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ORIGINAL PAPER

Development of genic cleavage markers in association with seed glucosinolate content in canola

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Abstract

Key message **The orthologues of *Arabidopsis* involved in seed glucosinolates metabolism within QTL confidence intervals were identified, and functional markers were developed to facilitate breeding for ultra-low glucosinolates in canola.**

Abstract Further reducing the content of seed glucosinolates will have a positive impact on the seed quality of canola (*Brassica napus*). In this study 43 quantitative trait loci (QTL) for seed glucosinolate (GSL) content in a low-GSL genetic background were mapped over seven environments in Germany and China in a doubled haploid population from a cross between two low-GSL oilseed rape parents with transgressive segregation. By anchoring these QTL to the reference genomes of *B. rapa* and *B. oleracea,* we identified 23 orthologues of Arabidopsis involved in GSL metabolism within the QTL confidence intervals. Sequence polymorphisms between the corresponding coding regions of the parental lines were used to develop cleaved amplified polymorphic site markers for two QTL-linked genes, *ISOPROPYLMALATE DEHYDROGENASE1* and *ADENOSINE 5′-PHOSPHOSULFATE REDUCTASE 3*. The genic cleavage markers were mapped in the DH population into the corresponding intervals of QTL explaining 3.36–6.88 and 4.55–8.67 % of the phenotypic variation for seed GSL, respectively. The markers will facilitate breeding for ultra-low seed GSL content in canola.

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Introduction

Brassica napus (AACC, $2n = 38$) is an allopolyploid that originated from spontaneous hybridisations between *B. rapa* (AA, $2n = 20$) and *B. oleracea* (CC, $2n = 18$) and is today one of the world's most important oil crops. The meal obtained after oil extraction contains 35–40 % of high-quality protein and is a valuable animal feed (Dimov et al. 2012; Leckband et al. 2002; Wanasundara 2011). However, high quantities of glucosinolates (GSL) and their degradation products, which occur naturally in the tissues of all *Brassica* species, can cause problems with palatability, along with thyroid, liver, and kidney abnormalities (Walker and Booth 2001) and especially limit the use of meal as a feed supplement for monogastric livestock.

After the identification of the Polish spring rapeseed variety "Bronowski" as a source of low seed GSL in 1969 (Kondra and Stefansson 1970), great efforts were made to introgress this trait into breeding materials worldwide. Accessions with low seed GSL content (less than 30 μmol g^{-1} meal), were initially released in Canada and became known as 'canola'. European winter oilseed rape

production was subsequently also converted to low GSL varieties having less than 18 μmol g^{-1} in whole seeds. Further reductions in GSL content would increase the ratio of oilseed rape/canola meal that can be used particularly for feeding of monogastric livestock.

The core pathway of GSL biosynthesis is well-known from the model crucifer *Arabidopsis*, a relative of *B. napus*. The genes involved in amino acid chain elongation, core structure and side-chain formation have been identified, along with the structural genes responsible for most biosynthetic steps (Wittstock and Halkier 2000; Bak and Feyereisen 2001; Grubb and Abel 2006; Mikkelsen et al. 2004; Piotrowski et al. 2004; Halkier and Gershenzon 2006). For instance, *ISOPROPYLMALATE DEHYDROGENASE1* (*IPMDH1*) is co-expressed with nearly all the genes known to be involved in aliphatic GSL biosynthesis (Gigolashvili et al. 2009; Binder et al. 2007; He et al. 2009), and the disruption of *IPMDH1* in *Arabidopsis* causes a dramatic decrease in the concentrations of GSL with side chains of four or more carbons (He et al. 2009). Another gene, *ADENOSINE 5′-PHOSPHOSULFATE REDUCTASE 3* (*APR3*), is involved in the synthesis of activated sulfate in the GSL biosynthesis network (Lee et al. 2011).

A large number of studies have identified quantitative trait loci (QTL) for seed GSL content in crucifers (Uzunova et al. 1995; Howell et al. 2003; Mahmood et al. 2003; Quijada et al. 2006; Sharpe and Lydiate 2003; Zhao and Meng 2003; Basunanda et al. 2007; Hasan et al. 2008; Harper et al. 2012; Javidfar and Cheng 2013; Li et al. 2014). However, previous efforts to determine the genetic basis of the low seed GSL trait in *B. napus* have focused on a small number of major QTL with large effects on the phenotypic variance. All of the above studies utilized crosses involving at least one parent with high seed GSL content, however. Reliable detection of minor QTL that segregate between different low-GSL materials was therefore masked by the strong effects of a few major QTL. Recently, the orthologues of *HIGH ALIPHATIC GLUCOSINOLATE 1* (*HAG1*), which controls aliphatic GSL biosynthesis in *A. thaliana*, were suggested as candidates for major QTL on A09, C02, C07 and C09 of rapeseed (Li et al. 2014; Hirai et al. 2007; Howell et al. 2003; Harper et al. 2012; Zhao and Meng 2003). These QTL form the basis of the major reduction in seed GSL content which has been achieved in worldwide canola breeding during the past three decades. On the other hand, further reductions in seed GSL require combination of these main-effect loci with additional QTL that have less prominent effects. Such loci are often overlooked in mapping studies that use parents carrying main-effect loci, making it difficult to implement them into breeding programmes. In this study, 43 QTL for seed GSL content were dissected over multiple environments in a doubled haploid (DH) population derived from two low-GSL parents. Among these QTL, cleaved amplified polymorphic site markers were developed for *B. napus* orthologues of the glucosinolate biosynthesis genes *IPMDH1* and *APR3*, both located within the confidence intervals of interesting QTL.

Materials and methods

Plant materials and phenotypic evaluation

A mapping population consisting of 261 DH lines was developed by microspore culture, using a single F1 plant derived from a cross between an inbred line of the European winter oilseed rape cultivar 'Express' (female) and the Chinese semi-winter breeding line 'SWU07' (male). The parents were selected because both have low seed GSL content (<30 μmol g^{-1} meal) when grown in their respective countries of origin, but previous QTL mapping studies in crosses with high-GSL parents (Basunanda et al. 2007; Fu et al. unpublished data) suggested they may carry different minor-effect QTL facilitating transgressive segregation.

The DH population was grown at Chongqing, China, for 5 years (from 2009 to 2013) and at Hohenlieth, Germany for 2 years (2008, 2012). These locations represent extremely different environments, ranging from the subtropical continental basin of the Yangtze River to the cool maritime climate of the Baltic Sea. A randomised complete block design with two replications was employed. Seeds were harvested from ten self-pollinated plants per genotype for quality analysis. Total seed GSL content from each seed sample was determined by near-infrared reflectance spectroscopy (NIRS), with two technical replicates. The seed GSL content (μmol g^{-1} meal) was calculated after subtracting oil content in seed, which also was determined by NIRS.

Statistical analysis

Analysis of variance (ANOVA) was performed for GSL over multiple environments using the GLM procedure of SAS (SAS Institute, SAS and Institute 2000). The broad-sense heritability (H^2) was calculated as follows: $H^2 = \sigma_g^2/(\sigma_g^2 + \sigma_{ge}^2/n + \sigma_e^2/nr)$, where σ_g^2, σ_{ge}^2 and σ_e^2 are estimates of the variances of genotype, genotype × environment interactions and error, respectively, n is the number of environments and r is the number of replications per environment (Hallauer and Miranda 1988). Pearson's product–moment correlation coefficient between traits of interest was calculated using the CORR procedure of SAS (SAS and Institute 2000).

Construction of linkage groups and QTL mapping

Genomic DNA was extracted from pooled leaves of ten plants of each DH line. Simple sequence repeat (SSR) primers were either obtained from public datasets (Lowe et al. 2004; Piquemal et al. 2005; http://ukcrop.net/ace/search/BrassicaDB; http://www.brassica.info/ssr/SSRinfo.htm; http://www.osbornlab.agronomy.wisc.edu/research/maps/ssrs.html) or designed according to the genome sequences of *Brassica* species and *Arabidopsis* (markers with prefix CEN, FM, POD, SWUC and YD).

Genetic linkage groups were constructed using the software JOINMAP 3.0 (Stam 1993) and assigned to chromosomes using published positions of common SSR markers (Suwabe et al. 2002; Gao et al. 2006; Long et al. 2007; Shi et al. 2009). QTL detection was performed with the composite interval mapping (CIM) procedure of the software WinQTL Cartographer 2.5 (Wang et al. 2005). A 1000-permutation test was performed to estimate a significance threshold of the test statistic for a QTL based upon a 5 % experiment-wise error rate (Churchill and Doerge 1994).

Alignment of QTL into reference genomes of *B. rapa* and *B. oleracea*

The QTL intervals of GSL were aligned into the reference genome of *B. rapa* (http://brassicadb.org) and *B. oleracea* (http://ocri-genomics.org) by BLAST analysis of the sequences of SSR markers linked with QTL or their primers when the full sequences were not available. The top significant hits returned by 'BLASTn' (*e* value $\leq$0.005) were used to infer the putative physical positions of these markers on the *B. oleracea* and *B. rapa* genomes. When a marker had multiple amplification loci on a same chromosome, the accurate position for a particular locus was determined manually by referring to the physical positions of its upstream and downstream markers. Potential candidate genes for GSL were identified by annotation analyses of genes within the physical boundaries of the QTL confidence intervals.

Gene sequencing and development of CAPS markers

The standard molecular cloning procedure of Sambrook and Russell (2001) was followed to isolate the genomic sequence of the candidate genes between the two parents. The sequences of *BnaIPMDH1-A02* and *BnaAPR3-A03* were aligned among clones using the software VectorNTI (www.invitrogen.com/VectorNTI).

The primer pairs for amplifying the complete open reading frame (ORF) were BnaIPMDH1-A02F (5′-ATGGCGG CAGCTTTACAAACGAAC-3′) and BnaIPMDH1-A02R (5′-TTAAACAGTAGCTGTAACTTTGGAGTCCAC-3′) for *BnaIPMDH1-A02*, and BnaAPR3-A03F (5′-ATGGC ACTAGCAATCAACGTTTCTTCATC-3′) and BnaAPR3-A 03R (5′-TTACCTAACAAGATTCAAGAAAGATGTCAA AGAATCA-3′) for *BnaAPR3-A03*. Based on the results of identified sequence polymorphisms, the amplified ORFs of *BnaIPMDH1-A02* and *BnaAPR3-A03* were digested by the restriction enzymes *Sac*I and *Hin*dIII, respectively, in each of the 261 DH lines and the two parental lines. The PCR products were digested for 1 h at 37 °C in a total volume of 20 µl with 5 units of *Sac*I or *Hin*dIII (Thermo Scientific). The digested PCR products were separated in 1.2 % agarose gels, stained with ethidium bromide and visualized under UV light.

Results

Variation for seed GSL content

Averaged over all years, the winter oilseed rape parental line 'Express' exhibited 29.02 and 40.03 µmol total GSL g^{-1} meal in selfed seeds from the field trials in Germany and China, respectively, while the semi-winter parental line 'SWU07' exhibited 16.02 and 28.87 µmol total GSL g^{-1} meal in Germany and China, respectively. A transgressive segregation of GSL was detected in the DH population, with normal distribution of values ranging from 10.56 to 88.64 µmol g^{-1} meal (Fig. 1). This indicates that the two parental lines carry different QTL for GSL. Although the overall values of GSL in Germany were lower than those of China, significant and positive correlations were detected between environments, with correlation coefficients ranging from 0.43 to 0.85 ($P < 0.01$) (Table S1).

The results of ANOVA revealed significant differences among genotypes, environments and genotype-by-environment interactions for GSL ($P < 0.01$) (Table 1), which was in accordance with the moderate heritability of GSL across environments ($H^2 = 68.89$ %).

Microsynteny analysis of QTL and identification of candidate genes

A total of 316 SSR markers were placed into 19 linkage groups. Via common markers these were subsequently assigned to the 19 *B. napus* chromosomes, designated A01–A10 and C01–C09. The genetic map spanned a genetic distance of 1198 cm, with an average distance of 3.79 cm between adjacent markers.

The QTL analysis via CIM procedure in the software WinQTL Cartographer 2.5 revealed a total of 43 individual QTL for seed GSL content in individual environments, located across seven chromosomes (A02, A03, A04, A07, A09, C03 and C08) and each explaining between 3.35

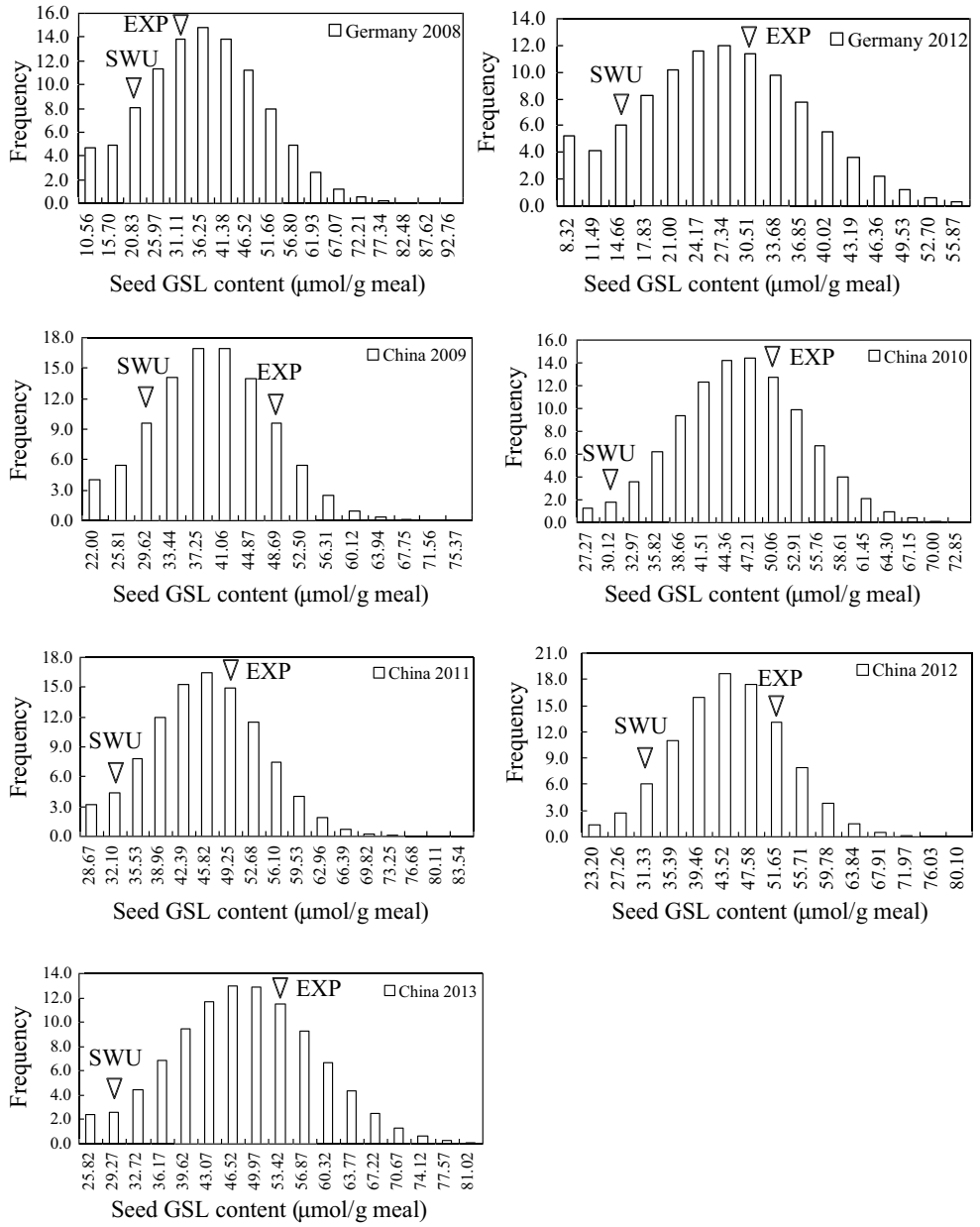

Fig. 1 Frequency distributions for seed GSL content in the DH population Express*SWU07, grown in seven environments in Germany and China from 2008 to 2013

Table 1 Analysis of variance and heritability for GSL content in DH population

Source	Df	Mean square
Genotype (*G*)	260	650.68*
Environment (*E*)	6	21,969.71*
$G \times E$	1287	67.55*

* Significance at $P = 0.01$

and 31.97 % of the phenotypic variation (Table S2). The confidence intervals for 31 single-environment QTL overlapped between Chinese and German environments (Table S2), suggesting that at these genome positions the same loci were influencing the GSL content under different environments.

In the physical genome regions of *B. rapa* and *B. oleracea,* delineated by SSR markers or SSR primers flanking the QTL peaks, a total of 23 orthologues

of Arabidopsis genes whose annotations suggest an involvement in GSL metabolism were found in 28 QTL regions (Tables S2, S3). In order to develop functional markers linked with GSL, these orthologues were sequenced to screen polymorphisms between two parents. Based on the discovered sequence polymorphisms, cleaved amplified polymorphic site (CAPS) markers were successfully developed within two orthologues, *BnaIPMDH1-A02* and *BnaAPR3-A03* (Fig. 2). The open reading frame of *BnaIPMDH1-A02* was represented by a component of 1902 and 1893 bp in 'Express' and 'SWU07', respectively, exhibiting more than 90 % sequence similarity against the sequence of *AtIPMDH1* (*AT5G14200*) from *Arabidopsis* and *BrIPMDH1-A02* (Bra023450) from *B. rapa*. The alignment of the *BnaIPMDH1-A02* sequence revealed 25 SNP polymorphisms and 1 insertion/deletion polymorphism between 'Express' and 'SWU07' across the entire coding region (Fig. S2). Among these, the presence of an SNP (T → C) at 871 bp of 'Express' and 873 bp of 'SWU07' resulted in the creation of a *Sac*I restriction site (5′… GAGCT ▼ C… 3′) in 'SWU07' (Fig. 2). This means that *Sac*I can digest the genomic sequence of *BnaIPMDH1-A02* from SWU07 into two fractions (873 and 1021 bp), whereas the *BnaIPMDH1-A02* sequence from 'Express' remains uncleaved (Fig. 3).

The *BnaAPR3-A03* sequences from 'Express' (1587 bp) and 'SWU07' (1579 bp) exhibited more than 85 and 90 % sequence similarity to *A. thaliana AtAPR3* (*AT4G21990*) and *B. rapa BrAPR3* (Bra019406), respectively. Amongst a number of SNP polymorphisms and 5 insertion/deletion polymorphisms between 'Express' and 'SWU07' (Fig. S3), the presence of one SNP (T → C) within the exon at 733 bp of the 'Express' sequence and 725 bp of the 'SWU07' sequence resulted in the creation of a *Hin*dIII restriction site (5′…A▼AGCTT…3′) in 'Express' but not in 'SWU07' (Fig. 2). This enabled *Hin*dIII to cleave the genomic sequence of *BnaAPR3-A03* in 'Express' into

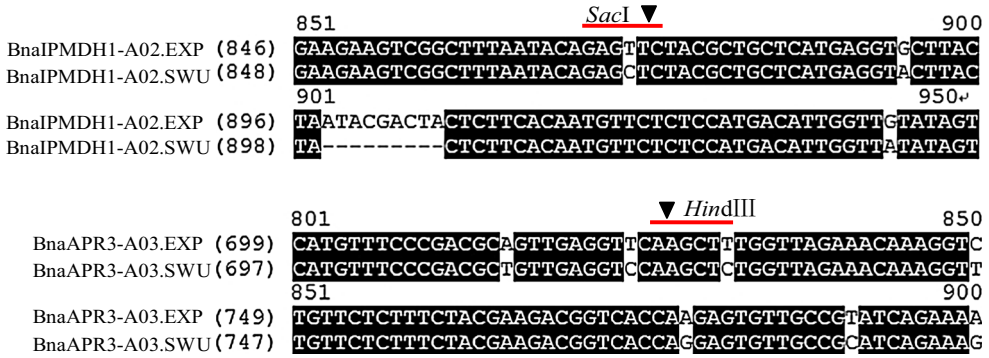

Fig. 2 Alignment of the partial sequence of *BnaIPMDH1-A02* and *BnaAPR3-A03* containing single nucleotide polymorphisms (SNPs) which resulted in the creation of the restriction sites for *Sac*I and *Hin*dIII, respectively, between Express and SWU07. *Lines* represent the restriction sites, while *arrows* indicate the positions of the cleavage sites in the respective sequences

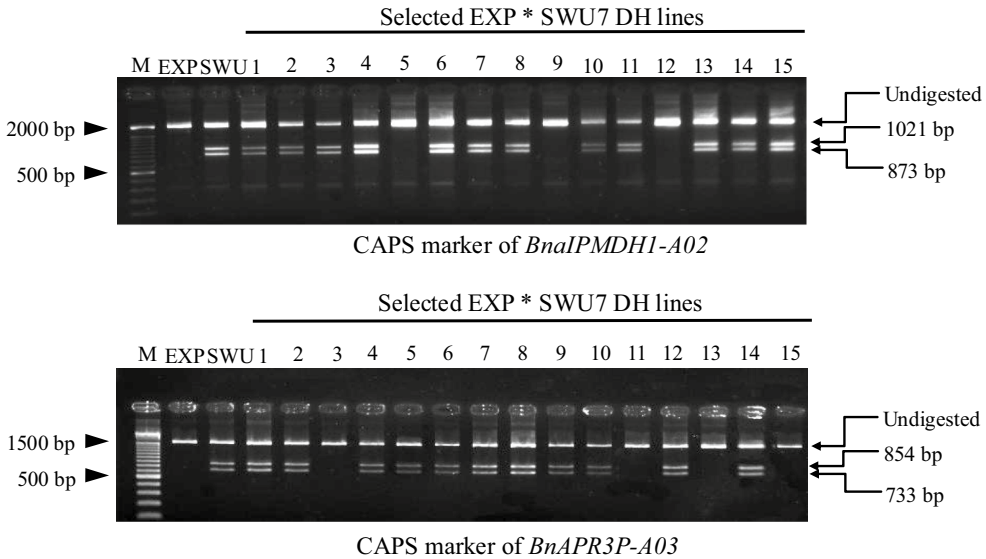

Fig. 3 Digestion of genomic sequence for *BnaIPMDH1-A02* and *BnaAPR3-A03* by *Sac*I and *Hin*dIII in the parental lines 'Express' and 'SWU07' along with DH *lines*

Table 2 QTL for GSL content on chromosomes A02 and A03 before and after integrating CAPS markers within *BnaIPMDH1-A02* and *BnaAPR3-A03*, respectively

QTL	Chr[a]	Env[b]	Add[c]	QTL mapping		QTL re-mapping with CAPS markers		
				Pos[d]	R^2 (%)[e]	Pos.	CAPS pos[f]	R^2 (%)
qGSLC09A02	A02	C2009	–	3.2–11.2	4.69	0–11.7	10.43	4.07
qGSLC10A02	A02	C2010	–			3.2–12.2	10.43	3.36
qGSLC11A02	A02	C2011	–	3.2–10	5.87	4.7–12.5	10.43	6.9
qGSLG08A02	A02	G2008	–	6–11	5.99	5.1–12.2	10.43	5.33
qGSLG12A02	A02	G2012	–	5.5–11	5.01	5.1–12.2	10.43	5.84
qGSLC13A02	A02	C2013	–			6.1–11.6	10.43	3.89
qGSLG08A03	A03	G2008	+	34.6–41.6	4.1	34.9–39.8	38.02	4.55
qGSLG12A03	A03	G2012	+	35–40.4	8.92	35.3–39.4	38.02	8.78
qGSLC09A03	A03	C2009	+	34.8–41.2	13.5	34.4–38.5	38.02	4.67
qGSLC10A03	A03	C2010	+			35.8–39.5	38.02	6.7

[a] Chromosome

[b] Environments for field trials: C, China; G, Germany; the suffix number represents the year

[c] Additive effect. The direction of additive effect is from the allele of 'Express', while a negative additive effect indicates an allelic contribution from 'SWU07'

[d] Length of 2-LOD score confidence interval

[e] Percentage of the phenotypic variance explained by each QTL

[f] Genetic map position of CAPS marker in linkage group

two fractions (733 and 854 bp), whereas the *BnaAPR3-A03* sequence from 'SWU07' remains undigested (Fig. 3).

In order to test the phenotypic effects on seed GSL content estimated by the two CAPS markers, we genotyped the DH population using both markers (Fig. 3). For the marker BnaIPMDH1-A02.CAPS, the GSL content in the DH group with the allele from 'Express' was significantly lower than that of 'SWU07' in all environments ($P < 0.01$), with the GSL reduced by an average of 1.65 μmol g^{-1} meal. For the marker BnaAPR3-A03.CAPS, the GSL content of the group containing the allele from 'SWU07' was significantly lower than that of the group carrying the 'Express' allele ($P < 0.01$), with the GSL reduced by 3.33 μmol g^{-1} meal. Furthermore, mapping of the CAPS markers back to the genetic map confirmed the localisation of genes. *BnaIPMDH1-A02* was located within a QTL cluster from 3.2 cm to 11.2 cm on A02 (between markers 'C2.141' and 'A2.246') (Table 2). The corresponding region harbours four overlapping QTL for seed GSL content, explaining 4.69–5.99 % of the phenotypic variation over environments (Table 2). *BnaAPR3-A03* was mapped within the QTL region from 34.6 to 41.6 cm on chromosome A03 (flanking markers, 'CNU146' and 'A3.1487b') (Table 2). This chromosome segment carries three overlapping QTL for seed GSL content with 4.1–13.5 % genetic effects of individual QTL over environments (Table 2). These findings show the association of both *BnaIPMDH1-A02* and *BnaAPR3-A03* with GSL content.

Discussion

A total of 43 GSL QTL were detected across seven environments in this study. Among these QTL, 41 were detected in the *B. napus* A-subgenome and only 2 in the C-subgenome. This bias possibly relates to the broader genetic variation within the A-subgenome of modern *B. napus* cultivars, which has been achieved through implementation of *B. rapa* germplasm via interspecific hybridisation (Wang et al. 2014). In accordance with this assumption, Qian et al. (2014) confirmed the presence of expansive blocks of conserved linkage disquilibrium surrounding major C-subgenome QTL for glucosinolate content in modern *B. napus* breeding materials, whereas the corresponding homoeologous regions in the A-subgenome were considerably more fragmented and diverse. A similar bias towards A-subgenome QTL for agronomic traits in *B. napus* was also reported by Shi et al. (2009). The majority of the detected QTL in our study were confirmed in highly distinct environments in China and Germany (Table S2). This result is in accordance with the moderate heritability and the correlation for GSL detected across environments.

Plant comparative mapping is a powerful tool which not only reveals the processes and rates of genome evolution, but also allows the transfer of genetic knowledge between species (Parkin et al. 2005). These advantages are particularly important in *Brassicaceae*, where a huge quantity of functional genomic information is available for the model

crucifer *A. thaliana*. The release of the genome sequences of *B. rapa* (Wang et al. 2011) and *B. oleracea* (Liu et al. 2014), the progenitor species of *B. napus*, opened the possibility to directly translate knowledge on important genes and pathways from Arabidopsis to oilseed rape, even before the availability of the recently released *B. napus* reference sequence (Chalhoub et al. 2014).

In this study, we identified candidate genes from the GSL biosynthesis pathway within the confidence intervals of QTL that segregate transgressively in low-GSL (double-low, canola quality) oilseed rape. This represents a first step towards potential cloning of genes having minor but positive effects on this valuable seed quality trait and provides useful CAPS markers for breeding of ultra-low GSL content in oilseed rape and canola meals. Although confirmation of the candidate genes will require additional functional analyses, for breeding purposes the availability of effective markers tightly linked to interesting QTL can be of considerable assistance both for identification of useful new diversity and for its introgression into elite materials by marker-assisted backcrossing. This is particularly true for traits like seed GSL content, where small-effect loci are often masked by a few large-effect QTL and field testing in multiple environments is necessary for accurate selection of ultra-low phenotypes.

Defatted rapeseed meal is enriched with a high-quality protein with a desirable amino acid composition similar to soybean protein. Reducing GSL content will further improve the nutritive value of meal in oilseed rape and canola meals. Although the low GSL trait in all current *B. napus* cultivars derives from the same founder accession, Bronowski (Krzymanski 1970), wide variances for the composition of GSL were detected in canola (Li et al. 2005). This suggests the presence of additional genetic factors besides the well-described major QTL. Hutcheson et al. (2000) developed an ultra-low GSL spring-type turnip rape (*B. rapa*) with a seed GSL content of only 4.2 μmol g^{-1} meal. This material derived from a cross between members of a low aliphatic GSL *B. rapa* breeding population (BC86-18) and the low indole GSL *B. rapa* breeding population DLY (Hutcheson et al. 2000). We also detected numerous minor-effects QTL for GSL content in a DH population derived from two lines of oilseed rape that each have low seed GSL. Those findings indicated variant alleles for seed GSL content in canola.

We performed a literature review for QTL reported in rapeseed in linkage mapping studies or by association approaches (Uzunova et al. 1995; Howell et al. 2003; Zhao and Meng 2003; Li et al. 2014) and compared the published QTL with those detected in this study. Four major QTL on chromosomes A09, C02, C07 and C09, which were detected independently in different studies (Uzunova et al. 1995; Howell et al. 2003; Zhao and Meng 2003), were positioned at four common regions at 3.2, 50.0, 39.9 and 2.8 Mb of A09, C02, C07 and C09, respectively, which were proved to be associated with homologues of the key gene controlling aliphatic glucosinolate biosynthesis (*HAG1*, At5g61420) (Li et al. 2014). Nevertheless, none of the 43 QTL detected in this study overlap or were nearby these major-effect loci. Routine phenotypic selection for low GSL content is generally performed in advanced, homogeneous breeding generations by use of high-throughput near-infrared spectroscopy (NIRS; Wittkop et al. 2009). Although this approach has been highly successful in the breeding of canola-quality *B. napus*, environmentally induced variation among low-GSL materials makes it challenging to implement new loci with additional small effects using conventional selection methods. The markers identified in this work and the plant materials that carry them, therefore, represent important resources to develop ultra-low GSL canola by pyramiding low GSL alleles with both major and minor effects. A previous study by Hasan et al. (2008) confirmed marker–trait associations of gene-linked SSR markers to seed glucosinolate content in genetically diverse *B. napus* germplasm. These included loci associated with homologues of *CYP79A2* and *MAM1/MAM3*, which we also identified within QTL intervals in the present study. Due to the narrow genetic background of the low GSL trait in current canola and oilseed rape cultivars, most of which carry common major-effect QTL from the same origin, we expect the novel minor QTL we describe in this work to be generally effective across a broad range Asian, North American and European breeding materials.

Author contribution statement Y.F. conducted all experiments and wrote the manuscript, K.L. designed primers, L.Q., J.M., D.W, X.P., X.X. participated in the field experiments and seed quality anlysis, W.Q. designed the experiment, J.L., M.F., F.D., W.Q. and R.S. directed the project and contributed to the writing.

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Conflict of interest The authors declare that they have no conflict of interest.

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