Genome-wide association analysis uncovers candidate genes for forage quality traits in Brassica napus stems

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Abstract

Background

Brassica napus (rapeseed) is an important oilseed crop and its leaves and stems can also be used as animal feed. Lignocellulose content is closely related to the nutritional quality and palatability of animal feed. However, quantitative trait loci (QTLs) associated with acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents in rapeseed stems have not yet been mapped.

Results

In this study, we used 494 *B. napus* accessions to perform genome-wide association studies (GWAS) of ADF and NDF contents. Ninety-two single-nucleotide polymorphisms (SNP) and 35 simple-sequence repeat (SSR) loci were significantly correlated with ADF and NDF contents, respectively, and six genetic loci associated with ADF and NDF contents were detected using both types of markers. We identified three candidate genes on chromosome A05 related to ADF content, including genes encoding chitinase-like protein 2 (CTL2) and two trichome birefringence-like 41 s (TBL41s). Seven genes on chromosomes A03 and A04 were related to NDF content, including genes encoding glycosyl hydrolase (GH), reversibly glycosylated polypeptide 1 (RGP), irregular xylem 12 (IRX12), trichome birefringence-like 34 (TBL34), galacturonosyltransferase 7 (GAUT7), cytokinesis defective 1 (CYT1), and LOB domain-containing protein 15 (LBD15). These candidate genes encode factors that likely participate in secondary cell wall formation and lignocellulose biosynthesis.

Conclusions

These findings lay the foundation for identifying genes related to forage quality traits and improving the efficiency of forage utilization in rapeseed, which will be beneficial for breeding new varieties for high-quality forage with low lignocellulose content.

Background

Brassica napus is not only an important source of edible oil, but it is also a forage crop whose leaves and stems are used for ruminant animal feed [1]. *Brassica* forage with low fiber and high protein contents is more similar to traditional forage, grass, legumes and herbage than other types of *Brassica* [2]. Following fermentation to form silage, rapeseed stems contained 4.78% crude protein, 1.04% ether extract, 45.59% crude fiber, 49.72% acid detergent fiber (ADF), 62.45% neutral detergent fiber (NDF), 9.17% acid detergent lignin (ADL), 0.63% Ca and 0.08% P contents, and showed improved palatability [3]. The weight of cattle increased after they were fed rapeseed stems [4]. However, rapeseed stems have poor digestibility due to the high cellulose and lignin contents in their cell walls, limiting the value of rapeseed straw for use as animal feed [5].

The cell wall is a composite of cellulose, hemicellulose, and lignin. The contents of the cell wall components ADF and NDF can be estimated using the Van Soest method [6]. NDF consists of the cellulose, hemicellulose and lignin released after neutral detergent treatment, and ADF consists of the cellulose and lignin released after acid detergent treatment. Cell wall digestibility is widely used to evaluate the nutritional quality of forage [7]. The ADF and NDF contents of crops are negatively related to the digestibility of forage, as well as its nutritional quality and palatability [8]. However, cellulose and lignin are important for the mechanical strength and structural integrity of the stem [9, 10], and correlated with lodging and disease resistance [11–13]. Therefore, it is important to balance the ADF and NDF contents in stems.

QTL mapping of cell wall components has been performed in various crops. Cardinal et al. (2005) [14] identified 64 QTLs related to lignin, ADF, and NDF contents in the leaf-sheaths and stalks of a maize (*Zea mays*) recombinant inbred line (RIL) population. Wang et al. (2016) identified 73, 41, and 82 single-nucleotide polymorphisms (SNPs) associated with ADF, NDF and in vitro dry matter digestibility (IVDMD), respectively in 368 maize inbred lines in seven different environments. Each significant SNP explained 4.2–6.2% of the phenotypic variation [7]. Another study [15] detected 24 SNPs associated with NDF on chromosomes

1, 2, 6, 7, 8, 9 and 10 and 7 SNPs associated with ADF content on chromosomes 3 and 4 in sorghum (*Sorghum bicolor*). In *B. napus*, most QTLs for ADF and NDF contents identified to date are associated with the seed coat. For example, Badani et al. (2006) [16] demonstrated that seed coat color is related to ADF contents. The main QTL for this trait was detected at the same position on chromosome N18 in all three populations examined; the second QTL for ADF contents was located on chromosome N13. Liu et al. (2013) [17] identified 7, 9, and 5 QTLs for seed coat lignin, cellulose, and hemicellulose contents in an RIL population of rapeseed, explaining 8.1–42.8%, 4.7–21.9% and 7.3–16.9% of phenotypic variation, respectively. However, to date, QTL mapping for ADF and NDF contents in rapeseed stems has not been reported.

In the current study, we used SNP markers from the *Brassica* Illumina 60K SNP array and simple-sequence repeat (SSR) markers to genotype 494 *B. napus* accessions. We performed genome-wide association studies (GWAS) to identify significant loci and candidate genes associated with ADF and NDF contents. This study provides an important basis for identifying and cloning genes related to ADF and NDF contents in *B. napus* stems.

Results

Phenotypic variation of ADF and NDF contents in B. napus

In this study, we measured ADF and NDF contents in the stems of 494 *B. napus* lines in both 2013 and 2014. These values showed continuous variation and approximated a normal distribution (Fig. 1). The phenotypic range of NDF content in 2013 was 70.34–79.85%, and that of ADF content was 54.87–62.86%. The variation coefficient of NDF content in 2013 and 2014 was 2.74% and 1.97%, respectively, and the variation coefficient of ADF content was 2.59% and 1.95%, respectively (Table 1). These results indicate that NDF and ADF contents are typical quantitative traits.

Table 1
Phenotypic variation of ADF and NDF contents in the stems of *B. napus* in 2013 and 2014

Trait	Year	Mean ± SD (%)	Range (%)	CV%
NDF	2013	75.21 ± 2.06	70.34–79.85	2.74
ADF	2014	77.27 ± 1.52	72.02–81.25	1.97
	2013	59.17 ± 1.53	54.87–62.86	2.59
	2014	59.31 ± 1.16	56.63–62.27	1.95

Gwas Using Snps

We performed GWAS of 494 *B. napus* accessions using the GLM and MLM models. For ADF content, based on the QQ plot, the *P* values detected by the K, K + PCA and K + Q models were close to the expected values, which had greater effects in reducing false positives than the other models (Fig. 2a). For NDF content, the P, K + PCA and K + Q models were better than the Q and K models (Fig. 2b).

We detected 11 SNPs that were significantly related to ADF content on chromosomes A05, A06, A07, and A09, which explained 3.90–5.32% of the phenotypic variation (Fig. 3 and Additional file 1). We identified the candidate gene *BnaA05g23000D* (*chitinase-like 2*, *CTL2*), which is located at position 17.4 Mb on chromosome A05. CTL2 binds to glucan-based polymers and regulates cellulose assembly in *Arabidopsis thaliana* [18, 19].

In addition, we identified 81 loci that were significantly associated with NDF on all chromosomes except A01, A08, and C06; these 81 loci contributed 3.21–6.21% of the phenotypic variation (Fig. 3 and Additional file 1). We also identified several candidate genes involved in lignocellulosic biosynthesis. *BnaA02g09490D* (*galacturonosyl transferase 12*, *GAUT12*), located at position 4.7 Mb on chromosome A02, is involved in xylan biosynthesis and lignin deposition during secondary cell wall formation [20]. *BnaA03g14000D* and *BnaA03g14010D* located at position about 6.4 Mb on chromosome A03, and

BnaA04g17560D and *BnaA04g17570D* at about 14.2 Mb on chromosome A04, encoding C4H involved in lignin biosynthesis [21]. We also found the MYB transcription factor gene *BnaA06g25640D* (*MYB103*) on chromosome A06. In *Arabidopsis*, MYB103 regulates syringyl lignin and cellulose biosynthesis in the cell wall [22, 23].

Gwas Using Ssrs

We identified 35 significant SSR loci in *B. napus*, including 21 for ADF content and 14 for NDF content (Table 2). The SSR loci that were significantly associated with ADF content were located on chromosomes A01, A03, A05, A06, A07, A08, C02, C03, C05, C06, C08 and Cnn_random. The SSR loci associated with NDF content were located on chromosomes A01, A03, A04, A05, A06, A07, C03, C05 and C06, which explained 0.72–2.22% of the phenotypic variation.

Table 2

SSR markers significantly associated with ADF and NDF contents in *B. napus*

Trait	Model	Marker	Chr	Position (bp)	*P* value	R^2 (%)
ADF	K+P	SWUA03_1847_278	A03	882,331	2.02E-02	1.14
		OL10_D01	A06	4,207,472	2.55E-02	0.99
		BRMS342	A08_random	466,448	1.55E-02	1.19
		BRMS322	C03	13,853,510	1.37E-02	1.24
	K+Q	CN71	A01	1,324,444	4.39E-02	0.85
		SWUA01_1041_204	A01	21,595,632	1.91E-02	1.20
		niab_ssr082	A05	18,279,544	4.88E-02	0.85
		CB10526	Ann_random	20,560,048	3.12E-02	0.95
		SWUA04_433_233	C02	20,010,508	1.79E-02	1.12
		SWUC372	C03	12,215,000	4.00E-03	1.91
		CB10065	C05	9,020,609	4.46E-02	0.81
		SWUA05_529_268	C05_random	3,379,694	3.61E-02	0.89
		SWUC055	C06	26,150,649	4.75E-03	1.61
		H081N08.7	C08	33,452,394	2.56E-02	1.04
		SWUC088	Cnn_random	26,653,269	2.49E-02	1.04
	K+P,K+Q	BRMS175	A01	21,483,281	5.82E-04	2.39
		SWUA03_1021_268	A03	13,286,992	3.41E-02	0.90
		cnu_ssr149	A06	8,050,632	1.75E-02	1.15
		sR0282	A07	1,641,514	1.05E-02	1.31
		BRMS036	A07_random	1,778,876	4.21E-02	0.84
		SWUC01_549	Cnn_random	67,548,285	1.25E-02	1.28
NDF	K+P	BRMS175	A01	21,483,281	1.85E-03	1.84
		SWUA01_1041_204	A01	21,595,632	2.28E-02	1.02
		SWUA03_1847_278	A03	882,331	2.51E-02	0.99
		SWUC576	A03	10,357,731	7.09E-04	2.19
		SWUA03_1021_268	A03	13,286,992	3.18E-02	0.88
		BRMS195	A04	16,916,234	1.27E-03	2.22
		cnu_ssr257	A05	3,211,956	2.74E-02	0.94
		OL10_D01	A06	4,207,472	4.41E-03	1.53
		cnu_ssr149	A06	8,050,632	2.49E-02	0.95
		BRMS221	A06	19,427,849	1.46E-02	1.15
		Sr0282	A07	1,641,514	3.50E-02	0.84
		BRMS322	C03	13,853,510	2.98E-02	0.91
		CB10065	C05	9,020,609	2.57E-02	0.93

	SWUC055	C06	26,150,649	4.98E-02	0.72

Common Associations Using Snps And Ssrs

We detected six genetic loci associated with ADF and NDF content using both SNPs and SSR markers (Table 3). The SSR associated with ADF content was located at position 18.3 Mb on chromosome A05 (niab_ssr082), which is close to SNP Bn-A05-p11322 (position 17.4 Mb on chromosome A05). Three common associations with NDF content were found on chromosomes A03 and A04: the SSR SWUC576 is close to SNP Bn-A03-p5268 (10.4 Mb on chromosome A03). The SNP Bn-A03-p5633 is also close to another SSR, SWUA03_1021_268, which are located at positions 13.7 Mb and 13.3 Mb on chromosome A03, respectively. Finally, the SSR BRMS195, located at position 16.9 Mb on chromosome A04, is located only 1.2 kb away from SNPs Bn-A04-p9172, Bn-A04-p9169 and Bn-A04-p35637 (located at 39.4 kb along this chromosome).

Table 3
Candidate genes associated with ADF and NDF contents detected using both SNP and SSR markers

Trait	Chr	SNP	Position	SSR	Position	Candidate gene	Annotation
ADF	A05	Bn-A05-p11322	17359502	niab_ssr082	182,79,544	*BnaA05g23000D*	*Chitinase-like protein 2* (*CTL2*)
NDF	A03		10352315	SWUC576	10,357,731	*BnaA05g24680D*	
	A04	Bn-A03-p5268	13723716	SWUA03_1021_268	13,286,992	*BnaA05g24710D*	*Trichome birefringence-like* (*TBL41*)
			16914980	BRMS195	16,916,234	*BnaA03g22360D*	*Trichome birefringence-like* (*TBL41*)
		Bn-A03-p5633	16953114			*BnaA03g27800D*	
			16955619			*BnaA04g21810D*	*Glycosyl hydrolase superfamily protein* (*GH*)
		Bn-A04-p9172				*BnaA04g21970D*	*Reversibly glycosylated polypeptide 1* (*RGP1*)
						BnaA04g22110D	*Irregular xylem 12* (*IRX12*)
		Bn-A04-p9169				*BnaA04g22820D*	
						BnaA04g23270D	*Trichome birefringence-like 34* (*TBL34*)
		Bn-A04-p35637					*Galacturonosyltransferase 7* (*GAUT7*)
							Cytokinesis defective 1 (*CYT1*)
							LOB domain-containing protein 15 (*LBD15*)

ADF: Acid detergent fiber; NDF: Neutral detergent fiber; SNP: single-nucleotide polymorphism; SSR: simple sequence repeat

Identification Of Candidate Genes

Based on the GWAS and SSR mapping results described above, we detected three candidate genes encoding chitinase-like protein 2 (CTL2) and two genes encoding trichome birefringence-like 41 (TBL41), which are related to ADF content; these enzymes are involved in cellulose biosynthesis and cell wall formation. We identified seven candidate genes for NDF content, including genes encoding glycosyl hydrolase (GH), reversibly glycosylated polypeptide 1 (RGP1), irregular xylem 12 (IRX12), trichome birefringence-like 34 (TBL34), galacturonosyltransferase 7 (GAUT7), cytokinesis defective 1 (CYT1) and LOB domain-containing protein 15 (LBD15). These enzymes might be involved in cellulose biosynthesis, lignin biosynthesis and secondary cell wall formation (Table 3).

Discussion

B. napus stems are currently an underutilized resource in China. A large proportion of *B. napus* stems are usually burned or chopped and incorporated into the soil, which pollutes the air and disrupts the ecological balance [24]. *B. napus* stems have huge potential for use as a source of fuel with high sulfur content, high calorific value and low moisture content, which would add value to the crop at the farm level [25]. In addition, *B. napus* stems could be used as feed to meet the current demands in light of insufficient forage. However, the low digestibility of rapeseed stems due to the high cellulose and lignin contents in the stem cell walls limits the value of this material. In the current study, we identified significant markers and candidate genes associated with ADF and NDF contents, which should facilitate the improvement of rapeseed varieties in the future. In addition, studying ADF and NDF contents in stems is essential for improving lodging and disease resistance for rapeseed breeding and cultivation.

Near-infrared (NIR) spectroscopy is a rapid, efficient, non-destructive approach for predicting chemical compound composition in numerous samples. This technique requires little or no sample pretreatment and does not alter the structure of the samples, which in turn reduces analytical costs and saves labor and time [26]. In addition, NIR results are accurate compared to other analytical techniques. NIR technology has been widely used to detect numerous nutrients and toxic elements in agricultural foods during agro-industrial production and to ensure product safety [27, 28]. NIR could also be used in the petrochemical industry for the determination of hydrocarbons, alcohols, ketones and nitrile compounds in organic solvents [29]. Paul et al. (2018) [30] detected microplastics in the soil using a combined NIR chemometric approach, which met the demands for high-throughput analysis of large sample volumes. In addition, NIR technology has been widely used to measure chemical compounds in plants, such as lignin in roots, and to identify important QTLs [31]. In a grain × sweet sorghum population, 17 and 14 QTLs associated with ADF and NDF contents, respectively, were detected using NIR [32]. Wang et al. (2016a) [7] established a NIR model to detect ADF, NDF, and IVDMD contents in maize stalks, as well as SNP markers that were associated with these traits. In the current study, we detected ADF and NDF contents in the stems of 494 *B. napus* accessions using NIR, a convenient, rapid, economical technique for high-throughput analysis.

In the current study, we identified candidate genes associated with ADF and NDF contents via GWAS using SNP and SSR markers. Three candidate genes were identified for ADF contents, including *CTL2* and two *TBL41* genes. CTL2 (*BnaA05g23000D*; chitinase-like 2) binds to glucan-based polymers and functions in cellulose biosynthesis [33]. *TBL41* (*BnaA05g24680D*; trichome birefringence 41) might be involved in cell wall formation. In *A. thaliana*, *TBL27* is required for xyloglucan acetylation [34], and *TBL3* contributes to cellulose biosynthesis [35]. Volker et al. (2010a) [36] proposed that TBL is a pectin-binding protein or bridging protein that binds to pectin and other cell wall polysaccharides based on sequence and structural similarities with rhamnogalacturonan acetylesterase (RGAE) of *Aspergillus aculeatus* and the protein LUSTRIN A-LIKE (*Oryza sativa*). The role of TBL41 needs to be further verified.

Among the candidate genes for controlling NDF content, *BnaA03g22360D*, which is homologous to *AT2G27500*, encodes a glucan endo-1,3-beta-glucosidase that participates in carbohydrate movement by hydrolyzing glycosidic bonds in cellulose and stabilizes protein morphology [37]. *BnaA03g27800D* encoding reversibly glycosylated polypeptide 1 (RGP1) is homologous to AT3G02230. RGP1 in pea (*Pisum sativum*) can be glycosylated by UDP-Glc, UDP-Xyl or UDP-Gal, and involved in xyloglucan and hemicellulose biosynthesis [38]. In addition, *BnaA04g21810D* (irregular xylem, *IRX12*), a laccase gene, is essential for lignin and secondary cell wall biosynthesis [39]. *BnaA04g21970D* (trichome birefringence-like 34, TBL34) encodes DUF231 (domain of unknown function 231). *TBL34* is expressed in xylem cells in *Arabidopsis* and mutation of this gene caused a significant reduction in the amount of acetyl groups in xylan. The *tbl34 tbl35 wsk1* (*ESKIMO1*) triple mutant exhibited collapsed xylem vessels and retarded plant growth, indicating that *TBL34* is essential for secondary cell wall biosynthesis and cellulose deposition [40].

Another candidate gene, *BnaA04g22110D*, encodes galacturonosyltransferase 7 (GAUT7), which plays an important role in polysaccharide biosynthesis in the cell wall matrix by interacting with GAUT1 [41]. *CYT1* (*BnaA04g22820D*) was identified at a position approximately 17.0 Mb away from chromosome A04. *CYT1*, which is also related to the monolignol S/G ratio, as described in a previous report, increases lignin content, and its previously reported position overlaps with the physical location determined in the current study [12]. The *cyt1* mutant exhibited a 5-fold decrease in cellulose contents in embryos, impairing cell

wall synthesis [42]. LBD15 (BnaA03g19070D, LOB domain-containing protein) regulates the expression of *VND7*, encoding a master regulator of tracheary element differentiation that functions via a positive feedback mechanism [43].

The functions and regulatory mechanisms of these candidate genes for NDF contents in *B. napus* should be clarified in the future. ADF and NDF contents are widely used to estimate the quality of feed. Our findings could facilitate the utilization of *B. napus* stems from plants with low ADF and NDF contents as feed, adding to the value of this important crop.

Conclusions

To date there is no research about the identification of ADF and NDF loci and candidate genes. In the study, 92 SNPs and 35 SSRs associated with ADF and NDF contents were identified using GWAS, respectively. And six common associations related to ADF and NDF contents were detected.

Based on the six common loci, three candidate genes (*CTL2* and two *TBL41s*) related to ADF contents and seven genes (*GH*, *RGP*, *IRX12*, *TBL34*, *GAUT7*, *CYT1* and *LBD15*) related to NDF contents were identified. This study will be useful for the utilization of *B. napus* stems as high-quality forage.

Methods

Plant materials and phenotypic evaluation

The seeds of 494 *B. napus* accessions were produced by our lab at Southwest University, Beibei, Chongqing, China. And these inbred lines of *B. napus* were cultivated in Xiema, Beibei, Chongqing, China in 2013 and 2014. All experiments were performed using a random block design, with two rows per line, 10 plants per row, 30-cm row spacing and 20-cm plant spacing. Field management was performed according to conventional methods.

The middle sections of *B. napus* stems were dried in a ventilated oven (65˚C) and ground in a mill for further analysis. ADF and NDF contents in stems were measured using the Van Soest method and determined by near infrared reflectance spectroscopy. The results were analyzed using the modified partial least-squares regression method. The determination coefficients for NDF and ADF were 0.892 and 0.891, respectively [44].

Genotyping With Snp Array And Ssr Markers

The *B. napus* accessions were genotyped using a *Brassica* 60K Illumina SNP array (http://www.illumina.com). SNPs with minimum allele frequency of < 0.05 were excluded from further study, leaving 31,468 SNPs for further analysis. In addition, 84 SSR markers were selected; dominant SSR alleles were scored as present (1) or absent (0) [45]. The physical locations of the SNP and SSR markers were identified as described by Wei et al. (2016) [46] and Wei et al. (2017a) [12].

Gwas And Candidate Gene Identification

The ADF and NDF traits were evaluated for two years with two biological repetitions. The best linear unbiased prediction of a trait with two replicates over two years was estimated using an R script (http://www.eXtension.org/pages/61006) based on a linear model. Population structure (Q) was performed using Structure software, setting the K value from 1 to 10, with three independent measurements for each value [47]. The relative kinship between the materials was calculated using TASSEL 5 [48]. Association analysis was performed using the general linear module (GLM) and mixed linear module (MLM) in TASSEL 5 for SNP markers and TASSEL 3 for SSR markers. In addition, a quantile–quantile (QQ) plot was generated based on the observed $-\log_{10}(P)$ and the expected P value, and a Manhattan plot was drawn using the R package qqman [49]. The GWAS threshold was set to $P < 3.17 \times 10^{-5}$ (1/ total SNPs, 31,468) for SNPs and $P < 0.05$ for SSRs. Candidate genes were identified by searching the 500 kb flanking region of each peak marker [12].

Abbreviations

ADF
acid detergent fiber
NDF
neutral detergent fiber
GWAS
genome-wide association studies
SNP
single-nucleotide polymorphisms
QTL
quantitative trait loci
CTL2
chitinase-like protein 2
TBL41
trichome birefringence-like 41 s
GH
glycosyl hydrolase
RGP
reversibly glycosylated polypeptide 1
IRX12
irregular xylem 12
TBL34
trichome birefringence-like 34
GAUT7
galacturonosyltransferase 7
CYT1
cytokinesis defective 1
LBD15
LOB domain-containing protein 15
RIL
recombinant inbred line
IVDMD
in vitro dry matter digestibility

Declarations

Availability of data and materials

The data sets used and/or analysed during the current study will be available upon reasonable request to the corresponding author.

Consent For Publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

L.W. designed the experiments; R.L., W.X., Y.Z., J.M. and A.L. performed the field and NIR experiments; J. L. and L.W. supervised the writing and editing of the article.

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Not Applicable

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Figures

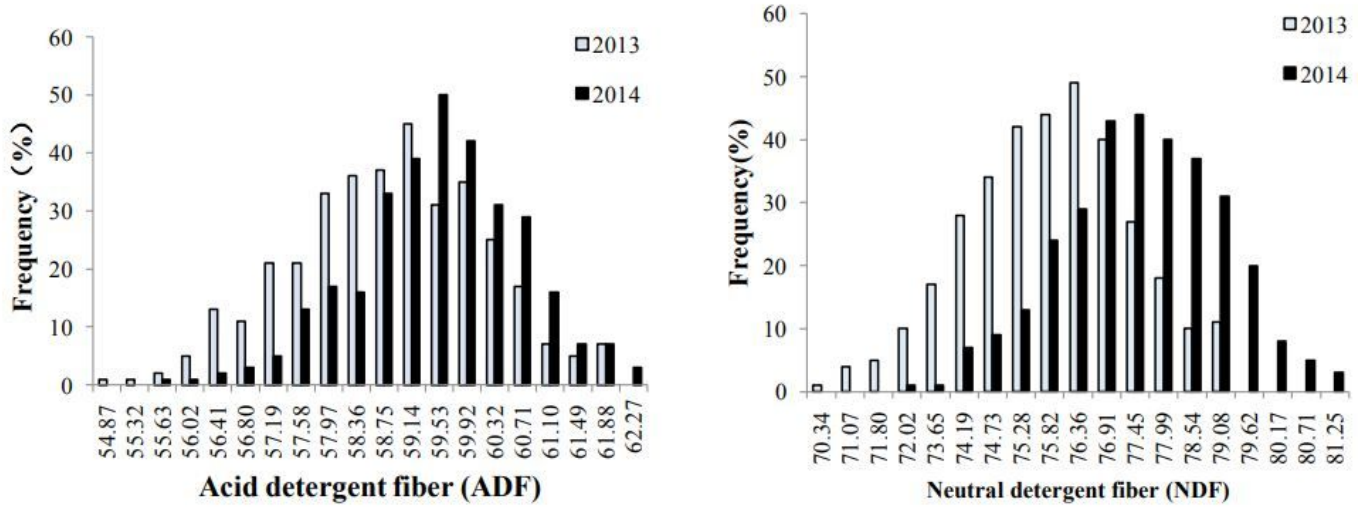

Figure 1

Phenotypic frequency distribution of ADF and NDF contents in the stems of B. napus in 2013 and 2014

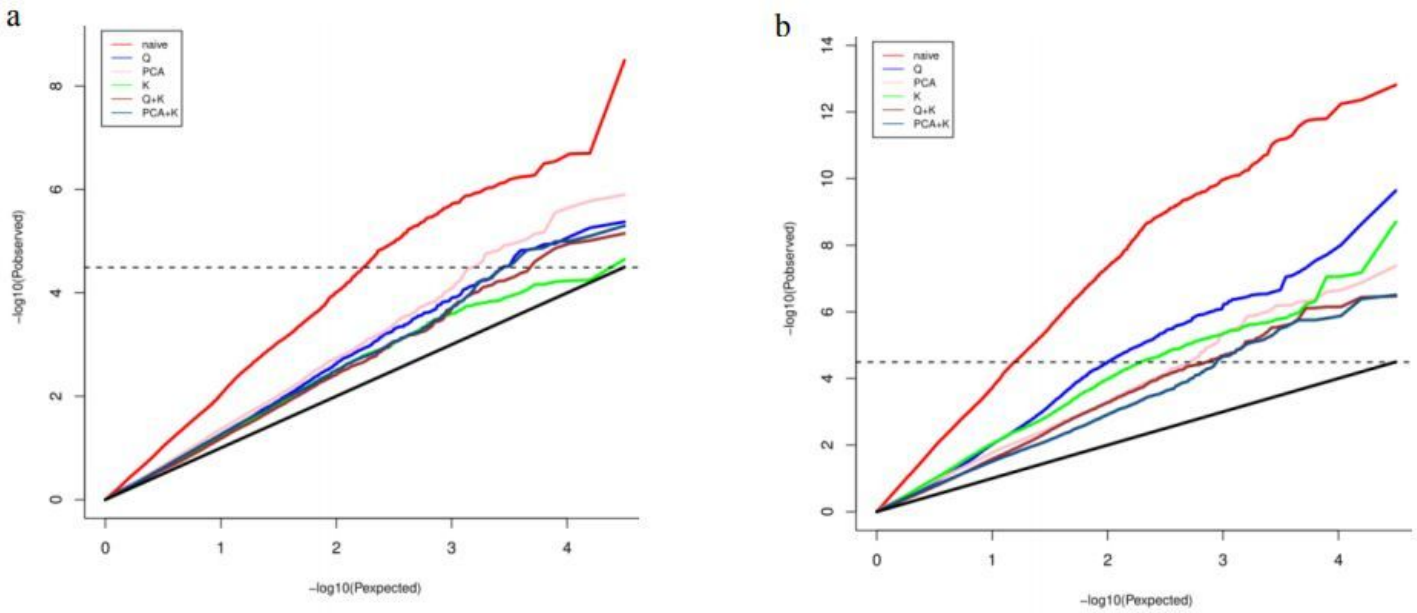

Figure 2

Quantile–quantile plots of ADF and NDF contents in B. napus stems

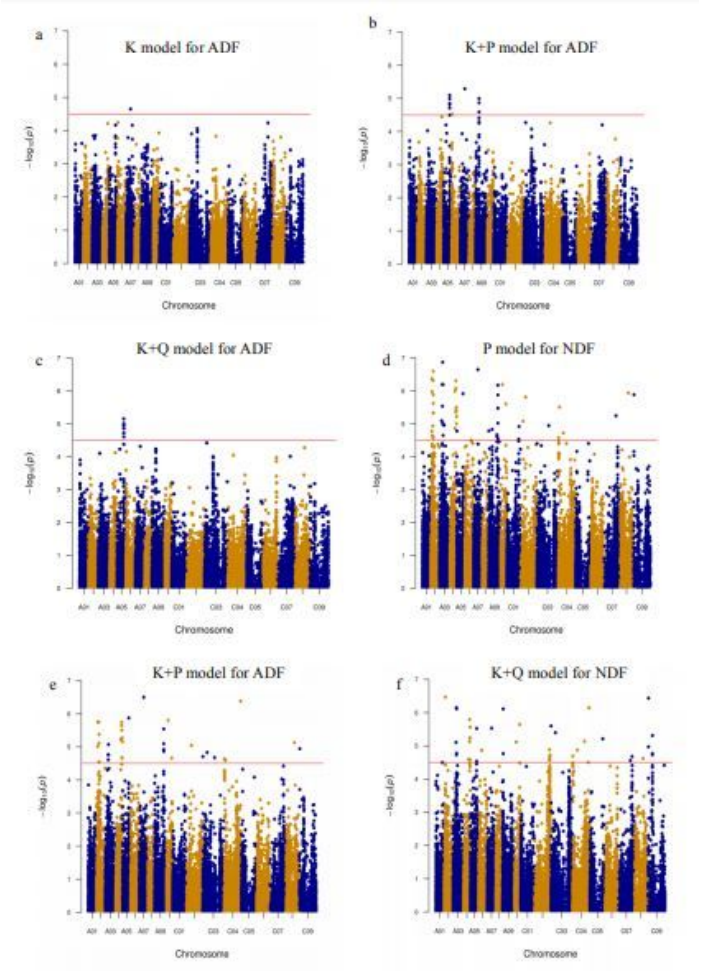

Figure 3

Manhattan plot of ADF and NDF contents in B. napus stems. (a) Manhattan plot of model K for ADF. (b) Manhattan plot of model K+P for ADF. (c) Manhattan plot of model K+Q for ADF. (d) Manhattan plot of model P for NDF. (e) Manhattan plot of model K+P for NDF. (f) Manhattan plot of model K+Q for NDF.

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