Plant, Cell and Environment (2008) **31**, 887–900 doi: 10.1111/j.1365-3040.2008.01791.x

Quantitative trait loci analysis of phytate and phosphate concentrations in seeds and leaves of *Brassica rapa*

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ABSTRACT

Phytate, being the major storage form of phosphorus in plants, is considered to be an anti-nutritional substance for human, because of its ability to complex essential micronutrients. In the present study, we describe the genetic analysis of phytate and phosphate concentrations in *Brassica rapa* using five segregating populations, involving eight parental accessions representing different cultivar groups. A total of 25 quantitative trait loci (QTL) affecting phytate and phosphate concentrations in seeds and leaves were detected, most of them located in linkage groups R01, R03, R06 and R07. Two QTL affecting seed phytate (SPHY), two QTL affecting seed phosphate (SPHO), one QTL affecting leaf phosphate and one major QTL affecting leaf phytate (LPHY) were detected in at least two populations. Co-localization of QTL suggested single or linked loci to be involved in the accumulation of phytate or phosphate in seeds or leaves. Some co-localizing QTL for SPHY and SPHO had parental alleles with effects in the same direction suggesting that they control the total phosphorus concentration. For other QTL, the allelic effect was opposite for phosphate and phytate, suggesting that these QTL are specific for the phytate pathway.

Key-words: micronutrients; quantitative trait loci.

INTRODUCTION

Phosphorus is an essential element for all living organisms. The major form in which phosphorus is stored in seeds of plants is *myo*-inositol-1,2,3,4,5,6-hexakisphosphate (IP6, or phytic acid), which releases phosphorus and *myo*-inositol during seed germination. Phytic acid especially accumulates in seeds in which it can account for up to several percent of seed dry weight and about 65–85% of seed total phosphorus (Raboy *et al*. 2001). The highly negatively charged phosphate groups in IP6 form a complex with various mineral cations, yielding phytate (Raboy 1997). Therefore, a high concentration of phytate in plant tissue causes problems, because essential elements are bound in phytate and hence not bioavailable for humans and animals. In non-ruminant animals (pig and chicken), a large portion of total phosphorus, supplied in feed, is excreted, contributing to soil and water pollution in the regions with high concentrations of non-ruminant animal production farms (Sharpley *et al*. 1994), which is a significant problem in some developed countries. Secondly, high phytate concentrations often cause micronutrient (iron and zinc) deficiencies in humans when their mineral supply largely depends on seed-derived diets such as cereals (Brown & Solomons 1991). The latter problem is most prominent in developing countries.

To reduce the phytate concentration in food and feed, and to increase the bioavailabilty of essential elements, breeding not only for low phytate accumulation but also for higher micronutrient concentrations is considered as a possible solution of this problem (Raboy *et al*. 2001; White & Broadley 2005). Low phytate mutants have been obtained after chemical mutagenesis in maize (Raboy *et al*. 2000), barley (Larson *et al*. 1998), rice (Larson *et al*. 2000), soybean (Wilcox *et al*. 2000) and wheat (Guttieri *et al*. 2004). These mutants are characterized by reduced phytate concentrations, which are matched by increased inorganic phosphorus concentrations, thereby retaining the same total phosphorus concentrations as in wild type. The biochemical and molecular characterization of the maize mutants revealed that these phenotypes (*lpa1, lpa2, lpa3*) are associated with the expression of different inositol phosphate synthases/kinases that play a role in the phytate biosynthesis pathway (Shi *et al*. 2003, 2005; Shukla, VanToai & Pratt 2004).

In addition to mutants, natural variation can serve as starting material for gene identification (Koornneef, Alonso-Blanco & Vreugdenhil 2004). Natural variation in *Arabidopsis* is abundantly present among the many accessions of this species found all around the world. Using a recombinant inbred line population derived from the accessions L*er* and Cvi, some genomic regions controlling

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phytate and phosphate contents in seeds and leaves, and mineral concentrations in seeds of *Arabidopsis* were identified (Bentsink *et al*. 2003; Vreugdenhil *et al*. 2004). It was found that the accumulation of phytate and certain minerals in seeds can be separated genetically, which indicates the possibility to breed for reduced phytate concentration without affecting micronutrient levels (Vreugdenhil *et al*. 2004).

The *Brassica* genus, comprising a large and diverse group of plant species, is closely related to *Arabidopsis thaliana*, which is a member of the same Brassicaceae family. *Brassica rapa* is an important vegetable and oil crop with variation for many different morphological characteristics. Within this species, genetic variation exists not only for agronomic characteristics but also for nutritional components (Zhao *et al*. 2005, 2007; Wu *et al*. 2007). In China, rapeseed oil represents approximately 35% of edible oil consumption, and in recent years, rapeseed meal accounts for about 25% of plant seed meal consumption by animals (Wang 2004). Today, 'double low' (low content of erucic acid and glucosinolates) commercial varieties of oilseed rape dominate the oilseed *Brassica* production area in the world, and their nutritional value is being improved. However, the phytate concentration is still about 2.5–3.0% in 'double low' rapeseed varieties, and the phosphorus in phytate represents 75–80% of seed total phosphorus concentration, which implies that the utility of seed meal is limited (Peng *et al*. 2001). In addition to their use as an oilseed crop, a wide range of *Brassica* species and varieties is also used as vegetables and provides a useful resource for phosphate and minerals. Phytate concentrations are generally low in non-seed tissues. Until now, the use of plant breeding to reduce phytate and increase the available minerals has not been exploited in *Brassica* species.

In the present study, we describe the genetic analysis of phytate and phosphate concentrations in *B. rapa* using five segregating populations. The aim was to identify the putative loci involved in the regulation of phytate and phosphate concentrations, which can be the basis for improvement of the nutritional quality of this important vegetable and oilseed crop species. Anchored single sequence repeat (SSR) markers will allow comparison of quantitative trait loci (QTL) between different genetic maps. The syntenic relationship of *Brassica* to the model plant *Arabidopsis* allows a direct comparison of map positions of the two species (Parkin *et al*. 2005; Schranz, Lysak & Mitchell-Olds 2006; Suwabe *et al*. 2006) and might assist the identification of candidate genes that are already known or will be known in *Arabidopsis*.

MATERIALS AND METHODS

Population development

Several mapping populations were developed from wide crosses between *B. rapa* accessions. The parents of crosses represent different cultivar groups in *B. rapa* that are polymorphic for both morphological traits and amplified fragment length polymorphism (AFLP) pattern (Zhao *et al*. 2005).

One F2/3 population (RC-CC F2/3) produced from selfing of a single F1 plant, resulting from a cross between a Rapid cycling line RC-144 (accession number: FIL501) and a vegetable-type Chinese cabbage line CC-156 (cultivar: Huang Yang Bai; accession number: VO2A0030), was analysed. This population consists of 178 F2 individuals. Seeds of F2 were grown in pots, and seedlings with four to five leaves were transferred to soil in a greenhouse of the Institute of Vegetables and Flowers, Chinese Academy of Agriculture Science during the spring of 2004. Full-grown leaves of F2 plants and mature F3 seeds were used for phytate and phosphate analysis.

Furthermore, three double haploid (DH) populations were developed from crosses between the oil-type Yellow sarson YS-143 (accession number: FIL500) and the vegetable-types Pak choi PC-175 (cultivar: Nai Bai Cai; accession number: VO2B0226), Asian vegetable turnip VT-115 (cultivar: Kairyou Hakata; accession number: CGN15199) and Mizuna MIZ-019 (cultivar: Bladmoes; accession number: CGN06790) using microspore culture. A total of 165 lines including 71 lines from population DH-38 (PC-175 X YS-143), 64 lines from population DH-30 (VT-115 X YS-143) and 30 lines from population DH-03 (MIZ-019 X YS-143) were analysed for phytate and phosphate in seeds and leaves. The construction of these lines, their genotyping and phenotyping are described elsewhere (Lou *et al*. 2007, 2008). These DH plants (5 plants per line) were grown in pots in a greenhouse under uniform soil conditions during autumn and winter (September to December) of 2004 in Wageningen University.

An additional large mapping population of 183 DH lines, called DH-CC, was developed by the Institute of Horticulture Science of Henan Academy of Agriculture Science in China. DH-CC is derived from a cross between a Chinese cabbage DH line obtained from the Japanese cultivar CC-Y177 and a Chinese cabbage DH line from the Chinese cultivar CC-Y195. For phytate and phosphate analysis, seeds were harvested from the 183 DH-CC lines, which were grown in pots, and seedlings with four to five leaves were transferred to open soil in a greenhouse during spring (January to April) of 2005 in the Institute of Vegetables and Flowers (Beijing), Chinese Academy of Agriculture Science.

Analysis of phytate and phosphate

The high-performance liquid chromatography (HPLC) analyses of phytate and phosphate in seeds and leaves were performed as described by Bentsink *et al*. (2003) with minor modifications.

Ten to fifteen mature seeds per line, harvested from individual F2 plants for the RC-CC F2/3 population and from 1 plant per line for DH-CC populations, were used. Before flowering, one leaf was sampled and lyophilized from each F2 plant for the F2/3 population. For the DH-38, DH-30 and DH-03 populations: single leaves were collected from each

of 4 plants per DH line, and leaves of two plants were ground together to represent one biological replication. One set of seeds, derived from the first tissue-culture-derived plants, was used to measure the phytate concentration; these data were not used for further QTL analysis because seed numbers were limited, and seeds were only available for 60 lines of DH-38, 30 lines of DH-30 and 19 lines of DH-03. Another set of seeds was harvested from 1 plant per line, which was used to determine the phytate concentrations for further QTL analysis. Two technical replicates were analysed for each biological measurement in all populations.

All samples were ground in a membrane disruptor for 20–60 s at 1500 r.p.m. The samples varied from 5.0 to 7.0 mg, and were extracted by boiling for 15 min in 0.5 mL of 0.5 N HCl and 50 mg L^{-1} cis-aconitate as an internal standard. Cis-aconitate was used as an internal standard, because it was undetectable in the samples, and its behaviour during the extraction procedure was expected to be similar to other anions present in the samples. The extracts were centrifuged at 17 000 ***g*** for 10 min. The supernatants were diluted 10 times (seeds) or five times (leaves) with ultrapure water, and 20 μL was analysed using a Dionex ICS2500 HPLC system (Dionex Corporation, Sunnyvale, CA, USA). Anions were separated on an AS 11 (4 × 250 mm) column at 30 °C, preceded by an AG 11 guard column, and eluted with a NaOH gradient. The elution profile was 0–3 min isocratic at 5 mM of NaOH, followed by a 3–15 min linear gradient with 5–100 mM of NaOH. After each run, the column was washed for 15–20 min with 0.5 M NaOH, followed by a 20–35 min equilibration at 5 mM. Flow rates were 1 mL min^{-1} throughout the run. Contaminating anions in the eluents were removed using an ion trap column (anion trap column) installed between the pump and the sample injection valve. Anions were determined by conductivity detection. Background conductivity was decreased using an anion self-regenerating suppressor, with water as a counterflow (5 mL min^{-1}), operated at 248 mA. Peaks were identified and quantified by co-elution with known standards.

Statistical analysis

Statistical analyses for distribution, correlation and variance [analysis of variance (ANOVA)] were performed in Genstat 8.1 (VSN International Ltd., Hemel Hempstead, UK). In DH-38, DH-30 and DH-03, broad-sense heritabilities of phytate and phosphate in seeds and leaves were estimated according to the formula $H^2 = V_G/(V_G + V_e)$, where V_G is the among-genotype variance component, and V_e is the residual (error) variance component of the ANOVA.

Construction of a genetic map of the RC-CC F2/F3 population

For the RC-CC F2/3 population, genomic DNA was extracted from fresh leaves of F2 plants according to the procedure described by van der Beek *et al*. (1992). Fresh leaf tissue was ground by shaking tubes containing leaf material and iron bullets in a Retsch shaker at a maximum speed (Retsch BV, Ochten, the Netherlands). The AFLP procedure was performed as described by Vos *et al*. (1995). Total genomic DNA was digested using two pairs of restriction enzymes, *EcoR* I/*Pst* I and *Mse* I, and ligated to adaptors. Preamplifications were performed with *EcoR* I + 1/*Mse* I + 1 primers or *Pst* I + 0/*Mse* I + 1. Five microliters of the 20-fold diluted preamplification product was used as a template for the selective amplification. Only *EcoR* I/*Pst* I primers were labelled with IRD-700 or IRD-800 fluorescent dyes at the 5′ end for the selective amplification. Following the selective amplification, the reaction products were mixed with an equal volume of formamide-loading buffer [98% formamide, 10 mM ethylenediaminetetraacetic acid pH 8.0, and 0.1% bromophenol blue], denatured for 5 min at 94 °C, cooled on ice, and run on a 5.5% denaturing polyacrylamide gel with a NEN Global Edition IR2 DNA analyser (Li-Cor Biosciences, Lincoln, NE, USA). The AFLP gel images were mainly scored as dominant markers on the basis of the presence or absence of the band at a corresponding position among the segregating population. When two polymorphic bands were derived from different parents within the same primer combination and segregate complementary, the two polymorphic bands were assigned as two alleles from one co-dominant marker. Two allelic segregating bands of this type were manually scored as one co-dominant marker, indicated by the addition of a suffix (a, b) to primer combinations used. Only clear and unambiguous bands in the range of 50–500 bp were scored for genotyping. Segregating AFLP markers in the mapping population were named according to the primer combinations employed, followed by the parental line from which they were derived.

Public SSR primer pair sequences information of *Brassica* was obtained from the *Brassica* information website (http://www.brassica.info) and previous publications (Suwabe *et al*. 2002; Kim *et al*. 2006).

Polymerase chain reactions (PCR) were performed in 96-well plates in a volume of 10 μL. The composition of the mix included one unit of Taq DNA polymerase, 5 mM of deoxyribonucleotide triphosphate, 2.5 μL 10× supertaq buffer and 50 ng of each primer (forward and reverse primers). DNA was present in the PCR to a concentration of 1 ng/μL. The PCR was performed on GeneAmp PCR system 9700 (Applied Bio-system, Foster City, CA, USA) with the following programme: 94 °C for 2 min; 10 cycles with 94 °C denaturation for 1 min, 65 °C annealing for 1 min, 72 °C elongation for 1.5 min, with a 1°C decrease in annealing temperature at each cycle; 30 cycles with 94 °C denaturation, 55 °C annealing, 72 °C elongation, 1 min each step; then a final elongation step of 5 min. The PCR products were loaded on 2% agarose electrophoresis gels with a loading buffer in 0.5× Tris-Borate-EDTA buffer. Alleles were scored as co-dominant markers visually, and bands of the same size were assumed to be identical. Multiple segregating loci detected with one SSR primer pair were indicated by the addition of a suffix (a, b) to the locus names.

Linkage analysis and map construction was carried out using the program Joinmap 3.0 (Kyazma, Wageningen, the Netherlands) (http://www.kyazma.nl). The initial step involved calculating the logarithm of odds (LOD) scores and pairwise recombination frequencies between markers. The segregating markers were grouped at a wide range of LOD scores (3.0–5.0) to identify the linkage groups. The Kosambi mapping function was adopted for map distance calculation. Linkage maps were visualized using Mapchart (Voorrips 2002). Linkage groups were assigned based on SSR markers that were also mapped in reference maps. The 10 linkage groups of *B. rapa* were named R01 to R10 (corresponding to A1-A10), respectively. The map orientation presented by Kim *et al*. (2006) and Lou *et al*. (2007) was taken as reference map. The orientation of R03 and R10 in these maps is inversed as compared with their orientation in the map presented by Choi *et al*. (2007) (http://www.brassica.info).

Quantitative trait loci analysis

The computer software MAPQTL 5.0 was employed to perform the QTL analysis (Plant Research International, Wageningen University and Research Centre, Wageningen, the Netherlands) using both interval mapping (IM) and multiple-QTL model mapping (MQM) methods as described in its reference manual (http://www.kyazma.nl/manuals/MapQTL5 Manual.pdf). The analysis started with the IM test to find the putative QTL by applying the permutation test to each data set (1000 repetitions) to decide the LOD thresholds ($P = 0.05$). Markers located in the vicinity of QTL were selected as an initial set of cofactors. MQM analysis was then performed to precisely locate the QTL after the automatic selection of cofactors. Only significant markers at $P < 0.02$ were used as cofactors in the multiple QTL detection. A map interval of 5 cM was used for both IM and MQM analyses. LOD 2.7 for F2/3, and 2.5 for DH-38, DH-30, DH-03 and DH-CC were used as a significance threshold for the presence of a candidate QTL. For each QTL, two-LOD support intervals were established as approximately 95% confidence intervals (Van Ooijen 1992). DH-38, DH-30 and DH-03 maps are described by Lou *et al*. (2008), and the DH-CC map is described by Wu (2007).

RESULTS

Construction of a genetic map of the RC-CC F2 population with AFLP and SSR markers

Screening of AFLP and SSR primer combinations detected polymorphisms between parents of the F2 population (RC-144 and CC-156); a total of 29 pairs of AFLPs and 16 SSRs (Table 1) were selected and used for genotyping the mapping population resulting in 321 AFLP and 17 SSR markers.

Of the 321 AFLP fragments, 16 bands (5.0%) showed a clearly alternating segregation in pairs of alleles, resulting in eight bi-allelic co-dominant markers. The F2 linkage map was based on 250 AFLP (from which five were co-dominant) and 17 SSR markers, representing 10 linkage groups covering a total map length of 920.9 cM (Table 2; Fig. 1). All the markers were arranged into 10 linkage groups, equal to the haploid chromosome number of *B. rapa*. Using the SSR markers, linkage groups could be assigned to R01–R10 of the international reference *B. rapa* map (Kim *et al*. 2006; Suwabe *et al*. 2006) and DH-30 and DH-38 maps (Lou *et al*. 2008). Most of the linkage groups showed no apparent clustering of linked markers, with the exception of R09. The number of markers in each linkage group varied from 7 (R01) to 41 (R09), with an average interval size of 4.35 cM ranging from an interval size of 2.21 cM in R09 to an interval size of 11.60 cM in R01 (Table 2). The maximum interval size was 23.3 cM in R08 and in R01 (Fig. 1). Of the 250 mapped markers, 77 (30.8%) deviated ($P \leq 0.01$) from the expected 3:1 (dominant loci)

Table 1. Primer combinations applied on the RC-CC F2/3 population

Types	Primer combinations			
AFLP	E31-AAA/M60-CTC	E32-AAC/M49-CAG	E32-AAC/M54-CCT	
	E32-AAC/M60-CTC	E32-AAC/M61-CTG	E33-AAG/M47-CAA	
	E33-AAG/M48-CAC	E33-AAG/M50-CAT	E33-AAG/M51-CCA	
	E33-AAG/M59-CTA	E34-AAT/M50-CAT	E35-ACA/M47-CAA	
	E35-ACA/M62-CTT	E36-ACC/M47-CAA	E37-ACG/M59-CTA	
	E37-ACG/M60-CTC	E38-ACT/M50-CAT	E38-ACT/M51-CCA	
	E38-ACT/M56-CGC	E38-ACT/M59-CTA	E38-ACT/M62-CTT	
	E39-AGA/M47-CAA	E39-AGA/M51-CCA	E41-AGG/M50-CAT	
	E41-AGG/M62-CTT	E42-AGT/M51-CCA	E44-ATC/M47-CAA	
	E44-ATC/M62-CTT			
SSR	BRMS096R01	Ra2G09R01	BRMS037	FLC2R02
	Na12H09R02	BRMS042R03	BRMS043R03	BRMS054R04
	Ra3H10R05	BRMS014R06	Ra2A01R07	BRMS036R07
	KS31100R07	Ra2E12R08	BRNS051R09	BRMS019R10

AFLP, amplified fragment length polymorphism; SSR, single sequence repeat.

Table 2. Characteristics of an F2 (CC-156 × RC-144) genetic map of *Brassica rapa*

Linkage group	Number of markers (AFLP + SSR)	Density (marker/cM)	Average interval (cM)	Number of distorted	Length (cM)
R01	7 (4 + 3)	0.09	11.60	1	81.2
R02	30 (28 + 2)	0.24	4.21	7	126.2
R03	32 (30 + 2)	0.26	3.78	10	120.8
R04	21 (20 + 1)	0.27	3.73	8	78.4
R05	26 (25 + 1)	0.35	2.90	9	75.3
R06	25 (24 + 1)	0.21	4.85	6	71.7
R07	23 (20 + 3)	0.22	4.52	9	111.6
R08	22 (21 + 1)	0.24	4.10	9	99.5
R09	41 (39 + 2)	0.45	2.21	13	90.7
R10	23 (22 + 1)	0.35	2.85	5	65.5
Sum/mean	250 (233 + 17)	0.28	4.35	77	920.9

AFLP, amplified fragment length polymophism; SSR, single sequence repeat.

or 1:2:1 (co-dominant loci) ratio, showing a distortion in the segregation values.

Variation in phytate and phosphate concentrations

To identify the genetic loci responsible for the genetic variation in phytate and phosphate accumulation, the concentration of both compounds was determined in seeds and (or) leaves of individuals in all segregating populations. The amounts of phytate and phosphate in seeds in DH-CC plants were higher (~2.0-fold), whereas leaf phosphate (LPHO) concentration was lower (~2.7-fold) in the F2 population, compared with plants of DH-38, DH-30 and DH-03, which may be caused by the different growing conditions. Seeds and leaves of DH-38, DH-30 and DH-03 were harvested from greenhouse-grown plants in the winter in Wageningen, whereas seeds and leaves of F2 plants and seeds of DH-CC were collected from greenhouse-grown plants in open soil in the spring in Beijing. The F2/3 and DH-CC populations also showed a larger variation in seed phosphate (SPHO) (~10-fold) than the other DH populations (~4–6-fold) (Table 3). Because the heritability estimates were high in DH-38, DH-30 and DH-03 [average 84% for seed phytate (SPHY), 74% for SPHO, 94% for leaf phytate (LPHY) and 81% for LPHO], the observed differences within populations are due to genetic differences.

In DH-38, DH-30 and DH-03, the variation coefficients for different traits were similar (around 0.25), except for SPHO in DH-30 (0.46), and LPHY in three DH populations (variation coefficient >0.50). In DH-CC, the variation coefficient for SPHY was lower (0.18), whereas the variation coefficient for SPHO was higher (0.57) than in all other populations.

Table 3 shows the correlation coefficients between phytate and phosphate concentrations in seeds and leaves. The correlation coefficient value between the two components in seeds and leaves was lower than that in the analysis of a collection of 160 accessions (Zhao *et al*. 2007); a significant positive correlation ($P < 0.05$) was only detected in DH-38 and in the F2/3 populations. No significant correlation was observed between phytate in seeds and phosphate in leaves, which implies that the phytate concentration in seeds might not represent the overall higher phosphorus status in the plant.

The variation between parental accessions was generally small. However, considerable variation and transgression were observed in most populations. For the DH-38, DH-30 and DH-03 populations, the frequency distributions of phytate and phosphate concentrations showed transgression in both directions except for the phytate concentration in DH-03 (Fig. 2). This implied that the parental accessions YS-143, PC-175, VT-115 and MIZ-019 carry alleles that decrease concentrations at some loci but increase concentrations at other loci. For the DH-CC population derived from a cross within the Chinese cabbage cultivar group, the amount of phytate in seeds revealed transgression towards higher concentrations, with the two parents having phytate concentrations in the lower range.

Identification of QTL for phytate and phosphate

To detect the association between molecular markers and phytate and phosphate concentrations, the QTL analysis was performed. Some loci significantly affecting the phytate and phosphate concentration in seeds and leaves were identified in all mapping populations (Table 4). In total, 25 QTL for phytate and phosphate concentrations in seeds and leaves were detected in five populations distributed over seven linkage groups. A large percentage of phenotypic variation (38.6–72.1%) was explained by a LPHY QTL at the upper part of R07, which was detected in DH-38, DH-30 and DH-03. For the other three traits (SPHY, SPHO and LPHO) in F2/3, DH-38 and DH-30 populations, the additive effects of QTL accounted for 26.2, 53.6 and 75.0% of the variation for SPHY, 31.3, 32.8 and 51.3% for SPHO, and 9.6, 28.1 and 46.2% for LPHO, respectively. In DH-CC, three QTL affecting the phytate and phosphate concentrations in seeds were detected, explaining only 12.7% of the variation for SPHY, and 16.1% of the variation for SPHO.

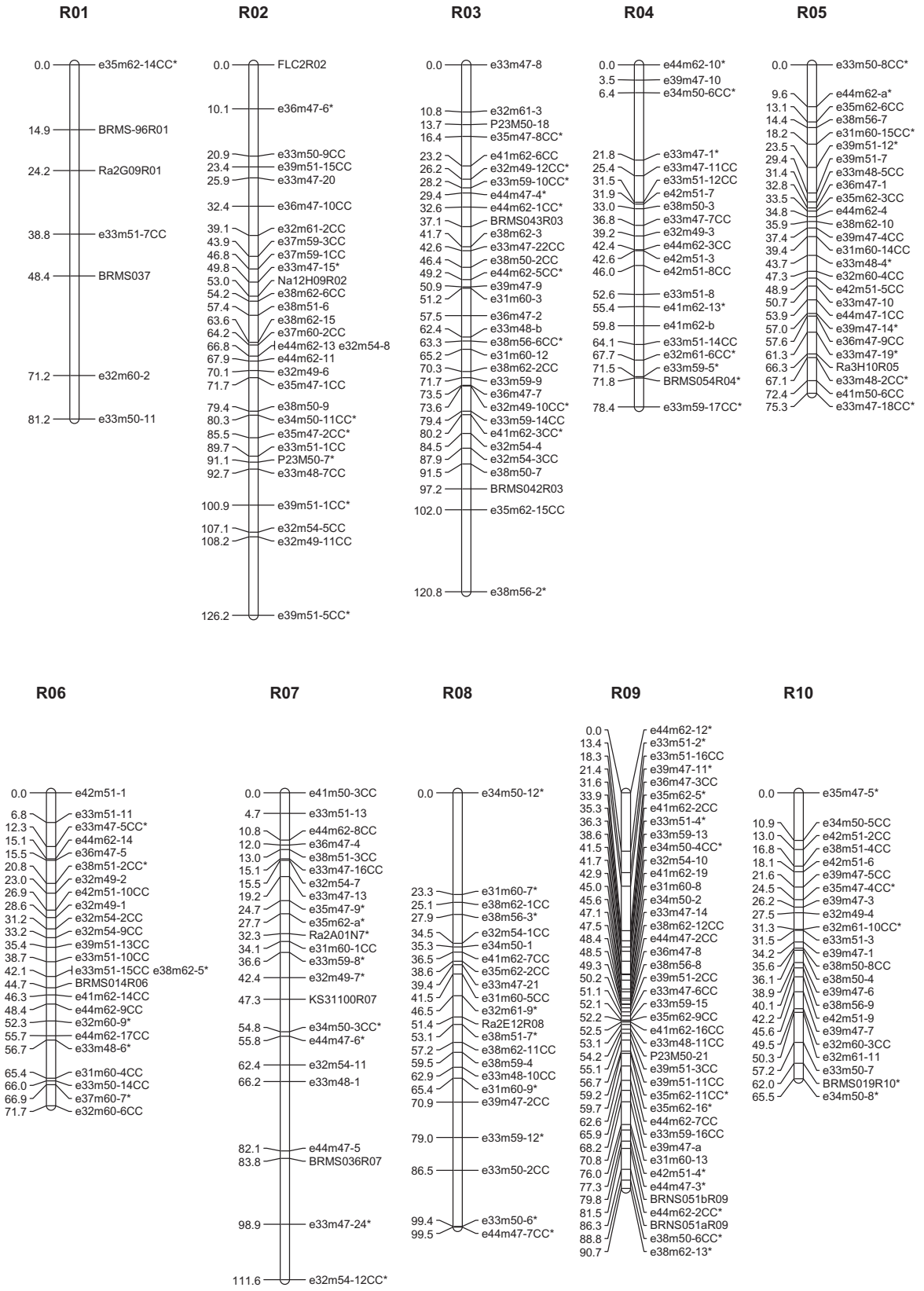

Figure 1. A linkage map of *Brassica rapa* based on the F2 (CC-156 × RC-144) population with amplified fragment length polymorphism and anchor single sequence repeat markers. Skewed marker loci are indicated with asterisk, indicating a significant level at $P \leq 0.01$.

Table 3. Statistical analysis of phytate and phosphate concentrations in five populations

Population	Statistical parameters	SPHY	SPHO	LPHY	LPHO	$R^{sphy/spho}$	$R^{lphy/lpho}$
F2/3	Range (mg g^{-1})	12.4–54.4	0.3–4.1	–	2.11–18.71	0.22*	–
	SD	9.14	0.65	–	2.77		
	Mean (mg g^{-1})	30.68	1.68	–	7.08		
	Variation coefficient	0.30	0.39	–	0.39		
DH-38	Range (mg g^{-1})	16.2–62.4	0.6–2.6	0.2–1.8	7.9–26.9	0.27*	0.36**
	SD	11.33	0.39	0.36	3.76		
	Mean (mg g^{-1})	32.95	1.22	0.56	15.97		
	Variation coefficient	0.34	0.32	0.64	0.24		
DH-30	Range (mg g^{-1})	13.6–38.9	0.6–3.9	0.1–2.1	8.8–35.1	0.14	–0.01
	SD	6.51	0.81	0.55	6.01		
	Mean (mg g^{-1})	26.31	1.78	0.85	22.94		
	Variation coefficient	0.25	0.46	0.65	0.26		
DH-03	Range (mg g^{-1})	21.5–54.8	0.5–2.4	0.4–2.8	10.5–34.4	–0.18	0.14
	SD	8.88	0.40	0.75	4.77		
	Mean (mg g^{-1})	33.29	1.35	1.39	19.70		
	Variation coefficient	0.27	0.29	0.53	0.24		
DH-CC	Range (mg g^{-1})	34.2–102.9	0.9–9.7	–	–	0.15	–
	SD	11.11	1.49	–	–		
	Mean (mg g^{-1})	61.54	2.62	–	–		
	Variation coefficient	0.18	0.57	–	–		

Significant correlation coefficient at $P < 0.05$* and $P < 0.01$**.
–, Not analysed.
SPHY, seed phytate; SPHO, seed phosphate; LPHY, leaf phytate; LPHO, leaf phosphate; $R^{sphy/spho}$ and $R^{lphy/lpho}$, correlation coefficient between SPHY and SPHO, and between LPHY and LPHO, respectively; DH, double haploid.

The locations of all significant QTL and their support intervals are indicated in Fig. 3, where the linkage maps were compared, based on the common SSR or AFLP markers. Some QTL affecting the same trait were detected in different populations in the same linkage group (R01, R03, R06 and R07). For SPHY, three QTL were identified at the upper part of R01, and four QTL were identified at the middle of R06 in multiple populations, which may represent only three different loci. For LPHO, three QTL explaining 9.6, 12.6 and 23.4% in F2/3, DH-38 and DH-30 were found in linkage group R03, which also may represent the same locus. One locus on R07 in the DH-38, DH-30 and DH-03 populations explained 59.7, 72.1 and 38.6% of the variation for LPHY, which appears to be the major locus responsible for the difference in phytate concentrations in leaves between YS-143, which hardly contains phytate in leaves, and the vegetable *B. rapa* parents. For SPHO, three QTL were detected in linkage group R01 in DH-38, DH-30 and DH-CC, respectively. However, we could not confirm whether these QTL have a similar position because of lack of common markers in the regions where these QTL were detected.

Three other SPHY QTL, one at the bottom of R02, another one at the lower part of R03 and one at the bottom of R06, were only detected in a single population and explained 11.0–22.3% of the variation. Additional QTL were detected for LPHO, one on R01 in DH-30 and one on R08 in DH-38, which explained 22.8 and 15.5% of the variation observed.

Because there is a low but positive correlation between the phytate and phosphate concentrations in leaves and seeds (Table 4), it was interesting to investigate whether QTL affecting both traits could be detected. Possible co-locations of QTL for SPHY, SPHO and LPHO in R01, SPHO and LPHO in R03, and SPHY and SPHO in R03 and R06 were observed in different populations. No genomic regions affecting all four traits simultaneously were detected in a single population. Some QTL, where the different parental alleles had either both positive or opposite effects on the trait, were detected for SPHY and SPHO. For example, the SPHY and SPHO QTL on R06 in DH-38 and on R01 in DH-30 co-localized, where the YS-143 allele increased the concentrations of both phytate and phosphate in seeds. In the DH-38 population, a QTL affecting both SPHY and SPHO was detected on R01. Here, the YS-143 allele decreased SPHY and increased SPHO. The YS-143 allele for the LPHY QTL (on R07) decreased the concentration in all DH populations (DH-38, DH-30 and DH-03) where it was detected. However, the YS-143 allele effects for LPHO (QTL on R01, R03 and R08) were either positive or negative, explaining the transgressive variation.

Only two possible co-locations of QTL for SPHY/SPHO and LPHY/LPHO were detected, corresponding to the absence of significant correlations between seeds and leaves. For this co-location, the different parental alleles had either both positive or opposite effects on the trait. The SPHO and LPHO QTL on R03 in DH-30 co-localized, whereas the YS-143 allele increased the phosphate concentration both in seeds and in leaves. The phenotypic effects for SPHY, SPHO and LPHO QTL on R01 in DH-30 were different: YS-143 alleles for SPHY/SPHO QTL decreased

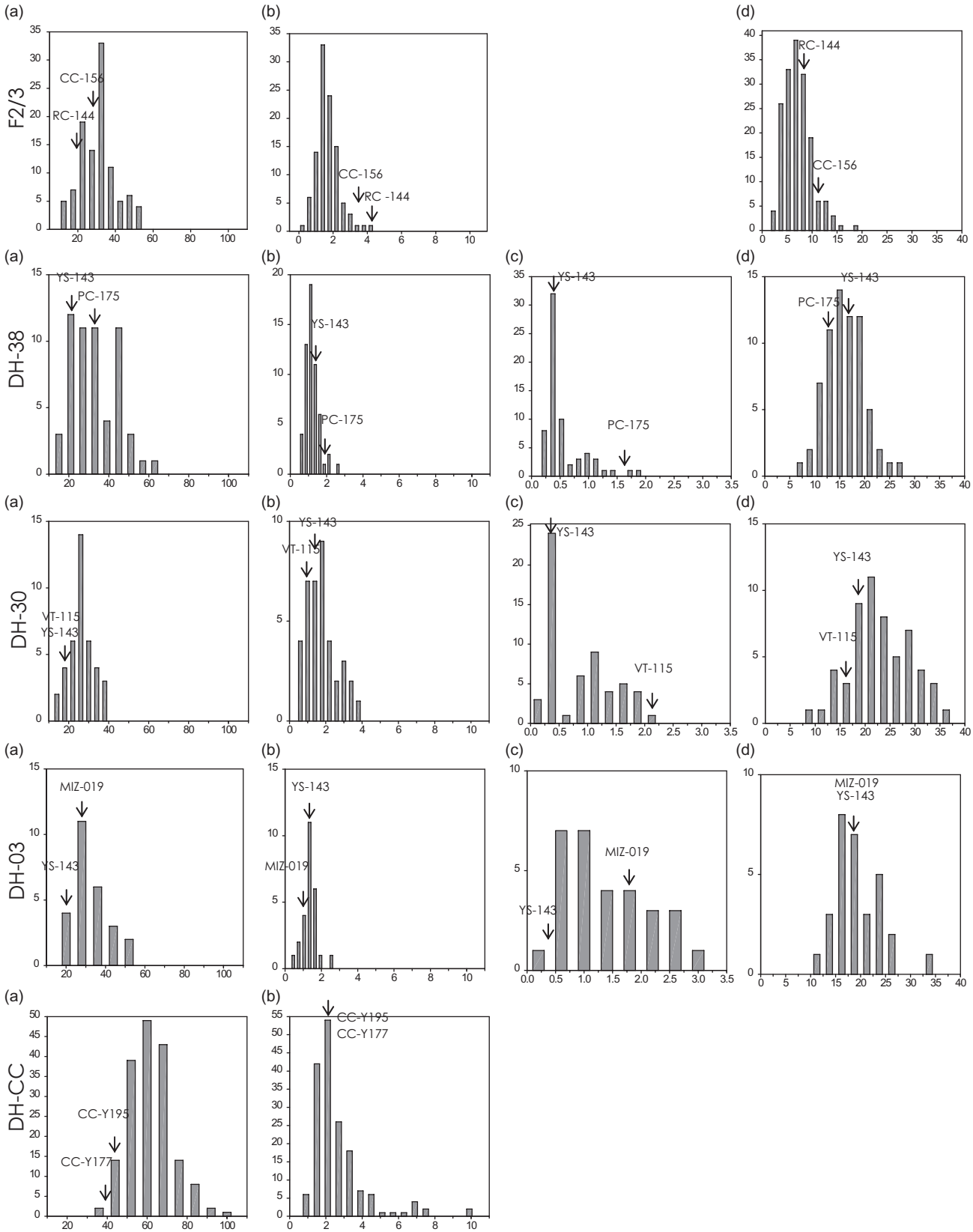

Figure 2. Frequency distribution of the concentration of seed phytate (a), seed phosphate (b), leaf phytate (c) and leaf phosphate (d) of five segregating populations. Arrows indicate the concentration in the parental lines. The horizontal axes indicate concentrations (mg g^{-1} DW); the vertical axes indicate the number of genotypes.

the concentration, whereas YS-143 alleles for the LPHO QTL increased the concentration.

DISCUSSION

The genetic regulation of phytate and phosphate concentrations in seeds and leaves was studied in five segregating populations involving eight parental accessions. In all populations except for DH-CC, the phytate concentration in seeds ranges from 12.4 to 62.4 mg g^{-1}, accounting for 1.2–6.2% of dry seed weight. The variation was higher than that (1.7–3.0%) in the collection of *B. rapa* accessions (Zhao *et al*. 2007) because of large transgression within the segregating populations. The concentration is comparable with that in other dry oil seeds such as linseed (3.7%), sesame (4.7%) and rapeseed (2.5%) (Lott *et al*. 2000), but higher than that in *Arabidopsis* (0.7–2.3%) (Bentsink *et al*. 2003). In DH-CC, the phytate concentration in seeds ranged from 5.0 to 7.0% (Fig. 2), which was higher than the concentration in the parental lines (CC-177 and CC-Y195) and individuals of the other population. This could be explained by the higher transgression, differences in moisture content of the seeds and environmental effects. In *Brassica napus* (Lickfett *et al*. 1999), it was also shown that the phytate and phosphate concentrations in seeds are affected by growth conditions. In the future, it would be interesting to investigate the phytate concentrations in one population grown at different locations to estimate the contribution of environmental and genetic variation to the total variation of the traits.

The genetic analysis for the traits showed that the direction of allelic effects could indeed explain the transgression observed in most cases, indicating that both parents of the populations have QTL alleles with positive and negative effects. For SPHO QTL in DH-38, DH-30 and DH-CC, and SPHY and LPHO in F2/3, the parents carried only one directional QTL, while the segregation for SPHO and LPHO in these populations was transgressive. Possibly, additional QTL with opposite phenotypic effects escaped detection in this study.

Because the maps contain bridging SSR and AFLP markers, especially DH-38, DH-30 and DH-03, which share one common parent, a comparison of linkage groups and QTL locations between different populations could be performed. Several QTL affecting the same trait co-localized in different populations. The SPHY QTL at the middle of R01 and R06 were detected in multiple populations, explaining 12.7–31.9% of the phenotypic variation, suggesting that these are two major QTL distinguishing SPHY concentrations in the oil-type parents compared with the vegetable parents. Some QTL affecting a particular trait were only found in a single population, suggesting

Table 4. Observed quantitative trait loci affecting phytate and phosphate concentrations in seeds and leaves in five populations

Population	Trait	Linkage group	Position (cM)	Nearest marker	LOD	Explained variance (%)	Effect
F2/3	SPHY	R01	24.15	Ra2G09R01	3.21	15.2	–
		R03	50.45	E38M62-2CC	2.55	11.0	–
	SPHO	R03	50.09	E38M62-2CC	3.20	13.0	–
		R09	48.41	E44M47-2CC	5.07	18.3	+
	LPHO	R03	69.54	E31M60-3	3.32	9.6	–
DH-38	SPHY	R01	31.95	E36M15M197.9Y	2.51	13.2	–
		R06	51.29	P23M48254.2y	3.35	18.1	–
		R06	86.20	P23M47254.2	4.03	22.3	+
	SPHO	R01	31.95	E36M15M197.9Y	2.65	15.3	+
		R06	86.20	P23M47254.2	3.47	17.5	+
	LPHY	R07	26.32	E32M16409.0	12.7	59.7	–
	LPHO	R03	39.50	E32M16496.7	2.63	12.6	–
		R08	46.63	E44M20190.4	3.09	15.5	+
DH-30	SPHY	R01	31.69	E32M19378.2	3.14	22.7	+
		R02	71.13	E34M16194.7	3.09	20.4	–
		R06	17.32	E34M15446.7y	3.95	31.9	+
	SPHO	R01	17.90	E34M15420.9Y	2.88	20.3	+
		R03	33.99	P23M48281.8	4.39	31.0	+
	LPHY	R07	36.31	BRMS018R07	12.6	72.1	–
	LPHO	R01	27.40	E34M16237.5Y	4.46	22.8	–
		R03	32.73	E46M16575.7	4.31	23.4	+
DH-03	LPHY	R07	24.85	E34M16221.9	3.17	38.6	–
DH-CC	SPHY	R06	77.75	E38M50-6	4.16	12.7	+
	SPHO	R01	46.71	E36M31-7	3.00	8.8	–
		R02	83.78	E33M56-2	2.47	7.3	–

Positive (+) effect indicates that from one parent (RC-144 for F2/3, YS-143 for DH-38, DH-30 and DH-03, and Y-195 for DH-CC), alleles at that marker increase the concentration of this trait; negative (–) effect indicates that from another parent (CC-156 for F2/3, PC-175 for DH-38, VT-115 for DH-30, MIZ-19 for DH-03, and Y-177 for DH-CC), alleles increase the concentration of this trait. Trait abbreviations are indicated in Table 3.

LOD, logarithm of odds; DH, double haploid; SPHY, seed phytate; SPHO, seed phosphate; LPHY, leaf phytate; LPHO, leaf phosphate.

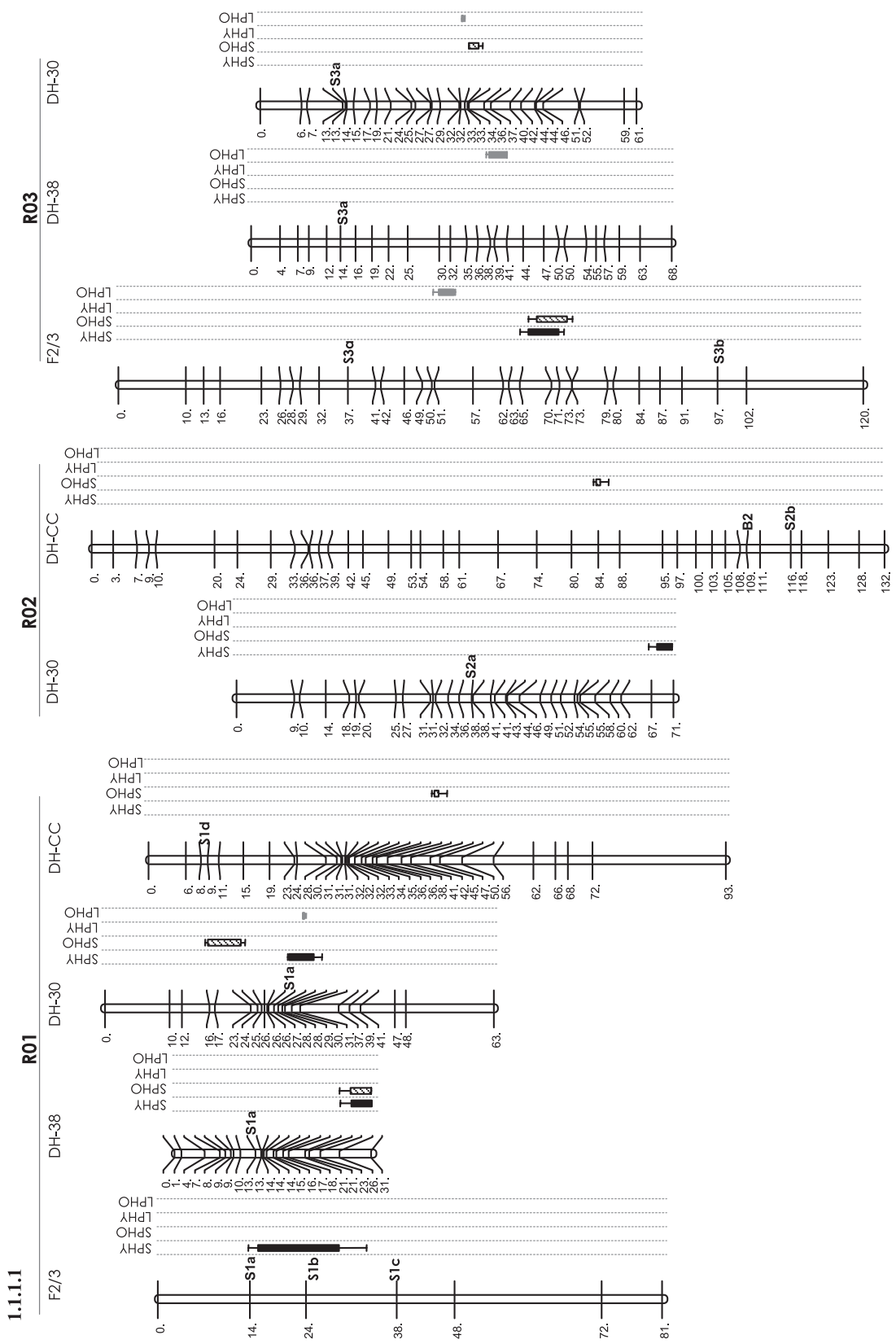

Figure 3. The genetic locations of quantitative trait loci (QTL) (different boxes) affecting seed phytate (SPHY), seed phosphate (SPHO), leaf phytate (LPHY) and leaf phosphate (LPHO) concentrations indicated above each column in five mapping populations. Boxes and whiskers represent 1-logarithm of odds (LOD) and 2-LOD confidence intervals (95%), respectively, for significant QTL. Linkage group designations followed the international R group of *Brassica rapa* (Kim *et al.* 2006; Suwabe *et al.* 2006). Markers of double haploid (DH) maps are the same as described in Lou *et al.* (2008) and Wu (2007). The position of the same linkage groups in different populations is compared based on common amplified fragment length polymorphism and single sequence repeat (B1–B10; S1–S10) markers. Centimorgan (cM) position is indicated at the left of each linkage group. Trait abbreviations are indicated in Table 3. S1a, BRMS096R01; S1b, Ra2G09; S1c, BRMS037; S1d, BRMS056; S2a, Na12H09; S2b, BrMAF-2; B2, BC-48; S3a, BRMS043; S3b, BRMS042; S6a, BRMS014; S6b, Na12H07; B6, BC51; S7a, BRMS018; S7b, O112E03; S7c, Ra2A01; S8, Ra2E12; S9, BRMS051.

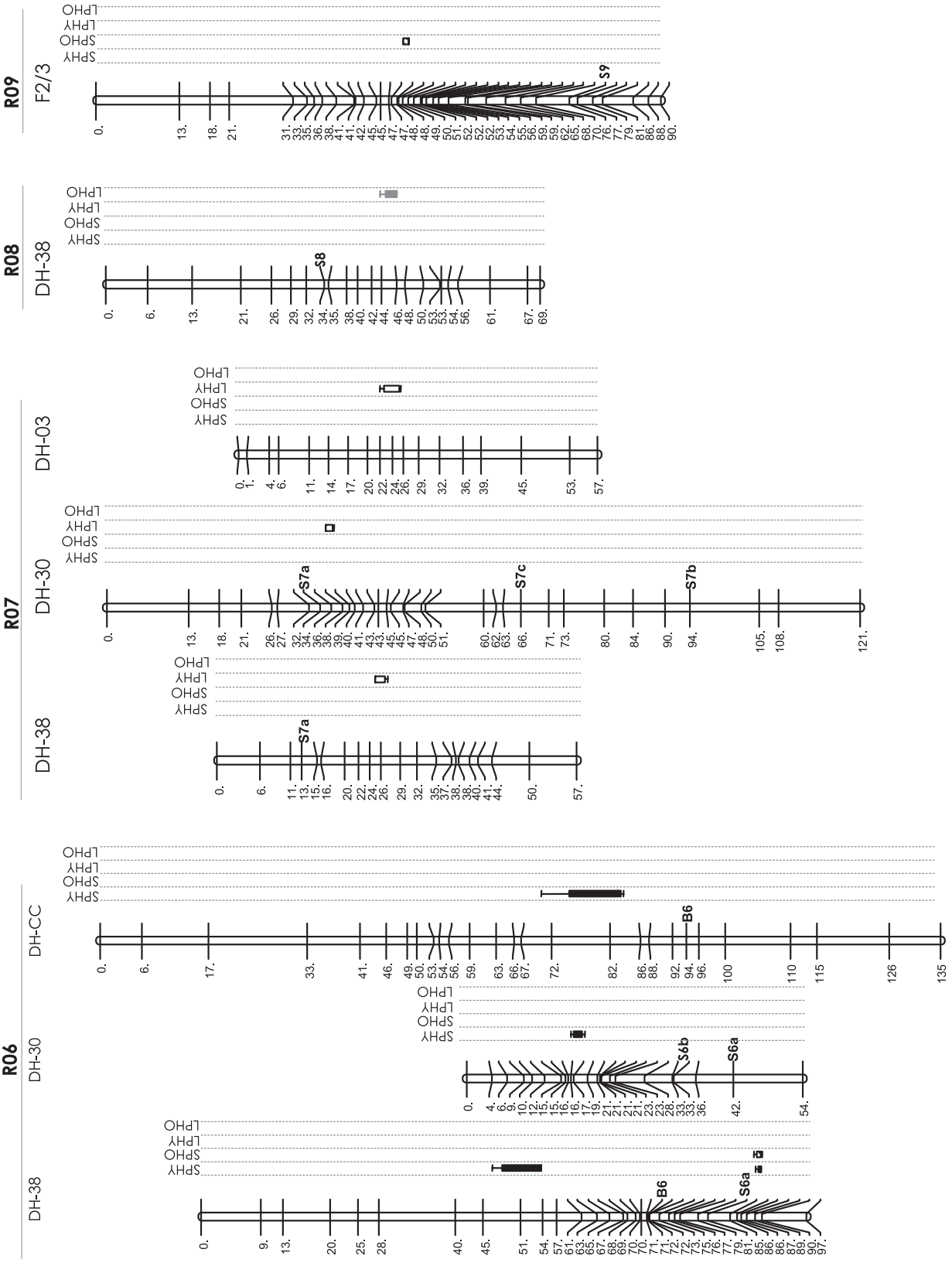

Figure 3. *Continued*

differences between populations (parents) or genotype × environment effects on the phytate and phosphate accumulation in seeds or leaves. The use of other accessions may identify additional loci affecting this trait. Possible co-locations of QTL for two or three traits were found in R01, R03 and R06 in different populations, suggesting that single loci are involved in the accumulation of phytate or phosphate in seeds or leaves. Such loci could control the overall phosphorus concentration in the plant or specifically in the different organs. It must be emphasized that the co-location of multiple QTL may indicate that a single gene underlies the QTL, but it may also imply that different but closely linked genes are involved. In this paper, comparisons of QTL positions across populations are also limited by the inaccuracy of QTL mapping, especially when a limited number of common markers are included.

The weak but positive correlation between phytate and phosphate within seeds and within leaves is in agreement with co-locations of QTL affecting these traits. Four QTL for SPHY/SPHO (on R01, R03 and R06) possibly co-located with each other within a specific population. Two of them (at the bottom of R01 and R06) only co-localized in DH-38, in which population the only strong correlation between the two traits was detected. For two SPHY/SPHO QTL, the allelic effects are in the same direction. This was also found for the major QTL on the top of chromosome 3 in *Arabidopsis* that controlled both the concentrations of phytate and phosphate in seeds and leaves. It was concluded that this QTL is a phosphorous accumulation QTL (Bentsink *et al*. 2003). For co-locating QTL with an apparent antagonistic effect (like QTL for SPHY/SPHO on R01 in DH-38), the biosynthesis of phytate may be altered, thus altering the ratio of phytate compared with the phosphate concentration, as has been described in the mutants of maize, barley and rice (Raboy *et al*. 2001). In DH-38, DH-30 and DH-03, we could not detect a LPHO QTL that co-localized with the major LPHY QTL on R07. This is possibly because of the low LPHY concentration in leaves, which implies that changes in the phytate concentration hardly affect phosphate level.

Based on homologous SSR loci, Suwabe *et al*. (2006) analysed the synteny between *B. rapa* and *Arabidopsis*. For QTL identified in this study on R01, R03, R06 and R07 of *B. rapa*, syntenic regions in each chromosome of *Arabidopsis* could be identified. Based on the comparative mapping between *B. napus* (the A genome component of *B. napus* N1–N10) and *Arabidopsis* (Parkin *et al*. 2005), Schranz *et al*. (2006) summarized the organization of *Arabidopsis* genomic blocks that make up the A genome in *B. rapa*. Some of the anchored SSRs on the SSR-based map (Suwabe *et al*. 2006) were also mapped in a sequenced-tagged map (Kim *et al*. 2006) and in this study, which allows the comparison of the maps presented in this study with the genomic blocks as defined by Schranz *et al*. (2006) and Parkin *et al*. (2005). This comparison makes it possible to directly compare the location of *B. rapa* phytate and phosphate QTL with those QTL identified in *Arabidopsis*. In *Arabidopsis*, five QTL affecting the phytate and phosphate concentrations in seeds and leaves were detected, one major QTL being located on the top of chromosome 3, and additional QTL being located on chromosomes 1, 2 and 4 (Bentsink *et al*. 2003). In Fig. 4, the synteny between

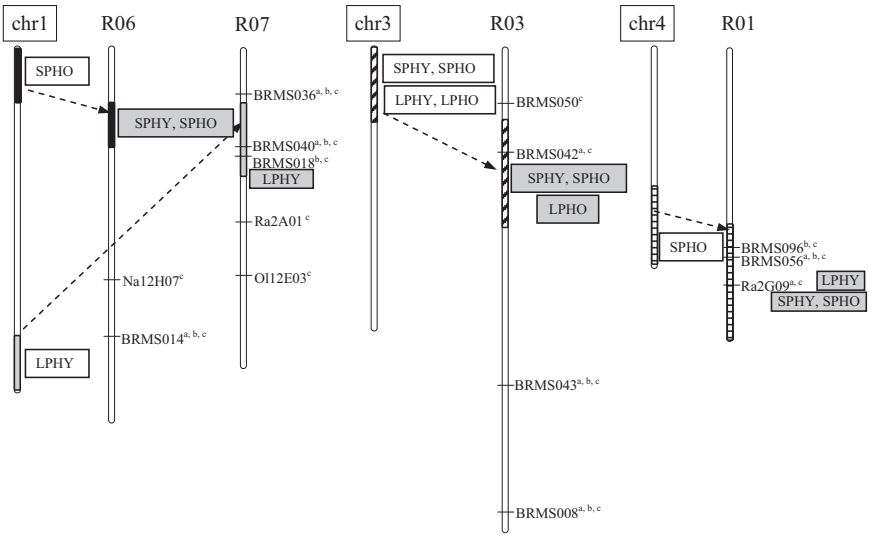

Figure 4. A comparative *Brassica rapa–Arabidopsis thaliana* map with phytate and phosphate quantitative trait loci (QTL). *B. rapa* linkage groups R03, R06 and R07 are presented with QTL positions (grey boxes) as identified in this study, and positions of single sequence repeats [a, mapped in Kim *et al*. (2006); b, mapped in Suwabe *et al*. (2006); c, mapped in this study] used for map comparison. The white boxes represent the QTL identified in *Arabidopsis* chromosomes (chr) 1, 3 and 4, which have been described by Bentsink *et al*. (2003). The synteny between *Arabidopsis* and *B. rapa* genomic blocks is indicated with similar patterns and shading [information from Parkin *et al*. (2005), Schranz *et al*. (2006) and Suwabe *et al*. (2006)]; only those syntenic blocks with QTL in both *Arabidopsis* and *B. rapa* are depicted. Trait abbreviations are indicated in Table 3. SPHY, seed phytate; SPHO, seed phosphate; LPHY, leaf phytate, LPHO, leaf phosphate.

Arabidopsis and *B. rapa* genomic blocks is depicted only for those syntenic blocks with QTL in both *Arabidopsis* and *B. rapa*. The QTL on R03 could be related to the major genomic region affecting phytate and phosphate on the top of chromosome 3 in *Arabidopsis*. The QTL on R01 and R07 are also possibly related to the QTL on chromosome 4 and 1 of *Arabidopsis*, respectively. However, we cannot reliably compare the SPHY/SPHO QTL on R06 of *B. rapa* with the SPHO QTL on the top of chromosome 1 in *Arabidopsis* because of lack of common SSR markers in these regions. Adding additional SSR and gene-targeted markers to the *B. rapa* linkage groups will improve the accuracy of identification of syntenic *Arabidopsis–B. rapa* QTL. For other QTL identified in this study and in *Arabidopsis*, we did not identify the QTL in syntenic regions.

Our results provide evidence for a genetic regulation of phytate and phosphate concentrations in seeds and leaves of *B. rapa*, and a preliminary genomic comparison with QTL identified in *Arabidopsis* at syntenic positions. The used populations will be further applied to perform genetic analysis for morphological traits, and are suitable to study the link between phytate concentration and plant vigour and seeds traits.

ACKNOWLEDGMENTS

We thank Prof. Sun Rifei and Xu Zeyong in the Chinese Academy of Agriculture Science for their supervision and support during the experiments conducted in China. We thank Prof. Zhang Xiaowei (Henan Academy of Agricultural Sciences, China) for the development of the DH-CC DH population; Jiangling Xiong, Xiaofei Song and Yao Wang are acknowledged for the testing of some SSR markers. We thank Dr. Chris Maliepaard for his assistance in map construction and heritability estimation. We are grateful to the staff in Plant Science Experimental Centre of Wageningen University, the Netherlands, and Yanguo Zhang and Yanling Liu at the Institute of Vegetables and Flowers (Beijing), Chinese Academy of Agriculture Science, China, for taking care of the plants and for their assistance. This project was sponsored by the Asian Facility (project AF01/CH/8 'Sino-Dutch Genomic Lab and Vegetable Research Center'), the Royal Dutch Academy of Sciences (KNAW), and a fellowship from an exchange student programme (Huygens) between the Chinese Scholarship Council in China and Nuffic in the Netherlands.

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Received 17 October 2007; received in revised form 13 December 2007; accepted for publication 07 January 2008

