QTL analysis of shoot elongation before winter in relation to vernalization requirement in the doubled haploid population L16 × Express617 (*Brassica napus* L.)

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Abstract In winter oilseed rape shoot elongation before winter is considered as a critical component of the complex trait winter hardiness. Genotypes with an enhanced shoot length before winter are very much prone to frost damage. However, it is not known to which extent shoot elongation before winter is related to vernalization requirement. Shoot length measured 3 months after sowing of winter oilseed rape in spring has been shown to be a good quantitative estimator for vernalization requirement. The main objective of this study was to analyze inheritance of shoot elongation before winter and in spring-sown field experiments using a doubled haploid population derived from the cross between two inbred lines. A SNP based molecular marker map was used to map QTL for shoot length and flowering time. Significant genotypic effects were detected for shoot length before winter and in the spring-sown environment, but there was no correlation between both traits. Six QTL for shoot length before winter contributed 49.2% to the phenotypic variance. For shoot length in the spring-sown environment three

QTL were detected that explained 68% of the phenotypic variance. Flowering time genes CONSTANS (BnaC09g41980D), FLC (BnaC09g42060D) and FT (BnaA02g12130D) were identified within C09 and A02 QTL confidence intervals. No collocation of QTL for shoot length before winter and for shoot length in the spring-sown environment was found. Results show that selection for reduced shoot length before winter would be effective and that this trait is inherited largely independent of vernalization requirement.

Keywords Winter hardiness · Flowering time · FLC · FT · CONSTANS · FRIGIDA

Introduction

In temperate regions, vernalization requirement is an important mechanism for frost tolerance of winter annual crops (Trischuk et al. 2014). In oilseed rape (*Brassica napus* L.) vernalization requirement prevents the development of frost sensitive shoots and floral structures before winter (Zografos and Sung 2012). An enhanced hypocotyl and epicotyl elongation before winter is considered to be negatively correlated with winter hardiness (Kulesza et al. 1986). However, it is not clear to which extent hypocotyl and shoot elongation before winter is correlated to vernalization requirement (Rapacz and Chilmonik 2000). Winter survival of spring genotypes, which typically lack vernalization

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requirement, usually is low compared to winter cultivars (Teutonico et al. 1993). Possible reason of low frost tolerance of spring type genotypes might be their limited capability to prevent shoot elongation during warm mid-winter spells (Rapacz and Chilmonik 2000). Key genetic factors regulating vernalization requirement and flowering time in oilseed rape are FLOWERING LOCUS C (FLC), FRIGIDA (FRI) and FLOWERING LOCUS T (FT). FLC and FRI are the two key genes in the vernalization pathway (Michaels and Amasino 1999; 2001; Shindo et al. 2005). FRI represses flowering through activating the expression of FLC (Choi et al. 2011). FLC is inactivated by prolonged cold temperatures (vernalization pathway). FLC inactivation removes a factor, which represses FT expression. Via the photoperiod pathway, FT is activated by the transcription factor *CONSTANS* (CO), which is only stably expressed under long day conditions (Luo et al. 2018). In the allopolyploid crop species *Brassica napus* flowering time pathway is much more complicated than in *Arabidopsis*. This is because of the presence of multiple copies of various flowering time genes (Schiessl et al. 2014). In the reference genome of *B. napus* (AC genome), nine *FLC* paralogs were found in the European winter oilseed cultivar Darmor-*bzh* (Hou et al. 2012; Zou et al. 2012; Chalhoub et al. 2014). Four FRI genes (Wang et al. 2011) and up to eight FT gene copies were identified in the *Brassica napus* genome (Chalhoub et al. 2014; Schiessl et al. 2014). At least four CO copies are present on A03, A10, C03 and C09 in the rapeseed genome (Chalhoub et al. 2014). To date it can only be speculated which of flowering time gene copies in the *Brassica napus* gene pool are functional and which are pseudogenes. Results obtained may depend very much on spring, winter or semi-winter type of plant material analyzed (Wang et al. 2009, 2011, 2012b; Schiessl et al. 2014; Yi et al. 2018; Raman et al. 2016; Shea et al. 2018). In previous experiments, vernalization requirement of winter oilseed rape has been analyzed in North-Western Germany in field experiments sown in spring by end of March or beginning of April (Ghanbari and Möllers 2018). In spring, the days are already quite warm, but temperatures during night still drop down to zero degrees Celsius or even below. In any case, the duration of the vernalization effective period in spring-sown field experiments is much shorter than during the winter from beginning of November until end of February. Genotypes with a strong vernalization requirement will not start to bolt in spring-sown field

experiments, whereas genotypes with a somewhat lower vernalization requirement may vary in their tendency to bolt and to flower. Hence, shoot length and inflorescence formation in spring-sown field experiments can be measured to quantify vernalization requirement. Results of these field experiments showed that there is large genetic variation for vernalization requirement among 19 analyzed genotypes (Ghanbari and Möllers 2018). The same genotypes were tested in autumn sown field experiments and significant genotypic differences were identified for shoot elongation before winter. Although the correlation between shoot length before winter and shoot length in the spring-sown environment was positive (Spearman's rank $r = 0.48^*$), a number of genotypes with reduced shoot elongation before winter and low vernalization requirement were identified (Ghanbari and Möllers 2018). Genotypes L16 and Express617 showed contrasting behavior in the two environments. Shoots of L16 remained short before winter and in the spring-sown environment, whereas Express617 was found to have longer shoots in both environments. To identify genetic factors controlling shoot elongation before winter and shoot length in the spring-sown environment a DH population derived from the cross between the two lines was tested in autumn and spring-sown field environments and in a greenhouse environment following artificial vernalization. A 60 K based SNP marker map was used to map QTL for shoot elongation and shoot diameter in the three environments.

Materials and methods

Plant material

A doubled haploid population, consisting of 151 inbred lines, derived from a cross between the resynthesized line L16 and the winter rapeseed line Express617 was used for a series of field and greenhouse trials. The resynthesized line L16 is an interspecific hybrid between a Chinese broccoli (*B. oleracea* convar. botrytis var. alboglabra) and a Chinese cabbage (*B. rapa* ssp. *pekinensis*; Girke 2002). L16 has high glucosinolate ($\sim 50 \mu\text{mol g}^{-1}$) content in the seed and less than 2% of erucic acid in the oil (Brandes 2016). This may indicate that L16 is not a true resynthesized line but rather a semi-synthesized line derived from a cross with a low erucic acid line or cultivar. Express617

is an inbred line of the double low quality winter oilseed rape cv. Express with low contents of erucic acid in seed oil and of glucosinolates in the seed. The two parents were selected based on their contrasting characteristics. L16 is late flowering with short shoot length before winter, whereas Express617 is comparatively early flowering with enhanced shoot length before winter (Ghanbari and Möllers 2018). The doubled haploid population was developed through microspore culture using F1 plants of the cross between L16 $\times$ Express617 as microspore donors (Brandes 2016).

Field and greenhouse experiments

The L16 $\times$ Express617 population and the parental lines were phenotypically characterized in field and greenhouse experiments. The field experiments were performed in two different environments. Those included normal sowing end of August/beginning of September and sowing in spring end of March/beginning of April. The L16 $\times$ Express617 population and its parental inbred lines were tested in autumn sown field experiments in four environments in North Western Germany. The environments in 2014/15 were Peine (Limagrain GmbH) and Einbeck (KWS Saat SE), and in 2015/16, it was Peine and Göttingen. Sowing was performed between 20 August and 4 September in Einbeck, Peine and Göttingen. Hundred seeds from each line were sown in small field-plots with double rows in Peine and Göttingen with 2 m length, 50 cm space between plots and plant-to-plant distance in the row was 10 cm. In Einbeck seeds were sown as one row with 3 m length and 80 cm space between plots and plant-to-plant distance in the row was 6 cm. All agronomic practices, such as fertilizer, herbicide and insecticide were applied at each location according to common practices. Three to four months after sowing, five representative plants were selected and harvested by cutting the stem below the crown. Harvesting time in 2014 for Peine and Einbeck were 8 and 24 December and in 2015 for Peine and Göttingen were 30 November 2015 and 11 January 2016, respectively. Shoot length from crown to shoot apex and shoot diameter at the crown were measured using a slide gauge and a metering rule before winter. Flowering time from first of January and plant height at end of flowering were recorded in the following spring at four locations. Data for begin of flowering and plant height at end of flowering was provided by

Haiko Brandes for autumn sown experiments at Göttingen and Peine in 2012 (Brandes 2016).

Spring-sown field experiments were performed to estimate vernalization requirement. Plant material was sown at four environments in North Western Germany. The environments were Göttingen with sowing dates 20 March 2014 and 24 March 2015 and Einbeck with sowing dates 4 April 2014 and 10 April 2015. All plants were grown by sowing 100 seeds using 2 m double rows and 50 cm space between plots and 10 cm between plants in Göttingen and 3 m single row with 80 cm between plots and plant-to-plant distance in the row was 6 cm in Einbeck. Three to four months after sowing, seven representative plants were selected and harvested by cutting the stem below the crown. In 2014 plants were harvested in Göttingen and Einbeck on 3 and 7 July, and in 2015 on 26 June and 13 July, respectively. Shoot length from the crown to shoot apex and shoot diameter at the crown were measured using measuring ruler and slide gauge. Percentage of tendency to form inflorescence was scored between 0 and 100% for absence or presence of visible buds or flowers on seven plants.

For the greenhouse experiments, plant material was sown in compost soil in 96-multipot trays (Quickpot QP 96 T, HerkuPlast Kubern GmbH, Ering). Each tray consisted of 96 plants and two experiments with each four plants per genotype were performed. Experiments were performed with three different vernalization treatments, i.e. no vernalization (0 weeks), 4 and 8 weeks vernalization treatments. In the greenhouse, each experiment was performed as a randomized complete block design with the entry of 4 adjacent plants as a group. Measurements were performed on individual plants. In each experiment, plants were allowed to grow in the greenhouse for 4 weeks until three to four leaf growth stage and were then transferred to a vernalization chamber adjusted to 4 °C and 8 h light. At the end of the vernalization treatment plants were cultivated in the greenhouse at around 20 °C. The experiments were performed from December (sowing) until June (harvest). Sowing of 8 weeks vernalization treatment was done 8 weeks ahead of sowing of the non-vernalization treatment. At end of vernalization, both treatments were cultivated together in the greenhouse under the same conditions. Compared to the 8 weeks vernalization, the 4 weeks vernalization treatment started with a 4 weeks' delay. The experiments were terminated 3 months after

sowing time in the 0 week vernalization treatment and 2 months following transfer to the greenhouse in the 4 and 8 weeks' treatments. In the all treatments, four plants per genotype were harvested by cutting the stem below the crown. Shoot length from crown to shoot apex and shoot diameter at the crown were measured using measuring ruler and slide gauge. In the 8 weeks vernalization treatment flowering time were recorded and shoot length was measured at beginning of flowering. Flowering time was not recorded following the 0 and 4 weeks' vernalization treatment, because genotypes did not flower or only some flowered following the 4 weeks vernalization treatment.

Linkage map, QTL mapping and identification of candidate genes

Genotyping of the DH population was performed using the Brassica Illumina Infinium 60 K SNP-chip and a linkage map was provided by Brandes (2016). The framework map used for QTL mapping consisted of 778 evenly distributed high-fidelity markers (ESM_1). QTL mapping was implemented using the WinQTL Cartographer software (ver. 2.5) (Wang et al. 2012a). QTL were initially detected with composite interval mapping (CIM) using stepwise regression model 6 for each trait, the LOD significance threshold ($\alpha = 0.05$) was estimated by 1000 permutation tests. A set of five markers for CIM was allowed as co-factor in forward and backward stepwise regression method in the analysis. It was done because other QTL especially major QTL would inflate the residual sum-of-squares, and reduce the power to detect more putative QTL in the neighboring region. Also it helps detecting false positive QTL. CIM tests were performed, at 1 cM steps with a 10 cM window size, to prevent any background markers within 10 cM of a putative QTL from being included in the final results. Peaks were treated as separate QTL when the distance was more than 10 cM and the minimum LOD value exceeds one between any two adjacent peaks. Subsequently, multiple interval mapping (MIM) (Kao et al. 1999; Silva et al. 2012) was applied to refine the QTL position, the QTL effect in detected QTL and also to search for more QTL, and to investigate epistatic effects among the detected QTL. The MIM model was built upon a priori model from CIM analysis and progressively refined using the BIC-M2 = $2\ln(n)$ criterion. QTL

positions that did not remain significant when fitted with others were then dropped from the model. QTL effects and their percentage of phenotypic variance explained by individual and all the QTL were estimated with the final model fitted in MIM. A one-LOD drop from the peak position was used as a confidence interval for each QTL. A QTL explaining more than 20% of the phenotypic variance was considered as major QTL.

To determine the physical position of major QTL and to identify candidate genes, sequences of flanking SNP markers were aligned against the Darmor-bzh *B. napus* reference genome v4.1 (<http://www.genoscope.cns.fr/brassicapnapus/>; Chalhoub et al. 2014). SNP marker sequences were provided by Isobel Parkin (AAFC, Saskatoon, Canada). Most likely positions were selected from the BLAT hits considering best matching and highest possible E-value as well as genetic map data information. *A. thaliana* candidate gene sequences were identified using the Araport11 annotation (Cheng et al. 2017). The assignment of the oilseed rape genes to the *Arabidopsis* genes using the protein sequences is described in Pucker et al. (2017). Identification of Reciprocal Best BLAST Hits (RBHs) between two sets of sequences (protein/DNAReciprocal) was performed as described in Pucker et al. (2016).

Statistical analysis

Analysis of variance and descriptive statistic were done by PLABSTAT (Utz 2011) and R (i386 3.0.3; R Core Team 2015). Analysis of variance was separately done for each environment in which combination of location and year was treated as experiment factor in each environment. Linear mixed-effect model (R version 3.1-125, package {nlme}) was applied to test significant difference between the genotypes for the traits, hence experiment and plant sample (sub-sample) were defined as random factor and genotype as fixed factor. The statistic model used for ANOVA is shown below.

$$X_{ijk} = \mu + g_i + e_j + g_i e_j + p_k : g_i e_j$$

where X_{ijk} is phenotypic observation of genotype i in experiment j and plant k , μ is a general mean, g_i and e_j are effects of genotype i and experiment j , $g_i e_j$ is residual error to test main effect of genotype i and

experiment j and $p_k: g_{ie_j}$ is sampling residual error to test g_{ie_j} effect. Broad sense heritability (h^2) for genotypes was calculated as follow:

$$h^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_{GE}^2/E + \sigma^2 p : ge/EP)$$

whereby σ_G^2 and σ_{GE}^2 are genotype and residual error variance components, respectively, E is the number of experiments and P is the number of measured plants. Genotypes mean across the experiments were used to calculate Spearman's rank correlation coefficients between the traits which was done by R software (i386 3.0.3).

Results

The analysis of variance showed significant effects for the genotype for all traits in the autumn and spring-sown field and in the greenhouse environments (Table 1). However, for shoot length before winter in the autumn sown environment variance components

for the effects of the experiment and the genotype $\times$ experiment interaction were tenfold and fourfold larger, respectively, than for the effect of the genotype. Heritability for shoot length before winter was moderate with 51%. Heritabilities for shoot diameter was low (29%) and it was high for plant height at end of flowering (73%) and flowering time (84%). In the autumn sown environment parental line L16 showed a reduced shoot length before winter and a delayed flowering time compared to Express617 (Table 2). Shoot length of the DH-population before winter ranged from 21 mm to 71 mm, which was about the same range as found for the two parents. There was a 12 days' difference among flowering time and 50 cm difference in plant height at the end of flowering in the DH population. In the spring-sown environment largest variance components were found for the effect of the genotype on shoot length (Table 1). L16 was with 218 mm much shorter than Express617 with 855 mm. A large variation for shoot length ranging from 87 mm to 1255 mm was found for

Table 1 Variance components and heritability of the L16 $\times$ Express617 population for shoot length, shoot diameter, begin of flowering, plant height at end of flowering and bud formation in the three environments

Environment	Trait	Variance components (σ^2)			Heritability (%)
		Genotype (G)	Experiment (E)	G $\times$ E	
Autumn sown	Shoot length ^a	35.9**	468**	151.9**	51
	Shoot diameter ^a	0.2**	9.01**	0.9**	29
	BOF [§]	4.1**	46.15**	4.14	84
	Plant height EOF ^a	61.4**	228**	46.7	73
Spring sown	Shoot length ^a	132083*	53745**	34581	94
	Shoot diameter ^a	10.93**	1.03*	10.25**	22
	Buds ^b	0.12**	0.01**	0.08**	80
GH 0 weeks	Shoot length ^a	53.4**	5.9**	20.7**	77
	Shoot diameter ^a	0.05**	0.04**	0.05**	43
GH 4 weeks	Shoot length ^a	7129**	5.8**	3009**	77
	Shoot diameter ^a	0.88**	0.04	9.02*	11
GH 8 weeks	Shoot length ^a	1670**	5.52	2153**	73
	Shoot diameter ^a	0.11**	0.07	0.14**	61
	BOF [#]	69**	0.15	186**	80

GH greenhouse

*, ** Significance at $P < 0.05$ and $P < 0.01$, respectively

^{a, b} Millimeter (mm) and percentage (%), respectively

[§]BOF/[§]EOF: begin/end of flowering (from first of January)

[#]BOF: begin of flowering after sowing

Table 2 Descriptive statistics of the parents and the L16 × Express617 population (n = 151) in the autumn and spring sown environment and in the greenhouse environment

Experiment	Trait	Parents		Doubled haploid population (n = 151)				
		L16	Express617	Min	Max	Mean	F-value	LSD5%
Autumn sown	Shoot length ^a	34	61	21	71	42	1.87**	18
	Shoot diameter ^a	10	9	8	14	11	1.41**	2.4
	BOF [§] (days)	118	113	112	124.1	119	6.02**	2.5
	Plant height EOF ^c	1591	1492	1343	1871	1612	3.63**	135
Spring sown	Shoot length ^a	218	855	87	1255	659	15.5**	264.8
	Shoot diameter ^a	23	26	17	29	23	1.2*	5.2
	Buds ^b	25	100	0	100	61	6.2**	4.2
GH 0 weeks	Shoot length ^a	21	48	15	71	33	4.32**	11.21
	Shoot diameter ^a	4	5	3	6	4	1.75**	0.78
GH 4 weeks	Shoot length ^a	32	425	28	400	126	4.25**	131
	Shoot diameter ^a	4.4	4.5	4	26	6	1.12**	1.1
GH 8 weeks	Shoot length ^a	332	459	270	518	370	3.66**	69.5
	Shoot diameter ^a	4.1	4.3	3	8	4	2.50**	2.5
	BOF [#] (days)	150	141	120	160	139	6.20**	10.1

GH, greenhouse

*, ** Significance at $P < 0.05$ and $P < 0.01$, respectively^a, ^b Millimeter (mm) and percentage (%), respectively[§]BOF/[§]EOF: begin/end of flowering (from first of January)[#]BOF: begin of flowering from sowing

the DH population (Table 2). Transgressive segregation for shoot length in the DH population was detected in both environments (c.f. LSD5% in Table 2). In the spring-sown environment heritabilities were high for shoot length and for the presence of flower buds and again low for shoot diameter (Table 1). In the greenhouse environment, highest heritabilities were obtained for shoot length without and after 4 and 8 weeks of vernalization (Table 1). Largest difference in shoot length between the two parent lines and in the DH population was found after 4 weeks of vernalization (Table 2). Frequency distributions for all traits were in all environments near normal (not shown), except for shoot length in the spring-sown environment (Fig. 1). Frequency distribution indicated the presence of four different groups which were not clearly separated. Significant transgression in the DH-population was only found to larger values. In the autumn sown environment flowering time was positively correlated with plant height at end of flowering, indicating that late flowering genotypes were taller (0.51**; Table 3). It was also positively correlated with shoot length and

the presence of buds in the spring-sown environment (0.54** and 0.53**, respectively). For the greenhouse experiment, close correlations were only detected between shoot length in the 4 weeks vernalization treatment and shoot length and presence of flower buds in the spring-sown environment (0.74** and 0.71**, respectively).

QTL mapping detected large number of QTL for the traits in the autumn sown environment (Table 4). QTL for shoot length, begin of flowering and end of flowering together explained almost 50% of the phenotypic variance for each trait (Table 4). The largest QTL Wi-Len-3 for shoot length before winter was located on chromosome A09 and explained 15.2% of the variance. The negative sign of the additive effect indicated that the allele increasing trait value was derived from Express617. This QTL did not collocate with any other QTL in this and the other two environments. However, both parents contributed QTL alleles increasing shoot length before winter. For begin of flowering QTL Wi-BOF-1 explained 23.7% of the phenotypic variance. This QTL collocated with QTL Wi-HGT-1 for plant height at end of

Fig. 1 Frequency distribution of shoot length in the spring sown environment

flowering. Both QTL had a negative additive effect, indicating that at this locus the QTL allele of Express617 was increasing days to flowering and plant height at end of flowering. The remaining five QTL for plant height at end of flowering had positive additive effects, which is consistent with taller plant height of L16 and the positive correlation to begin of flowering in this environment (Table 3).

In the spring-sown environment three QTL for shoot length were identified (Table 5). Thereby, QTL Sp-Len-1 on chromosome A02 and Sp-Len-3 on chromosome C09 were major QTL explaining each more than 25% of the phenotypic variance. QTL Sp-Len-1 on A02 collocated with QTL Sp-Bud-1 for the presence of flower buds, and both QTL had positive additive effects with the L16 allele increasing both traits. QTL Sp-Len-3 collocated with QTL Sp-Bud-3 on chromosome C09. Both QTL on C09 had negative additive effects, with the Express617 allele at this position enhancing shoot length and the presence of flower buds. Confidence intervals of minor QTL Sp-Len-2 for shoot length and of QTL Sp-Bud-2 for bud formation on A07 did overlap, but did have opposite additive effects. None of the three QTL for shoot length and the presence of flower buds in the spring-sown environment did collocate with QTL for shoot length in the autumn sown environment. But QTL Sp-Len-1 and Sp-Bud-1 on A02 did collocate with opposite additive effects with QTL Wi-BOF-1 for begin of flowering and QTL Wi-HGT-1 for plant

height at end of flowering in the autumn sown environment. In the winter environment the L16 allele at this locus led to a reduced plant height at end of flowering and earlier begin of flowering, which is consistent with the consideration that reduced vernalization requirement leads to earlier flowering. Noticeably, this relationship was not observed for the QTL Sp-Len-3 on C09. In the spring environment six QTL were detected for shoot diameter. Both parents contributed alleles for increased shoot diameter. However, none of the QTL did collocate with QTL for the same trait in the autumn sown environment and with QTL for shoot length in the spring and autumn sown environment.

QTL mapping using data from the greenhouse environment did not reveal any consistent QTL for the 0, 4, and 8 weeks vernalization treatments (Table 6). However, consistent QTL positions were detected for shoot length at C04 of the 4 and 8 weeks vernalization treatments. In both treatments, the Express617 allele increased trait value. However, the largest QTL GH-Len-H5 for shoot length on C09 after 4 weeks vernalization was not detected after 8 weeks, but this QTL did collocate with QTL GH-Flw-F4 for begin of flowering. The Express617 allele at this position increased shoot length and reduced time to flowering. QTL for shoot length at C04 in the greenhouse environment were detected neither in the autumn nor in the spring-sown environment. However, the largest QTL GH-Len-H5 for shoot length on C09 mapped at

Table 3 Spearman's rank correlation of the traits in the L16 × Express617 population (n = 151)

Environment	Trait	Autumn sown			Spring sown			Greenhouse						
		SL	SD	BOF [§]	PH	SL	SD	Buds	0 week		4 weeks		8 weeks	
Autumn sown	SD		0.10											
	BOF [§]	− 0.02	− 0.08											
	PH EOF [§]	0.25**	− 0.13	0.51**										
	SL	0.11	0.09	− 0.54**	− 0.27									
	SD	− 0.12	0.06	0.11	0.15	− 0.29								
Spring sown	Buds	0.08	0.08	− 0.53**	− 0.30**	0.93**	− 0.29**							
	SL	0.31**	0.01	− 0.1	0.01	0.22**	− 0.17	0.17						
	SD	− 0.18*	0.17*	− 0.12	− 0.04	0.02	0.17	0.02	− 0.06					
	SL	0.17*	0.08	− 0.4**	− 0.21**	0.74**	− 0.3	0.71**	0.37**	− 0.03				
	SD	− 0.05	0.16*	0.02	0.16*	− 0.03	0.1	− 0.04	− 0.13	0.24**	0.23*			
GH 0 week	SL	0.18*	0.22**	− 0.06	0.06	0.19*	− 0.04	0.18	0.16	− 0.02	0.33**	− 0.06		
	SD	− 0.11	0.11	− 0.06	− 0.03	0.17*	− 0.003	0.2	− 0.16	0.23**	0.04	0.07	0.14	
	BOF [#]	− 0.06	− 0.08	0.23*	− 0.01	− 0.33**	0.14	− 0.36**	− 0.34**	− 0.05	− 0.26**	0.04	− 0.36**	0.01

GH greenhouse, SL shoot length, SD shoot diameter, PH plant height

*, **, *** Significance at $P < 0.05$ and $P < 0.01$, respectively[§]BOF/[§]EOF: begin/end of flowering (from first of January)[#]BOF: begin of flowering from sowing

Table 4 QTL mapped for the traits in the autumn sown environment in the L16 × Express617 population (n = 151)

Trait	QTL name	Chromosome	Position (cM)	CI ^a (cM)	LOD	Add. effect ^b	R ^{2c}	TR ^{2d}
Shoot length	Wi-Len-1	A07	53.2	50.1–62.3	5.31	2.42	5.1	49.2
	Wi-Len-2	A08	43.12	35.8–46.5	2.78	– 1.88	3.6	
	Wi-Len-3	A09	107.4	102.1–111.1	11.5	– 3.55	15.2	
	Wi-Len-4	C01	5	1–7	3.96	– 2.2	5	
	Wi-Len-5	C04	60.04	56.1–63.7	5.75	2.64	8.3	
	Wi-Len-6	C09	57.5	45–60.2	3.85	– 2.1	7	
	Wi-Len-7	A08*C01	–	–	5.46	– 2.02	5.1	
Shoot diameter	Wi-Dim-1	A07	67.2	66.2–69.2	3.73	0.33	1.03	8.13
	Wi-Dim-2	C04	1.01	0–6.1	2.43	– 0.27	7.1	
Begin of flowering	Wi-BOF-1	A02	52.2	51.3–54.9	9.24	– 0.93	23.7	47.1
	Wi-BOF-2	A03	6.7	3.5–8	3.79	– 0.41	2.7	
	Wi-BOF-3	A03	111.4	111.3–115.7	4.26	0.58	10.5	
	Wi-BOF-4	C04	100.9	97–102.7	3.79	0.55	10.2	
Plant height EOF	Wi-HGT-1	A02	50.7	42.1–52.9	5.63	– 29	10.8	47.8
	Wi-HGT-2	C01	12.4	6–15	4.27	24	4	
	Wi-HGT-3	C03	187.2	186.3–188.8	7.15	33	13.8	
	Wi-HGT-4	C04	100.9	94.6–101.8	4.81	28.2	8.5	
	Wi-HGT-5	C05	42.9	40.5–48.9	5.23	28.2	8.5	
	Wi-HGT-6	C08	81.09	80.2–92.7	2.86	20.2	2.2	

^a1-LOD Confidence interval^b{+} or {–} indicates that the trait value is increased by the allele derived from L16 or Express617, respectively^cR² is the percentage of phenotypic variance explained by each QTL^dTR² is the percentage of phenotypic variance explained by all QTL

similar position in the spring-sown environment (Table 5) and both QTL had same direction of the additive effect.

The physical regions around the major QTL for shoot length in the spring-sown environment were inspected for candidate genes. The major QTL for shoot length on C09 in the spring-sown field environment and in the greenhouse environment after 4 weeks vernalization spanned a confidence interval from 125 to 134.3 cM (Table 5). This corresponded to a region from 43,704,159 to 45,278,391 bp on the physical map (Fig. 2). Within this region the flowering time genes *CONSTANS* (BnaC09g41980D) and *FLC* (BnaC09g42060D) were identified (ESM_2). A little upstream of the confidence interval at 46 Mbp another two *FLC* copies (BnaC09g46500D and BnaC09g46540D; ESM_2) were found. The confidence interval of the second major QTL for shoot length on A02 in the spring-sown environment ranged from 44 to 52.7 cM and for bud formation it ranged from 52.3 to 54.1 cM. Within this narrower

confidence interval, a FT gene (BnaA02g12130D) was located at 6375,936 bp of the physical map (Fig. 3; ESM_3).

Discussion

Climate warming requires breeding of winter rapeseed cultivars allowing early and vigorous plant development in spring, and which nevertheless are sufficiently frost tolerant in winter. Shoot elongation before winter has been identified as one important factor for frost tolerance and significant genotypic differences for this trait have been described (Ghanbari and Möllers 2018, and references therein). Hence, genetic combination of early and vigorous plant development in spring with a reduced shoot elongation before winter appears as useful trait combination. Shoot length measured in spring-sown environments proved to be a good quantitative estimate of vernalization requirement in the present study. Heritability for this trait was above

Table 5 QTL mapped for the traits in the spring sown environment in the L16 × Express617 population (n = 151)

Trait	QTL name	Chromosome	Position (cM)	CI ^a (cM)	LOD	Add. effect ^b	R ^{2c}	TR ^{2d}
Shoot length	Sp-Len-1	A02	52.7	44–52.7	20.8	189.6	25.7	68
	Sp-Len-2	A07	78.4	70.5–85.2	3.03	73.7	6.8	
	Sp-Len-3	C09	128.7	125–131	8.35	– 223.4	35.5	
Shoot diameter	Sp-Dim-1	A01	3.74	3–8	2.45	– 0.48	2.6	48.4
	Sp-Dim-2	A06	62.93	59–66	5.98	– 0.74	11.7	
	Sp-Dim-3	A09	18.48	16–24.8	4.36	– 0.65	10.7	
	Sp-Dim-4	A10	14.2	13.3–17.5	6.95	0.88	13.9	
	Sp-Dim-5	C01	13.4	10.3–15.5	3.82	0.58	6	
	Sp-Dim-6	C09	31.9	29–35	2.66	– 0.47	4.4	
Buds	Sp-Bud-1	A02	52.9	52.3–54.1	20.15	22	22.3	60.1
	Sp-Bud-2	A07	90.26	80.8–91.3	4.01	– 8	4.4	
	Sp-Bud-3	C09	128.7	127.5–130	16.96	– 24	33.4	

^a1-LOD Confidence interval^b{+} or {–} indicates that the trait value is increased by the allele derived from L16 or Express617, respectively^cR² percentage of the phenotypic variance by each QTL^dTR² percentage of the phenotypic variance explained by all QTL

90% in this and in the earlier experiment (Ghanbari and Möllers 2018). However, it has not been clear to which extent the degree of vernalization requirement is influencing shoot elongation before winter. Results of the present study show that vernalization requirement measured as shoot length in the spring-sown environment is not correlated with shoot elongation before winter (c.f. Table 3). Furthermore, two major and a minor QTL for shoot elongation and bud formation were detected in the spring-sown environment and which were missing in the autumn sown environment (Tables 4, 5). In total six QTL with additive effects for shoot elongation before winter were detected which were not found in the spring-sown environment. This indicates that breeding for reduced shoot length can be effective independent of vernalization requirement.

Frequency distribution of the shoot length in the spring sown environment (Fig. 1) indicated the presence of four different groups as one would expect for two major QTL segregating in a DH-population (Fig. 1 and c.f. Table 5). Despite high heritability for this trait (Table 1) the four groups were not clearly separated, which could be explained by the third minor QTL on A07 segregating in this population. The frequency distribution for shoot length showed significant transgression only to larger values, which is in line with the observation that the two QTL alleles on

A02 and A07 increasing shoot length were derived from L16. The largest QTL for shoot length in the spring-sown environment explaining 35.5% of the phenotypic variance was located on C09. Investigating the physical region within the confidence interval revealed the presence of a *CONSTANS* (CO; BnaC09g41980D) and a *FLC* (BnaC09g42060D) gene. Little upstream the QTL confidence interval another two *FLC* gene copies were detected. It remains unclear which of those three *FLC* copies are functional and which may represent pseudogenes. Since CO is a central day-length regulator necessary for flowering initiation it appears meaningful that both QTL are responsible for shoot elongation and flowering initiation in this DH population. Resolution of QTL mapping is not good enough to conclude which of the three *FLC* loci are involved in trait expression. Mutations in anyone of the three *FLC* loci could affect the total functional BnFLC dosage. As mentioned above, there is also a *FRI* locus (BnaC09g27290D) present on C09 which is however located far away at 29 Mbp. Obviously, none of the four *FRI* loci significantly affect shoot elongation and flower bud formation in the L16 × Express617 DH population in the spring-sown environment. Either *FRI* has no influence on shoot elongation and flowering time in spring-sown environment or there is no significant difference between the *FRI* alleles at the four loci in

Table 6 QTL mapped for the traits in the greenhouse environment in the L16 × Express617 population (n = 151)

Treatment	Trait	QTL name	Chromosome	Position (cM)	CI ^a (cM)	LOD	Add. effect ^b	R ^{2c}	TR ^{2d}
0 week	Shoot length	Gh-Len-N1	A06	49.4	46.6–55.5	2.73	– 2.29	7.8	16.2
		Gh-Len-N2	A09	3.35	1–11	3.03	2.40	8.4	
	Shoot diameter	Gh-Dim-N1	A03	72.4	69–75	2.67	0.08	7.1	18
		Gh-Dim-N2	C07	46.7	42–51	3.98	– 0.1	10.9	
4 weeks	Shoot length	Gh-Len-H1	A02	45.8	45.3–48.4	7.36	31.7	14.6	68.5
		Gh-Len-H2	A07	91.6	85–111	2.78	19.1	5.2	
		Gh-Len-H3	A08	37.5	35.2–38.7	3.6	– 23.2	6	
		Gh-Len-H4	C04	75.9	74–85	2.8	– 19.4	4	
		Gh-Len-H5	C09	134.2	131–134.3	11.9	– 41.6	21	
		Gh-Len-H6	A02*C09			3.7	2.18	9	
		Gh-Len-H7	C04*C09			4.24	– 23.5	8.7	
	Shoot diameter	Gh-Dim-H1	A02	52.9	41.5–52	4.21	0.97	10.3	17.1
		Gh-Dim-H2	A09	25.24	20–35	3.2	0.75	6.8	
8 weeks	Shoot length	Gh-Len-F1	A10	19.21	15.4–20	2.9	20.1	7	13.3
		Gh-Len-F2	C04	82.67	80.7–92	2.75	– 18.6	6.3	
	Shoot diameter	Gh-Dim-F1	A07	24.5	15.2–26	16.26	– 0.32	10.6	73.0
		Gh-Dim-F2	C04	33.6	25–35	28.1	– 2.8	62.4	
	BOF [#]	Gh-Flw-F1	A10	50.69	48–56	3.19	– 2.7	6.3	27.5
		Gh-Flw-F2	C03	63.3	62.4–64	2.7	– 2.86	2.9	
		Gh-Flw-F3	C03	92.87	87.9–93	4.94	4.04	9.5	
		Gh-Flw-F4	C09	133.2	131–134	3.2	2.8	8.8	

^a1-LOD Confidence interval^b{+} or {–} indicates that the trait value is increased by the allele derived from L16 or Express617, respectively^cR² percentage of the phenotypic variance explained by each QTL^dTR² percentage of the phenotypic variance explained by all QTL[#]BOF: begin of flowering (from sowing time)

the DH population. In other winter oilseed rape populations, different flowering time loci may contribute to phenotypic variation for shoot length and flowering time (Raman et al. 2013). In a genome wide association study with 182 *Brassica napus* accessions Raman et al. (2016) reported that BnFLC.A02 explained up to 22% of variation in flowering time of non-vernalized samples, while this effect completely disappeared on vernalized samples. Chen et al. (2018) produced and analyzed two pairs of near-isogenic rapeseed lines that showed large differences in flowering time under both winter and spring conditions. Their data confirmed that BnFLC.A2 and BnFLC.C2 are causal genes for quantitative variations in flowering time. A large insertion in BnFLC.A2 resulted in a loss-of-function-mutation (Bnflc.A2).

Through homoeologous recombination between A02 and C02 significantly earlier flowering lines were generated that carried the homozygous Bnflc.a2 allele in both chromosomes A02 and C02. In a genome wide association study using 158 European winter type oilseed rape inbred lines Schiessl et al. (2015) revealed that FLC was absent from the candidate regions associated with flowering time, indicating that vernalization requirement has been fulfilled in the different field test environments. In the Tapidor and Ningyou7 DH population a major QTL for flowering time in the spring cropped environment was mapped on A10 which collocated with the BnFLCA10 locus (Long et al. 2007). Using resynthesized oilseed rape generated from interspecific crosses between the two diploid ancestor species *Brassica rapa* and *Brassica*

Fig. 2 Genetic (cM) and physical map (Mbp) of chromosome C09 with positions of QTL and candidate genes. {+} or {-} indicates that the trait value is increased by the allele derived

from L16 or Express617, respectively. For QTL and candidate gene explanation see Tables 4 to 6 and text

oleracea in crosses with current cultivars, additional variation in flowering time genes is introduced in the oilseed rape gene pool. Line L16 used in the present study is an example for this enhanced genetic diversity.

Investigating genotype specific vernalization requirement in greenhouse experiments with different vernalization treatments showed that the 4 weeks vernalization treatment was most suitable to predict vernalization requirement as determined in spring-sown field experiments. The correlation for shoot length and bud formation was close between the spring-sown environment and the 4 weeks vernalization treatment (Table 3). Furthermore, the three major

QTL for shoot length in the spring-sown environment were detected at very similar positions in the greenhouse experiment and the direction of the additive effects were identical. The 4 weeks vernalization treatment led to the detection of two more QTL with additive effects and with epistatic effects. This may have resulted from the more standardized conditions in the greenhouse experiment with respect to soil type, sowing depth, fertilizer treatment, watering, etc. There were significant genetic differences for shoot diameter in the autumn, spring and greenhouse environment. However, heritabilities were mostly low and correlations to shoot length before winter and vernalization requirement were in general very low. Obviously,

Fig. 3 Genetic (cM) and physical map (Mbp) of chromosome A02 with position of QTL and candidate gene. {+} or {−} indicates that the trait value is increased by the allele derived

from L16 or Express617, respectively. For QTL and candidate gene explanation see Tables 4, 5, 6 and text

shoot elongation before winter and vernalization requirement has no significant effect on shoot diameter.

Enhanced hypocotyl elongation is like enhanced epicotyl (shoot) elongation before winter presumably related to reduced frost tolerance. Hypocotyl elongation has not been measured in the present studies, but in a genome wide association study Luo et al. (2017) suggested candidate gene BnaC07g46770D among others as a negative regulator of hypocotyl elongation and of flowering time. In the present study, no significant QTL for shoot elongation was detected on C07 in the autumn and in the spring-sown environment. This suggests that epicotyl and hypocotyl elongation before winter are inherited largely independent from each other.

In conclusion, the results show that in the DH population L16 × Express617 the FLC/CO and the FT gene copy on C09 and A02, respectively, significantly determine vernalization requirement in spring-sown field experiments. There is significant genetic variation for shoot length before winter and selection for reduced shoot length can be effective and is largely independent of vernalization requirement. Greenhouse experiments performed under controlled conditions with 4 weeks of artificial vernalization can be used to predict genotype specific vernalization requirements. Further studies have to show if in other genotypes same or different flowering time loci affect shoot elongation before winter and vernalization requirements. Considering that the FLC locus in *Arabidopsis* regulates developmental pathways

throughout the plants life cycle (Deng et al. 2011), the question remains which of the various flowering time loci and alleles are required to achieve maximum seed yield in different environments (Long et al. 2007; Schiessl et al. 2015; Raman et al. 2019; Tyagi et al. 2018).

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Author contribution CM designed the experiments and all authors performed the experiments. MG and CM analyzed the data. CM and MG wrote the manuscript and all authors agreed on the final manuscript.

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