (WM255; Fargro) at 0.4 g L^{-1}. Potting mixes were made in bulk using a paddle mixer (CM-type model 156; St Moritz Nurseries). Three seeds of each accession were hand sown directly into a 1-L compressed polystyrene pot of dimensions 11 × 11 × 12 cm (Desch Plantpak), containing the appropriate potting mix, at a depth of approximately 0.5 cm. Pots were irrigated with deionized water as required and were kept on metal mesh-covered benches, which were free draining. All seedlings except one were removed shortly after emergence.

The experimental design was constructed from two separate incomplete-block designs, one for three replicates of the 376 DFS accessions and a second for nine replicates of the 74 F_1 cultivars. For the DFS accessions, an alpha design (Patterson and Williams, 1976) was initially constructed for three replicates of 420 treatments in 60 blocks per replicate, each block containing seven plots. The last 44 (i.e. 420–376) treatments were deleted from each replicate such that each block contained either six or seven of the 376 accessions. Similarly, for the F_1 cultivars, an alpha design (Patterson and Williams, 1976) was initially constructed for nine replicates of 80 treatments in 20 blocks per replicate, each block containing four plots. The last six (i.e. 80–74) treatments were deleted from each replicate such that each block contained either three or four of the 74 F_1 cultivars. The final design was constructed by combining one replicate of the DFS treatments with three replicates of the F_1 cultivars, merging blocks containing six DFS accessions with those containing four F_1 cultivars, and blocks containing seven DFS accessions with those containing three F_1 cultivars. This resulted in three replicates of 60 blocks, with 58 blocks each containing 10 treatment plots and two blocks each containing nine treatment plots, repeated across six separate occasions (i.e. one replicate comprised two occasions), with two benches per occasion, and 15 blocks per bench. Each treatment plot consisted of four individual pots of the specified accession/cultivar, two at each $[P]_{ext}$. Plant shoots were sampled after 39, 47, 49, 49, 42, and 37 d growth from seed on occasions 1 to 6, respectively, at similar developmental stages. In total, up to 355 DFS accessions and 74 F_1 cultivars were sampled during *GE1* (Supplemental Table S1). Remaining DFS accessions could not be cultivated under these conditions. Shoot FW, representing all above ground biomass, was recorded immediately, shoot DW after conventional oven drying at 60°C for 72 h. Dry shoot tissues were subsequently milled (Apex type 529AA mill; Apex Construction). Total shoot Ca and Mg concentrations were determined using a commercial foliar analysis laboratory (Yara Phosyn).

A variance components model with a random term [(occasion/bench/block/plot/pot) + ($[P]_{ext}$ × accession)] and no defined fixed factors was used to allocate sources of variation in shoot FW and DW, and shoot Ca and Mg, using REML procedures (Patterson and Thompson, 1971; Robinson, 1987; Welham and Thompson, 1997). Subsequently, accession means were estimated using the [($[P]_{ext}$ × accession)] term as a fixed factor, retaining [(occasion/bench/block/plot/pot)] as a random factor. A Wald test statistic (W) was calculated so that significant variation sources could be identified using a χ^2 function based on the appropriate degrees of freedom. All statistical analyses were performed using GenStat (Release 9.1.0.147; VSN International).

Experiment 2 (FE1)

FE1 was conducted between May 2004 and May 2005, on a sandy loam Inceptisol, in Wharf Ground of Warwick HRI, Wellesbourne, UK (latitude 52°12′30″ N, longitude 1°36′39″ W, 45 m above sea level). This is a Wick series soil in the English classification (Whitfield, 1974). The field had not received P and K fertilizers for >20 years but it was regularly cropped during that period. Unfertilized, the soil had an Olsen extractable P (Olsen et al., 1954) of approximately 20 mg L^{-1} (Greenwood et al., 2005). The experiment was originally run on two separate occasions. Within each occasion, 72 F_1 cultivars were arranged across three replicates of the four $[P]_{ext}$ treatments using an alpha design (Patterson and Williams, 1976). Thus, for each occasion, 12 replicates of the 72 F_1 cultivars were arranged within eight blocks per replicate, and with nine plots per block. For the purposes of horticultural best practice, on each occasion, the three replicates of the four $[P]_{ext}$ treatments were allocated to three adjacent strips of plots, orientated in a north-south direction, with strip 1 the most easterly and strip 3 the most westerly. Each strip was subdivided into eight areas of dimension 6 × 1 m, with pairs of adjacent areas (i.e. area-pairs) allocated to each different $[P]_{ext}$ treatment in a systematic arrangement from lowest to highest, and with the direction of increasing $[P]_{ext}$ alternating from one strip to the next. Each of these areas were further subdivided into four subareas—north-east, north-west, south-east, and south-west—and the eight blocks each of nine cultivars were randomly allocated to the eight subareas within the pairs of areas for each $[P]_{ext}$ treatment. Each subarea consisted of three rows, spaced 0.20 m apart and running in a north-south direction, with each row containing three plots, and each plot containing four plants of the specified cultivar (planted at a spacing of 0.20 m within row). All plants were raised from seeds in module trays of plug size 2.4 × 2.4 × 4.2 cm, for 43 to 56 d in compost as described for *GE1* with 6 mg L^{-1} of added P. Guard rows of *B. oleracea* var. *capitata* 'Impuls' F_1 hybrid were planted around each subplot. Occasions 1 and 2 were transplanted on June 29, 2004, and September 8, 2004, respectively. Subsequently, it became possible to incorporate a fully replicated third occasion of the experiment. Occasion 3 was therefore transplanted on April 6, 2005 using the same design as originally used for occasion 1.

Each of the $[P]_{ext}$ treatments was imposed by addition of triple superphosphate (21% P) equivalent to 0, 298, 1,125, or 2,713 kg ha^{-1} in Spring 2002. Triple superphosphate was incorporated to a depth of 0.10 m using a power harrow (Greenwood et al., 2005). There was an annual overall dressing of 289 kg N ha^{-1} and 250 kg K_2O ha^{-1} in 2004 and 2005. Soils of each subplot were analyzed in 2005 post harvest, using standard practices, at 0.00 to 0.30 m depth, and confirmed that appropriate P gradients had been established. Irrigation, pesticide, and herbicide applications were applied according to horticultural best practice. Plant shoots were sampled after 101, 97, and 93 d growth on occasions 1 to 3, respectively. These timings were chosen to represent precommercial maturity. The field-grown plants grew more slowly than those in the glasshouse, although they produced a higher biomass over the longer growth period deployed. Typically, two of the four shoots were sampled at random and pooled per experimental unit. Shoot FW and DW were measured, and dry shoot material was milled through a mesh of diameter 0.5 mm (Junior Laboratory Mill Size 5; Christy and Norris Engineers). Shoot Ca and Mg was determined using the micro Kjeldahl method; approximately 0.1 g subsample of dried plant material was digested for 1 h, following the addition of 1 mL of hydrogen peroxide and 2 mL of a H_2SO_4/Se catalyst (Bradstreet, 1965). Inductively coupled plasma emission spectrometry (JY Ultima 2; Jobin Yvon) was used to determine mineral concentrations in digested shoot material.

A variance components model with a random term [(occasion/replicate/area-pair/block/plot) + ($[P]_{ext}$ × (varietas/cultivar))], and no defined fixed factors, was used to allocate sources of variation in shoot FW and DW, and shoot Ca and Mg, using REML in procedures in GenStat. N.B. each area-pair comprised eight blocks for a single $[P]_{ext}$. Subsequently, varietas and cultivar means were estimated using [($[P]_{ext}$*(varietas/cultivar))] as fixed factors, retaining [(occasion/replicate/area-pair/block/plot)] as a random factor. Wald tests were conducted as described previously.

Experiment 3 (GE2)

GE2 was conducted between February and July 2005 in the same glasshouse compartment, and using identical growth conditions, to those described in *GE1*. The experiment was arranged following an alpha design (Patterson and Williams, 1976) for nine replicates of 100 lines arranged in 10 blocks per replicate, each block containing 10 plots. The 100 lines comprised 90 AGDH lines plus the A12DHd and GDDH33 parents of the AGDH population, and eight reference F_1 hybrid cultivars (Supplemental Table S2). Each block of 10 plots contained nine of the 90 AGDH lines plus one of the parent or reference cultivars. The experiment was physically arranged over three separate occasions, with three replicates per occasion. Each plot contained four individual pots, with two pots at each of the two $[P]_{ext}$. Plant shoots were sampled after 50, 50, and 34 d growth from seed on occasions 1 to 3, respectively, at similar developmental stages. Shoot FW and DW were measured, and dry shoot material was milled, as described previously. Shoot Ca and Mg was determined on a DW basis, as described in *FE1*. A variance components model with a random term [(occasion/replicate/block/plot/pot) + ($[P]_{ext}$ × accession)] and with no defined fixed factors, was used to allocate sources of variation in shoot FW and DW, and shoot Ca and Mg, using REML in procedures in GenStat. Subsequently, accession means were estimated using [accession] as a

fixed factor, retaining [(occasion/replicate/block/plot/pot) + $[P]_{ext}$] as a random factor. Wald tests were conducted as described previously.

QTL analysis was conducted on the estimated trait means using 88 loci spaced at 10.26 ±7.1 cM SD intervals across the nine chromosomes (Supplemental Table S4). Estimates of marker mean and QTL positions were conducted in QTL Cartographer 2.0 (S. Wang, C.J. Basten, and Z.-B. Zeng, 2001–2004, Windows QTL Cartographer 2.0, Department of Statistics, North Carolina State University, Raleigh, NC). The composite interval mapping (CIM) option was used, with five background cofactors determined by forward stepwise regression and a 10-cM window, and with genome scanning set to 2 cM. This procedure estimated the LOD score and additive effect explained every 2 cM along each chromosome. Significant LOD thresholds for QTL ($P < 0.05$) were determined empirically based on 1,000 permutations and ranged from 2.69 to 2.96 for all shoot Ca and Mg traits in experiments *GE2* and *FE2* (described below). The additive effect of each QTL was reported relative to the contribution of alleles from the female A12DHd parent. The population-wide additive genetic variation (V_A), approximating narrow-sense heritability, was determined from the outputs of the REML analyses described previously. The proportion of V_A explained by each QTL ($\%V_A$) was calculated as r^2 values within the CIM output. QTL mapping was also performed using marker regression analysis in QTL Café (Kearsey and Hyne, 1994). For each chromosome, the presence and absence of QTL was tested using 5,000 simulations per analysis.

Experiment 4 (FE2)

FE2 was conducted between March and May 2006 at the same field site described in *FE1*. The experiment was arranged as for a single occasion of *FE1*, with three replicates of 72 cultivars (62 AGDH lines plus the two AGDH population parents and eight reference F_1 hybrid cultivars) at four $[P]_{ext}$ levels arranged following an alpha design (Patterson and Williams, 1976) for 12 replicates of 72 treatments in eight blocks per replicate with nine plots per block. The physical arrangement of the experiments was as described for a single occasion of *FE1*. Plant shoots were sampled after 105 d of growth. Typically, two of the four shoots were sampled at random and pooled per experimental unit. Shoot FW and DW were measured, and dry shoot material was milled, as described in *GE1*. Shoot Ca and Mg was determined on a DW basis, as described in *FE1*. A variance components model with a random term of [(replicate/area-pair/block/plot) + ($[P]_{ext}$ × accession)] and with no defined fixed factors, was used to allocate sources of variation in shoot FW and DW, and shoot Ca and Mg, using REML in procedures in GenStat. Subsequently, accession means were estimated using [($[P]_{ext}$ × accession)] as a fixed factor, retaining [(replicate/area-pair/block/plot)] as a random factor. Wald tests were conducted as described previously. All further QTL analyses were as described in *GE2*.

Experiment 5 (GE3)

GE3 was conducted between March and May 2006 in the same glasshouse compartment, and using identical growth conditions, to those described in *GE1*. The design comprised three replicates of 30 accessions, including 20 AGSLs, A12DHd, and GD33DH, and eight reference F_1s, grown at two $[P]_{ext}$ treatments. Two individual pots of each cultivar, representing a single experimental unit, were sown within each plot at each $[P]_{ext}$. The pots were arranged randomly over a single occasion, with $[P]_{ext}$ as a subplot treatment. Plant shoots were sampled after 39 d growth from seed. Shoot FW and DW were measured, and dry shoot material was milled, as described previously. Shoot Ca and Mg was determined on a DW basis, as described in *FE1*. A variance components model with a random term of [[$P]_{ext}$ × accession)], and with no defined fixed factors, was used to allocate sources of variation in shoot FW and DW, and shoot Ca and Mg, using REML in procedures in GenStat. Subsequently, accession means were estimated using [accession] as a fixed factor, retaining [($[P]_{ext}$)] as a random factor. A Wald test was used to indicate significant variation between lines.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. DFS accessions and F_1 hybrid cultivars of *B. oleracea*, and their trait means, in glasshouse (*GE1*) and field (*FE1*) experiments.

Supplemental Table S2. The AGDH mapping population and reference accessions of *B. oleracea*, and their trait means, in glasshouse (*GE2*) and field (*FE2*) experiments.

Supplemental Table S3. The AGSL substitution population and reference accessions of *B. oleracea* used in glasshouse experiment *GE3*.

Supplemental Table S4. Linkage map of 906 cM for the AGDH mapping population developed using markers developed from RFLP, AFLP, and microsatellite markers segregating in 206 lines generated through anther culture of the F_1 of a cross between A12DHd and GDDH33 (Sebastian et al., 2000; G.R. Teakle, unpublished data).

Supplemental Table S5. One hundred six gene models (TAIR6) in Arabidopsis (*Arabidopsis thaliana*) on chromosome 5, between positions 24,854,600 and 25,262,500 bases, corresponding to the region contained between 43 and 47 cM on chromosome C9 in *B. oleracea*.

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LITERATURE CITED

Allender CJ, Allainguillaume J, Lynn J, King GJ (2007) Simple sequence repeats reveal uneven distribution of genetic diversity in chloroplast genomes of *Brassica oleracea* L. and (n = 9) wild relatives. Theor Appl Genet **114:** 609–618

Barker GC, Larson TR, Graham IA, Lynn JR, King GJ (2007) Novel insights into seed fatty acid synthesis and modification pathways from genetic diversity and quantitative trait loci analysis of the *Brassica* C genome. Plant Physiol **144:** 1827–1842

Baxter I, Ouzzani M, Orcun S, Kennedy B, Jandhyala SS, Salt DE (2007) Purdue ionomics information management system: an integrated functional genomics platform. Plant Physiol **143:** 600–611

Bettey M, Finch-Savage WE, King GJ, Lynn JR (2000) Quantitative genetic analysis of seed vigour and pre-emergence seedling growth traits in *Brassica oleracea*. New Phytol **148:** 277–286

Bohuon EJR, Keith DJ, Parkin IAP, Sharpe AG, Lydiate DJ (1996) Alignment of the conserved C genomes of *Brassica oleracea* and *Brassica napus*. Theor Appl Genet **93:** 833–839

Bradstreet RB (1965) The Kjeldahl Method for Organic Nitrogen. Academic Press, London

Broadley MR, Bowen HC, Cotterill HL, Hammond JP, Meacham MC, Mead A, White PJ (2003) Variation in the shoot calcium content of angiosperms. J Exp Bot **54:** 1431–1446

Broadley MR, Bowen HC, Cotterill HL, Hammond JP, Meacham MC, Mead A, White PJ (2004) Phylogenetic variation in the shoot mineral concentration of angiosperms. J Exp Bot **55:** 321–336

Broadley MR, Mead A, White PJ (2006a) Reply to Davis (2006) commentary. J Hortic Sci Biotechnol **81:** 554–555

Broadley MR, White PJ, Bryson RJ, Meacham MC, Bowen HC, Johnson SE, Hawkesford MJ, McGrath SP, Zhao FJ, Breward N, et al (2006b) Biofortification of U.K. food crops with selenium (Se). Proc Nutr Soc **65:** 169–181

Cogan NOI, Lynn JR, King GJ, Kearsey MJ, Newbury HJ, Puddephat IJ (2002) Identification of genetic factors controlling the efficiency of *Agrobacterium rhizogenes*-mediated transformation in *Brassica oleracea* by QTL analysis. Theor Appl Genet **105:** 568–576

Cogan NOI, Newbury HJ, Oldacres AM, Lynn JR, Kearsey MJ, King GJ, Puddephat IJ (2004) Identification and characterisation of QTL controlling *Agrobacterium*-mediated stable and transient transformation in *Brassica oleracea*. Plant Biotechnol J **2:** 59–69

Davis DR (2006) Commentary on: 'Historical variation in the mineral composition of edible horticultural products' [White, P.J. and Broadley, M.R. (2005) *Journal of Horticultural Science & Biotechnology*, 80, 660–667]. J Hortic Sci Biotechnol **81:** 553–554

Davis DR, Epp MD, Riordan HD (2004) Changes in USDA food concentration data for 43 garden crops, 1950 to 1999. J Am Coll Nutr **23:** 669–682

Farnham MW, Grusak MA, Wang M (2000) Calcium and magnesium concentration of inbred and hybrid broccoli heads. J Am Soc Hortic Sci **125:** 344–349

Garvin DF, Welch RM, Finley JW (2006) Historical shifts in the seed

mineral micronutrient concentration of US hard red winter wheat germplasm. J Sci Food Agric **86:** 2213–2220

Graham R, Sendadhira D, Beebe S, Iglesias C, Monasterio I (1999) Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. Field Crops Res **60:** 57–80

Greenwood DJ, Stellacci AM, Meacham MC, Broadley MR, White PJ (2005) Components of P response of different *Brassica oleracea* genotypes are reproducible in different environments. Crop Sci **45:** 1728–1735

Greenwood DJ, Stellacci AM, Meacham MC, Broadley MR, White PJ (2006) Relative values of physiological parameters of P response of different genotypes can be measured in experiments with only two P treatments. Plant Soil **281:** 159–179

Grusak MA, Cakmak I (2005) Methods to improve the crop-delivery of minerals to humans and livestock. *In* MR Broadley, PJ White, eds, Plant Nutritional Genomics. Blackwell Publishing, Oxford, pp 265–286

Grusak MA, DellaPenna D (1999) Improving the nutrient composition of plants to enhance human nutrition and health. Annu Rev Plant Physiol Plant Mol Biol **50:** 133–161

Gums JG (2004) Magnesium in cardiovascular and other disorders. Am J Health Syst Pharm **61:** 1569–1576

Guzmán-Maldonado SH, Acosta-Gallegos J, Paredes-López O (2000) Protein and mineral content of a novel collection of wild and weedy common bean (*Phaseolus vulgaris* L). J Sci Food Agric **80:** 1874–1881

Guzmán-Maldonado SH, Martínez O, Acosta-Gallegos JA, Guevara-Lara F, Paredes-López O (2003) Putative quantitative trait loci for physical and chemical components of common bean. Crop Sci **43:** 1029–1035

Hall NM, Griffiths H, Corlett JA, Jones HG, Lynn J, King GJ (2005) Relationships between water-use traits and photosynthesis in *Brassica oleracea* resolved by quantitative genetic analysis. Plant Breed **124:** 557–564

Ibrikci H, Knewtson SJB, Grusak MA (2003) Chickpea leaves as a vegetable green for humans: evaluation of mineral composition. J Sci Food Agric **83:** 945–950

Islam FMA, Basford KE, Jara C, Redden RJ, Beebe S (2002) Seed compositional and disease resistance differences among gene pools in cultivated common bean. Genet Resour Crop Evol **49:** 285–293

Kearsey MJ, Hyne V (1994) QTL analysis: a simple 'marker-regression' approach. Theor Appl Genet **89:** 698–702

Kim JS, Chung Y, King GJ, Jin M, Yang TJ, Jin YM, Kim HI, Park BS (2006) A sequence-tagged linkage map of *Brassica rapa*. Genetics **174:** 29–39

Kopsell DE, Kopsell DA, Lefsrud MG, Curran-Celentano J (2004) Variability in elemental accumulations among shooty *Brassica oleracea* cultivars and selections. J Plant Nutr **27:** 1813–1826

Lawrence MJ, Marshall DF, Davies P (1995a) Genetics of genetic conservation. 1. Sample-size when collecting germplasm. Euphytica **84:** 89–99

Lawrence MJ, Marshall DF, Davies P (1995b) Genetics of genetic conservation. 2. Sample-size when collecting seed of cross-pollinating species and the information that can be obtained from the evaluation of material held in gene banks. Euphytica **84:** 101–107

Lysak MA, Koch MA, Pecinka A, Schubert I (2005) Chromosome triplication found across the tribe *Brassiceae*. Genome Res **15:** 516–525

Monasterio I, Graham RD (2000) Breeding for trace minerals in wheat. Food Nutr Bull **21:** 392–396

Morris J, Hawthorne KM, Hotze T, Abrams SA, Hirschi KD (2008) Nutritional impact of elevated calcium transport in carrots. Proc Natl Acad Sci USA **105:** 1431–1435

National Research Council (1989) Recommended Dietary Allowances, Ed 10. Subcommittee on the Ed 10 of the RDAs, Food and Nutrition Board, Commission on Life Sciences, National Research Council. National Academy Press, Washington, DC

Olsen SR, Cole CV, Watanabe FS, Dean LA (1954) Estimation of Available Phosphorus in Soil by Extraction with Sodium Bicarbonate (Circular 39). United States Department of Agriculture, Washington, DC

Parkin IAP, Gulden SM, Sharpe AG, Lukens L, Trick M, Osborn TC, Lydiate DJ (2005) Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. Genetics **171:** 765–781

Patterson HD, Thompson R (1971) Recovery of inter-block information when block sizes are unequal. Biometrika **58:** 545–554

Patterson HD, Williams ER (1976) A new class of resolvable incomplete block designs. Biometrika **63:** 83–92

Payne KA, Bowen HC, Hammond JP, Hampton CR, Lynn JR, Mead A, Swarup K, Bennett MJ, White PJ, Broadley MR (2004) Natural genetic variation in caesium (Cs) accumulation by *Arabidopsis thaliana*. New Phytol **162:** 535–548

Rae AM, Howell EC, Kearsey MJ (1999) More QTL for flowering time revealed by substitution lines in *Brassica oleracea*. Heredity **83:** 586–596

Robinson DL (1987) Estimation and use of variance components. Statistician **36:** 3–14

Rosa EAS, Haneklaus SH, Schnug E (2002) Mineral content of primary and secondary inflorescences of eleven broccoli cultivars grown in early and late seasons. J Plant Nutr **25:** 1741–1751

Sebastian RL, Howell EC, King GJ, Marshall DF, Kearsey MJ (2000) An integrated AFLP and RFLP *Brassica oleracea* linkage map from two morphologically distinct doubled-haploid mapping populations. Theor Appl Genet **100:** 75–81

Shaul O (2002) Magnesium transport and function in plants: the tip of the iceberg. Biometals **15:** 309–323

Thacher TD, Fischer PR, Strand MA, Pettifor JM (2006) Nutritional rickets around the world: causes and future directions. Ann Trop Paediatr **26:** 1–16

Vreugdenhil D, Aarts MGM, Koornneef M, Nelissen H, Ernst WHO (2004) Natural variation and QTL analysis for cationic mineral content in seeds of *Arabidopsis thaliana*. Plant Cell Environ **27:** 828–839

Watanabe T, Broadley MR, Jansen S, White PJ, Takada J, Satake K, Takamatsu T, Jaya Tuah S, Osaki M (2007) Evolutionary control of shoot element composition in plants. New Phytol **174:** 516–523

Wiesenberger G, Steinleitner K, Malli R, Graier WF, Vormann J, Schweyen RJ, Stadler JA (2007) Mg^{2+} deprivation elicits rapid Ca^{2+} uptake and activates Ca^{2+}/calcineurin signaling in *Saccharomyces cerevisiae*. Eukaryot Cell **6:** 592–599

Welch RM, Graham RD (2004) Breeding for micronutrients in staple food crops from a human nutrition perspective. J Exp Bot **55:** 353–364

Welham SJ, Thompson R (1997) Likelihood ratio tests for fixed model terms using residual maximum likelihood. J Roy Statist Soc Ser B Methodological **59:** 701–714

White PJ (2005) Calcium. *In* MR Broadley, PJ White, eds, Plant Nutritional Genomics. Blackwell Publishing, Oxford, pp 66–86

White PJ, Broadley MR (2003) Calcium in plants. Ann Bot **92:** 487–511

White PJ, Broadley MR (2005a) Biofortifying crops with essential mineral elements. Trends Plant Sci **10:** 586–593

White PJ, Broadley MR (2005b) Historical variation in the mineral composition of edible horticultural products. J Hortic Sci Biotechnol **80:** 660–667

Whitfield WAD (1974) The soils of the National Vegetable Research Station, Wellesbourne. *In* Report of the National Vegetable Research Station for 1973. Herald Press, Stratford-upon-Avon, UK, pp 21–30