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ORIGINAL PAPER

A knockout mutation in the lignin biosynthesis gene *CCR1* explains a major QTL for acid detergent lignin content in *Brassica napus* seeds

Liezhao Liu · Anna Stein · Benjamin Wittkop · Pouya Sarvari · Jiana Li · Xingying Yan · Felix Dreyer · Martin Frauen · Wolfgang Friedt · Rod J. Snowdon

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Abstract Seed coat phenolic compounds represent important antinutritive fibre components that cause a considerable reduction in value of seed meals from oilseed rape (*Brassica napus*). The nutritionally most important fibre compound is acid detergent lignin (ADL), to which a significant contribution is made by phenylpropanoid-derived lignin precursors. In this study, we used bulked-segregant analysis in a population of recombinant inbred lines (RILs) from a cross of the Chinese oilseed rape lines GH06 (yellow seed, low ADL) and P174 (black seed, high ADL) to identify markers with tight linkage to a major quantitative trait locus (QTL) for seed ADL content. Fine mapping of the QTL was performed in a backcross population comprising 872 BC_1F_2 plants from a cross of an F_7 RIL from the above-mentioned population, which was heterozygous for this major QTL and P174. A 3:1 phenotypic segregation for seed ADL content indicated that a single, dominant, major locus causes a substantial reduction in ADL. This locus was successively narrowed to 0.75 cM using *in silico* markers derived from a homologous *Brassica rapa* sequence contig spanning the QTL. Subsequently, we located a *B. rapa* orthologue of the key lignin biosynthesis gene *CINNAMOYL CO-A REDUCTASE 1* (*CCR1*) only 600 kbp (0.75 cM) upstream of the nearest linked marker. Sequencing of PCR amplicons, covering the full-length coding sequences of *Bna.CCR1* homologues, revealed a locus in P174 whose sequence corresponds to the *Brassica oleracea* wild-type allele from chromosome C8. In GH06, however, this allele is replaced by a homologue derived from chromosome A9 that contains a loss-of-function frameshift mutation in exon 1. Genetic and physical map data infer that this loss-of-function allele has replaced a functional *Bna.CCR1* locus on chromosome C8 in GH06 by homoeologous non-reciprocal translocation.

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L. Liu and A. Stein contributed equally to the work.

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L. Liu · A. Stein · B. Wittkop · P. Sarvari · W. Friedt · R. J. Snowdon (✉)
Department of Plant Breeding, IFZ Research Centre for Biosystems, Land Use and Nutrition, Justus Liebig University, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany
e-mail: rod.snowdon@agrar.uni-giessen.de

L. Liu · J. Li · X. Yan
College of Agronomy and Biotechnology, Southwest University, Beibei, Chongqing 400716, China

F. Dreyer · M. Frauen
Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, 24363 Hohenlieth, Germany

Introduction

Oilseed rape/canola (*Brassica napus* L., genome AACC) is widely grown throughout the world for vegetable oil and biodiesel production, and after oil extraction it also provides a high-quality meal used primarily for livestock feeding (Downey and Rakow 1987). In contrast to other *Brassica* species, which show a range of seed colour from bright yellow to black, *B. napus* seeds normally have a dark pigmentation. Nevertheless, breeding for yellow seed colour is considered advantageous for the meal quality because yellow-seeded genotypes have a thinner seed coat associated with higher protein content (Wittkop et al. 2009)

and reduced quantities of non-energetic fibre and antinutritional polyphenolics (Simbaya et al. 1995). Hence, substantial efforts have been made to introgress genes imparting yellow seed colour into *B. napus* via interspecific hybridisation (e.g. Shirzadegan 1986; Van Deynze and Pauls 1994; Rashid et al. 1994; Tang et al. 1997; Li et al. 1998, 2011).

Seed colour is difficult to use in breeding programmes as a morphological marker for improved meal quality, however, because seed coat pigment biosynthesis is complex and highly sensitive to temperature, light intensity and other abiotic factors (Boesewinkel and Bouman 1995). Genetic studies of seed colour in different genetic backgrounds often revealed different major-effect quantitative trait loci (QTL; Tang et al. 1997; Li et al. 1998; Badani et al. 2006; Liu et al. 2005; Xiao et al. 2007; Fu et al. 2007), along with allelic variation among black-seeded crossing partners at major seed colour loci and other contributing QTL (Badani et al. 2006; Fu et al. 2007; Snowdon et al. 2010). This complexity of inheritance and the environmental instability of seed pigmentation complicate introgression of the yellow-seed trait into elite breeding lines. Early reports described the dominance of the black seed trait over yellow seed colour in *B. napus*, and suggested control by three independent gene loci (Shirzadegan 1986; Van Deynze et al. 1993). However, further analysis of other yellow-seed sources revealed that different major loci can be involved and that semi-dominant or dominant inheritance of yellow seed colour over black seed colour is also possible (Tang et al. 1997; Li et al. 1998).

Biosynthesis of lignin and its phenylpropanoid precursors in xylem and stem tissues has been studied extensively in numerous model and crop plants (see Bonawitz and Chapple 2010 for a recent review). In contrast, seed coat phenylpropanoid biosynthesis is less well understood, although the seeds from many crops play a huge role both in livestock feeding and in human nutrition. Whereas insufficient dietary fibre is commonly associated with increased obesity in human populations, high quantities of indigestible, non-energetic fibre are considered undesirable in livestock feeds. On the other hand, seed coat compounds have obvious roles in protection of the embryo against damage by biotic and abiotic stress factors (reviewed by Boesewinkel and Bouman 1995).

Marles and Gruber (2004) found that most of the lignin-related compounds in seeds of the closely related *Brassica carinata* are found in the testa. In accordance with this result, experiments with *B. napus* seeds showed that de-hulling achieves considerably reduced crude fibre and condensed tannin contents in the extraction meal from different oilseed rape varieties (Matthäus 1998). We have observed that near-infrared reflectance spectroscopy (NIRS) measurements of acid detergent lignin (ADL) in *B. napus* seeds correlate strongly to concentrations of phenylpropanoid-derived lignin precursors measured by mass spectrometry analysis of seed coat preparations (A. Frolov and B. Wittkop, unpublished results). Hence, our NIRS estimates of ADL from whole, intact *B. napus* seeds can be regarded as quantitative measurements of lignin-related metabolites in the seed coat. By NIRS analysis of genetically diverse *B. napus* accessions, we found considerable phenotypic variation for ADL content, and a considerably greater heritability of ADL content than of seed colour (Snowdon et al. 2010). Most importantly, genotypes with very similar seed colour can show large variation in seed ADL, implying that seed colour alone is not always suitable as a selection marker for meal digestibility.

In different studies, the two homoeologous *B. napus* chromosomes A9 and C8 have been reported to contain a major QTL with a large effect on seed colour in different genetic backgrounds (Li et al. 1998; Badani et al. 2006; Fu et al. 2007). Recently, the major QTL on chromosome A9 was found to coincide with a corresponding major QTL for seed ADL content (Snowdon et al. 2010). Previous studies of seed colour and antinutritive phenolics in *Brassica* oilseeds focused on Arabidopsis *transparent testa* (*tt*) or *tannin-deficient seed* (*tds*) mutations as potential candidate gene targets for improved meal quality (e.g. Marles et al. 2003; Badani et al. 2006; Burdzinski and Wendell 2007; Wei et al. 2007; Xu et al. 2007; Akhov et al. 2009; Auger et al. 2009; Chai et al. 2009). Most *TT* and *TDS* genes are involved in flavonoid biosynthesis. In contrast, little attention has been paid to the phenylpropanoid biosynthesis pathway. In this study, we exploited genomic DNA sequence data from the *Brassica* A-genome of *Brassica rapa* for sequence-based fine mapping of a major QTL for seed ADL content in *B. napus*. By *in silico* chromosome walking across the QTL, we identified a key phenylpropanoid biosynthesis pathway gene within the QTL. DNA sequence analysis uncovered functional mutations that appear to explain the low-ADL phenotype.

Materials and methods

Mapping populations

A population of 232 F_9 recombinant inbred lines (RILs) was derived by single seed descent (SSD) from F_2 offspring of a cross between the Chinese semi-winter oilseed rape parental lines, GH06 (yellow seeds, low ADL) and P174 (black seeds, high ADL). The pedigree of GH06 includes a yellow-seeded genotype derived from interspecific hybridisation between *B. napus* and *Brassica juncea*, and another yellow-seeded *B. napus* genotype developed by radiation mutagenesis of a dark-seeded breeding line.

QTL mapping of seed colour and fibre traits was performed using data collected from field trials of the RIL population in four different environments over 2 years as follows: 2008 in Giessen (central Germany), Hohenlieth (northern Germany) and Chongqing (southwest China), and 2009 again in Giessen. For the trials in Germany in 2008, the population was sown in the greenhouse in winter and transplanted to the field in early spring, after vernalisation, when plants reached the four-leaf stage. In Chongqing in 2008, the seeds were sown in nursery beds in autumn and transplanted to the field 1 month later. In Giessen in 2009, the population was sown directly in the field in spring. All trials were performed with a plot size of 4.5 m^2 (1.5 m $\times$ 3 m) with five or six rows per plot depending on the location.

Based on the results of genetic mapping and QTL analysis, a backcross population for subsequent fine mapping was derived from a cross of an F_7 plant of the yellow-seeded, low-ADL inbred line RIL138, derived from the cross GH06*P174, with the black-seeded, high-ADL parent P174. Phenotypic analysis of F_2–F_3 progenies from the selected RIL138 plant revealed that is was heterozygous for the major QTL controlling ADL content and seed colour (see results), although all markers surrounding this QTL in RIL138 were derived from the black-seeded parent P174. Hence, we expected the resulting backcross to have a high resolution for fine mapping. A heterozygous, high-ADL BC_1 plant from RIL138 $\times$ P174 was selected for selfing to produce BC_1F_2 seeds. A total of 872 BC_1F_2 plants were sown in the greenhouse and transplanted to the field in Giessen in 2010. We collected open-pollinated seed samples from all individual BC_1F_2 plants to avoid micro-environmental temperature and humidity effects from self-pollination bags. NIRS analysis of ADL content was performed in two technical replicates.

DNA marker screening

Genomic DNA from the RILs and parental lines was extracted from young leaf tissue using the method described by Doyle and Doyle (1990). Genomic DNA samples from the BC_1F_2 population were extracted using a BioSprint 96 magnetic bead extraction system (Qiagen, Hilden, Germany) with corresponding BioSprint 96 DNA Plant Kits, in accordance to the manufacturer's instructions. For bulked-segregant analysis, equimolar DNA samples from ten low-ADL and ten high-ADL RILs, respectively, were pooled into low- and high-ADL bulks. The two bulked samples and the two parents were used to generate amplified fragment length polymorphism (AFLP; Vos et al. 1995) and simple sequence repeat (SSR) fingerprints to identify markers tightly linked to QTL for ADL. AFLP analysis was carried out using *Eco*RI/*Mse*I AFLP kits from Invitrogen (Carlsbad, CA, USA), in accordance with the manufacturer's instructions. A total of 1,024 selective AFLP primer combinations were applied with fluorescently labelled selective primers in the two bulks and parents, with dual IRD 700/800 fluorescent fragment detection on a LI-COR 4200 DNA Analyzer. Detection of SSR marker fragments was performed with a fluorescently labelled universal M13 primer, as described by Berg and Olaisen (1994). *Brassica* SSR primer pairs were obtained from publicly available collections listed at http://www.brassica.info/ and by *in silico* SSR detection from *B. rapa* sequences available at the *Brassica* Genome Gateway (http://brassica.bbsrc.ac.uk/) and the BRAD *Brassica* Database (http://brassicadb.org/), respectively. Primer sequences for the informative SSR markers are provided as electronic supplementary material (ESM 1).

Bulked-segregant analysis and fine mapping

Two AFLP markers tightly linked with seed ADL content were selected for cloning and sequencing after re-amplification with unlabelled AFLP primers. The target fragments were extracted from silver-stained polyacrylamide gels by boiling in 50 µL of sterile double-distilled water. The corresponding AFLP selective primers and 5 µL of the supernatant as template were used to re-amplify the recovered DNA fragment. Cloned PCR products containing inserts of the expected size were sequenced, and sequences were BLASTed to the *B. rapa* sequences available at BRGB, South Korea (http://www.brassica-rapa.org/BRGP/index.jsp) and BRAD, China (http://brassicadb.org/brad/) to identify corresponding *Brassica* genomic sequences. These were subsequently used to mine SSR markers in close proximity to the sequences of the trait-linked AFLP markers, using the *in silico* SSR finder software WebSat (Martins et al. 2009).

Codominant *Brassica* SSR markers associated with ADL content in the RIL population were re-mapped in the BC_1F_2 population, and sequences of all markers flanking the major QTL for ADL content were identified in corresponding sequence scaffolds from *B. rapa* chromosome A9 for comparison of the genetic and physical maps across the QTL region. An *in silico* chromosome walking approach was used to narrow the QTL confidence interval, using the physical sequence of *B. rapa* chromosome A9 to successively select *in silico* SSR markers with reduced recombination frequencies to the causal locus. For fine mapping, backcross progenies were screened for recombinations of marker alleles from the low-ADL parent in recessive, high-ADL plants.

Trait analysis

Measurements for seed colour and fibre components were obtained using an NIR System 6500 with WinISI II

software (FOSS GmbH, Rellingen, Germany). Phenotype values for seed colour (visual light absorbance), ADL (% seed dry weight), acid detergent fibre content (ADF, % seed dry weight) and neutral detergent fibre content (NDF, % seed dry weight) were extrapolated from NIRS spectra using calibrations developed specifically for the measurement of these traits in *B. napus* (Wittkop et al. 2009). NIRS-derived estimates for each trait and genotype were averaged over two technical repetitions. In the RIL population, mean trait values from up to three self-pollinated plants from each genotype over all four environments were used as the basis for the subsequent QTL analysis of seed colour and fibre components, while single-plant analyses were performed in the BC_1F_2 population. Cellulose concentrations were calculated as the difference between NDF (predominantly phenylpropanoid compounds, cellulose and hemicellulose) and ADF (predominantly phenylpropanoid compounds and hemicellulose), and hemicellulose concentration as the difference between ADF and ADL (phenylpropanoids). Statistical analysis of the phenotype data was performed using the software package SPSS 13.0.

Linkage analysis and QTL mapping

Genetic linkage analysis was performed using JoinMap 3.0 (Van Ooijen and Voorrips 2001), with the mapping function of Kosambi (1994) and a minimum logarithm of odds (LOD) score of 3.0. Detection of QTL for seed colour, cellulose, hemicellulose and ADL was performed in the RIL population by composite interval mapping using WinQTLCart2.5 (Wang et al. 2006; see http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). The LOD threshold for the detection of significant QTL was set by permutation analysis with 300 permutations.

454 amplicon sequencing and structural analysis of *Bna.CCR1* homologues

A 454 amplicon sequencing approach was employed to survey DNA sequence variation over homologous *B. napus* copies of the phenylpropanoid biosynthesis candidate gene *CCR1* in P174 and GH06, respectively. Using genomic *CCR1* sequences from *B. rapa* (*BraA.CCR1*) and *Brassica oleracea* (*BolC.CCR1*), which each consist of five exons, we designed barcoded fusion PCR primers to generate amplicons of ~450–500 bp in length, from both A and C genome homologues, covering exons 1 and 2 in a single amplicon and exons 3, 4 and 5 with one amplicon each. Details of the tailed fusion primer sequences are provided in the electronic supplementary material (ESM 2).

After quantification, normalisation and equimolar pooling, the barcoded amplicons were sequenced in both directions by SeqIT (Kaiserslautern, Germany), as part of a larger pooled sequencing reaction on a 1/8th sequencing plate of a 454-FLX genome sequencer (454 Life Sciences, Branford, CT, USA). The resulting sequence reads were clipped to remove emulsion PCR primer sequences, 4 bp key sequences and (after sorting into genotype-specific pools) MID barcodes. The sequences were then processed with the Sequencher® version 4.10 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA) as follows:

For each genotype, the sequences were individually grouped into amplicon-specific pools, according to their corresponding forward/reverse primer sequences and trimmed to remove M13/T7 overhang sequences. Subsequently, the raw reads were assembled into locus-specific contigs using a preferential 3′ gap alignment with a stringent 95% minimum match and a minimum overlap of 20 bp. The chromosomal origin of each locus-specific contig was identified by aligning to *B. rapa* and *B. oleracea* chromosomes via the online BLAST available at http://brassicadb.org/brad/. For structural analysis, the sequences were trimmed to exons corresponding to the coding DNA sequence for *BraA.CCR1* and subsequently translated to the protein sequence. Positions of conserved functional domains were derived by protein BLAST analysis with translated amino acid sequences from full-length plant *CCR1* coding sequences available in the NCBI database.

Results

Inheritance of seed coat ADL content

Considerable variation was found in the expression of seed coat pigmentation phenotypes of individual RILs between German and Chinese environments, with genotypes that produced bright yellow seeds in China having considerably darker seeds at the two locations in Germany. In contrast, the fibre traits showed much less genotype-by-environment (G*E) interaction, reflecting the higher heritability of seed fibre components compared to seed pigmentation.

Correlations among seed traits in the RIL and BC_1F_2 populations are shown in Table 1. Although the seed colour showed high variation between the Chinese and German environments, the seed ADL content was still significantly positively correlated with seed colour in both the RIL population ($R^2 = 0.67$, $P < 0.01$) and the BC_1F_2 population ($R^2 = 0.82$, $P < 0.01$). The correlations of ADL to cellulose and hemicellulose content were also significant ($P < 0.01$), though considerably lower in both populations.

Segregation patterns for seed colour and fibre traits in the RIL and BC_1F_2 populations are shown in Fig. 1. Cellulose and hemicellulose, which are primarily embryonic traits and thus independent of seed colour, showed continuous normal distributions. For both of these traits,

Table 1 Correlations between seed components and seed colour in a RIL population (above diagonal) and a BC_1F_2 population (below diagonal) derived from the cross GH06 (yellow seeds, low seed ADL) × P174 (black seeds, high seed ADL)

Seed fractions	Seed ADL (% DW)	Seed colour	Oil (% DW)	Protein (% DW)	Cellulose (% DW)	Hemicellulose (% DW)
Seed ADL (% DW)	–	0.67**	0.06	−0.30**	0.14*	−0.23**
Seed coat colour	0.82**	–	0.20**	−0.29**	0.17**	−0.03
Oil (% DW)	−0.04	−0.18**	–	−0.50**	−0.19**	−0.82**
Protein (% DW)	−0.09	−0.05	−0.58**	–	−0.34**	0.42**
Cellulose (% DW)	0.25**	0.11**	0.05	−0.31**	–	−0.22**
Hemicellulose (% DW)	−0.25**	−0.13**	−0.63**	0.56**	−0.60**	–

* Significant correlation at $P < 0.05$ (two-tailed)

** Significant correlation at $P < 0.01$ (two-tailed)

very little difference was observed between the parental lines and strong transgressive segregation was evident. The phenotypic distributions for seed colour also suggested a polygenic inheritance with environmental influence, although in both populations the distribution deviated significantly from normality. In contrast, the clear bimodal segregation for ADL content in the BC_1F_2 corresponds to a simple Mendelian segregation with dominant inheritance of a single major locus conferring reduced ADL content ($\chi^2_{(3:1)} = 0.523 < \chi^2_{0.05}$). The low-ADL phenotype of heterozygous F_1 seeds from the cross between P174 and GH06 also corresponds to a dominant inheritance of low ADL in this cross. On the other hand, however, full dominance is not exhibited over all high-ADL *B. napus* parents (data not shown). A strong maternal effect on seed ADL, as expected for a trait primarily expressed in the testa, was not observed in reciprocal crosses between GH06 and P174.

Bulked-segregant analysis

Of the 1,024 AFLP primer combinations tested in the bulked-segregant analysis, 202 exhibited polymorphic amplification products between bulked DNA samples from ten high-ADL RILs and ten low-ADL RILs, respectively. Testing of these 202 selected primer combinations in ten selected low-ADL and ten high-ADL RILs identified only two polymorphisms associated to the seed ADL phenotype. The AFLP primer combination E46/M35 generated a 362 bp dominant marker specific to lines with high-ADL content, while primer combination E35/M43 amplified a codominant marker with a 395 bp allele in high-ADL lines and a 399 bp allele in low-ADL lines, respectively. Genotyping of these two AFLP markers in the entire RIL population revealed a genetic distance of 1.84 cM between them and confirmed their correlation to seed ADL content.

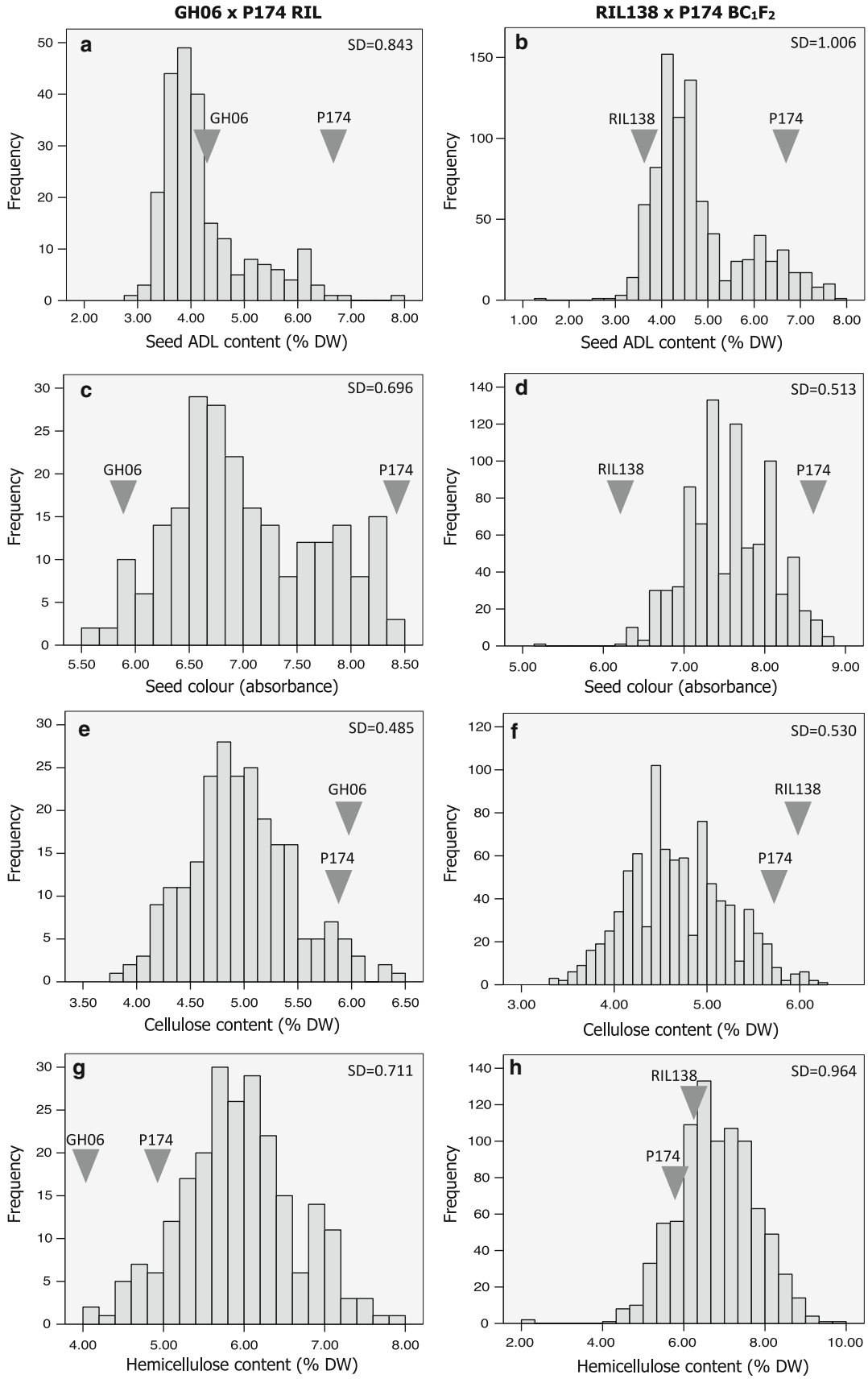
Identification and mapping of ADL-associated markers

BLAST alignments of the cloned AFLP sequences to the Chinese *B. rapa* sequence database BRAD (accessed in January 2011) revealed 100% matches of both ADL-associated markers to *B. rapa* chromosome A9 scaffold 000074. The physical distance between the markers was a mere 173 kb, despite the relatively large genetic distance of 2.6 cM between them in the RIL population. Based on the sequence information from *B. rapa* scaffold 000074, we designed two SCAR markers corresponding to the respective AFLP markers, and screened 18 *in silico* SSR primer pairs from the neighbouring chromosome region. Five of the SSRs showed polymorphisms between the mapping parents and the ADL bulks, and were henceforth genotyped in the entire RIL population. In addition, 101 *Brassica* SSR primer pairs with previously mapped loci on *B. napus* chromosome A9 were also used for bulk analysis. Seven of these SSR markers, which closely matched the segregation pattern for high- and low-ADL content in the bulk lines, were subsequently used to genotype the RIL population.

Linkage mapping of the ADL-associated markers from chromosome A9 revealed that the identified markers segregated into two separate linkage groups (Fig. 2). The first of these linkage groups (designated RIL_A9-1) contained ten markers covering 19.7 cM, whereas the second linkage group (RIL_A9-2) had a length of 9.3 cM and contained the two AFLP-derived SCAR markers. Interestingly, RIL_A9-1 contains an 8.5 cM gap that corresponds closely to the length of RIL_A9-2, and alignment of the two groups to the *B. rapa* physical map of chromosome A9 (between 2.5 and 3.5 Mbp) revealed that the sequence of RIL_A9-2 indeed corresponds to the *B. rapa* sequence between the markers CN79_145 and KBrH095F22.3_230, which flank the gap on RIL_A9-1 (Fig. 2).

QTL analysis and fine mapping

QTL analysis by composite interval mapping in the linkage maps for RIL_A9-1 and RIL_A9-2 revealed highly significant QTL for ADL content and seed colour (LOD = 25.3 and 20.0, respectively). These QTL colocalise at the same position on RIL_A9-1 (Fig. 2),


GH06 x P174 RIL
RIL138 x P174 BC1F2
a
SD=0.843
GH06
P174
Frequency
Seed ADL content (% DW)
b
SD=1.006
RIL138
P174
Frequency
Seed ADL content (% DW)
c
SD=0.696
GH06
P174
Frequency
Seed colour (absorbance)
d
SD=0.513
RIL138
P174
Frequency
Seed colour (absorbance)
e
SD=0.485
GH06
P174
Frequency
Cellulose content (% DW)
f
SD=0.530
RIL138
P174
Frequency
Cellulose content (% DW)
g
SD=0.711
GH06
P174
Frequency
Hemicellulose content (% DW)
h
SD=0.964
RIL138
P174
Frequency
Hemicellulose content (% DW)

◀ **Fig. 1** Histograms showing phenotype distributions for **a**, **b** ADL content, **c**, **d** seed colour, **e**, **f** cellulose content and **g**, **h** hemicellulose content in seeds from **a** 232 RILs from the cross GH06 × P174, and **b** 872 BC_1F_2 plants from the backcross of RIL138 × P174. *Arrows* show the mean values for the parental lines GH06, P174 and RIL138, respectively. *SD* standard deviation

explaining 39.3 and 31.0%, respectively, of the phenotypic variation for ADL content and seed colour in the RIL population. Negative additive effects of −0.48 and −0.52 for seed colour and ADL content, respectively, indicate that the allele from parent P174 increases both seed coat pigmentation and ADL. The QTL peak spans the two SCAR markers developed from the bulked-segregant AFLP analysis.

For fine mapping, 200 BC_1F_2 plants with high seed ADL content were analysed with all polymorphic codominant markers spanning the QTL region on RIL_A9-2. The segregation pattern in the BC_1F_2 population revealed a single locus (hereinafter referred to as *ADL*) with dominance of the low-ADL allele. Consequently, codominant markers involved in recombination events with the *ADL* locus must be heterozygous in high-ADL BC_1F_2 individuals, whereas these in turn must be homozygous-recessive at *ADL*. Conversely, markers cosegregating with *ADL* must be homozygous for marker alleles from the high-ADL parent in all high-ADL BC_1F_2 plants. Pairwise genetic distances in relationship to the *ADL* locus could, thus, be calculated based on frequencies of such recombinations. This allowed reconstruction of an 8 cM long linkage group, designated BC_1F_2_A9-2, corresponding to RIL_A9-2 but

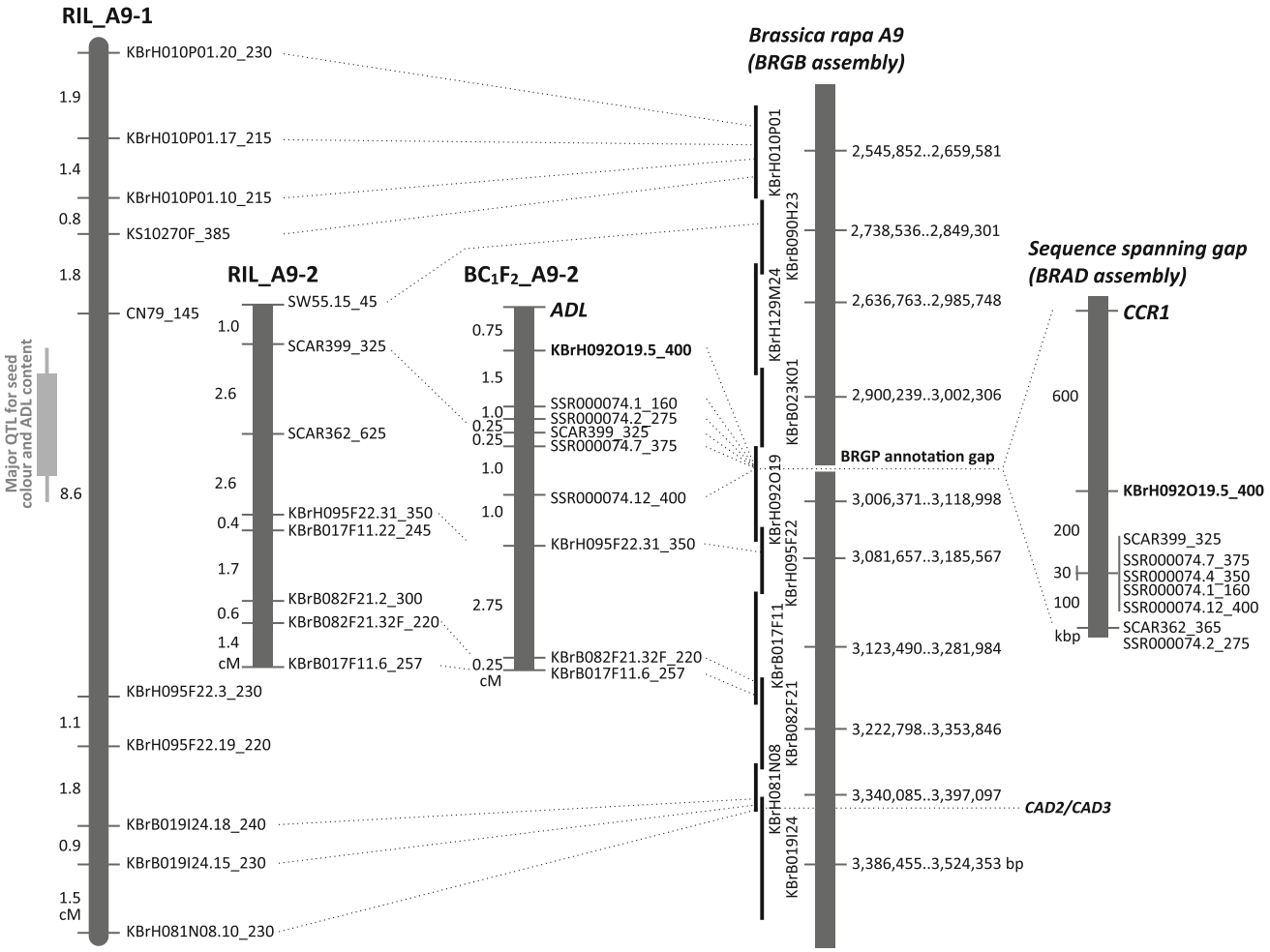


Fig. 2 Comparison of the genetic and physical maps for the region of *Brassica napus* chromosome A9 spanning the major QTL for seed ADL content and seed colour in the RIL population from GH06 × P174, and the corresponding *ADL* locus in a BC_1F_2 population derived from the heterozygous RIL138 backcrossed with P174. Alignment of marker sequences to the *B. rapa* chromosome A9 sequence assembly revealed that linkage group RIL_A9-2, which was remapped with additional markers as BC_1F_2_A9-2 in the BC_1F_2 population, corresponds to the gap in linkage group RIL_A9_1. The lack of linkage between markers on these two groups is explained by a putative homoeologous non-reciprocal translocation of the chromosome block represented by RIL_A9-2 to chromosome C8 in the parental line GH06

including *ADL* as a Mendelian locus along with additional markers in closer proximity to *ADL*. The nearest SSR marker we were able to locate in BC_1F_2_A9-2 was KBrH090O19.5, which mapped 0.75 cM below the *ADL* locus. Thirty-three further *in silico* SSR markers were tested from the 1 Mbp physical sequence interval between KBrH090O19.5 and CN79_145, the most proximal marker on the other side of the putative translocation breakpoint on RIL_A9-1. Unexpectedly, all but 12 of these putative SSRs failed to amplify and the 12 that did amplify were all monomorphic between the parental lines. This meant that the *ADL* locus mapped at the proximal end of BC_1F_2_A9-2, a few centimorgan upstream from the position of the QTL for seed ADL content on RIL_A9-2. This discrepancy may result from less accurate mapping of RIL_A9-1 due to the lower population size and the presence of both dominant and codominant markers, whereas only codominant markers were used to map *ADL* on BC_1F_2_A9-2.

CINNAMOYL CO-A REDUCTASE 1 is a positional and functional candidate gene for the ADL locus

Based on the fine-mapping results, we expected the causative gene or genes for the *ADL* locus to be located in the vicinity upstream of the marker KBrH090O19.5 on *B. napus* chromosome A9. Due to the high homology of the A-genome chromosomes between *B. rapa* and *B. napus*, and the strong conservation of marker order between our *B. napus* genetic map and the available *B. rapa* genome sequence, we queried the BRGP and BRAD databases to locate nearby candidate genes. No known *TT* genes were found in the vicinity of KBrH090O19.5. On the other hand, only 600 kbp upstream of KBrH090O19.5, we located a *B. rapa* orthologue of the major lignin biosynthesis gene, *CINNAMOYL CO-A REDUCTASE 1* (*BraA.CCR1*), in the BRAD gene annotation. This gene was, therefore, revealed for the first time as an important positional candidate for a key role in the regulation of seed coat fibre content in *B. napus*. Unexpectedly, the sequence containing *BraA.CCR1* was not found in the BRGP assembly of the same region of *B. rapa* chromosome A9, which instead exhibited a gap of around 260 kbp at the corresponding position in comparison to the matching BRAD sequence scaffold (accessed in May 2011). In the BRAD annotation, the region in question also contained, besides *BraA.CCR1*, five codominant markers that mapped to the putative translocation represented by linkage group BC_1F_2_A9-2 and are at the same time associated with the major QTL for seed ADL content on chromosome A9.

Identification of functional mutations in *Bna.CCR1* homologues from GH06

Amplicon sequencing of the complete lengths of all five *Bna.CCR1* exons in GH06 and P174 revealed sequence contigs corresponding to *Brassica* homologues from chromosomes A9 of *B. rapa* and C8 of *B. oleracea*. Figure 3 compares the translated protein sequences derived from the coding regions of these homologues from GH06 and P174 with the corresponding *B. rapa* and *B. oleracea* reference sequences. In the black-seeded, high-ADL *B. napus* parent P174, we identified homologues corresponding to both the A9 locus from *B. rapa* (*BraA.CCR1.A9*) and the C8 locus from *B. oleracea* (*BolC.CCR1.C8*), whereas the latter locus was not found in GH06. Instead, GH06 contains a knockout allele that corresponds to the homologous *BraA.CCR1.A9* sequence but carries a loss-of-function frameshift (fs) mutation in exon 1. We propose that this *bnaA.ccr1.A9-fs* knockout is the causal dominant allele for the *ADL* locus in this cross.

The translated protein sequence of the chromosome C8 allele in P174 (*BnaC.CCR1.C8*) is identical to that from *B. oleracea* chromosome C8 (*BolC.CCR1.C8*). This differs to the predicted protein from *BraA.CCR1.A9* by a P > A

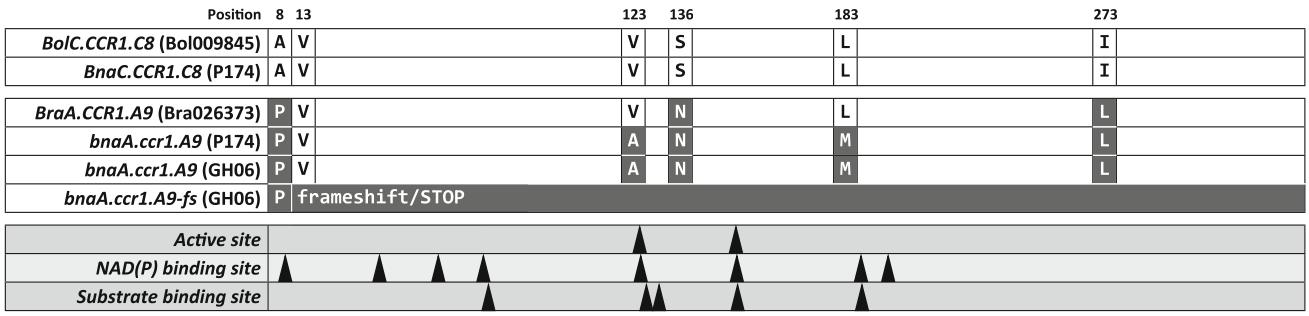


Fig. 3 Positions of amino acid alterations in the derived protein sequences of *BnaCCR1* homologues from chromosomes C8 and A9 of the dark-seeded, high-ADL *B. napus* line P174 and the yellow-seeded low-ADL mutant GH06. The protein sequences are aligned to the *BolC.CCR1* and *BraA.CCR1* reference sequences from *B. oleracea* chromosome C8 (Bol009845) and *B. rapa* chromosome A9 (Bra026373), respectively. *Boxes* show putative functional mutations adjacent to enzyme active sites, NAD(P) binding sites and/or substrate binding sites, respectively

substitution at amino acid position 8, an N > S substitution at position 136 and an L > I substitution at position 273. While this last substitution occurs in a non-conserved terminal protein domain, particularly the proline–alanine substitution at position 8 is expected to significantly alter the binding properties of the protein near the first predicted NAD(P) binding site of the enzyme. The strong conservation of the protein sequences from the chromosome C8 allele from both *B. napus* and *B. oleracea* wild types suggests that *BnaC.CCR1.C8* encodes a functional CCR1 enzyme in P174.

The *B. napus* A-genome allele corresponding to *BraA.CCR1.A9* in both P174 and GH06 additionally carries two putatively functional amino acid substitutions: V > A at position 123 and L > M at position 183. Both of these changes are likely to further influence the adjacent substrate and NAD(P) binding sites, while the former mutation is also adjacent to the enzyme active site. We, therefore, suggest that the native A-genome allele found on chromosome A9 in both P174 and GH06 (hereinafter designated *bnaA.ccr1.A9*) is likely to encode a protein with impaired function and possibly an altered substrate specificity compared to the putative wild-type enzyme encoded by *BolC.CCR1.C8* and *BnaC.CCR1.C8*. Besides the *bnaA.ccr1.A9-fs* knockout GH06 possesses only this functionally impaired locus.

Discussion

Oilseed rape and canola extraction meals after oil extraction contain around 40% high-quality protein (dry weight after oil extraction) with a favourable composition of amino acids, including comparatively high contents of the essential sulphuric amino acids, methionine and cysteine. Modern oilseed rape varieties with "double-low" (00 or canola-quality) seeds, having no erucic acid in the seed oil and low concentrations of glucosinolates in the extraction meal, therefore represent a valuable feedstuff for livestock nutrition. On the other hand, the extraction meal contains a relatively large proportion of non-energetic crude fibre, particularly ADL, along with condensed tannins that reduce the protein digestibility. This strongly limits the quantities of oilseed rape meal that can be used in feed rations for monogastric livestock. Reductions in the seed ADL content would significantly increase the proportion that could be included in poultry and swine feed mixtures, considerably improving the overall economical value of the crop. Interestingly, low-ADL lines identified in this study tended to have an increased protein content, with no corresponding effect on oil content despite the strong negative correlation between oil and protein content. This coincides with the findings of Wittkop et al. (2009) in other *B. napus* germplasm and contrasts the general belief that a reduction of seed coat thickness in light-seeded *B. napus* lines should result in a pleiotropic increase of both oil and protein content, due to the proportionally greater contribution of the embryo to the seed. In fact, the observed tendency for higher protein in low-ADL lines, with no corresponding increase in oil content, suggests a direct biochemical relationship between the seed coat and the biosynthesis of storage proteins in the embryo. Biosynthesis of the *B. napus* seed storage protein *napin* competes with the phenylpropanoid biosynthesis pathway for methionine. An accumulation of free methionine in the embryos of low-ADL genotypes (unpublished observations) might feasibly cause a feedback into increased storage proteins. The negative correlation between ADL and protein content has a dual positive effect on the nutritional composition of the seed meal for animal nutrition, by simultaneously increasing the energy content and the digestibility of the meal. Identification of genes directly influencing ADL content, independently of the flavonoid pathway, will enable considerably more targeted approaches for identification and use of genetic resources for this trait in practical breeding.

Inheritance of seed colour and ADL content

Strong correlations were observed between seed colour and ADL content, but not between ADL and cellulose or hemicellulose contents. These findings confer to our expectation that seed phenylpropanoids contributing to antinutritive ADL are largely confined to the testa, whereas overall seed contents of cellulose and hemicellulose should also contain a significant contribution from embryo cell walls. Oil content in the embryos was highly negatively correlated with hemicellulose content in the two populations. This is presumably due to competition for carbon partitioning between the two pathways, confirming that a substantial proportion of the hemicellulose is embryo derived. Interestingly, the lack of a clear maternal effect on the *ADL* locus suggests that the regulation of phenylpropanoid biosynthesis in the testa may be influenced by genes expressed in the seed embryo or endosperm.

A non-reciprocal translocation involving the *bnaA.ccr1.A9_fs* mutation putatively disrupts seed coat phenylpropanoid biosynthesis

We identified the important lignin biosynthesis gene *CCR1* as a positional candidate with very close physical vicinity to markers that are tightly linked to the major dominant locus for low seed coat ADL content in *B. napus*. Furthermore, we discovered that a functional *BnaC.CCR1* locus on chromosome C8 appears to have been replaced in

GH06 by a loss-of-function frameshift mutant allele. These findings implicate the *bnaA.ccr1.A9_fs* knockout as the effector of the *ADL* locus.

Unexpected discrepancies between our detailed genetic maps and two alternative physical maps, corresponding to the chromosome region containing the *ADL* locus in *B. rapa*, suggest that translocation as the likely mechanism for the replacement of *BnaC.CCR1.C8*. The linkage groups RIL_A9-1 and RIL_A9-2 showed no genetic linkage, although they represent physically adjacent sequences on chromosome A9 (Fig. 2). These observations are consistent with a homoeologous non-reciprocal translocation (HNRT; Udall et al. 2005) of a section of chromosome A9 to its homoeologue C8 in the yellow-seeded parent GH06. Figure 4 presents a model explaining how an HNRT and accompanying knockout mutation could give rise to the DNA sequence, mapping and phenotype anomalies in GH06 and its low-ADL progenies. The pedigree of GH06 includes a yellow-seeded line derived from resynthesised *B. napus* and another ancestor originating from radiation mutagenesis. HNRT events are common during resynthesis of *B. napus* (Udall et al. 2005; Szadkowski et al. 2010), while gene knockouts caused by deletions are a common consequence of radiation mutation. According to Nicolas et al. (2009), the chromosome arms at the top of the partially homoeologous *B. napus* chromosomes A9 and C8 are particularly prone to homoeologous recombination.

CCR1, also known in Arabidopsis as *IRREGULAR XYLEM 4* (*IRX4*; At1g15950), is the first specific committed step in the biosynthesis of monolignols that in xylem are later assembled into cell wall lignin (Lacombe et al. 1997; Piquemal et al. 1998). Xylem cell walls of Arabidopsis *irx4* (*ccr1*) mutants were found to contain 50% less lignin than wild-type plants, although their cellulose and hemicellulose content remained unchanged (Jones et al. 2001). This phenotype is consistent with the seed phenotypes seen in low seed ADL progenies from the yellow-seeded oilseed rape genotype GH06.

Few studies have specifically investigated the role of phenylpropanoid biosynthesis genes in seed testa development. In one rare example, in which no distinction was made between *CCR* homologues, Bezold et al. (2005) confirmed that hull-less mutants of pumpkin (*Cucurbita pepo* L.) show considerably lower expression of *CCR* and other genes involved in secondary cell wall biosynthesis. In Arabidopsis, the laccase gene *LAC15* (*TRANSPARENT TESTA 10*) was found by Liang et al. (2006) to play a role in monolignol polymerisation in seeds. However, *lac15/tt10* mutants also exhibit an accumulation of soluble proanthocyanidins (Pourcel et al. 2005), an association that we have not observed in *B. napus* genotypes with low seed ADL content (Snowdon et al. 2010). The role of *CCR1* in xylem lignification has been investigated in considerable detail, however, microarray data show that the same gene is also expressed at moderate to strong levels in Arabidopsis seeds during the mid to late stages of seed development (data from the Arabidopsis eFP Browser, http://bbc.botany.utoronto.ca/; Winter et al. 2007). In other *B. napus* genotypes, we confirmed a strong expression of *CCR1* during seed development by quantitative real-time PCR (W. Bekele, C. Obermeier and R. Snowdon, unpublished data). In Arabidopsis, the seed expression is particularly pronounced in the peripheral endosperm and the testa (data from https://www.genevestigator.com/; Hruz et al. 2008), supporting its participation in seed coat phenylpropanoid biosynthesis. Our low-ADL phenotype is not associated

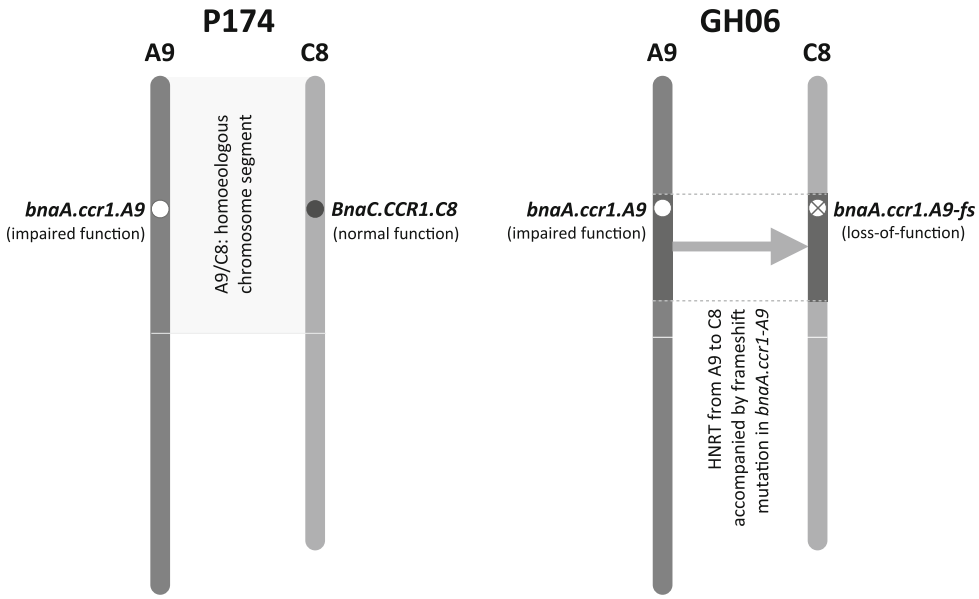


Fig. 4 Explanation for the replacement of the functional *BnaC.CCR1.C8* locus on *B. napus* chromosome C8 with a functionally impaired homologue from chromosome A9 in GH06 by a homoeologous non-reciprocal translocation (HNRT). The native *bnaA.ccr1.A9* alleles in both P174 and GH06 share functional mutations affecting amino acids near the enzyme active sites that are expected to impair the enzyme activity. Furthermore, the translocated *bnaA.ccr1.A9-fs* allele carries a frameshift mutation (fs) that causes complete loss-of-function due to a stop codon in exon 1 (cf. Fig. 3)

with any developmental defects like those seen in Arabidopsis *irx4* mutants, hence it is likely that the *bnaA.ccr1.A9-fs* mutant knocks out a homologue with seed-specific expression and that independent homologues are responsible in *B. napus* for lignin biosynthesis in xylem tissues.

CCR1 encodes the enzymatic reduction of hydroxycinnamoyl CoA esters to their corresponding cinnamoyl aldehydes during monolignol biosynthesis. In *Medicago truncatula*, Zhou et al. (2010) found that the enzyme encoded by *CCR1* prefers feruloyl CoA as a substrate, generating S and G lignin subunits in the presence of coniferaldehyde 5-hydroxylase (F5H), 5-hydroxyconiferaldehye 5-*O*-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD). The substrate preference was confirmed by Lauvergeat et al. (2001), who found that *CCR1* is five times more efficient than *CCR2* in Arabidopsis with feruloyl CoA and sinapoyl CoA as a substrate. This suggests a primary role of *CCR1* in constitutive phenylpropanoid biosynthesis, while *CCR2* is more active in the synthesis of phenolic compounds in response to biotic or abiotic stress (Lauvergeat et al. 2001).

BnCAD2/CAD3 may also be involved in seed-specific phenylpropanoid biosynthesis

The multiple members of the *CAD* gene family encode NADPH-dependent oxidoreductases catalysing reduction of various phenylpropenyl aldehyde derivatives (Mansell et al. 1974). This multigene family exhibits high sequence diversity, but relatively broad functional redundancy (Kim et al. 2004). In Arabidopsis, the xylem-expressed homologues *AtCAD4* (At3g19450) and *AtCAD5* (At4g34230), which are the most closely related family members to proven *CAD* genes from other species, were found by Kim et al. (2004) to show the highest enzymatic activity on phenylpropenyl aldehyde substrates. In contrast, the tandemly duplicated homologues *AtCAD2* (At2g21730) and *AtCAD3* (At2g21890) were found to have far lower catalytic activity. The physiological function of these two *CAD* family members has yet to be established; however, *AtCAD2* was found to use caffeyl aldehyde most effectively as a substrate, whereas *AtCAD3* had a slight preference for *p*-coumaryl and 5-hydroxyconiferyl aldehydes (Kim et al. 2004).

Somewhat surprisingly, both *AtCAD2* and *AtCAD3* show strong expression levels in the peripheral endosperm of developing embryos (data from https://www.genevestigator.com/). The spatial and temporal proximity of *AtCCR1*, *AtCAD2* and *AtCAD3* gene expression at the periphery of developing embryos are consistent with a possible joint role in seed coat phenylpropanoid biosynthesis. Most interestingly, we located a *Brassica* orthologue of the tandem *CAD2/CAD3* duplication ~660 kbp downstream of KBrH090O19.5 in *B. rapa* (BRAD annotation). This raises the question of whether *BnCAD2/CAD3* may not be a similarly suitable positional candidate for the *ADL* locus in *B. napus*. However, by mapping of markers closely flanking *CAD2/CAD3* in the *B. rapa* genome sequence, we established that the *Bna.CAD2/CAD3* locus does not correspond with the *ADL* locus, instead mapping around 13 cM away on chromosome A9 (Fig. 2). Furthermore, disruption of *CAD* function on its own does not necessarily have a negative impact on overall lignin content (e.g. Chabannes et al. 2001; Kim et al. 2004), although the lignin composition may be altered (e.g. Halpin et al. 1998; Patten et al. 2005). Hence, disruption of *Bna.CAD2/CAD3* alone might not be expected to cause the dominant, low seed coat ADL phenotype of GH06 and its progenies.

Interaction of *Bna.CCR1* and *Bna.CAD2/CAD3* in seed coat phenylpropanoid biosynthesis

Nevertheless, results from other studies strongly suggest that disrupted activity of both *Bna.CCR1* and *Bna.CAD2/CAD3* in seeds may be required for the drastic, dominantly inherited reduction of seed coat ADL that we observed in GH06 and its low-ADL progenies. In particular, simultaneous reduction of CCR and CAD enzymatic activity can reduce lignin in xylem by 50% compared to wild-type levels (Thévenin et al. 2011). Chabannes et al. (2001) reported that transgenic silencing of *CCR1* by itself induced a strong reduction in lignin content in tobacco, however, only an intermediate effect was seen in hemizygous plants. This corresponds to a codominant inheritance of wild type and silenced alleles due to a gene dosage effect. On the other hand, the low-lignin phenotype was fully dominant in hemizygous *CCR1/ccr1* plants that were also hemizygous for a silenced copy of *CAD*, whereas the *cad* mutation on its own (either in homozygous or hemizygous form) had no negative effect on lignin content. Chabannes et al. (2001) concluded that the ectopic expression of *ccr1* mutants depends on the genetic background in which the mutation occurs, and that a double mutant with non-functional *ccr1* + *cad* can show drastic (dominant) reduction in lignin content even when both mutations are present in hemizygous form.

These observations fit perfectly into the context of our own data, if we assume that *Bna.CCR1* loss-of-function underlies the *ADL* locus in GH06. Figure 5 gives a hypothetical inheritance model for the *ADL* locus based on the knowledge of the interaction between *ccr1* and *cad* mutations in xylem. According to the results of Chabannes et al. (2001), the mutant *bnaA.ccr1.A9-fs* from GH06 would be expected to be completely dominant over a functional *BnaC.CCR1.C8* from P174 only when the nearby

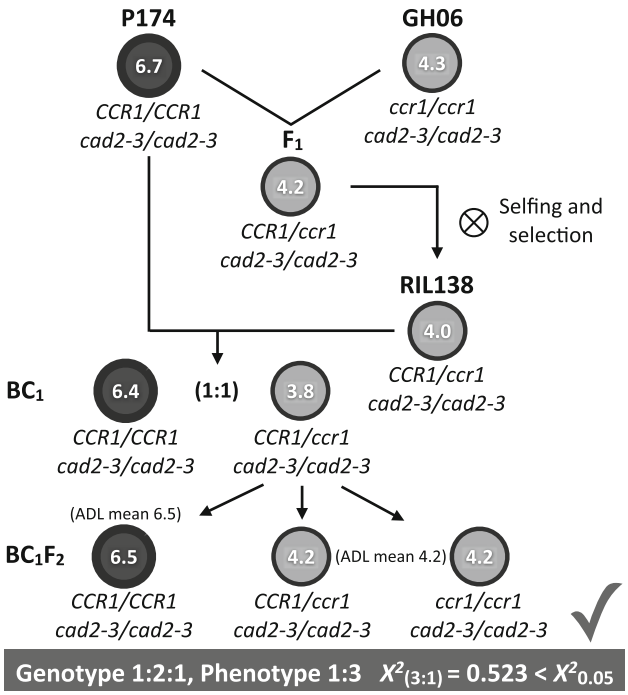


Fig. 5 Hypothesis explaining the observed BC_1F_2 segregation with the *bnaA.ccr1.A9-fs* mutation in GH06 accompanied by a putative *bna.cad2-3* mutation in both parents. Observed seed ADL contents (% DW) in each generation are shown *within the circles*. The *bnaA.ccr1.A9-fs* knockout mutation is only expected to be dominant in the presence of a non-functional *bna.cad2/cad3* (cf. Chabannes et al. 2001). Disruption of *CAD* alone does not disrupt phenylpropanoid biosynthesis, hence P174 has a normal, high-ADL phenotype. All *cad2-3* mutants containing the *bnaA.ccr1.A9-fs* knockout in either homozygous or hemizygous form should have ~50% total seed ADL, but show a normal developmental phenotype

Bna.CAD2/CAD3 locus is also silenced or non-functional in both parents. A *bna.cad2/cad3* mutation on its own should have no phenotypic effect on seed coat ADL in P174 due to the presence of a functional copy of *BnaC.CCR1.C8*. Furthermore, in crosses of high-ADL *B. napus* lines with normal *Bna.CAD2/CAD3*, only partial dominance or codominance of *bnaA.ccr1* should be observed. Crosses of GH06 to genetically diverse black-seeded lines often show an incomplete dominance consistent with this theory.

Use of *bna.ccr1* and *bna.cad2/cad3* mutants for breeding

Diagnostic markers for the *bnaA.ccr1.A9-fs* knockout mutation are expected to be particularly useful for marker-assisted selection of oilseed rape with reduced seed fibre content. Whereas NIRS is commonly used in selection for other important seed quality traits, many breeders do not have access to effective NIRS calibrations for important seed fibre compounds. Gene-based markers, therefore, offer a promising alternative for uncovering allelic variation in breeding materials and introgressing promising alleles for low seed fibre content into elite breeding lines. Whereas previous efforts to reduce fibre content in oilseed rape relied heavily on selection based on the environmentally unstable yellow-seed trait, marker-assisted selection for functional seed coat phenylpropanoid mutants may provide a means for effective improvement of meal quality independently of seed colour. Combination of markers for *bna.ccr1* and *bna.cad2/cad3* mutations may also facilitate breeding of hybrid cultivars that take advantage of the dominant inheritance of the low seed ADL trait derived from GH06.

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