Effect of vernalization on turnip formation and other morphological traits in *Brassica rapa*

Thesis Report

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Abstract

In this study, the effect of vernalization on turnip traits and other morphological traits in *Brassica rapa* is described and an association mapping approach was used to find markers associated with the measured traits. Therefore 34 turnip accessions representing both fodder and vegetable types from different geographic origins and 7 non-turnip accessions were grown after three different vernalization treatments (0, 4 and 8 weeks vernalization) and phenotypic data on turnip, leaf and flowering traits was obtained. The accessions were genotyped with 40 SSR markers, including five turnip candidate genes.

The exposure of seedlings to cold for either four or eight weeks resulted in smaller, more elongated, a deeper growth into the soil and color changes of the peel. The smaller turnip diameter and the lower weight of vernalized turnips is most probably caused by the delayed turnip swelling onset and the slower growth. Different effects of vernalization on flowering and leaf traits were observed.

In total 128 significant allele-trait associations were found by a General Linear Model approach. Of the five turnip candidate genes primers, only nm_60 was proven to be associated with turnip traits (turnip weight and width).

Keywords: *Brassica rapa*, phenotypic variation, vernalization, association mapping

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Introduction

Brassica rapa

Brassica rapa is one of the approximately 3710 species within the Brassicaceae family (Beilstein et al. 2008). *B. rapa* is a diploid species of the *Brassica* genus, which consists of three diploid species: *B. rapa* (2n = 20; genome composition AA), *B. nigra* (2n = 16; genome composition BB) and *B. oleracea* (2n = 18; genome composition CC) and three amphidiploids: *B. juncea* (2n = 36; genome composition AABB), *B. napus* (2n = 38; genome composition AACC) and *B. carinata* (2n = 34; genome composition BBCC). Each of these amphidiploids is evolved through interspecific hybridization between two of the elementary diploids. The relationship between the species and their genomes are clearly displayed in the 'triangle of U' (Figure 1) (U 1935).

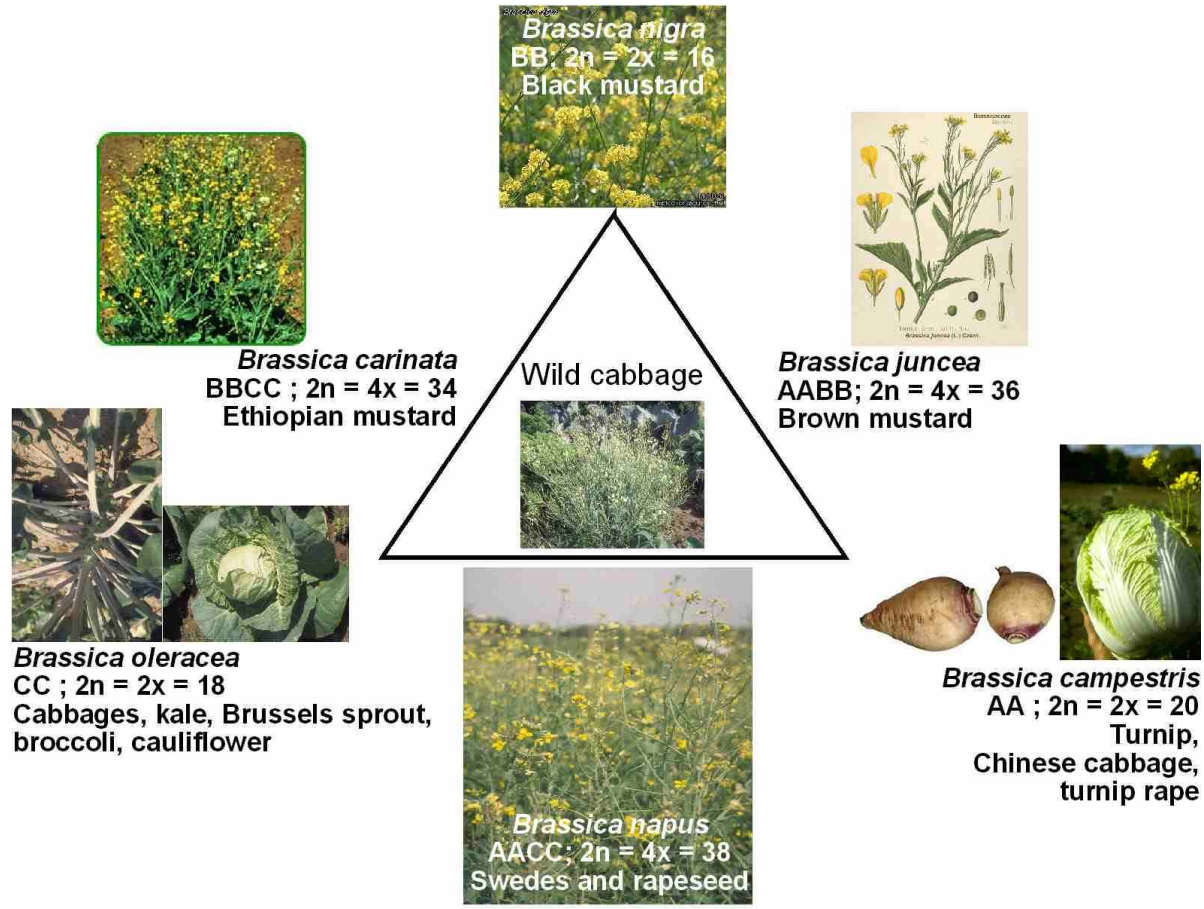


Figure 1. Schematic overview of the relationship between the different species of the *Brassica* genus in the 'triangle of U'.

Based on several studies using molecule markers, two centers of variation are identified in *B. rapa*, Europe and Asia (Song et al. 1988; Zhao et al. 2005; Zhao et al. 2009). Whether

these centers of variation are also the centers of origin or if there is one common center of origin is not clear to date. Turnip and turnip rape (*oleiferous* forms) are the types most abundant in the domestication in Europe and the leafy vegetable types like Chinese cabbage and pak choi are found mainly in Asia (Zhao et al. 2005). However, in Asia there are also turnip rapes and turnips. The variation within *B. rapa* in the two centers of variation likely arose independently from each other (Zhao et al. 2005).

All the different organs (leaf, swollen stem, swollen root, inflorescence or seeds) of *B. rapa* are of interest for consumers, a lot of morphological variation has evolved during domestication history. Based on the organ that is used these morphotypes are classified into leafy, turnip and oil types (Figure 2). *B. rapa* consists of approximately 15 different subspecies or morphotypes (Bonnema et al. 2009; Diederichsen 2001). The best known *B. rapa* subspecies are turnip (*B. rapa* L. subsp. *rapifera*), Chinese cabbage (*B. rapa* L. subsp. *pekinensis*), oil rape (*B. rapa* L. subsp. *oleifera*) and pak choi (*B. rapa* L. subsp. *chinensis*).

Based on DNA fingerprints, a morphotype from the same region is often closer related to an other morphotype from the same region than to the same morphotype from a different region (Zhao et al. 2005) which suggest that different morphotypes were selected independently in those regions.

Figure 2. Different morphotypes of *B. rapa*. From left to right: leafy (Chinese cabbage), turnip (vegetable turnip) and oil type

Zhao et al. (2010a) investigated a large core collection of *B. rapa* accessions. These accessions originate from the WUR core collection and the VIR collection. The accessions from the WUR collection were obtained from the Dutch Crop Genetic Resources Center (CGN) and the Institute of Vegetables and Flowers and the Oil Crop research Institute from the Chinese Academy of Agricultural Sciences (CAAS). The lines of the VIR collection were

obtained from the Vavilov Institute of Plant Industry (VIR) in Russia. Based on the data of 13 SSR markers and one CAPS marker profiled over 239 *B. rapa* accessions, Zhao et al. (2010a) found five subgroups. The first subgroup (S1) consists of annual oil types (yellow sarson and summer oil). Group two (S2) consists of 67 accessions, all Chinese cabbages. The third subgroup (S3) is mainly composed of pak chois and a few Chinese cabbage, Chinese turnip rape and neep greens accessions. Group four (S4) consists of European turnips and some oil types. The last subgroup (S5) is composed of the Pakistani winter oils, other oil types and Japanese turnips.

Based on the distribution of the accessions over the five subgroups, the two core collections are not evenly represented in each subgroup (Zhao et al. 2010a), so a combination of these two core collections for association mapping is an improvement compared to only the WUR core collection. Because some morphotypes are still underrepresented in the combined collection, addition of material from other gene banks is preferred. This will result in core collection that harbors the complete genetic variation present in *B. rapa* species (Zhao et al. 2010a).

Vernalization

Vernalization is the prolonged exposure of plants to temperatures between 0ºC and 7 ºC. Various effects of vernalization have been reported in *B. rapa*. Flowering and bolting of turnip plants was strongly induced by four weeks of vernalization at 3 ºC (Takahashi et al. 1994). The effect of vernalization was inhibited if the plants were subsequently grown under short day conditions. It is known that turnip accessions need a relative longer vernalization period compared to other *B. rapa* accessions (Bonnema, personal communication).

The induction of flowering by vernalization was also observed in other members of the Brassicea family like Arabidopsis and Radish (Carolus 1936; Cheon and Saito 2004; Koornneef et al. 2004; Napp-Zinn 1985). Other morphological responses to vernalization in *B. rapa* are not described in literature. But from *A. thaliana* is known that the leaves of vernalized plants are smaller and more erect (Hopkins et al. 2008).

A lot of quantitative traits loci (QTL) related to morphological traits (flowering, leafy traits and growth-related traits) in *B. rapa* were reported (Lou et al. 2007; Zhao et al. 2007), but these QTL are not related to the effect of vernalization on the morphology of *B. rapa*. Only a few QTL specific for the morphology of *B. rapa* under vernalized conditions are detected (Nini 2008). These QTL, related to flowering time, leaf traits and developmental

traits are found on linkage group A04, A07, A08 and A10. Less QTL were found after 31 days of vernalization compared to 18 days of vernalization.

Turnip formation

Little is known about what determines which storage organ a plant is using. Within *B. rapa* several types of storage organs are found: oleiferous seeds, fleshy leaves, enlarged stems like broccoletto's or enlarged hypocotyls and roots, called turnip. It is most likely that the turnip is composed of both the swollen primary root and an enlarged hypocotyl (Zhang, personal communication). A lot of variation in the morphology of turnips is present between different accessions (Zhang, personal communication). The initiation and the growth of the storage root is essential for the quality and the yield (Reid and English 2000), but till now the genes underlying the process of turnip formation are not fully unraveled (Iwata et al. 2004). To understand what makes a plant using a certain storage organ, the process of the formation of the storage organs should be understood. Although the whole genome of the *B. rapa* is sequenced (Zhang, personal communication; www.brassica-rapa.org).

Lu et al. (2008) found 18 QTL correlated with root growth in *B. rapa* in a F_2 population of the cross between AJH97-2 (S_3 of the Chinese Cabbage cultivar Aijiaohuang) and QSH97-24 (S_3 of the Chinese vegetable turnip cultivar Qishihai). Since their linkage groups were not assigned to the *B. rapa* chromosomes, it is not possible to compare these QTL regions to those in other studies. One major QTL (TuQTL-1) explaining up to 40% of the variation was found by Lou et al. (2007) in a BC_1 and a double haploid population of a cross between a yellow sarson accession (YS-143) and a Japanese vegetable turnip (VT-115). Till now, these two researches are the only published information on QTL related to the turnip formation in *B. rapa.*

In *Brassica napus* QTL related to turnip formation were found by a group at the University of Kiel (Lange et al. 2010, submitted). They also identified a number of candidate genes associated with storage root formation based on cDNA libraries of upregulated genes during secondary root growth.

In the same study where TuQTL-1 was detected, a lot of other QTL of morphological traits, like flowering time, seed weight, seed color, plant height, leaf shape etc. were found (Lou et al. 2007). TuQTL-1 was co-located with FLQTL-2 (the major flowering time QTL, explaining up to 60% of the variation) on the top of linkage group A02. It is not known whether the co-location of TuQTL-1 and FLQTL-2 is due to tight linkage, pleiotropy or epistasy. A phenotypic correlation between flowering time and turnip formation in F_2

populations was only observed if the parents of that population differed greatly in flowering time (Vos 2009), so there was no correlation between turnip formation and flowering time in all segregating population.

Many of the QTL positions for flowering time in *B. rapa* seem to correspond with map position of *FLC* paralogues (Lou et al. 2007). Based on QTL analysis in *Brassica* and *A. thaliana*, *FLCs* and *FRI* are proven to be genes controlling the effect of vernalization on flowering in *A. thaliana* and *B. rapa* (Lee and Amasino 1995; Okazaki et al. 2007; Osborn et al. 1997; Schranz et al. 2006). There are in total four *FLC* mapped in *B. rapa*: *BrFLC1, BrFLC2, BrFLC3 and BrFLC5*. *BrFLC* genes show high homology to *AtFLC*, although size of the putative proteins of *BrFLC* is different (Kim et al. 2007). Of the *BrFLC* paralogues, *BrFLC2* was located on A02 and co-localized with the major QTLs FTQTL-2 and TuQTL-1 (Lou et al. 2007). If *B. rapa* plants were vernalized, the gene expression of *BrFLC2* was much lower, resulting in a shorter flowering time (Kim et al. 2007; Zhao et al. 2010b). The same effect was found on *FLC* in *A. thaliana (Koornneef* et al. *2004; Schranz* et al. *2006)* The explained variation by the FLQTL-2 was much lower after vernalization (Lou et al. 2007), which is in agreement with the decrease of *FLC* expression (Zhao et al. 2010b). If the TuQTL-1/FLQTL-2 co-location is caused by pleiotropy or epistasy, vernalization is supposed to have an effect on the turnip traits controlled by TuQTL-1.

Association mapping

If two alleles or markers at different loci are observed more or less frequent than expected based on the random formation of haplotypes, the loci segregate not independent from each other. This is called Linkage Disequilibrium (LD) and is present in both natural and breeding populations (Flint-Garcia et al. 2003; Mackay and Powell 2007). LD is a powerful tool to link the observed phenotypes to genetic diversity. This marker-trait association based on LD is called association mapping. By plotting the LD against the genetic of physical distance, an estimate of the average distance between recombinations is obtained. This is a measure for the average spacing of the markers over the genome that would be needed for an association mapping study. This distance is in *B. rapa* approximately 10 to 15 cM (Del Carpio, personal communication) when calculated over the whole genome. However in certain regions it decreases to several tens of kb.

For association mapping, no segregating populations of a biparental cross are needed, but large collections are required, so a broader phenotypic variance can be studied. One further advantage is that the mapping resolution is higher compared to that in controlled

crosses. (Gupta et al. 2005; Myles et al. 2009). The major disadvantage of association mapping is the occurrence of false positive associations due to the population structure. So observed marker-trait associations are not only caused by physical/genetic linkage but also by population cofounding. Association mapping can be successful in the identification of loci associated to a certain phenotypic trait (D'hoop et al. 2008; Simko et al. 2004; Zhao et al. 2007).

SNP (single nucleotide polymorphism) or SSR (single sequence repeat) markers are often used for association mapping studies. These types of markers are used because they are highly conserved throughout related genomes, highly polymorphic and they are anchored on a reference genome map (Collard et al. 2005). If the markers are anchored on a reference map, the results of different studies can be compared. This property is the main reason why SSR markers are used in this experiment. In general, a marker density of four times the chromosome number is stated to be a good starting point for a association study (Zhu et al. 2008), but many factors like length of the chromosome, genetic and phenotypic diversity and availability of marker systems have their impact on the results of the study. By using multi allelic SSRs the number of markers needed is much lower than by using biallelic SNPs. Many SSR markers have been developed for *B. rapa* to construct genetic maps (Choi et al. 2007; LING et al. 2007; Suwabe et al. 2002) and their occurrence in the genome is abundant (Jin, in preparation).

Scope of thesis

One aim of this thesis is to investigate the effects of different vernalization conditions on turnip formation and other morphological traits in a diverse selection of turnip accessions. To examine this, phenotypic measurements on turnip traits, flowering traits and some leafy traits will be performed on selected accessions after zero, four and eight weeks vernalization at 4ºC.

An additional aim is to define the population structure within the used turnip accessions using genotype information. Therefore SSR markers covering all linkage groups and some focusing on candidate genes related to turnip formation and flowering time will be used for the calculation of the population structure.

An additional aim is to combine molecular marker-trait data with the population structure to identify markers associated with the traits of interest.

Material and Methods

Plant materials

This study consists of two parts. One part is the greenhouse experiment for the phenotypic observations on the effect of vernalization and another part is the molecular analysis with SSR markers.

Phenotypic measurements

For the phenotyping under different vernalization conditions, 41 turnip accessions were selected: representing the geographic and morphological variation as much as possible, according to Zhao et al. (2010a) (Table 1). Only 41 turnip accessions were chosen because only a limited number could be grown due to space in the greenhouse and available time for scoring. Twenty-six accessions were chosen from the WUR collection, based on their phylogenetic distance (Zhao et al. 2010a; Zhao et al. 2007; Zhao et al. 2005). If some accessions were closely related to each other, only one was selected. All nine turnip accessions of the VIR collection were included in the experiment. Additionally seven new potentially interesting turnip accessions from Russia were added to the experiment. Because they arrived too late, phenotypic traits are measured only on non-vernalized plants.

Beside the turnip accessions, seven accessions of other morphotypes were included in the experiment to see the effect of vernalization on these *B. rapa* morphotypes. These groups of cultivars are: yellow sarson (YS), pak choi (PC), oil seed rape (OR), winter oil (WO), Broccoletto (BRO) and Chinese cabbage (CC). The complete list of all the accessions can be found in Table 1.

Genotyping

All turnip accessions in the WUR collection and the VIR collection (n = 54) were used for the molecular data analysis. The seven turnip accessions from Russia and the eight non turnip accessions were also included. Beside those accessions, CC-chiifu and 3 other recently obtained turnip accessions from Korea (W-red round-T, W-early T and Tk-198-T) were also used for genotyping (Table 1). For accessions which were not grown for phenotypic measurements only two plants were planted for DNA isolation.

Table 1. List with all the accessions used in this study. If an accession is used for phenotypic observations, it has a number in the first column.

Accession number used in experimental layout of phenotyping experiment (Appendix I)	Accession	morpho-type*	Collection	Cultivar name	Accession No.	Country of origin	Subtaxa in gene bank
1	T-1050V	VT	VIR	Volynskij	1050	Ukraine	rapa
2	T-1283V	VT	VIR	Zolotoj shar	1283	Netherlands	rapa
3	T-163V	PC	VIR	Local	163	China	rapa
4	T-307V	VT	VIR	Osterzundomskij	307	Russia	rapa
5	T-385V	VT	VIR	Bartfeldskij	385	Ukraine	rapa
6	T-738V	VT	VIR	Karelskaya	738	Russia	rapa
7	T-821V	VT	VIR	Grobovskaya	821	Russia	rapa
8	T-826V	VT	VIR	Milanskaya belaya	826	Russia	rapa
9	T-830V	VT	VIR	Petrovskaya	830	Russia	rapa
10	W-FT-004	FT	WUR	Lange Gele Bortfelder	CGN06678	Denmark	fodder turnip
11	W-FT-047	FT	WUR	Moskovskij	CGN06866	Soviet Union	fodder turnip
12	W-FT-051	FT	WUR	Krasnaja	CGN07164	Russia	fodder turnip
13	W-FT-056	FT	WUR	Daisy; Bladraap	CGN07179	France	fodder turnip
14	W-FT-086	FT	WUR		CGN07223	Pakistan	fodder turnip
15	W-FT-097	FT	WUR	Buko; Bladraap	CGN11010	Germany	fodder turnip
16	W-VT-007	VT	WUR	Maiskaja	CGN06710	Soviet Union	vegetable turnip
17	W-VT-008	VT	WUR	Pusa Chandrina	CGN06711	India	vegetable turnip
18	W-VT-009	VT	WUR	Ronde Rode -Tsutsui	CGN06717	Japan	vegetable turnip
19	W-VT-010	VT	WUR	Platte Ronde Blauwkop Ingesneden Blad- Lila Ker	CGN06718	Hungary	vegetable turnip
20	W-VT-012	VT	WUR	Ronde Rode Heelblad-Yurugu Red	CGN06720	Japan	vegetable turnip
21	W-VT-013	VT	WUR	Ronde Rode Heelblad-Scarlet Ball	CGN06721	Japan	vegetable turnip
22	W-VT-014	VT	WUR	Platte Witte Blauwkop Heelblad-Milan	CGN06722	Italy	vegetable turnip
23	W-VT-017	VT	WUR	Platte Witte Meirapen	CGN06732	Netherlands	vegetable turnip
24	W-VT-018	VT	WUR	Goudbal; Golden Ball	CGN06774	Netherlands	vegetable turnip
25	W-VT-044	VT	WUR	Soloveckaja	CGN06859	Soviet Union	vegetable turnip

26	W-VT-052	VT	WUR	Hilversumse; Marteau	CGN07166	Netherlands	vegetable turnip
27	W-VT-053	VT	WUR	Teltower Kleine	CGN07167		vegetable turnip
28	W-VT-089	VT	WUR	D`Auvergne Hative	CGN10995	France	vegetable turnip
29	W-VT-091	VT	WUR	Snowball; Blanc Rond de Jersey	CGN10999	United Kingdom	vegetable turnip
30	W-VT-115	VT	WUR	Kairyou Hakata	CGN15199	Japan	vegetable turnip
31	W-VT117	VT	WUR	Toya	CGN15201	Japan	vegetable turnip
32	W-VT-120	VT	WUR	Platte Gele Boterknol	CGN15210	Netherlands	vegetable turnip
33	W-VT-123	VT	WUR	Terauchi-Kabu	CGN15220	Japan	vegetable turnip
34	W-VT-137	VT	WUR		CGN20735	Uzbekistan	vegetable turnip
35	YS-143	SO	WUR		FIL500	India	spring yellow sarson
36	WO-083	WO	WUR		CGN07220	Pakistan	Chinese cabbage
37	PC-105	PC	WUR	BRA 77/72	CGN15171	China	pakchoi
38	PC-175	PC	WUR		VO2B0226	China	Chinese cabbage chinensis
39	OR-213	OR	WUR	Huang po tian you cai	OCRI0235	China	
40	BRO-025	BRO	WUR	Natalino	CGN06823	Italy	broccoletto
41	CC-113	CC	WUR	Bei jing 106	CGN15195	China	Chinese cabbage
	CC-chifu**	CC					
	SM-14	VT	RSAU	JE 4-11		Russia	
	SM-15	VT	RSAU	Petrovskaya 1-122		Russia	
	SM-16	VT	RSAU	JJE 2-1		Russia	
	SM-17	VT	RSAU	Iz 2-11		Israel	
	SM-18	VT	RSAU	Petrovskaya 34-12		Russia	
	SM-19	VT	RSAU	Petrovskaya 1-35		Russia	
	SM-20	FT	RSAU	ECD 04-0126		Russia	
	Tk-198-T**					Korea	
	W-early-T**					Korea	
	W-FT-001	FT	WUR	Halflange Witte Blauwkop Ingesneden Blad-Barenza	CGN06669	Netherlands	fodder turnip
	W-FT-002	FT	WUR	Grote Ronde Witte Roodkop-Norfolk; De Norfolk a Collet Rouge	CGN06673	United Kingdom	fodder turnip
	W-FT-003	FT	WUR	Lange Witte Roodkop	CGN06675	Netherlands	fodder turnip
	W-FT-005	FT	WUR	Ochsenhorner	CGN06688	Germany	fodder turnip
	W-FT-088	FT	WUR	Blauwkop Heelblad-Oliekannetjes	CGN10985	Netherlands	fodder turnip
	W-red round-T*					Korea	

W-VT-006	VT	WUR	Pusa Chandrina	CGN06709	India	vegetable turnip
W-VT-011	VT	WUR	Platte Witte Blauwkop Ingesneden Blad-Siniaja	CGN06719	Soviet Union	vegetable turnip
W-VT-015	VT	WUR	Bianca Lodigiana; Italiaanse Witte	CGN06724	Italy	vegetable turnip
W-VT-045	VT	WUR	Milanskaja; Italiaanse Witte	CGN06860	Italy	vegetable turnip
W-VT-090	VT	WUR	De Croissy	CGN10996	France	vegetable turnip
W-VT-092	VT	WUR	Amerikaanse Witte Roodkop Heelblad	CGN11000	Netherlands	vegetable turnip
W-VT-116	VT	WUR	Nagasaki Aka	CGN15200	Japan	vegetable turnip
W-VT-119	VT	WUR	Roodkop-Pfalzer	CGN15209	Netherlands	vegetable turnip
W-Wenzhoupancai	VT	WUR			China	rapa

* CC Chinese Cabbage
PC pakchoi
BRO broccoletto
NG mizuna/Komatsuna/neep green
T vegetable turnip/fodder turnip
OR Chinese turnip rape
WO winter turnip rape
SO yellow sarson

** Kindly provided by Dr. Jungu Lee

Experimental setup and design

The seeds are sown first in 6 cm petri dishes on three layers of wet filter paper. Three treatments were applied to the plants: no vernalization, 4 weeks vernalization and 8 weeks vernalization. Of each accession, 9 seeds are sown per treatment to be sure that four replicates could be transplanted

The seeds were germinated at room temperature for approximately three days. As soon as the cotyledons and to a small extend also hypocotyl were developed (Figure 3), the petri dishes were placed in a cold room at 4ºC for vernalization in the dark. The three different treatments were sown with intervals of four weeks so all seedlings can be transplanted at the same moment. All seedlings were transplanted to jiffy pots on the ninth of February. The pots were placed in the greenhouse at 18ºC. After three weeks, four average, vigorous seedlings per accession per treatment are transplanted to 17 cm pots and placed according to the experimental layout (Appendix I).

For the plants which were only needed for DNA isolation, only four seeds were sown and two seedlings were transplanted. These plants were not vernalized.

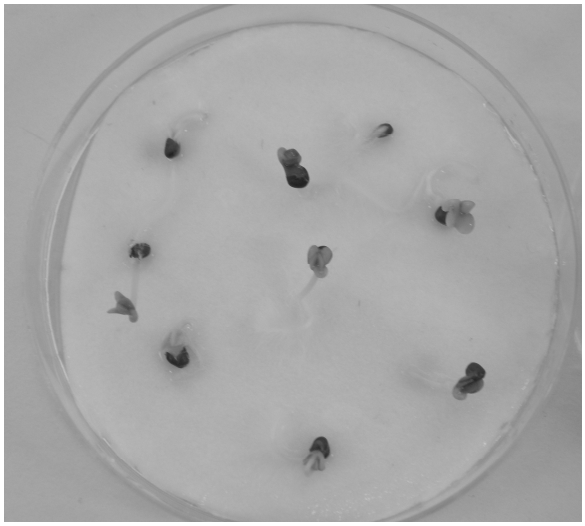


Figure 3. Stage at which seedlings were put at 4ºC.

The plants were placed on tables according to a complete randomized block design with four blocks. Every block contains one of the four replicates of each treatment. The exact layout of the experiment can be found in Appendix I. To keep the plants equally spaced if a plant was missing, plants from the 'Russian' turnip accessions or plants which will only be used for genotyping were placed in the open space.

Phenotyping

Turnip traits

Since the effect of vernalization on turnip formation is the main aim of this study, the phenotypic scoring was focused on the turnip traits. The scored turnip traits were shape (TS), diameter (TWi), weight (TWe), color of the upper part and the lower part (TCu and TCl), swelling onset date (TSo), depth of growing of the turnip (TD), and smoothness of the skin (TSs) (Table 2). The turnip shape was scored on an ordinal scale of 1-6 (1 = oval, 2 = round to oval, 3 = round, 4 = oval to long, 5 = long, 6 = very long). The color of the turnip was also scored on an ordinal scale: 1 = white, 2 = green/white, 3 = cream, 4 = yellow/light brown, 5 = light purple/pink, 6 = purple, 7 = dark purple, 8 = red, 9 = dark red. The width of the turnip was determined by measuring the diameter (in cm) at the widest point with a caliper. The onset of turnip swelling is set if the diameter of the turnips exceeds 5 mm. The days needed from transplanting till the onset of the swelling of the turnip were counted. The depth at which the turnip grows was scored at an ordinal scale of 1-3 (1 = complete underground, 2 = intermediate, 3 = complete above ground). The smoothness of the tuber skin was also measured on an ordinal scale of 1 – 3 (1 = very smooth, 2 = intermediate, 3 = very wrinkled skin). Pictorial representations of each ordinals scale can be found in Appendix II.

TWi was measured every two weeks and the first measurement took place three weeks after transplanting. The TWi was measured seven times. The TSo was checked three times a week, starting four weeks after transplanting and continued till all turnips were swollen. TC, TS, TWe, TSn, TD and the TSs will be determined at harvest, approximately 100 days after transplanting.

Flowering traits

The following flowering traits were measured; flowering time (FT), bolting, (FB), flower color (FC) and the shape of the flower (FS) (Table 2).The flowering time was measured as the number of days between transplanting and the opening of the first flower. Bolting was quantified by measuring the length of the flower branch at the moment the first flower appears. The color of the flowers was measured on an ordinal scale of 1 – 5 (1 = orange, 2 = dark yellow, 3 = yellow, 4 = medium yellow, 5 = light yellow). The shape of the flowers was also measured on an ordinal scale of 1 – 5 (1 = open, 2 = not touching, 3 = touching, 4 = overlapping, 5 = strong overlapping). Pictorial representations of these ordinal scales are presented in appendix II. All plants were scored three times a week whether the first flower

opens. If that was the case, FB, FC and FS were determined. This measurement started three weeks after transplanting because some of the vernalized non turnip accessions are supposed to flower already 20 days after vernalization (Zhao et al. 2007).

Leaf traits

Based on the observations of Hopkins et al. (2008) that leaves of vernalized *A. thaliana* plants are smaller and more erect, some leafy traits will also be measured in this study. The following traits were measured: Lamina length (LL), lamina width (LWi), petiole length (PL) (Figure 4), petiole width (PW), leaf erectness (LEr), leaf color (LCo), leaf surface (LSu), leaf trichomes (LTr) shape of the leaf tip (LTi), leaf edge shape (LEs), lamina attitude (LA), moment of senescence of the third leaf (LSe) and the number of leaves at the moment of flowering (LN) (Table 2).

One fully developed leaf (third or fourth leaf) of each plant in three out of four blocks was picked and scanned approximately 40 days after transplanting. Images were analyzed with the software program ImageJ v. 1.43 (http://rsb.info.nih.gov/ij/) to measure the LL, LW, PL and the PW. Based on the LL and LW the leaf index (LI) was calculated. LI is the ratio between the lamina length and the lamina width. The fresh picked leaves were also used for scoring the LSu, LTr, LTi and LEs. The scoring of the leaf surface was on an ordinal scale of 1 – 3 (1 = very smooth, 2 = intermediate wrinkled, 3 = very wrinkled). The habit of the leaf tip was scored from 1 – 3 (1= very sharp, 3 = round) and leaf edge shape from 1 – 4 (1 = entire, 2 = slightly serrated, 3 = intermediate serrated, 4 = much serrated). The level of curling of the lamina was scored from 1 – 7 (1 = extreme deep down curling, 4 = straight, 7 = extreme deep up curling).

The leaf color was measured with a SPAD meter (Minolta SPAD 502 Chlorophyll Meter) approximately 40 days after transplanting on one fully developed leaf of every plant in three out of four blocks. Per leaf, five spots were measured and one average value was calculated by the meter.

The leaf development of six accessions (T-1283V, W-VT-117, W-VT-115, W-VT-052, YS-143, and PC-105) was also investigated by measuring the LL, LW and the PL twice a week with a ruler. Only six accessions were used because the time was limited. The measurements started two weeks after transplanting. The same leaf (third) was measured every time and measurements continued till senescence of the leaf. The date of senescence (LSe) was also recorded and the number of days till senescence of the third leaf was calculated. Only these accessions were also used for the determination of the erectness of the

leaves LEr. This was done on an ordinal scale of 1 – 4 (1 = horizontal, 2 = light upright, 3 = leaf has angle of approximately 45°, 4 = steep upright) on a fully developed leaf.

The leaf number was scored at the moment of flowering. So when the first flowers opened, the number of leaves was also counted. At the moment of harvest (100 days after transplanting), the weight of the leaf and stem fraction (LWe) was also determined.

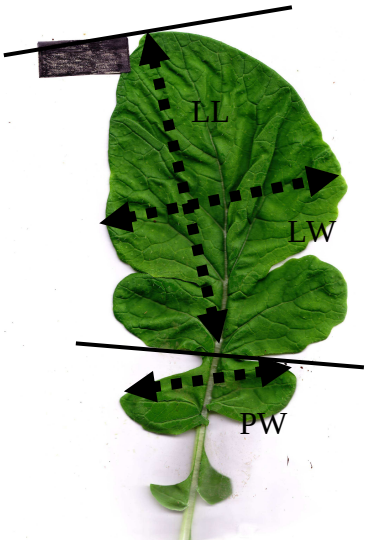


Figure 4. Pictorial representation of measurements on lamina and petiole. The smaller lobes were not included in the lamina length measurement.

Table 2. List of phenotypic scored traits. Pictorial representations of each ordinal scale can be found in Appendix II.

Trait name	Abbreviation	Description	measurement protocol
Turnip shape	TSh	Form of the turnip	ordinal; 1-6
Turnip width	TWi	Diameter of the turnip at the widest point, measured by a caliper	cm
Turnip color upper part	TCu	Dominating color of the upper part of the turnip	ordinal; 1-9
Turnip color lower part	TCl	Dominating color of the lower part of the turnip	ordinal; 1-9
Turnip swelling onset	TSo	Number of days between transplanting and the start of the swelling of the turnip	# days
Turnip growing depth	TD	Depth in the soil at which the turnip grows	ordinal; 1-3
Turnip smoothness	TSs	Smoothness of the turnip skin	ordinal; 1-3
Turnip weight	Twe	the weight of an individual turnip 100 days after transplanting	g
Flowering time	FT	Number of days between transplanting and the appearance of the first flower	# days
Bolting	FB	Extent of length growth of the flowering branches	cm
Flower shape	FS	Shape of the flower	ordinal; 1- 5
Flower color	FC	Color of the flower	ordinal; 1- 5
Lamina length	LL	From tip of lamina to the bottom of lamina (Figure 4)	cm
Lamina width	LWi	Lamina width at the widest point (Figure 4A)	cm
Leaf index	LI	Ratio of LL to LW: LL/LW	ratio
Petiole length	PL	From base of petiole to bottom of lamina (Figure 4A)	cm

Petiole width	PW	Width of petiole at widest point (including lobes) (Figure 4)	cm
Leaf erectness	LEr	The angle between the leaf and the soil	ordinal; 1 – 4
Leaf color	LCo	Amount of chlorophyll in the leaves	
Leaf surface	LSu	Level of wrinkling of the leaf	ordinal; 1 – 3
Leaf trichomes	LTr	Absence or presence of trichomes on the leaf	ordinal; 0 and 1
Leaf tip shape	LTi	Form of the leaf tip	ordinal; 1 – 3
Leaf edge shape	LEs	Level of serration of the lamina edge	ordinal; 1 – 4
Lamina attitude	LA	Curling of the leaf blade	ordinal; 1 – 7
Number of leaves	LN	Number of leaves at the moment of flowering	# leaves
Leaf and stem weight	LWe	Weight of the stem and leaves	g
Senescence of leaf	LSe	Days after transplanting the 3rd leaf is senescent	# days

Genotyping

DNA isolation

DNA of all turnip accessions from the WUR core collection and the VIR core collection was isolated by Zhao et al. (2010a). The use of this DNA fits better in the time schedule because we do not have to wait till the plants have enough material for a DNA extraction. This already isolated DNA is the pooled DNA of ten plants per accession. Genomic DNA of the 66 accessions used in this study was additionally isolated as a control and to have a backup in case there is not sufficient DNA available. Twelve of the 66 accessions in this study were not used by Zhao et al. (2010a) but used for present marker analysis.

Of each accession grown in present experiment, the genomic DNA of eight plants was isolated as eight separate samples. Of each plant, two leaf discs were taken from one of the younger leaves (not the third leave, because that is used for measuring the leave development). The DNA isolation was performed according the RETCH 1.3 protocol (Gert van Arkel & Maarten Nijenhuis) (Appendix I).

SSR marker analysis

A touch-down PCR program was used (Table 3). The advantage of a touch-down PCR program is that the amplification of the correct product over any non-specific product is stimulated. The PCR protocol can be found in Appendix IV. In total 45 SSR markers were tested (Appendix V). By choosing the markers, an even distribution over the genome is aimed to obtain reliable population structure calculation. Furthermore as many as possible markers in interesting regions were chosen (e.g. FTQTL-2). Additionally five markers physically

linked to turnip candidate genes were designed (Lange et al. 2010, submitted) 0.5 µl Of the PCR product was loaded on a 5.5% polyacrylamide gel and analyzed with Global Edition IR2 DNA analyzer (Li-Cor Biosciences, Lincoln, NE). The protocol for the Li-Cor analyzing can be found in Appendix VI.

Table 3. PCR touch-down program for SSR markers.

# cycles	Temperature (℃)	Time (min)
1	94	3
10	94	1
	65-56	1
	72	1.5
30	94	1
	55	1
	72	1
1	72	5
Hold	10	∞

Data Analysis

Population structure

The program STRUCTRURE 2.3.3 (http://pritch.bsd.uchicago.edu/software) was used to identify the population structure of the used accessions. This program uses a Bayesian approach to calculate the probability of a certain number of pre-set number of populations (K), based on the allele frequencies (Falush et al. 2003; Pritchard et al. 2000). An admixture model was used, which allows a mixed ancestry and independent allele frequencies were assumed between the populations. The number of subpopulations was set to vary between 1 and 10 and for each fixed number of sub-populations, 2 independent MCMCs (Markov Chain Monte Carlo) were run using 600,000 iterations for each, and the first 100,000 iterations were discarded as burn-in.

Both marker data of informative markers (Appendix V) obtained for this study and marker data of the study of Zhao et al. (2010a) were used as input. Accessions were assigned to a subpopulation when the fraction explained variation by that group was larger than the variation explained by any of the other sub-populations.

After the number of sub populations was revealed, all indentified single sub-populations were used for testing whether a sub-population was present within the sub-populations. The non turnip lines which were included in determining the population structure were not included in this calculation, because they were initially assigned to different sub-

populations (Zhao et al. 2010a). Same settings were used for the computation as described previously.

Phenotypic variation

Phenotypic data was analyzed by using the statistical program Genstat v13 (VSN International Ltd., Oxford, UK). Ordinal scored traits were analyzed using a Kruskall-Wallis ANOVA and for the continue data the normal ANOVA procedure was used. If a trait had no normal distribution of the variance, the data was transformed with either a log (base e) or a standardize transformation to get a normal distribution of the variance. Traits were tested for a significant influence of the sub-population (ignoring vernalization treatment), the vernalization treatment (ignoring sub-population) and for a interaction between vernalization and sub-population. If plants were not flowering at the moment of harvest (100 days after transplanting), the flowering time was set to 200 days after transplanting.

The measurements in time were analyzed with the '*Repeated Measurements'* menu, choosing the option '*Correlation Models by REML'*. To get better balanced data, the differences for the day of transplanting were ignored and set to the average number of days after transplanting.

To visualize the correlation between the phenotypic traits for the three different vernalization treatments, a principal component analysis (PCA) was performed. The PCA analysis was conducted by using the software program R (www.r-project.org). A plot was made with two principal components (most of the time the two components having the highest cumulative explained variance) as axis and arrows representing every trait. The length of the arrow indicates the percentage of variation explained by the two principle components on the axis. The angle between the arrows indicates their correlation.

Association mapping

To correlate the phenotypic data with the marker data, the program TASSEL V.3.0. was used. A General Linear Model (GLM) approach was used, taking the population structure into account. The statistical model for this model is: Trait = *population structure* + *marker effect* + *residual*; in which the population structure serves as a covariate. Alleles with rare frequencies (<0.10) were filtered out of the dataset. An F-test was performed for each

association between a trait and a locus. A p-value of 0.001 was used as a threshold for significance.

Additionally also a Mixed Linear Model (MLM) was used to calculate the probabilities of each possible marker-trait association. This model takes beside the population structure and the markers also the random effects into account, so it incorporates also information about relationships among individuals. The statistical model of a MLM model is: Trait = *population structure* + *marker effect* + *Individuals* + *residual.* Settings for the MLM were the same as for the GLM approach.

Results

Validation of used DNA

DNA was isolated from young leaves from four week old plants grown in the greenhouse. The concentration of the isolated DNA was determined and ranged between 10 and 130 ng/µl. To compare whether these newly isolated DNA samples of each accession give similar allelic patterns upon screening with SSR compared to the DNA of Zhao et al. (2010a), four markers were tested. The four tested markers displayed in total 25 alleles when screened over 61 accessions using the DNA stock of Zhao *et al*. (2010a). This DNA stock consists of the pooled DNA of 10 plants. Only 18 alleles were detected when screened over exact the same 61 accessions of using isolated DNA in this study (pooled DNA of four plans/accessions). All these 18 alleles were also detected when the DNA of Zhao et al. (2010a) was used.

If for one accession a band was visible for a certain allele in only one of the two DNA stocks, this was counted as a mismatch. The number of mismatches per marker differed between only 1 and 23. Markers with a low number of alleles (marker 362 and est-18) and with many accessions having that allele had only a few mismatches. The more rare alleles per marker, the more mismatches; likely these rare alleles were not detected in the mixed sample. In Figure 5 an illustration of the difference in complexity between a SSR with many alleles and one with only two alleles in the accessions screened is given.

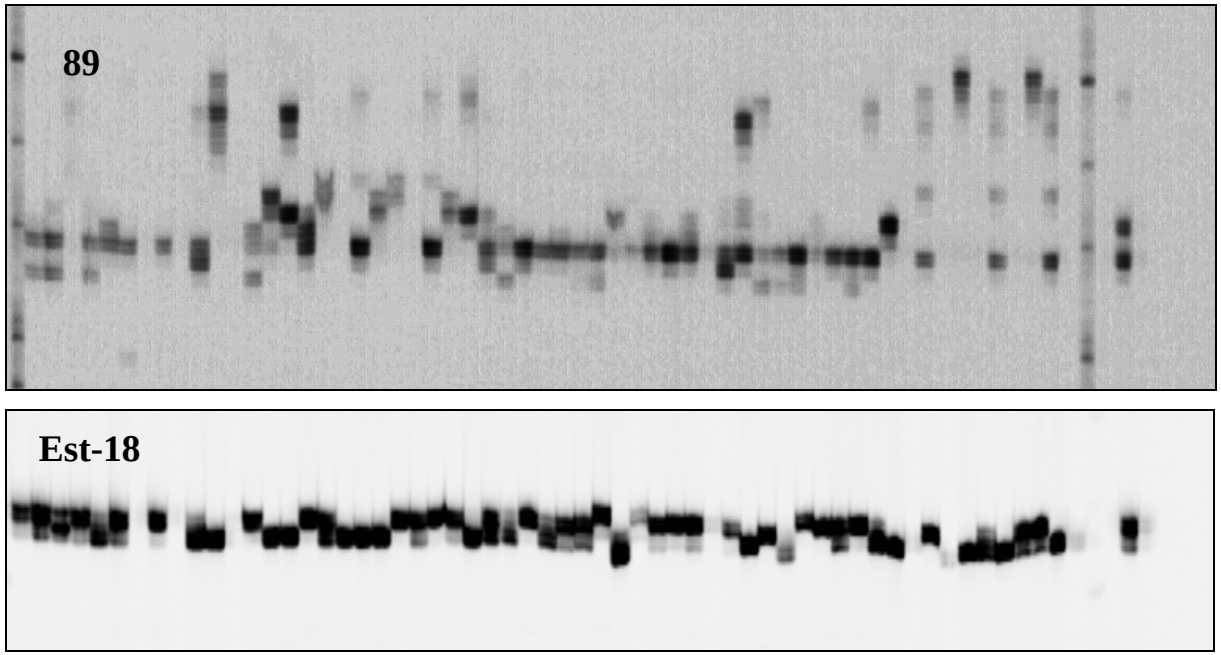


Figure 5. Examples of gels of two of the SSR markers used, screened over 61 accessions; SSR 89 and est-18. Marker 89 displays many alleles differing in intensity and est-18 has only two alleles. For both gels, DNA of my own study was used.

Table 4. Comparison in allele detection per SSR marker between the two used DNA stocks for the 61 accessions. For each allele the number of accessions having that allele and the number of mismatches between the two stocks per accession are shown. If for one accession an allele was present in one of the stocks and this allele was not present for that accession in the other stock, this was counted as a mismatch. Both the number of mismatches per allele and total number of mismatches per marker (sum of all the alleles) is given. The number of mismatches per allele shows the difference between the two DNA stocks.

SSR marker	272					362			96													est-18	
Allele (length in bp)	269	354	360	362	368	373	387	390	306	309	32	314	316	318	320	322	327	330	333	336	339	215	217
# of accessions* in stock Zhao with allele	54	2	14	11	40	2	10	50	15	33	34	7	18	3	31	20	3	2	7	8	9	32	39
# of accessions* in stock de Visser with allele	54	3	17	17	38	1	7	58	0	24	32	0	0	0	44	35	5	4	9	10	0	34	39
# of accessions* with mismatches per allele	1	1	11	9	17	1	5	9	15	15	14	7	18	3	22	23	1	3	7	8	9	3	2
Total number of mismatches per marker	39					15			145													5	

The same 61 accessions were used.

Population structure

In total 29 SSR markers of the 45 tested markers were informative (visible PCR products without a ladder pattern or more than 15 alleles) for the selected accessions. These informative markers were combined with the data of 11 SSR markers of Zhao et al. (2010a) (Appendix V) and used for calculating the population structure. Two main populations were found by STRUCTURE within the used accessions (Figure 6). The portion variation of each accession explained by each subpopulation is shown in Table 5.

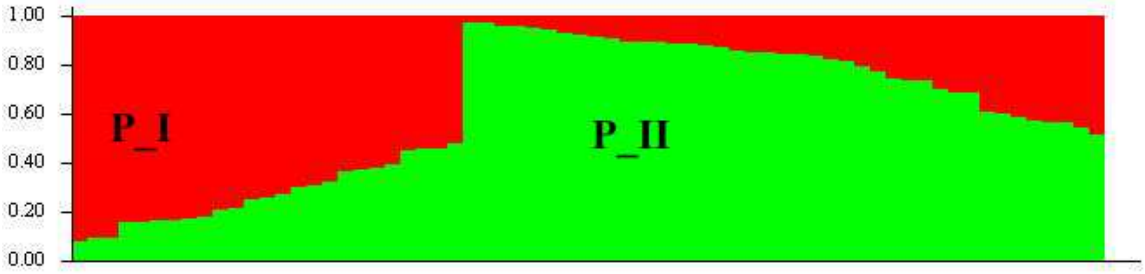


Figure 6. Result of population structure calculation assuming two population (K=2) named P_I (Asian turnips, n = 25) and P_II (European turnips, n = 41).

The first sub-population (P_I) consists of all turnips from Asia (n = 25), but also some accessions from the Soviet Union region and one accession from the Netherlands. The second sub-population (P_II) consists of 42 accessions: all European turnips and some turnips from Russia. Two accessions from India were also assigned to this sub-population.

The non turnip accession which were also included in the experiment were mostly grouped in P_I except BRO-025 and PC-105, however PC-105 was very admixed (0.479/0.521), while Bro-025 fitted well in group P_II (0.22/0.78)

Calculating sub-population within the two sub-populations gave no result. For both sub-populations, a population structure without any sub-groups was the most probable.

Table 5. List of sub-population to which each accession belongs (based on 40 SSR markers) including the fraction of the variance which is explained by each sub-group. Additionally the sub-group to which each accession was assigned by Zhao et al. (2007 and 21010a) is shown (study of 2007 also with structure-membership fractions). The colors indicate the to which sub-group each accession belongs: gold = P_I, dark green = P_II, blue = 1*, yellow = 2*, red = 3*, light green = 4*

accession	country of origin	structure-membership fractions in this study		Sub popu-lation in this study	structure-membership fractions in Zhao et al. (2007)				Sub popu-lation in study Zhao et al. (2007)*	Sub popu-lation in study Zhao et al. (2010a)**
W-VT-115	Japan	0.918	0.082	P_I	0.478	0.221	0.164	0.137	1	S1 a
W-early-T		0.906	0.094	P_I					N.D.	N.D.
Tk-198-T		0.902	0.098	P_I					N.D.	N.D.
PC-175	China	0.841	0.159	P_I	0.865	0.008	0.031	0.095	1 b	S4 b
W-VT-012	Japan	0.841	0.159	P_I	0.378	0.175	0.206	0.241	1	S3
CC-chifu		0.832	0.168	P_I					ND	N.D.
YS-143	India	0.829	0.171	P_I	0.005	0.004	0.987	0.004	3 b	S1 b
w-wenzhoupancai	China	0.824	0.176	P_I					N.D.	S1 a
W-VT117	Japan	0.821	0.179	P_I	0.519	0.079	0.201	0.201	1	S5
W-VT-116	Japan	0.788	0.212	P_I	0.636	0.032	0.188	0.144	1	S4 a
W-VT-009	Japan	0.782	0.218	P_I	0.512	0.211	0.071	0.206	1	S5
W-red round-T		0.746	0.254	P_I					N.D.	N.D.
SM-14	Russia	0.738	0.262	P_I					N.D.	N.D.
W-VT-013	Japan	0.723	0.277	P_I	0.492	0.153	0.224	0.131	1	S5
OR-213	China	0.699	0.301	P_I	0.539	0.048	0.008	0.405	1 b	S3 b
W-VT-123	Japan	0.69	0.31	P_I	0.445	0.059	0.245	0.252	1	S5
W-FT-001	Netherlands	0.678	0.322	P_I	0.072	0.806	0.009	0.113	2 a	S4 a
CC-113	China	0.633	0.367	P_I	0.012	0.016	0.041	0.932	4 b	S2 b
WO-083	Pakistan	0.626	0.374	P_I	0.319	0.324	0.207	0.151	2 b	S3 b
T-163V	China	0.62	0.38	P_I					N.D.	S5
SM-16	Russia	0.604	0.396	P_I					N.D.	N.D.
W-VT-137	Uzbekistan	0.543	0.457	P_I	0.154	0.595	0.084	0.168	2 a	S5
SM-19	Russia	0.54	0.46	P_I					N.D.	N.D.
W-FT-086	Pakistan	0.54	0.46	P_I	0.397	0.249	0.238	0.116	1	S2
W-FT-051	Russia	0.516	0.484	P_I	0.32	0.175	0.324	0.181	1	S5
PC-105	China	0.479	0.521	P_II	0.433	0.215	0.048	0.304	1 b	S3 b
SM-20	Russia	0.457	0.543	P_II					N.D.	N.D.
SM-17	Israel	0.434	0.566	P_II					N.D.	N.D.
SM-15	Russia	0.429	0.571	P_II					N.D.	N.D.
W-VT-008	India	0.428	0.572	P_II	0.162	0.792	0.013	0.033	2	S4
T-385V	Ukraine	0.414	0.586	P_II					N.D.	S4
T-738V	Russia	0.394	0.606	P_II					N.D.	S4
SM-18	Russia	0.391	0.609	P_II					N.D.	N.D.
W-VT-052	Netherlands	0.309	0.691	P_II	0.098	0.699	0.026	0.177	2	S4
T-821V	Russia	0.308	0.692	P_II					N.D.	S4
W-VT-007	Soviet Union	0.298	0.702	P_II	0.413	0.539	0.02	0.029	2	S4
W-FT-056	France	0.26	0.74	P_II	0.05	0.408	0.008	0.533	4	S4
W-VT-090	France	0.259	0.741	P_II	0.066	0.77	0.006	0.158	2	S4
W-FT-004	Denmark	0.251	0.749	P_II	0.044	0.846	0.035	0.075	2	S4
BRO-025	Italy	0.222	0.778	P_II	0.164	0.764	0.033	0.039	2	S4
W-FT-097	Germany	0.204	0.796	P_II	0.049	0.691	0.007	0.253	2	S4

T-830V	Russia	0.185	0.815	P_II					N.D.	S4
W-VT-091	United Kingdom	0.177	0.823	P_II	0.02	0.859	0.04	0.081	2	S4
W-VT-010	Hungary	0.163	0.837	P_II	0.196	0.761	0.009	0.034	2	S4
W-FT-003	Netherlands	0.157	0.843	P_II	0.046	0.774	0.005	0.175	2	S4
W-VT-092	Netherlands	0.157	0.843	P_II	0.01	0.972	0.008	0.01	2	S4
W-FT-088	Netherlands	0.147	0.853	P_II	0.02	0.894	0.034	0.052	2	S4
W-VT-017	Netherlands	0.143	0.857	P_II	0.022	0.865	0.016	0.097	2	S4
W-VT-089	France	0.139	0.861	P_II	0.035	0.853	0.016	0.095	2	S4
W-VT-053		0.125	0.875	P_II	0.059	0.804	0.013	0.124	2	S4
W-FT-002	United Kingdom	0.119	0.881	P_II	0.041	0.921	0.007	0.032	2	S4
T-1283V	Netherlands	0.112	0.888	P_II					N.D.	S4
W-FT-005	Germany	0.11	0.89	P_II	0.118	0.784	0.019	0.078	2	S4
W-VT-006	India	0.107	0.893	P_II	0.025	0.85	0.008	0.117	2	S4
W-VT-018	Netherlands	0.107	0.893	P_II	0.097	0.756	0.046	0.101	2	S4
W-VT-014	Italy	0.102	0.898	P_II	0.058	0.785	0.066	0.091	2	S4
T-826V	Russia	0.091	0.909	P_II					N.D.	S4
W-VT-044	Soviet Union	0.084	0.916	P_II	0.192	0.643	0.12	0.045	2	S4
W-VT-119	Netherlands	0.076	0.924	P_II	0.135	0.731	0.042	0.092	2	S4
W-VT-120	Netherlands	0.07	0.93	P_II	0.037	0.881	0.04	0.041	2	S4
T-307V	Russia	0.057	0.943	P_II					N.D.	S4
W-FT-047	Soviet Union	0.046	0.954	P_II	0.123	0.767	0.007	0.103	2	S4
T-1050V	Ukraine	0.04	0.96	P_II					N.D.	S4
W-VT-045	Italy	0.039	0.961	P_II	0.012	0.971	0.007	0.01	2	S4
W-VT-015	Italy	0.028	0.972	P_II	0.014	0.822	0.008	0.156	2	S4
W-VT-011	Soviet Union	0.022	0.978	P_II	0.178	0.776	0.025	0.021	2	S4

a In studies of Zhao et al. in a different sub group

b non turnip accession

* 1 = Pak choi and Asian turnips, 2 = European turnips, 3 = Annual oil types and 4 = Chinese cabbages

** S1 = Annual oil types, S2 = Chinese cabbages, S3 = Pak choi, S4 = European turnips and S5 = winter oils and Asian turnips.

Phenotypic variation

According to the population structure proposed in previous paragraph, all turnip accessions were divided into P_I or P_II. The non turnip accessions were placed in a separate group. Phenotypic traits were analyzed separately per sub-group. Means and standard deviations of each trait were calculated for the three vernalization treatments per sub-group and can be found in Appendix VII. All turnip traits were significantly influenced by the vernalization ($P<0.05$). A significant difference for weight, width, shape and smoothness of the turnips was observed between the two sub-populations. Accessions from P_II had a larger turnip diameter (Figure 7a) and a higher turnip weight compared to accessions from P_I. The onset of the turnip growth was significantly delayed by the vernalization.

The weight of the turnips appeared to be normally distributed for the European turnips (P_II) in all vernalization treatments. The turnip weight of the unvernalized Asian turnips was also normally distributed. The eight and four weeks vernalized Asian turnips however showed a left skewed distribution for turnip weight. P_II had a slightly higher variation in diameter compared to P_I. In P_I the variation in turnip diameter increased if the plants were vernalized while P_II showed the opposite trend (Figure 7a). Taking all traits into account, there is no clear trend in the variation per treatment or subpopulation.

The prolonged exposure of the seedlings to cold resulted in a significant smaller diameter of the turnips which was more evident in the Asian turnips (P_I) (Figure 7a). Beside that, the turnips of both sub-populations had a more wrinkled surface, were more elongated and growing deeper in the soil if they were vernalized.

The moment of Flowering and the bolting were significantly influenced by the length of the vernalization period. Plants which were vernalized start to flower earlier and had shorter flower stalks. None of the flowering traits showed a significant difference between the sub-populations. Neither the sub-population nor the length of vernalization had significant influence on the shape (Figure 7b) and the color of the flowers.

Most of the leaf traits were significantly influenced by the subpopulation (Appendix VII). Only the length of the leaf and the petiole, the surface and color of the leaf and the weight of the leaves (including flower stalk) were independent from the subpopulation to which the accessions belong. The significant effect of vernalization on the leaves was limited to the length, color, presence or absence of trichomes, erectness of the leaves and the number of leaves at the moment of flowering. The vernalized plants had smaller lamina length, but the lamina width did not differ significantly due to the vernalization although the variation was rather small for the lamina width. (Figure 7c). Leaf shape is another example of a trait with a very narrow distribution but the differences are still not significant between subpopulations or treatments (Figure 7d).

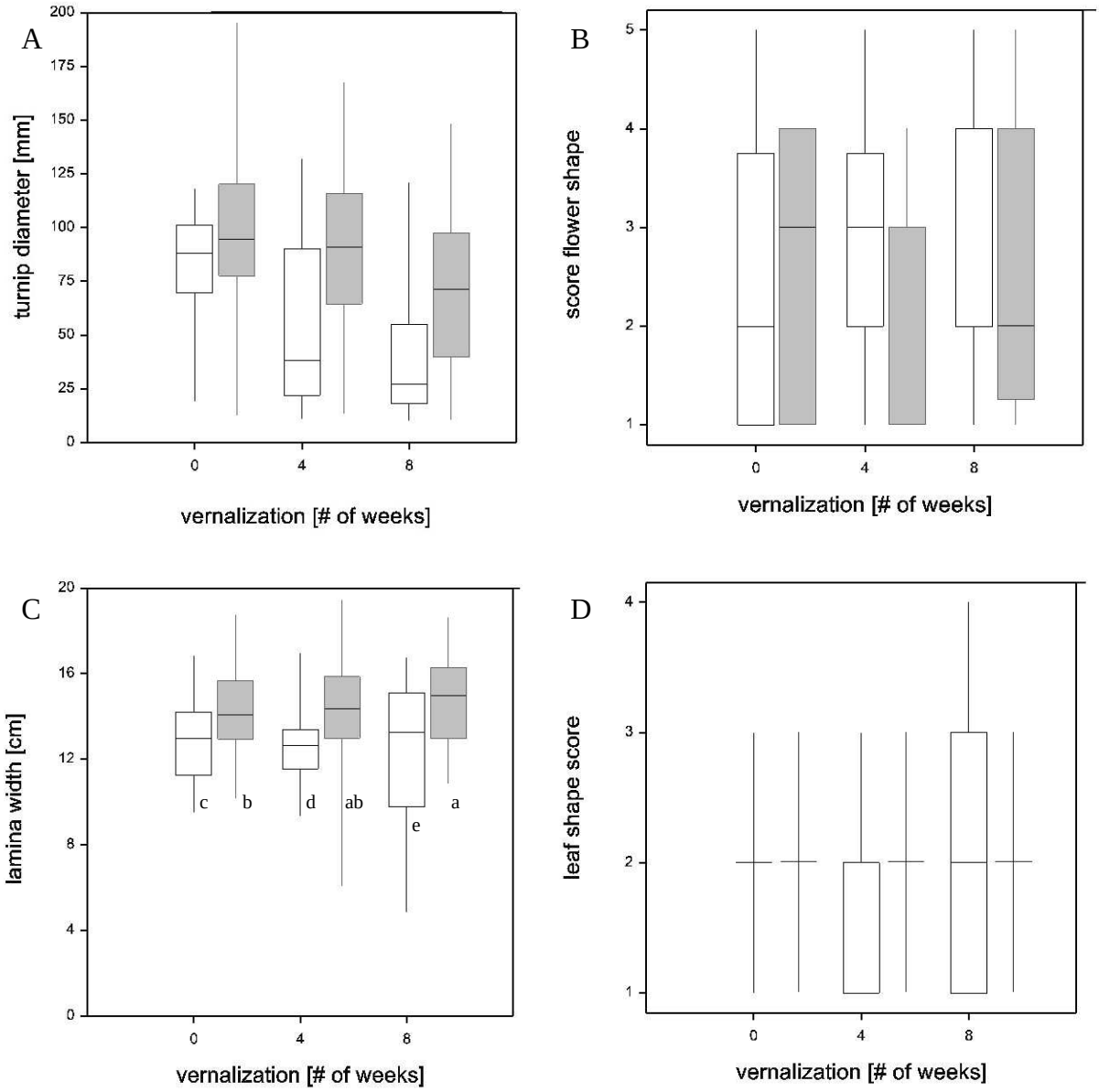


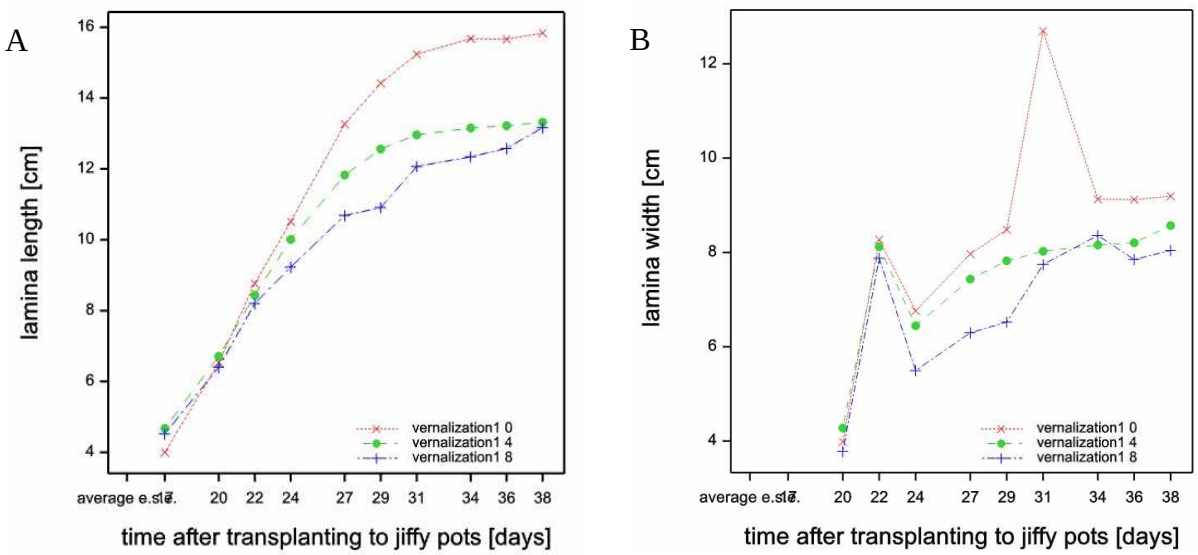
Figure 7. Box-plots of four different traits over two sub-populations and three vernalization treatments. White bars represent P_I (n = 25) and the grey bars P_II (n = 41). A = TWi (significant influence of population and vernalization P<0.05)), B = FS, C = LWi (significant influence of population and interaction between population and treatment P<0.05) and D = LS. The letters in diagram C indicate the interaction between vernalization and population; different letters indicate a significant difference (F-test, P = 0.05).

Developmental measurements

The average values over four plants per accession per treatment of the four traits (LL, LWi, PL and TWi) which were measured in time are shown in Figure 8. LL, LWi and PL were measured for only six accessions (T-1283V, W-VT-117, W-VT-115, W-VT-052, YS-143,

and PC-105) and the TWi was measured for all turnip accessions (n = 36). For all four traits it is obvious that vernalization causes a smaller and/or slower growth of either the leaf or the turnip. The initial growth speed (22 days after transplanting) is for LL and LWi the same in vernalized condition as in the unvernalized conditions. The length of the petiole is already increasing less in plants which were vernalized for 8 weeks compared to the two other treatments. For the turnip diameter the same trend is observed; the 8 weeks vernalization resulted in delayed growth rate. The leaf traits were measured till leaves were fully expanded and started to senescence. Both four and eight weeks vernalized plants had the same final lamina length which was smaller than that of the unvernalized plants. At the moment of harvest the turnips were not full grown, but the longer the plants were vernalized, the smaller the turnips.

By testing the whole growing curve for the effects of vernalization, only the development of LWi was not significantly affected by the vernalization (F-test, P <0.05); the vernalization caused a significant different growth for LL, PL and TWi. A large outlier of the lamina width of the 0 weeks vernalization treatment at day 31 was observed and a decrease of the lamina width from day 22 till day 24 for all the three treatments.



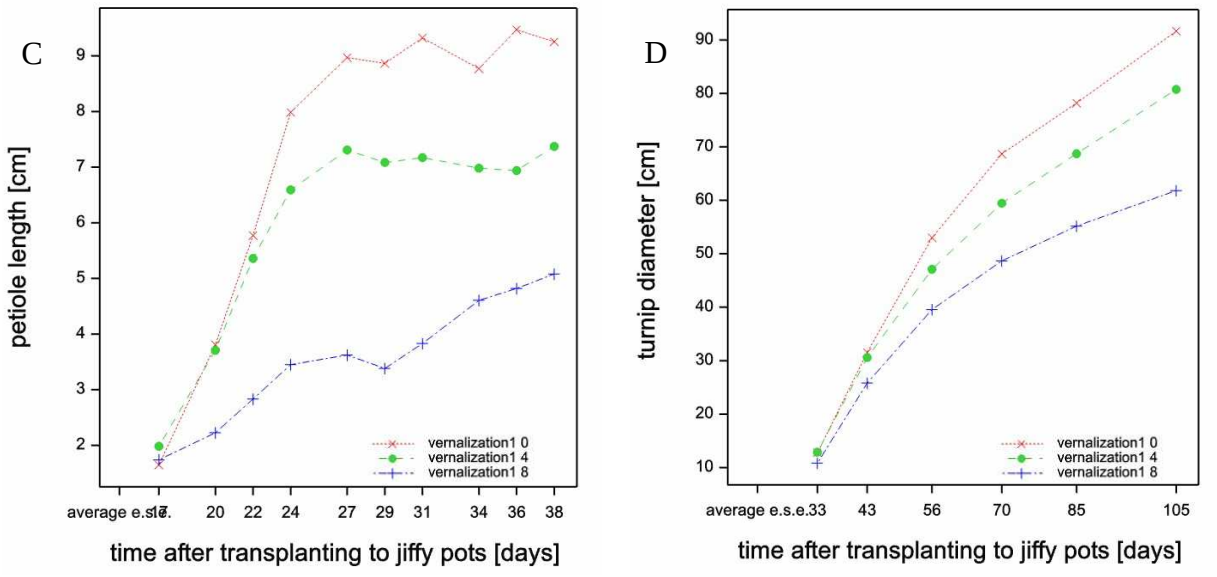


Figure 8. Plots of the development of the lamina length (A), lamina width (B), petiole length (C) and turnip diameter (D) showing the effect of the three vernalization treatments on the growth. Plots A till C show the average of six accessions (T-1283V, W-VT-117, W-VT-115, W-VT-052, YS-143, and PC-105) and plot D shows the average turnip diameter for all the turnip accessions. Four plants per treatment per accession were used as a replicate.

Correlation between morphological traits

Correlations between all morphological traits scored and derived traits calculated from those traits (LI, root/shoot ratio) and p-value of all correlations are included in Appendix VIII. Flowering traits were hardly correlated with other flower traits or other traits. Only the bolting was correlated with the days to flowering (FT).

Turnip traits were correlated very often significantly with other turnip traits (Table 6, Appendix VII). Many of the traits related to the size and shape of the turnip are all correlated with each other: TWi, TD, TSo and TWe. The upper and the lower turnip color are also very tightly correlated, what means that the same color combinations were observed frequent.

Quite some leafy traits appeared to be correlated with other leafy traits or with turnip traits. LEs is significantly correlated with LL, LS, LTr LWi and PW. The PW is also correlated with the LTr, LWi, LCo and the LI. Other interesting correlations are LI – LTr and LWi – Lco. The correlations between a leaf trait and a turnip trait are mostly related to the turnip color or to the number of leaves at the moment of flowering (Appendix VIII)

Table 6. Number of significant correlations (p = 0.05) between and within groups of traits.

# of correlations	Flower	Leafy	Turnip
Flower	1		
Leafy	2	17	
Turnip	6	18	19

A graphic restitution of the correlations between traits is given by the PCA plot in Figure 9. The first two principle components represent only 33% of the variation. Three real distinct groups with traits are present which are indicated with red circles in Figure 9. One cluster contains only turnip traits: TWe, TWi and TD. Also a clear cluster with leaf traits is present, consisting of LW, PW, LL Les and LTr. The last cluster consists of turnip, leaf and flower traits: TSh, TSs, LA and FS.

The same three clusters were also observed when data of only one treatment was used. So vernalization had similar effect on all the traits within one cluster. All other traits not grouped in one of these clusters showed very variable correlations relative to each other for the different vernalization treatments (data not shown)

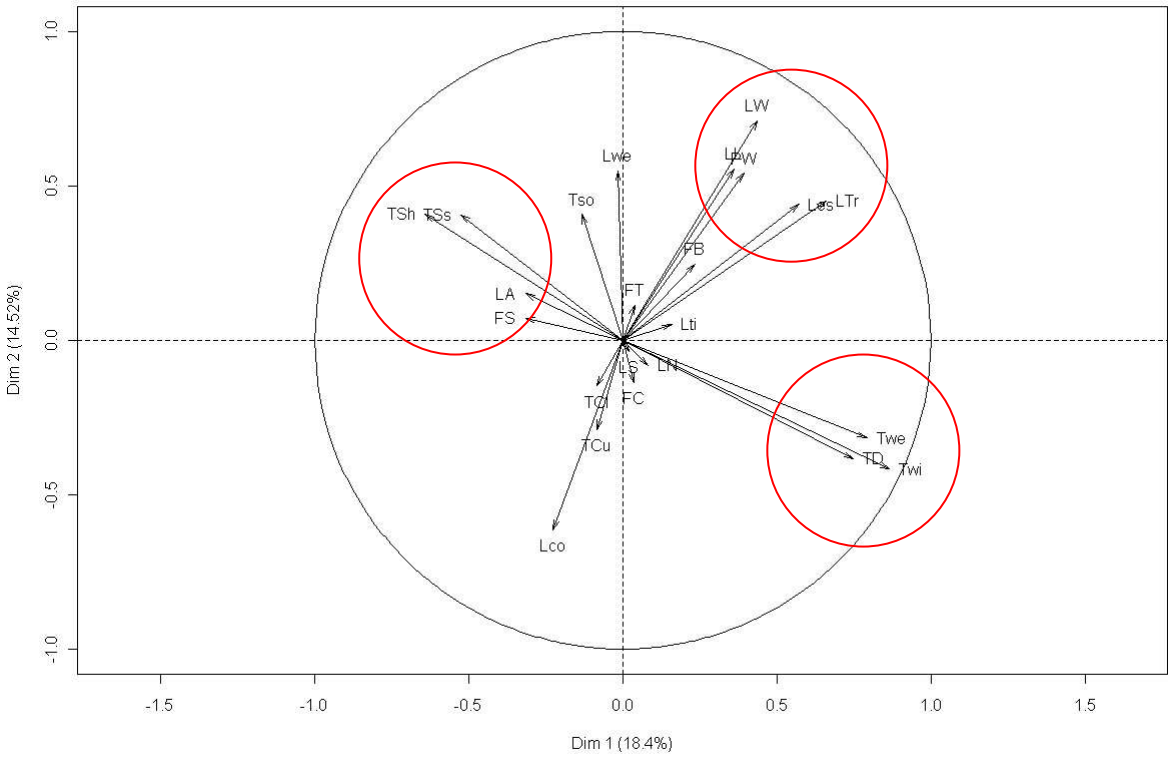


Figure 9. PCA plot of all traits based on all three treatments. Dim 1 and Dim 2 on the X and Y axis represent the amount of variation explained by either the first or the second principle component. Between brackets the total amount of variation explained by each principle component is displayed. The red circles highlight the clusters of traits. For abbreviation of traits, see Table 2.

Association mapping

Number of marker loci associated with traits at a test level of 0.01 was given for the two used approaches, GLM and MLM in Table 7. For this study, profiles of 40 SSR markers were used, based on DNA samples from mixtures of ten plants per accession. For the 12 new accessions, the DNA was a mixture of four plants per accession. All accessions of which phenotypic data was obtained were used, so both turnip and non turnip accessions. For nearly all traits, the number of associated SSR markers decreased after including a correction for random effects in the model (MLM). The number of associated loci per trait ranged between 2 and 11 in the GLM and between 0 and 5 in the MLM (Table 7). Every trait had at least two significant associations in the GLM. For only 15 traits a significant association with one or more markers was revealed by using the MLM.

The same marker-trait associations were only revealed under different vernalization treatments for a limited number of traits under the assumption of the GLM (Table 7). Only two of these multiple occurrence of a marker-trait association were also observed using the MLM (TWi - BRMS-050 and TSo – BR384t). BRMS-050 was also associated with TWe_0, TWe_4, TD_8, LWe_4, PW_0, Les_0, Les_4 and FS_4. BR384t appeared to have also an association with TWi_0, LN_0 and FB_8. A complete overview of all significant marker-trait associations under both the models can be found in Appendix IX.

Table 7. Overview of number of loci significantly associated with the 24 traits studied (P<0.01). Two models were used: GLM (assuming a linear effect of the marker corrected for the population structure) and MLM (a GLM model which is corrected for random effects). Markers for which a trait-marker association was detected for more than one vernalization treatment are shown on the right side of this table.

	# of markers per model		markers in common for the three different vernalization treatments	
Trait name	**GLM**	**MLM**	**GLM**	**MLM**
Turnip width	7	2	BRMS-042-2t, GOL3, BRMS-050	BRMS-050
Turnip weight	6	3	BRMS-042-2t, GOL3, BRMS-050, nw_60	-
Turnip shape	4	2	-	-
Turnip swelling onset	6	5	BR343t, BR384t	BR384t
Turnip color upper part	9	1	BRH04D11flc2t, BRMS-043t, Na12A01m, Na12H07t, Br326t	-
Turnip color lower part	7	0	BRH04D11flc2t, BRH80C09flc3, BRMS-043t	-
Turnip growing depth	4	0	-	-

	# of markers per model		markers in common for the three different vernalization treatments	
Trait name	**GLM**	**MLM**	**GLM**	**MLM**
Turnip smoothness	3	0	-	-
Flowering time	5	4	BRH04D11flc2t	-
Bolting	3	0	-	-
Flower shape	5	0	-	-
Flower color	2	3	-	-
Lamina length	7	0	Na12A01m, Ra2A01, Ra2E12t, BRMS-042-2t	-
Lamina width	7	1	BRH80C09flc3, Ra2E12t	-
Petiole width	4	2	-	-
Leaf erectness	6	0	-	-
Leaf color	3	0	-	-
Leaf surface	5	0	-	-
Leaf trichomes	4	3	-	-
Leaf tip shape	3	1	-	-
Leaf edge shape	6	4	Br308t, BRMS-034t, BRMS-050, Na12A01m	-
Lamina attitude	6	1	-	-
Number of leaves	11	1	BRMS-042-2t	-
Leaf and stem weight	5	2	-	-
Total number of found association	**128**	**35**	**29**	**2**

To investigate the effect of the absence or presence of a allele, the distribution of a trait is plotted in absence or presence of the allele (Figure 10). Four significant marker trait associations were selected for further study: BRH04D11flc2t_204 - FT_0, GOL3_259 - TWi_0, BRH04D11flc2t _244 - TCl_4 and BR321_152 - TCu_0. BRH04D11flc2t_204 was selected because the already in literature described relation between this marker and flowering time (Lou et al. 2007). GOL3_259 was chosen because this allele appeared to be correlated with turnip width and turnip weight for several vernalization treatments. An other allele of the marker BRH04D11flc2t was selected because the association with the turnip color and this allele was unexpected. BR321_152 – Tcu_0 was chosen because BR321 was one of the markers which had only a few significant marker-trait associations. The average flowering time increased in absence of the allele BRH04D11flc2t_204 from 136 days to 167 days. This main effect was mainly due to the European subgroup (P_II) since the Asian subgroup showed the opposite trend (Figure 10A). So the distribution of this trait was really different

for the two sub populations but the correction for sub populations in the GLM procedure corrects for this. All the other plotted traits show also that the presence of the associated allele gives different trait scores.

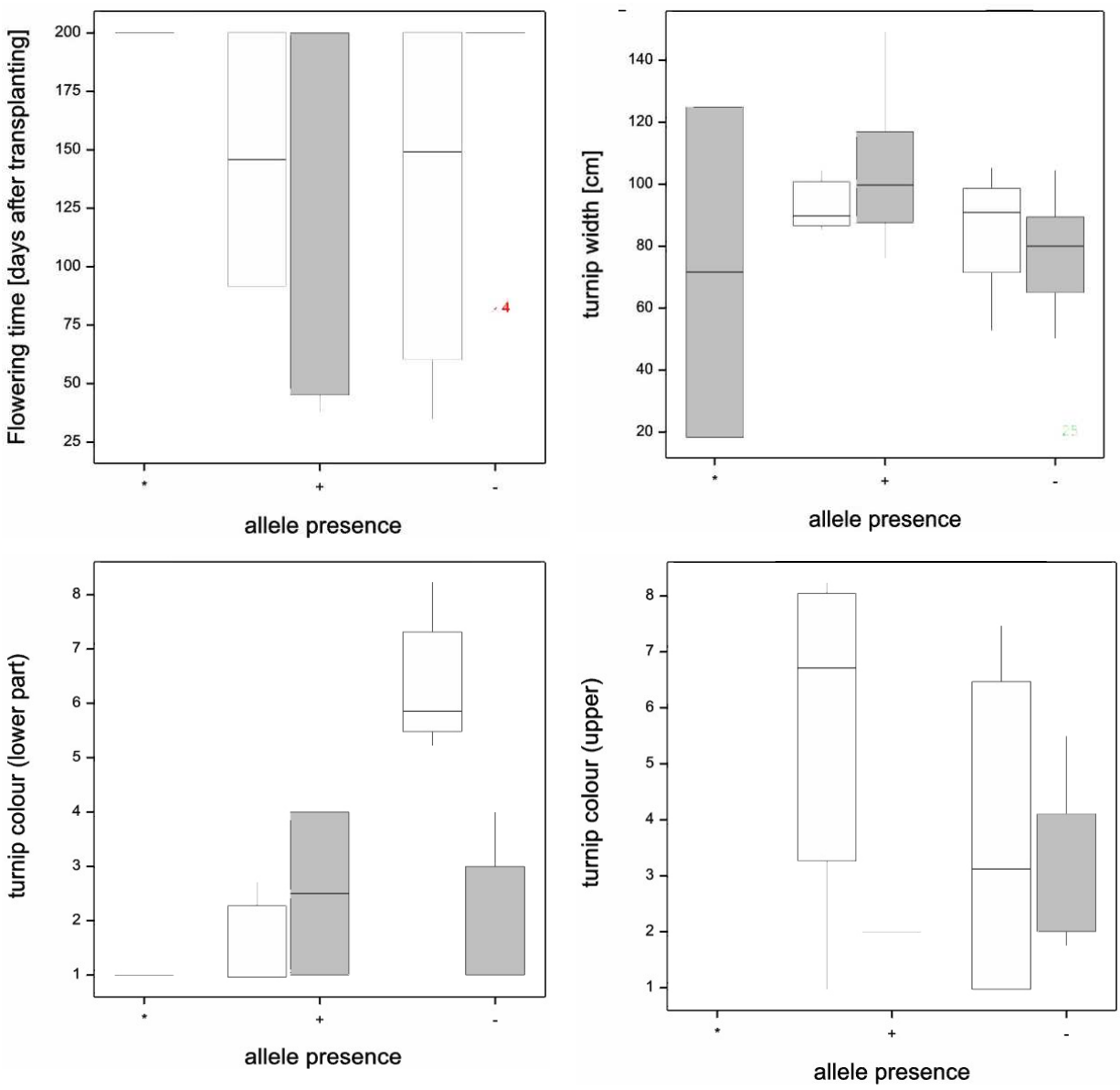


Figure 10. Distribution of traits in the absence or presence of the associated allele: BRH04D11flc2t_204 - FT_0 (A), GOL3_259 - TWi_0 (B), BRH04D11flc2t _244 - TCl_4 (C) and BR321_152 - TCu_0 (D). The absence (-) or presence (+) of the alleles is plotted on the X-axis. In case the information about the allele was unknown for one or more accessions, a asterisk is shown on the X-axis. Traits are plotted per sub population separately. The white bars represent P_I (Asian turnips, n = 25) and the grey bars indicate P_II (European turnips, n = 41).

Discussion

Large differences were observed in the marker data based on the DNA isolated in this study (four plants/accession) and the DNA isolated in the study of Zhao et al. (2010a) (ten plants/accession). The large differences between the DNA stock of Zhao et al. (2010a) and the DNA stock, isolated in this study might be due to heterogeneity of the accessions, since most *B. rapa* species are self incompatible. Especially when alleles are occurring in less than half of the accessions, many differences were observed (Table 4). The reason for this might be that these rarer alleles are not detected because the DNA of four plants was pooled in this study, instead of the DNA of ten plants, so statistically less chance to detect. If there were a high frequency of rare alleles for the four selected markers in the collection of accessions, one would expect that pooling ten instead of four plants result in a higher total number of alleles. As the total number of detected alleles is nearly the same; 440 versus 431, it is concluded that there are hardly any rare alleles present in the products of the four selected markers. So rare alleles are not the reason for the mismatches between the two DNA pools.

Calculation of the population structure based on both the scoring of my study (DNA of accessions present in study of Zhao et al. (2010a) were used if possible) and the study of Zhao et al. (2010a) (results of 11 SSR markers) results in only a few differences in population structure compared to the study of Zhao et al. (2007) (Table 5). In my study nine accessions were added of which the DNA was only pooled for four plants and 29 more markers were used, but the same population structure was obtained. This indicated that although the results are obtained by a different person and with a denser coverage of markers, the results are the same, which eliminates the explanation that the observed difference between the two DNA stocks might be caused by the different person who did the work.

Most probably the differences are caused by the heterogeneity of the plants. Since *B. rapa* is self incompatible, all accessions are heterogeneous. By using the pooled DNA of ten plants compared to only four, only two more rare alleles were observed but many differences are present although the total number of detected alleles was the same (Table 4). Heterogeneity is therefore the most probable reason for the differences between the two DNA pools. Therefore the isolated DNA of this study is dependable and can be used for further studies.

At the moment, much effort is invested in obtaining fixed lines of *B. rapa* accessions (Bonnema, personal communication). Using fixed lines for association studies would

eliminate the problems related to heterogeneous DNA and is therefore preferred over working with heterogeneous lines.

The result of the population structure is really comparable to the studies of Zhao et al. (2007) (Table 1). Zhao et al (2007) analyzed 160 accessions, all from the WUR collection and used 233 polymorphic AFLP marker profiles of one single plant per accession. Less markers were used in my study compared to Zhao et al. (2007), but the DNA was pooled for either ten (accessions present in study of Zhao et al. (2010)) or four (new lines). Differences between this study and the other two studies are indicated with a 'a' in the last column. Only three accessions are assigned to a different sub population. FT-001 is a Dutch fodder turnip accession but is now assigned to the group of Asian turnips (membership fraction P_I = 0.678). In the data of Zhao et al. (2007), this turnip fits very well in the European turnip group (membership_fraction 2 = 0.808). The fraction explained variance by P_I is not very high, so the limited number of markers (n = 40) are most probably the reason that FT-001 is assigned to a different sub population. It is unexpected that this Dutch accession belongs to the sub population with Asian turnips.

VT-137 has also a membership fraction for P_I of only 0.543, which is just higher than 0.5. This Turnip from Uzbekistan may have a high level of genetic admixture between European and Asian gene pools, which gives both populations nearly equal probabilities. It is also possible that the numbers of markers used in this study was not sufficient. FT-056 was assigned to the group of the Chinese cabbages in the study of Zhao et al. (2007), which is unlikely. By looking principally to only turnip accessions, the program will force this accession to one of the two sub-population. FT-056 had also a very high membership fraction for the European turnip (Zhao et al. 2007), so this accession is now assigned to the European group. This accession was also grouped in the European turnips in the study of Del Carpio (2010) in which also a high number of markers was used (n = 412). In conclusion, calculating the population structure based on only a limited number of markers for pooled DNA (the approach in this study) gives comparable results to a population structure with a high number of markers on DNA of single plants.

It would have been better to run the data of the study of Zhao et al. (2007) again, but now only including the accessions which are also part of this study. This would result in a better comparison between the two studies.

The non turnip accessions are forced by the program to one of the two sub groups although they were previously assigned to a different sub group. This outcome is not reliable due to the limited number of non turnip accessions in this study.

Most of the SM Lines from Russia were assigned to P_II, but the structure-membership fractions of P_II were close to 0.5. These might indicate that these lines have a high amount of admixture between the Asian and the European group. It is advised to use more markers for these highly admixed lines to assign these lines more precisely to either the Asian or the European turnips.

The distribution of leaf treats was very different for the two subgroups. A number of the turnip traits also had different distributions in the two sub populations. A PCA plot for all the turnip or all leaf traits with all the accessions (all three vernalization treatments combined) should also reveal a separation between the two sub populations. Such a plot is shown in Figure 11 for all the turnip traits. Here it is clearly shown that most of the turnips of the Asian subgroup are grouped together based on only the turnip traits, so the values of the different principle components were really different per subgroup.

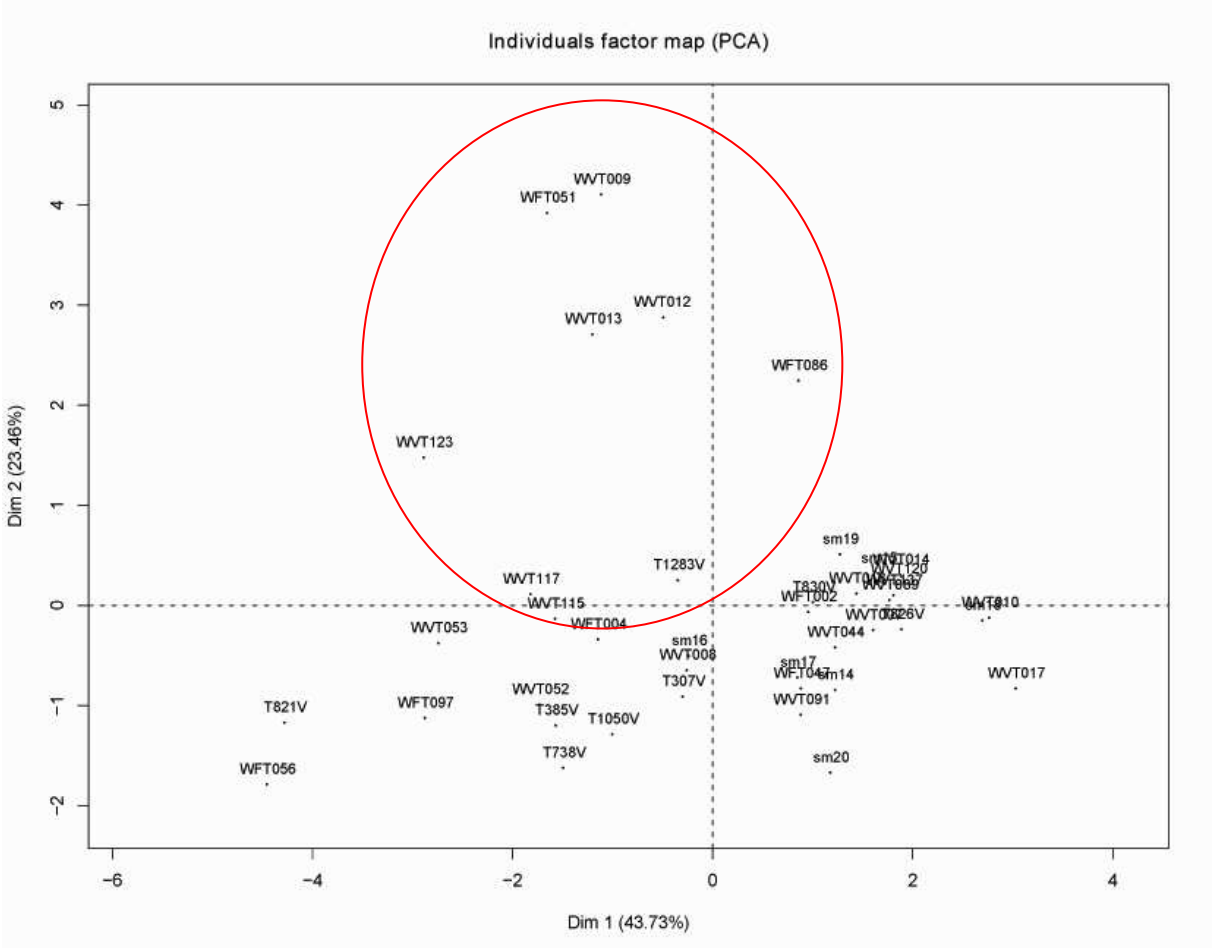


Figure 11. PCA plot in which the individual accessions are shown for only the turnip traits. The majority of the Asian turnips (P_I) is grouped together at the upper side of the plot, as indicated with the red circle. Dim 1 and Dim 2 on the X and Y axis represent the amount of variation explained by either the first or the second principle component. Between brackets the total amount of variation explained by each principle component is displayed.

Effects of vernalization

In agreement with observations by Takahashi et al. (1994), the decrease of flowering time for vernalized *B. rapa* plants was also clearly found (Figure 12) and appeared to be significant (Appendix VII). Many other significant effects of vernalization were found in this study.

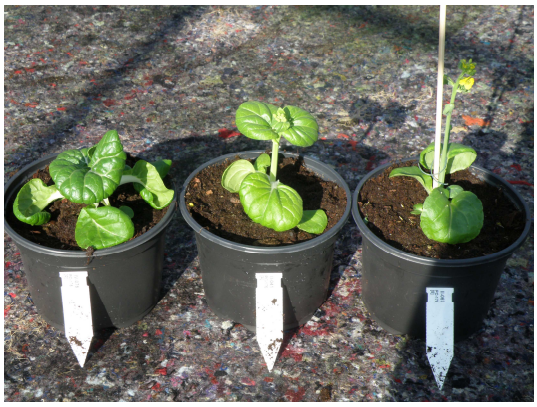


Figure 12. Example of effect of vernalization on the flowering time of PC-175. Left plant is unvernalized, middle vernalized for four weeks and right plant was vernalized for eight weeks. All three plants are from the cultivar PC-175.

The observation that leaves of Arabidopsis are smaller and more erect upon vernalization (Hopkins et al. 2008), was also apparent in *B. rapa*. Some other traits (LTr, LCo and LN) appeared to be influenced by vernalization in *B. rapa.* Vernalized *B. rapa* plants seemed to have less number of leaves compared with non-vernalized plants. This might be due to that the leaf number was counted at the moment of flowering and the vernalized plants took less time to grow till flowering. Leaf color change in vernalized plants, indicated by the decrease in chlorophyll content, may suggest that vernalized plants were less vigorous.

Many turnip traits were proven to be influenced by the vernalization (Appendix VII). It is know that an early cold spill in the spring causes a yield decrease (Lou, personal communication), but the effects of vernalization on turnip traits are not described in literature yet.

Figure 13. Illustration of the correlation between flowering time and turnip size for the cultivar VT-117. Left plant is unvernalized, middle vernalized for four weeks and right plant was vernalized for eight weeks.

Strong correlations between FT and TWe/TWi were observed, resulting in colocolization of FQTL-1 with FLQTL-2 on the top of R02 (Lou et al. 2007). Similar results were found by Raynaud (2010) and Vos (2009) when studying progeny of crosses between accessions which had quite different flowering time. During greenhouse observations this trend was also observed (Figure 13). For all these studies VT-115 was one of the parents. As shown in Table 8, for this specific accession the correlation between FT and TWe/TWi under different vernalizations is high. This specific combination of traits is not present in many other turnip accessions, resulting in the absence of a significant correlation between FT and TWe/TWi if a broader set of accessions is studied under different vernalization treatments (0, 4 and 8 weeks vernalization). (Table 13).

Table 8. Average TWe, TWi and FT for VT-115 for all three vernalization treatments. Correlation coefficient between TWe - FT and TWi – FT are respectively 0.76 and 69.

	Vernalization [weeks]	TWe	TWi	FT
W-VT-115	0	308	100	58.75
	4	40	40	32.75
	8	22	26.8	48.5

It is more likely that the larger turnip size of unvernalized turnips is caused by the earlier turnip swelling onset (TSo). Since there is a strong correlation between TSo and TWe/TWi (Table 13) and the swelling onset is significantly delayed by vernalization (Table 12) the delayed TSo in vernalized plants causes a longer growing period for the turnips so they can grow bigger. The difference in turning swelling onset is caused by the differences between genotypes. The growing rate of the turnip diameter is also influenced by the vernalization (Figure 8D), so this factor will also have influence on the final turnip width and weight. A few very striking correlations concerning the turnip traits are the correlation TSh – FSh, LN – TWe, LN – TWi and TC(l/u) – LEs. Expected correlations like FT – FN and FT – Twe/TWi were not observed. Pooling the three vernalization treatments may be a reason for the lack of these expected correlations. Additionally calculation the correlations for each vernalization treatment separately would have been better. By using ordinal scales, less accurate data is achieved and therefore the chance on false positive correlations is higher.

Comparing the results of the PCA plot obtained by R-package with the correlations obtained by Genstat, a striking difference was observed. Some of the traits very close correlated in the PCA are not significantly correlated at all in the output of Genstat (Figure 9, Appendix VII). TSh and TSs are the best example of this. They are even in all the vernalization treatments close related (data not show), but Genstat calculates only a correlation coefficient of 0.13 (P = 0.29) (Appendix VII). After double checking the data, this discrepancy still remained. The only explanation is that both programs deal in a different way with missing values and this causes differences in the correlations.

Association mapping

In total 128 significant trait-allele associations were found between 278 alleles from 40 SSR markers and 23 traits (Table 7). For most of the markers more than one trait was found to be associated per marker and even more than on trait found was to be associated per allele sometimes (Appendix IX). Because BRH04D11flc2t is designed based on a well studied flowering time gene BrFLC2 (Kim et al. 2007; Zhao et al. 2010b) ,it is used as an example to illustrate the outcome of this study. BRH04D11flc2t was found to be correlated with a flowering QTL (Lou et al. 2007) and *BrFLC2* was suggested as a candidate gene for this QTL (Zhao et al. 2010b).

In this study, when using GLM approach beside flowering time in the unvernalized plants (FT_0) and eight weeks vernalized plants (FT_8), BRH04D11flc2t was also associated

with LN_0, LA_8, LTr_0, TD_4, TSh_4, LWe_4, TWi_4, FB_4, TCl_0, TCl_4, TCu_4, TCu_8 and TSo_8. Since a couple of these traits (TWi, LN) are found to be related to this flowering locus in both this study and by Lou et al. (2007), it is suspected that this *BrFLC2* candidate gene is also co-regulating many other traits, especially traits influenced by vernalization (Zhao et al. 2010b). However, using the more restricted MLM approach, only two traits are still correlated with BRH04D11flc2t: LA_8 and TSo_4. Significant association with flowering time was no longer observed.

This example shows the complexity of the associating mapping output; the linkage of a certain marker with a certain trait found in previous studies (Lou et al. 2007; Vos 2009; Zhao et al. 2010b; Zhao et al. 2007) is confirmed in this study under the GLM assumption, but for every marker many more association with high diverse traits are found. Using the MLM shows no longer a confirmation of the already described marker trait association, therefore it is doubtful whether MLM is the right approach.

In case markers are found to be associated with a trait under several vernalization treatments by both models (BRMS-050 – TWi and BR383t – Tso), it is likely that these marker-trait associations show the true positions of a gene. To investigate whether all the associations found with only the GLM approach are valid, the distribution of some traits was plotted in case of absence or presence of the associated allele (Figure 10). The absence or presence of the alleles resulted in a quite different distribution of the trait. This was even the case for unexpected associations like the association between turnip color (TCl_4) and BRH04D11flc2t (Figure 10), so it is likely that all the detected marker trait observations are indicating at least a genomic region related to trait. Because lots of marker-trait associations were detected with a quite low density of random markers , this could suggest that the LD is the investigated turnip accessions stretches over a very long region. Testing markers of interest with a found association on a population would reveal more information about the effect of an allele on the trait.

Since most of the accessions were turnips from only two sub populations the used accessions may contain too less genotypic variation which is not sufficient compensated by adding the population structure into the model. If no data is used from other morphotypes, still population cofoundings occur due to the population structure. This would imply that although a trend in the trait distribution of an associated allele is found (like is shown in Figure 10), this would not have been significant if the used model would be correcting for the limited genotypic variation in a more suitable way.

Some studies suggest that an appropriate number of markers was used (D'hoop et al. 2008; Zhu et al. 2008), especially because also a couple of candidate genes is used and most markers were multiallelic, but other studies suggest that a higher number of markers or even complete sequence data of regions of interest is needed to get reliable results (Hansen et al. 2001). Because on average only four markers per linkage group were used in this study and the mapping position of some markers are still unknown, the association between the chromosomal region and the trait could not be precisely located. If a higher amount of markers with known map positions is used, more precise information will become available about the association.

Of the three turnip growth candidate genes, only marker nw_60 was associated with turnip traits TWe, TWi and TCu. Like all the other markers, nw_60 was also related to many other traits (Appendix IX). Comparing these association mapping results with the outcome of the correlation test, it is observed that most of these traits are significantly correlated with each other.

Conclusions

Using only 40 SSR markers was already sufficient to obtain a reliable population structure. Pooling the plants of only four instead of 10 plants had not many influence on the detection of rare alleles, because rare alleles were not very abundant in the screened accessions. The effects of vernalization was significant for all turnip traits. Vernalized turnips had a smaller diameter, lower weight and more wrinkled. The distribution of many flowering and leaf traits was also significantly different under the different vernalization treatments. Many significant correlations were found between the different traits, but a correlation between flowering time and turnip weight was not detected. 128 significant marker-trait associations were detected under the assumption of the MLM and 35 under the assumption of the GLM model.

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Wageningen, November 2010
Joan

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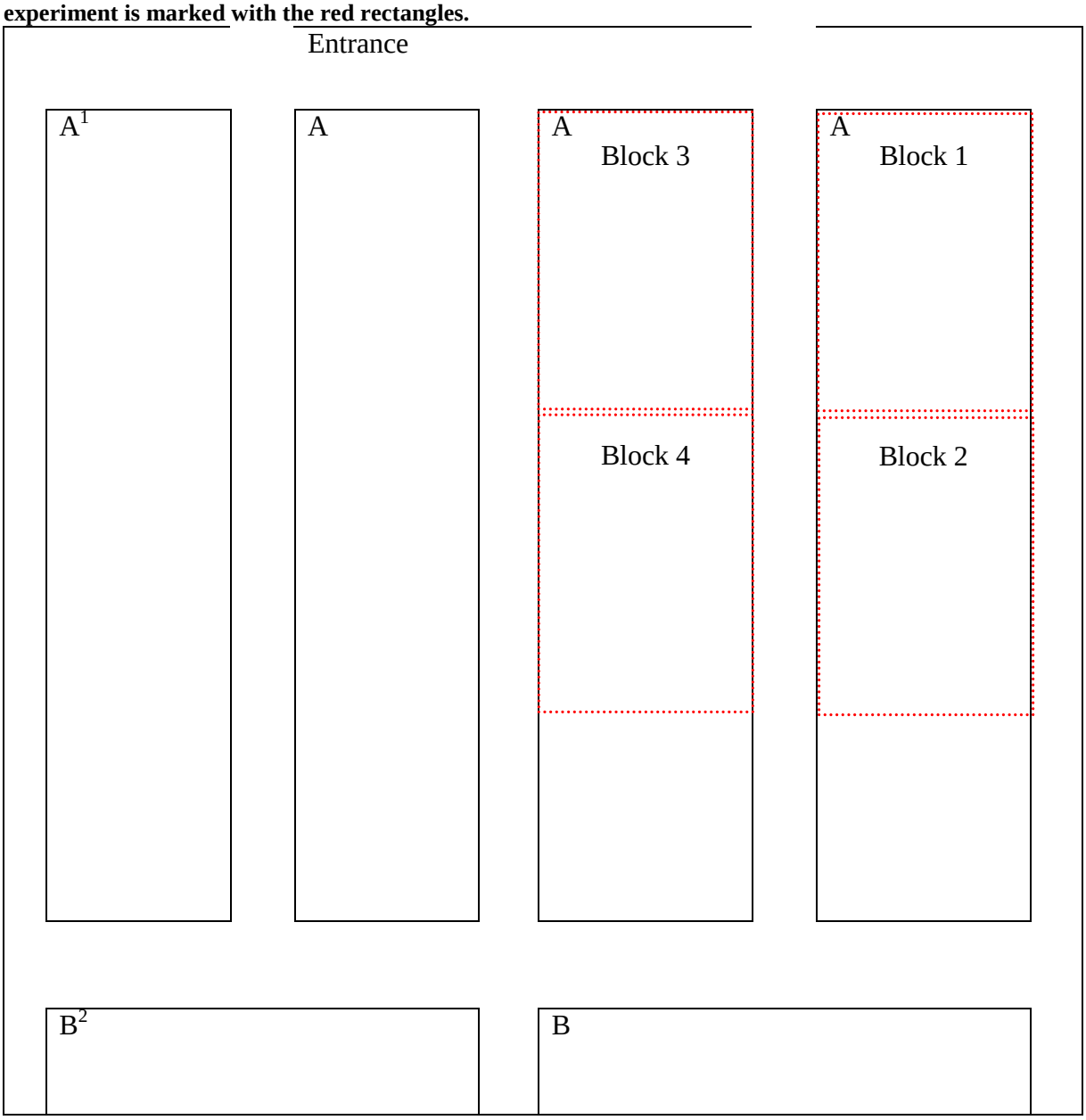
Appendix I: Layout of the experiment

Overview spacing of experiment within the greenhouse compartment (Compartment 11, Nergena Greenhouse, Bornsesteeg 10 Wageningen).

Table 9. Overview greenhouse compartment 11. The space in this compartment which is used for my experiment is marked with the red rectangles.



[1]Large tables (2m * 11m)
[2]Small tables in the back of the compartment (1m * 4m)

Each block is organized in the same order; Since half of the table can be reached from one side, the table is widthwise splitted in two for continuation of the plot numbering (Figure 14).

1 15 2	**2** 35 1	**3** 7 3	**4** 38 3				
5 23 2	**6** 20 3	**7** 22 3	**8** 40 1	**123** 32 3	**122** 39 3	**121** 37 2	
9 3 3	**10** 27 1	**11** 27 3	**12** 1 3	**120** 4 1	**119** 41 2	**118** 16 2	**117** 29 3
13 30 2	**14** 16 1	**15** 32 1	**16** 38 1	**116** 12 2	**115** 2 1	**114** 26 3	**113** 29 2
17 25 2	**18** 12 3	**19** 28 3	**20** 7 1	**112** 24 1	**111** 31 2	**110** 7 2	**109** 28 2
21 40 3	**22** 4 3	**23** 1 2	**24** 32 2	**108** 6 1	**107** 31 3	**106** 11 1	**105** 39 1
25 36 3	**26** 23 3	**27** 34 1	**28** 21 3	**104** 19 2	**103** 12 1	**102** 18 3	**101** 41 3
29 39 2	**30** 21 1	**31** 8 3	**11** 2	**100** 3 2	**99** 27 2	**98** 36 1	**97** 41 1
33 9 1	**34** 25 3	**35** 5 2	**36** 29 1	**96** 26 2	**95** 20 2	**94** 18 2	**93** 6 3
37 3 1	**38** 9 3	**39** 31 1	**40** 37 1	**92** 13 1	**91** 15 3	**90** 6 2	**89** 13 3
41 20 1	**42** 15 1	**43** 24 2	**44** 22 1	**88** 34 2	**87** 40 2	**86** 26 1	**85** 24 3
45 30 3	**46** 14 3	**47** 28 1	**48** 33 2	**84** 10 2	**83** 14 1	**82** 35 2	**81** 19 1
49 22 2	**50** 11 3	**51** 33 3	**52** 17 1	**80** 4 2	**79** 35 3	**78** 10 1	**77** 21 2
53 30 1	**54** 16 3	**55** 23 1	**56** 2 3	**76** 17 2	**75** 5 3	**74** 14 2	**73** 8 2
57 36 2	**58** 19 3	**59** 25 1	**60** 10 3	**72** 13 2	**71** 37 3	**70** 18 1	**69** 2 2

61	**62**	**63**	**64**	**68**	**67**	**66**	**65**
33	5	8	17	34	1	38	9
1	1	1	3	3	1	2	2

Figure 14. Layout of block 1. The number in bold are the plot numbers. The number below the plot number is the cultivar and the last number indicates the treatment (1 = 8 weeks vernalization, 2 = 4 weeks vernalization, 3 = no vernalization).

The complete division of the all the plants over the four blocks is shown in Table 10.

Table 10. Complete overview of which plant is in what plot number in which block. The upper number of each plot number is the accession number (Table 1) and the lower number the treatment (1 = 8 weeks vernalization, 2 = 4 weeks vernalization, 3 = no vernalization)

Plot nr	block 1	block2	block 3	block 4	Plot nr	block 1	block2	block 3	block 4
1	15	7	39	5	*20*	7	23	14	29
	2	3	2	2		1	2	3	2
2	35	30	11	4	*21*	40	3	12	28
	1	1	3	1		3	1	1	3
3	7	23	22	13	*22*	4	7	41	30
	3	3	3	2		3	2	3	3
4	38	17	15	17	*23*	1	39	19	19
	3	1	2	1		2	3	3	1
5	23	2	32	36	*24*	32	11	3	3
	2	2	1	2		2	3	2	3
6	20	8	30	29	*25*	36	23	32	11
	3	2	2	3		3	1	2	3
7	22	26	30	27	*26*	23	33	10	18
	3	1	3	1		3	2	1	3
8	40	1	39	20	*27*	34	10	40	9
	1	2	1	2		1	3	1	1
9	3	6	2	27	*28*	21	19	20	22
	3	1	2	2		3	3	2	2
10	27	36	17	1	*29*	39	28	19	13
	1	2	3	1		2	1	1	3
11	27	18	1	23	*30*	21	5	37	10
	3	3	1	2		1	2	3	3
12	1	8	38	16	*31*	8	17	14	36
	3	3	1	2		3	3	2	3
13	30	34	29	11	*32*	11	4	27	25
	2	2	1	1		2	1	2	3
14	16	30	30	32	*33*	9	19	23	40
	1	2	1	2		1	1	3	2
15	32	24	22	14	*34*	25	26	26	10
	1	3	1	1		3	2	3	2
16	38	20	40	14	*35*	5	4	1	16
	1	3	2	3		2	2	3	3
17	25	13	4	2	*36*	29	14	10	1
	2	2	1	2		1	3	2	2
18	12	28	16	36	*37*	3	39	34	7
	3	3	3	1		1	1	1	2
19	28	28	40	21	*38*	9	25	8	38
	3	2	3	3		3	2	2	1

Plot nr	block 1	block2	block 3	block 4
39	31	6	34	34
	1	2	3	2
40	37	12	37	8
	1	1	2	1
41	20	38	24	11
	1	2	2	2
42	15	21	16	29
	1	2	2	1
43	24	37	21	20
	2	1	3	3
44	22	24	5	39
	1	2	1	2
45	30	2	21	31
	3	3	1	3
46	14	29	28	31
	3	3	3	2
47	28	31	7	24
	1	3	2	1
48	33	29	7	6
	2	1	1	1
49	22	37	15	18
	2	3	1	1
50	11	31	25	9
	3	1	2	3
51	33	32	4	40
	3	2	2	3
52	17	20	6	21
	1	2	3	2
53	30	41	12	26
	1	1	3	1
54	16	35	33	16
	3	3	2	1
55	23	36	29	34
	1	1	3	3
56	2	3	15	30
	3	3	3	2
57	36	1	9	3
	2	1	3	1
58	19	33	35	32
	3	3	1	1
59	25	15	22	6
	1	1	2	2
60	10	9	11	38
	3	3	1	2
61	33	4	20	21
	1	3	3	1
62	5	18	18	12
	1	2	3	1
63	8	41	10	6
	1	2	3	3

Plot nr	block 1	block2	block 3	block 4
64	17	22	33	1
	3	1	1	3
65	9	24	24	17
	2	1	1	2
66	38	35	32	12
	2	2	3	3
67	1	38	8	8
	1	1	3	3
68	34	22	6	9
	3	3	2	2
69	2	27	9	34
	2	3	1	1
70	18	22	1	8
	1	2	2	2
71	37	15	35	24
	3	3	2	2
72	13	19	41	37
	2	2	2	2
73	8	21	27	5
	2	1	3	1
74	14	18	7	25
	2	1	3	1
75	5	16	25	3
	3	3	3	2
76	17	32	18	38
	2	1	2	3
77	21	13	24	37
	2	1	3	3
78	10	9	17	12
	1	2	2	2
79	35	30	21	37
	3	3	2	1
80	4	20	26	18
	2	1	1	2
81	19	36	11	28
	1	3	2	1
82	35	40	9	17
	2	2	2	3
83	14	5	33	2
	1	3	3	1
84	10	21	2	28
	2	3	3	2
85	24	27	39	23
	3	1	3	3
86	26	12	34	24
	1	3	2	3
87	40	10	3	7
	2	2	1	1
88	34	27	13	14
	2	2	2	2

Plot nr	block 1	block2	block 3	block 4
89	13	11	41	2
	3	2	1	3
90	6	29	38	19
	2	2	3	3
91	15	11	35	15
	3	1	3	3
92	13	17	5	19
	1	2	3	2
93	6	10	13	7
	3	1	1	3
94	18	9	36	23
	2	1	1	1
95	20	40	5	15
	2	1	2	2
96	26	25	29	30
	2	1	2	1
97	41	12	4	13
	1	2	3	1
98	36	5	37	4
	1	1	1	2
99	27	41	28	26
	2	3	1	3
100	3	1	14	31
	2	3	1	1
101	41	34	8	22
	3	3	1	3
102	18	6	25	15
	3	3	1	1
103	12	8	19	4
	1	1	2	3
104	19	16	36	10
	2	2	3	1
105	39	7	31	32
	1	1	3	3
106	11	26	27	5
	1	3	1	3
107	31	38	31	35
	3	3	1	3
108	6	39	6	40
	1	2	1	1
109	28	35	31	41
	2	1	2	3
110	7	32	38	25
	2	3	2	2
111	31	15	12	22
	2	2	2	1
112	24	2	28	20
	1	1	2	1
113	29	3	23	26
	2	2	2	2

Plot nr	block 1	block2	block 3	block 4
114	26	40	26	39
	3	3	2	1
115	2	13	36	33
	1	3	2	2
116	12	25	13	33
	2	3	3	1
117	29	16	20	27
	3	1	1	3
118	16	14	18	33
	2	1	1	3
119	41	31	16	35
	2	2	1	2
120	4	14	23	41
	1	2	1	1
121	37	33	17	35
	2	1	1	1
122	39	34	3	41
	3	1	3	2
123	32	37	2	39
	3	2	1	3

Appendix II. Pictorial representations of all ordinal scales.

1 = oval

2= round to oval

3= round

4 = oval to long

5 = long

6 = very long

Figure 15. Ordinal scale for scoring the turnip shape.

1 = white (lower half)

2 = greenish (upper half)

3 = cream

4 = yellow\light brown

5 = light purple/pink (lower half)

6 = purple (lower half)

7 = dark purple (upper half)

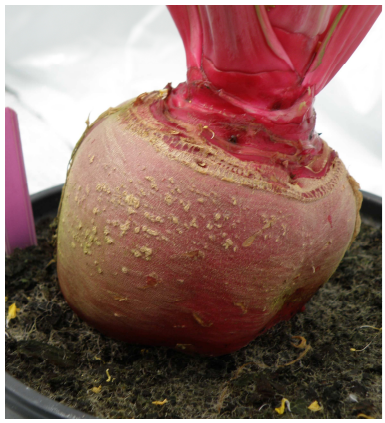
8 = red (lover half)

9 = dark red (upper half)

Figure 16. Pictures of the nine different scoring colors for turnips. These nine colors are scored twice per turnip: once for the upper half and once for the lower half.

1 = complete underground

2 = intermediate



3 = complete above ground

Figure 17. Three classes of growing depth of turnips

1 = very smooth 2 = intermediate 3 = very wrinkled

Figure 18. Ordinal scale for the classification of the three different levels of smoothness of the turnip scored in this experiment.

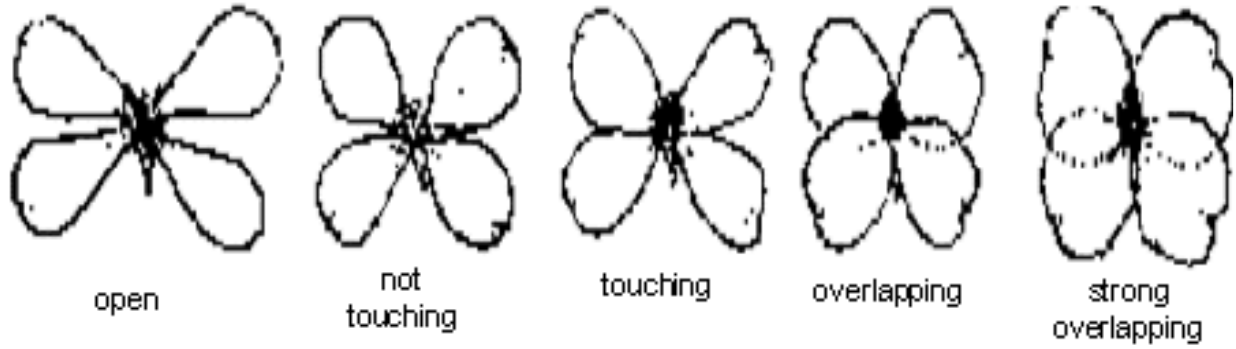


Figure 19. Different shapes of *B. rapa* flowers.

1 = orange 2 = dark yellow 3 = yellow 4 = medium yellow 5 = light yellow

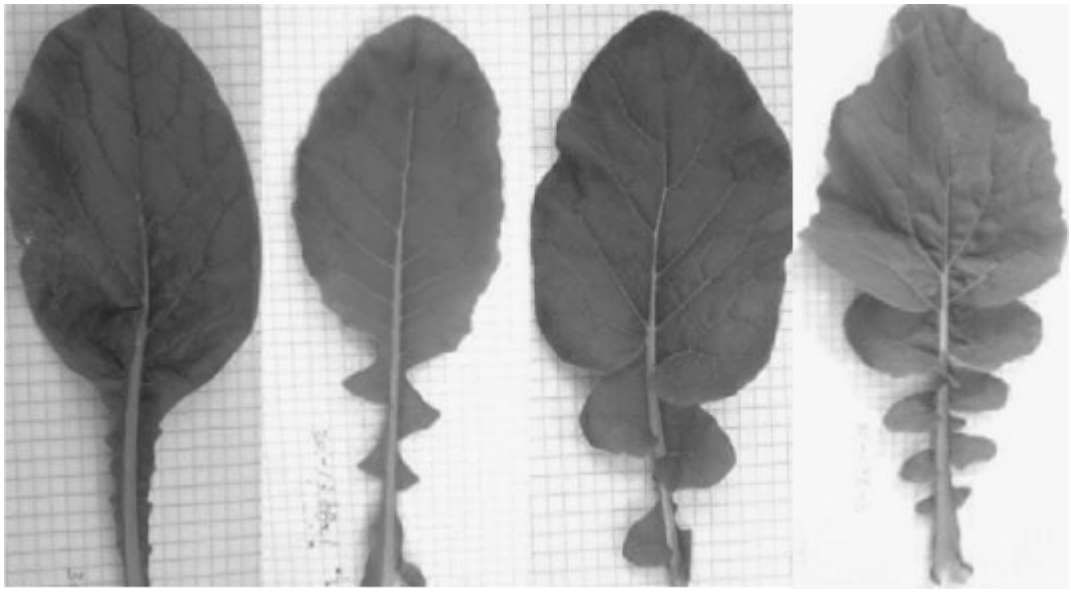
Figure 20. The five different scoring colors of the flower color.

1 = very smooth 2 = intermediate 3 = very wrinkled

Figure 21. Different classes of smoothness of the leaf surface.

1 = very sharp 2 = intermediate 3 = round

Figure 22. Classification of the form of the leaf tip.



1 = entire 2 = slightly serrated 3 = intermediate serrated 4 = very serrated

Figure 23. Four different classes of leaf edge serrating (Lou et al. 2007).

1 = extreme deep down curling

2 = deep down curling

3 = little down curling

4 = straight

5 = little up curling

Figure 24. Different classes of the lamina attitude used for scoring.

Appendix III: DNA isolation protocol

RETCH 1.3 protocol (Gert van Arkel & Maarten Nijenhuis)

I always use this protocol for young/ not mature plant material. I have only use these on non frozen, fresh young tissue.

Day before harvesting the plant material:

Put the 96 micronic tubes in a blue holder and mark them well

Add 2 steel balls to every tube (*simple device ask Johan*)

Check if there is enough Isolation buffer and other solutions *(volumes)*

DNA isolation day:

Put the water bath on and set it to 65°C
Put the 70% ethanol to the -20°C
Put the isolation buffer at 65°C (pipetting becomes easier)

Put two leaf disc in a tube by closing the tube

Place the already done tubes onto ice leave 2 tubes empty for calibration.

Put the entire blue holder with the tubes into liquid nitrogen

Place the blue holder without the transparent lid into a black and white adapter. The 2 round extensions should be facing one way and fixate in the RETCH machine.

Check the boxes with samples if they are really fixed.

Mill the samples for 30 seconds at 30 Hz for three times and changing the direction of the holders

Put the entire blue holder with the tubes back into liquid nitrogen

Centrifuge the boxes briefly in order to get the powder in the bottom of the tube

Add 400 µl of isolation buffer to the tubes once finished.

Place the blue holder without the transparent lid in the special designed clamp and tight the 4 nuts *(This will prevent that the lids will pop off after incubation at 65°C)*

Incubate at 65°C in a water bath for 60 minutes

Cool the clamp containing the tubes for 15 minutes on ice-water

Work in the fumehood for these steps.

Add 400 µl of chloroform : isoamyalcohol (24 : 1) to the tubes and mix by inversion for 5 minutes
(Machine is in the 1.132 Lab)

Separate the phases by centrifuging at 6000 rpm for 20 minutes

During the centrifuging fill new tubes with 280 µl isopropanol

Pipette 2x 175 µl of the water phase in tube that contains 280 µl isopropanol

Mix the content carefully by inversion *(Look if you can see the something moving inside the solution)*
Pellet the DNA by centrifuging for 8 minutes at 6000 rpm

Discard the supernatant

Add 300 µl of ice cold 70% ethanol

Centrifuge for 5 minutes at 6000 rpm

Discard the supernatant and let the pellet dry overnight in the fumehood

Next day dissolve the pellet in 100 µl of MQ containing 4 µg/ml RNase

Let the tubes containing DNA stand on the lab bench for 3 hours
(The RNase will get rid off the RNA in your DNA sample)

Check the DNA quality of a 1% agarose gel by putting:

2 µl of isolated DNA
8 µl of MilliQ
2 µl of loading buffer

Load 12 µl on the gel

Check the DNA concentration on the NANOdrop

The manual is in the Brassica protocol folder

Concentration: is in ng/µl

260/280: a value of 1.8 is considered as pure DNA values of 2.0 or higher are considered as pure RNA

Lyses buffer **500 ml**

Tris-HCl 1M (pH 7.5)	100 ml
EDTA 0.5 M (pH 8.0)	5 ml
NaCl 5 M	200 ml
MQ	195 ml
CTAB	10 g

Extraction buffer **500 ml**

Tris-HCl 1M (pH 7.5)	50 ml
EDTA 0.5 M (pH 8.0)	5 ml
MQ	445 ml
Sorbitol	31.9 g

Sarkosyl 5%

Isolation buffer **200 ml**

Lyses buffer	84.0 ml
Extraction buffer	84.0 ml
Sarkosyl 5%	33.5 ml
Sodium bisulfide	500 mg

Appendix IV: PCR reaction

First of all you need to book a PCR machine in one of the agenda's for the time you are going to use it

Making a PCR mastermix (this is a mix of: primers, dNTP's, Taq buffer, Taq polymerase and MilliQ)

Get a bucket or tray with ice and *work on ice the entire time.*

Get the next solutions from the -20.

- Primers (foreward and reverse)
- dNTP's
- Super Taq buffer 10X
- Super Taq (polymerase)

Place the Super Taq (polymerase) immediately on ice.

Get a new Eppendorf tube and clearly write down mastermix on it.

When the solutions are thawing make the calculations for the mastermix in your lab journal.

This reaction will be used for 3 µl DNA sample

Mastermix	1X	48X
Primer forward	1.0	48.0
Primer reverse	1.0	48.0
dNTP's	1.0	48.0
Super Taq buffer 10X	2.5	120.0
Super Taq (polymerase)	0.2	9.6
MilliQ	16.3 +	782.4
Total	22.0 µl	

Put 22.0 µl of the mastermix inside a well of a PCR plate.

DNA template and MilliQ are variable; increasing the amount of DNA with 1.0 µl (4.0) will decrease
the amount of MilliQ with 1.0 µl (15.3 µl). *When changing the volume of the MilliQ and DNA the*
mastermix volume changes to so in this example to 21.0 µl. (Sometimes you will need more DNA
for a PCR reaction because otherwise the reaction will not work).

Add the 3.0 µl of DNA template/sample (look at the example Mastermix above.

The total volume inside a well of a PCR plate is now 25 µl.
Heat seal the plate with a seal next to the green sealing machine *(check the seal).*

Appendix V: SSR markers

Table 11. Overview of the SSR markers which are used in this study. The yellow indicated markers are already screened by Zhao *et a.*. (2010a) for most of the accessions. Markers which appeared to be informative are indicated with an asterisk in the column 'labcode'.

Marker	Linkage group (WU-integrated)	Lab code	Position (cM)-WU map	LG-reference map	Labelled primer	Reference	For	Rev
BRMS-043t	3	3*	59.089		IRD800	Suwabe et al. (2002)	GCGATGTTTTTTCTTCAGTGTC	TTAATCCCTACCCACAATTTCC
Na10D09	4	4*		R04, R05	IRD800	Lowe et al. (2003)/ jinsun Kim	AAGAACGTCAAGATCCTCTGC	ACCACCACGGTAGTAGAGCG
BRMS-014	6	6		R06	IRD800	Suwabe et al. (2002)	CCGTAAGGAATATTGAGGCA	TCCCAATTCTCAAACGGTA
BRMS-040	7	7*	23.4		IRD800	Suwabe et al. (2002)	TCGGATTTGCATGTTCCTGACT	CCGATACACAACCAGCCAACTC
Ra2E12t	8	8*	77.732		IRD800	Lowe et al. (2003)/ jinsun Kim	TGTCAGTGTGTCCACTTCGC	AAGAGAAACCCAATAAAGTAGAACC
Na12A01m	6	14*	70.2		IRD800	Lowe et et al. (2003)	GCATGCTCTTGATGAACGAA	GCTTCAACCTCTCAATCGCT
BRMS-018t	7	15	25.7		IRD800	Suwabe et al. (2002)	TCCCACGCCTTCTAGCCTTC	ACCGGAGCTTTTCTGTTGCC
BRMS-051t	9	16*	69.2		IRD700	Suwabe et al. (2002)	GGCCAAGCCACTACTGCTCAGA	GCGGAGAGTGAGGGAGTTATGG
BRMS-033		20		R08	IRD700	Suwabe et al. (2002)	CCTCCTTGTGCTTTCCCTGGAGACG	GCGGAAACGAACACTCCTCCATGT
BRMS-007t	5	23	41.863		IRD700	Suwabe et al. (2002)	AAATTGTTTCTCTTCCCCAT	GTGTTAGGGAGCTGGAGAAT
BRMS-008	3	27	0		IRD700	Suwabe et al. (2002)	AGGACACCAGGCACCATATA	CATTGTTGTCTTGGGAGAGC
BRMS-054	4	30*		R04	IRD800	Suwabe et al. (2002)/Jinsun Kim	CAATCTCTGCAACAAACAAATG	TTCCTCAGCATCAAGTAACCTC
BRMS-088		32*		R08	IRD800	Suwabe et al. (2002)/Jinsun Kim	TATCGGTACTGATTCGCTCTTCAAC	ATCAGGTTGTTTATTTGAGAGCAGATT
BRMS-096	1	33		R01	IRD700	Suwabe et al. (2002)	AGTCGAGATCTCGTTCGTGTCTCCC	AGTCGAGATCTCGTTCGTGTCTCCC

Marker	Linkage group (WU-integrated)	Lab code	Position (cM)-WU map	LG-reference map	Labelled primer	Reference	For	Rev
Na10A08	9	34*		O5,n9	IRD800	Lowe et et al. (2003)	CATGGTTAAAACAATGGCCC	CAAGAAACACCATCATTTCTCA
Na12H09t	2	40	65.149		IRD800	Lowe et et al. (2003)	AGGCGTCTATCTCGAAATGC	CGTTTTTCAGAATCTCGTTGC
Ol12F02	9	46		N9, N15	IRD800	Lowe et et al. (2003)	GGCCCATTGATATGGAGATG	CATTTCTCAATGATGAATAGT
BRMS-031t	1	56	64.409		IRD800	Suwabe et al. 2002	TGCCACCAATGACAATGACACTATC	GATGCACTGGGACCACTTACATTTT
Ol11B05	3	85	64.019		IRD700	Lowe et et al. (2003)	TCGCGACGTTGTTTTGTTC	ACCATCTTCCTCGACCCTG
Ra2A01	7	89*		N7	IRD800	Lowe et et al. (2003)	TTCAAAGGATAAGGGCATCG	TCTTCTTCTTTTGTTGTCTTCCG
Ra3H10	5	93		R05	IRD700	Lowe et et al. (2003)/ jinsun Kim	TAATCGCGATCTGGATTCAC	ATCACAACAGCGACGAGGTC
BRH80A08flc1t	10	95*	58.0		IRD700	Jinsun kim	TTCCCAAGCTTGCTGGTACT	GAGATTTCCCTCGCTTGATG
BRH80C09flc3	2	96*		R02	IRD700	Jinsun kim	CAGTGAAGTTCAACCGCAGTGAAA	CATGAGTGAACATAAAACAGTGAAA
BC105t	1	198	5.8		IRD700	Ma Rongcai	GACGCCTCAATTGCTTACTT	AGGGAATGAGGATGGGTCTG
BRMS-042-2t	3	210*	120.123		IRD800	Suwabe et al. (2002)	AGCTCCCGACAGCAACAAAAGA	TTCGCTTCCTTTTCTGGGAATG
F3H-SSR2t	3	256*	124.467		IRD800	Dunia	GTCATCTCCAGGTAAATCCA	TCTTGAACAACCTCTCCCTA
chsssr13	10	272*	2.5		IRD700	Dunia	AGTGAGACGAAGTCCAGAAA	GCATAATCTTAGGCAACCAG
EJU6R10	10	362*	63.1		IRD800	Choi et al. (2007)	TCTCTCACCTGCCTTGTCT	ACTCCTCGGTAATGCCTC
ENA13i	2	363	22.428		IRD700	Choi et al. (2007)	CTGCGTTCCAGTACCCTCTC	TCATCCTTAATGGTCCTGCC
ENA23h	2	367*	42.248		IRD800	Choi et al. (2007)	GCTGTGCCAGTTCCTCTTTC	TCATTCCAAATGGCCTTACC
GOL3		382*		R08	IRD700	Choi et al. (2007)	ACT CAC TTT TGT TGG GCG TC	GGA GCC GCT TTC TCT ACC TT
KBRH143D22		383*		R08	IRD800	Choi et al. (2007)	GAT GTG ATA CTT TGG CGA CGG	TGA AGG ATA ATA TGG TCT TGG CC
nw_51		392			IRD800	Lange et al. (2009)	GATTTATGCTCTGATCCCCAAG	GCTACAATTAGATCGGCTCCTG
nw_55		396*			IRD800	Lange et al. (2009)	ACTTGGGATTTTCTCGTCACCAC	TAAAGCGACTTTCGTTCAGACA

Marker	Linkage group (WU-integrated)	Lab code	Position (cM)-WU map	LG-reference map	Labelled primer	Reference	For	Rev
nw_57		398*			IRD700	Lange et al. (2009)	AGAGTGGCAAACCAATGCTCT	AATCCTCTTTTCCTCCAAGCC
nw_60		401*			IRD700	Lange et al. (2009)	CCACAAGTACGATGAAGG	ACGTAACATGACCAGGCAAAT
nw_61		402			IRD700	Lange et al. (2009)	GGTCCATCTTGACTTACATTATATTT	CAGCCAATCTTGTTGCTTTA
Br308t	1	est-2*	4.4		IRD800		ACAAACTAAACACCGACGAC	AGAGAGAATGAGAATGCGAA
BR325	4	est-19	47.384		IRD700		ATCAGTTGGATCTTTCCAGA	GTCATCTCTTTCTCGGTGAG
Br326t	2	est-20*	56.439		IRD700		CGTTTGATGGGAGTTATTGT	ACTGAGCAGCTGGACTGTAT
BR343t	9	est-37*	61.0		IRD700		GGTAGATACAGGTTTGGCTG	GACAAGTAAAGAGCTCCCAA
Br344t	6	est-38	60.5		IRD800		TTTGATTGATTCGCTTTGAT	ACAACTATCCAATTAGCCGA
Br340t	9	est-40*	80.7		IRD700		AATCCCTCTCTCTCATTTCA	TGGATTACTCCATCCAAAAC
BR356t	3	est-50*	68.34		IRD700		GCATCTCAGCCTTACAACTT	AGCAAGAACCCAGAAACATA
BR378t	5	est-72*	28.742		IRD800		TTCATCCATCCATCTTTCTC	ATGATTCCTCCATGTTCATC
BRMS-034t	5	5*	16.007		IRD800	Suwabe et al. (2002)	GATCAAATAACGAACGGAGAGA	GAGCCAAGAAAGGACCTAAGAT
BRMS-050	3	21*	115.319		IRD700	Suwabe et al. (2002)	AACTTTGCTTCCACTGATTTTT	TTGCTTAACGCTAAATCCATAT
Na12H07t	6	73*	62.8		IRD805	Lowe et al. 2003	GCGGCATTAGTTGTCAGTCC	TCGTTGATTTACATACATGCG
BRH04D11flc2t	2	94*	10.293		IRD700	Jinsun kim	CAGTGAAGTTCAACCGCAGTA	CATGAGTGAACATAAAACAGTGAAA
BC7t	6	176*	0.0		IRD804	Ma Rongcai	AGTTGGCCCCATTTCATTGTTAT	CATCTTGACGGCCTCCATCTCCA
BR319t	8	est-13*	33.351		IRD700		TCTATGATCATGGCTTCCTC	TCTCCGGTGTAGAGTTTGTT
BR321	9	est-15*	67.4		IRD700		CCTTATCCCATCTCTCCTCT	GAGATCAAAGTCGTAGTGGC
Br323	2	est-17*	13.74		IRD801		GTGGTGAACGTGCTTAAGAT	ACGAGCTGGTTGAAAGTTTA
Br360	3	est-54*	31.456		IRD802		CATCGTCGTCTCCAATACTA	GAGTTGAGATCGTTCCTCTG

Marker	Linkage group (WU-integrated)	Lab code	Position (cM)-WU map	LG-reference map	Labelled primer	Reference	For	Rev
Br372	7	est-66*	12.4		IRD800		AACGTAGTCACCAACGAAAC	TCTGAGAAAAGAAGGAGCTG
BR384t	4	est-78*	38.961		IRD803		TTCAATCACTTCTTCGTTTG	GAAGTACAGAAACAGCACC

Appendix VI: LI-COR Protocol

Before starting: Clean the gel making items with water, then with ethanol and dry them with a new paper check glass plates carefully
Check if the **94° C** heater near the window is **switched on**
Put equal volume of formamide loading buffer to the PCR product close the seal again and mix on vortex

The writing on the glass plates should not be readable when they are placed on the lab table (*mirror writing*)

Place the two spacers on the large glass plate (*back-plate*) and leave some space on the bottom of the plate (*this is easier for the cleaning of the glass plates at the end*)
lace the other smaller plate (*front-plate*) on top of the back plate (*which have the spacers and do not move the spacers, the writing on the front plate is now readable*)

Place the two black holders at each side and make sure that they make contact with the glass plate on the bottom and the top

First screw the middle nut and then the bottom nut (*not to tight*)

Make the gel solution: 20 ml Long Ranger solution
15 µl TEMED (*add in the small fume hood*)
150 µl APS (*add in the small fume hood, smells like rotten eggs*)

Pour the gel and let the capillary do the work (*maybe gently tap on the glass with your finger*)

Place the top spacer in the gel and lock it with the black / transparent holder (*screw the top nuts not to tight*)

Let the gel polymerize for 1½ hour

Place the in the transparent holder on the lab table, so the gel is standing vertical and put a paper comb 1 mm in the gel (*from now on be very careful not to move the comb*)

Screw the top compartment at the top of the front glass plate (*not to tight*)

Place a silver paper at the back of the back plate.

Place the gel inside the LICOR machine

Make a new buffer: 100ml 10× buffer
900 ml distilled water (*green tap*)

First fill the top compartment to the maximum that is indicated with buffer

Then fill the bottom compartment with the remaining buffer

Run the calibration program, don't change the parameters only fill in the name of the folder and the file name

When running the program make sure that the indicated values left and right are having not more then 50 units of difference

Let the gel heat up until 45° C

Heat the samples in the 94° C heater for 1 minute and directly place in cooling block from fridge

Open the door of the LICOR machine

Clean the space between the comb by spraying with a syringe (*this is now filled with ureum which will interfere loading of a gel*)

Load **0.7 µl of IRD 800 product**

Load 1.0 µl of the IRD 800 size marker (*At least 3 size markers should be included one at each side and one in the middle*)

Close the top compartment and the bottom compartment and then close the door of the LICOR machine and press enter when the machine indicate this

Let the machine run for 5 minutes

Now load **0.5 µl** of **IRD 700** product

Load 1.0 µl of the IRD 700 size marker (*At least 3 size markers should be included one at each side and one in the middle*)

Close the top compartment and then close the door of the LICOR machine and press enter when the machine indicate this

Let the gel run for 3 - 4 hours

The LICOR will shutdown automatically

Appendix VII: Summary of all phenotypic traits measured

Table 12. Phenotypic values of subpopulations. Per subpopulation, the average and the standard deviation are given for each vernalization treatment. The values of the non turnip accession are additionally shown for comparison. Trait abbreviations can be found in Table 2. The significance difference between P_I and P_II and/or vernalization treatments of a trait are indicated in the first column.

	P_I						P_II						non turnip accessions					
	0		4		8		0		4		8		0		4		8	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
TSh: 2	2.6	1.4	3.5	1.6	3.9	1.3	3.0	1.5	2.9	1.5	3.4	1.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Twi: 1,2	83.2	25.3	53.8	37.4	44.0	34.8	96.9	34.5	88.4	36.3	70.0	35.9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Tcu: 2	6.1	11.1	5.1	2.8	4.8	2.8	2.9	1.4	2.9	1.4	2.6	1.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
TCl: 2	3.9	2.7	4.2	2.7	4.1	2.4	1.9	1.4	2.0	1.4	2.0	1.3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Tso: 1,2	27.4	5.8	28.2	8.8	29.8	7.1	32.6	5.4	30.5	5.8	33.1	6.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
TD: 1,2	2.6	0.7	2.2	0.7	1.9	0.7	2.7	0.5	2.6	0.7	2.3	0.7	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
TSs: 1,2	1.9	0.7	2.2	0.6	2.2	0.5	1.8	0.7	1.9	0.6	2.2	0.6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Twe: 1,2	265.6	137.3	124.4	147.5	100.6	153.9	455.3	665.4	310.7	204.4	202.8	202.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
FT: 2	60.8	31.9	34.4	15.7	31.9	15.5	57.7	25.5	38.1	14.8	40.5	22.2	61.4	23.9	32.5	8.5	30.6	6.3
FB: 2	74.9	42.0	55.9	33.7	41.2	21.1	94.6	42.8	67.0	29.0	57.3	36.8	71.9	38.9	45.6	26.9	34.7	12.7
FS	2.3	1.6	2.8	1.2	3.2	1.4	2.6	1.5	2.6	1.1	2.5	1.3	2.3	1.4	2.6	1.3	2.8	1.4
FC	3.2	1.0	3.8	4.4	3.2	0.8	3.2	0.4	3.2	0.8	3.1	1.3	3.3	0.7	3.2	0.8	3.3	1.0
LL: 2	22.6	4.4	20.4	3.9	19.1	4.8	21.2	3.1	20.1	2.4	20.1	2.6	17.3	6.5	15.4	6.3	13.4	6.0
Lwi: 1,3	12.7 c	1.9	12.7 d	1.8	12.1 e	3.5	14.3 a	1.9	14.4 ab	2.3	14.8 b	2.1	12.5	3.3	10.9	4.4	9.8	4.1
LI: 1,2	1.8	0.4	1.6	0.4	1.7	0.5	1.5	0.3	1.4	0.2	1.4	0.2	1.4	0.4	1.5	0.5	1.4	0.3
PL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
PW: 1	4.0	2.2	3.7	1.9	4.2	1.9	4.9	2.5	5.6	2.4	5.5	3.0	3.0	2.1	3.2	2.5	3.0	2.1
Ler*: 1,2	4.0	0.0	2.9	0.6	1.5	0.8	2.6	1.2	2.9	0.6	3.0	1.1	2.4	0.7	2.3	0.5	2.0	0.5
Lco: 2	32.1	4.3	31.3	4.6	29.7	4.9	31.2	4.0	30.9	3.9	30.0	3.3	35.9	8.9	34.1	9.1	34.5	8.4
LS	1.9	0.6	1.8	0.7	2.2	0.9	2.0	0.4	2.1	0.5	2.0	0.6	2.0	0.7	1.9	0.8	1.8	0.8
LTr: 1,2	0.5	0.5	0.4	0.5	0.2	0.4	0.9	0.3	0.9	0.3	0.8	0.4	0.4	0.5	0.4	0.5	0.3	0.5

Lti: 1	2.5	0.9	2.4	0.7	2.6	1.4	2.8	0.9	2.9	0.7	2.5	0.8	2.8	0.5	2.7	1.1	2.5	1.3
Les: 1	2.2	1.1	2.2	1.2	2.0	1.2	2.8	0.9	3.1	1.0	2.9	1.0	2.0	1.2	2.1	1.2	1.9	1.1
LA: 1	2.7	1.0	3.1	0.8	3.1	1.2	2.6	0.9	2.7	1.1	2.6	1.0	2.4	1.1	3.0	1.2	2.9	0.9
LN: 1,2	19.0	5.5	14.0	22.5	6.6	2.2	18.0	6.5	9.9	2.8	10.6	5.0	18.3	15.1	7.3	3.5	5.0	1.9
Lwe: 3	280,7 abc	200.2	273,4 c	181.4	223,1 c	112.6	242,6 bc	216.0	287,8 ab	213.8	292,2 a	192.7	215.2	170.8	140.5	138.1	89.3	50.9
Lse*: 1	33.3	6.1	28.9	3.2	27.8	4.8	36.4	6.1	30.9	5.9	38.3	10.9	38.9	4.5	33.9	10.7	38.0	6.2

N.D.	no observations
*	measured only on six accessions; see material and methods
1	significant difference between two populations (P<0.05). The non turnip accessions are no part of the test
2	significant difference between three vernalization treatments (P<0.05). The non turnip accessions are not included in the test
3	significant interaction between population and vernalization treatment (P<0.05)

Appendix VIII: Correlations between morphological traits

Table 13a. Part I of table with all correlations between morphological traits measured on all genotypes in the three vernalization treatments and their P-values. Yellow traits are traits related to flowering, red means related to leafy traits and the trais indicated in green are turnip traits. Correlations are given below the diagnal. The P-values of the correlations are shown in het upper half. Significant correlations are highlighted with a colour, which is explained below the table.

	FB	FC	FS	FT	LA	LI	LL	LN	LS	LTr	LWi	Lco	Les	Lti	Lwe	PW
FB	*	0.36	0.06	0.03	0.11	0.62	0.04	<0.01	0.27	0.57	0.29	0.47	0.17	0.99	0.11	0.12
FC	0.11	*	0.40	0.61	0.36	0.06	0.06	0.61	0.68	0.16	0.81	0.24	0.50	0.20	0.77	0.78
FS	-0.23	-0.11	*	0.80	0.20	0.04	0.23	0.41	0.72	0.65	0.21	0.75	0.56	0.94	0.07	0.60
FT	0.27	-0.06	0.03	*	0.94	0.95	0.74	0.13	0.40	0.71	0.81	0.98	0.28	0.58	0.80	0.18
LA	0.20	0.11	0.16	-0.01	*	0.74	0.59	0.48	0.17	0.04	0.87	0.70	0.18	0.24	0.97	0.08
LI	0.06	-0.23	-0.25	0.01	0.04	*	<0.01	0.69	0.95	0.01	<0.01	0.16	0.00	0.44	0.09	<0.01
LL	0.25	-0.23	-0.15	0.04	0.07	0.63	*	0.57	0.82	0.04	0.10	0.15	<0.01	0.53	0.48	0.06
LN	0.48	0.06	-0.10	0.19	-0.09	-0.05	0.07	*	0.62	0.23	0.45	0.53	0.64	0.36	<0.01	0.38
LS	-0.14	-0.05	0.05	0.10	0.17	0.01	-0.03	-0.06	*	0.33	0.85	0.89	0.05	<0.01	0.11	0.34
LTr	0.07	-0.17	-0.06	-0.05	-0.25	-0.30	-0.26	0.15	0.12	*	0.34	0.38	<0.01	0.88	0.11	<0.01
LWi	0.13	0.03	0.16	0.03	-0.02	-0.60	0.21	0.10	-0.02	0.12	*	<0.01	0.04	0.90	0.14	<0.01
Lco	-0.09	0.15	0.04	0.00	0.05	0.18	-0.18	-0.08	-0.02	-0.11	-0.44	*	0.32	0.96	0.63	<0.01
Les	-0.17	0.08	-0.07	-0.14	-0.17	-0.51	-0.43	0.06	0.24	0.53	0.26	-0.12	*	0.47	0.21	<0.01
Lti	0.00	-0.16	0.01	0.07	0.15	0.10	0.08	-0.11	0.34	0.02	0.02	0.01	-0.09	*	0.06	0.10
Lwe	0.20	0.04	0.22	-0.03	0.01	-0.21	-0.09	0.39	0.20	0.20	0.18	0.06	0.16	0.23	*	0.39
PW	-0.19	0.03	-0.07	-0.17	-0.21	-0.42	-0.23	0.11	0.12	0.34	0.34	-0.39	0.68	-0.20	0.11	*
TCl	-0.12	-0.23	0.24	-0.15	-0.02	0.31	0.16	0.01	0.18	-0.19	-0.26	0.05	-0.27	0.02	0.04	-0.17
TCu	-0.04	-0.03	0.07	-0.23	0.02	0.39	0.22	0.08	0.17	-0.32	-0.32	0.11	-0.26	0.06	0.04	-0.21
TD	-0.01	0.06	-0.22	0.18	-0.39	-0.03	-0.01	0.42	0.00	0.15	0.00	-0.02	0.24	0.07	0.02	0.19
TSh	0.07	0.16	0.34	-0.05	0.35	-0.06	-0.07	-0.23	-0.10	-0.12	-0.02	0.03	-0.11	-0.03	0.15	-0.11
TSs	0.06	-0.04	0.08	0.13	0.11	-0.36	-0.27	-0.09	0.10	0.15	0.22	-0.12	0.15	0.07	0.11	0.05
Tso	-0.07	-0.03	-0.02	-0.15	0.13	-0.22	-0.17	-0.22	-0.05	0.07	0.14	-0.11	0.24	-0.16	-0.05	0.20
Twe	0.29	0.06	-0.32	0.17	-0.27	0.14	0.22	0.50	0.07	0.21	-0.01	0.08	0.07	-0.11	0.09	0.03
Twi	0.24	-0.01	-0.18	0.20	-0.25	0.01	0.20	0.53	0.08	0.13	0.18	-0.07	0.04	-0.04	0.05	0.07
root_shoot_ratio	0.16	0.08	-0.34	0.26	-0.24	0.14	0.15	0.39	0.03	0.14	-0.09	0.01	0.02	-0.19	-0.27	0.02

correlaton between flower and leaf traits
correlation between flower traits
correlation between leaf traits

Table 13b. Part II of the table with all correlations between morphological traits measured on all genotypes in the three vernalization treatments and their P-values. Yellow traits are traits related to flowering, red means related to leafy traits and the trais indicated in green are turnip traits. In the lower diognal of the table, the correlations are given. The P-values of the correlations are shown in het upper diognal.

	TCl	TCu	TD	TSh	TSs	Tso	Twe	Twi	root_shoot_ratio
FB	0.33	0.73	0.94	0.55	0.62	0.55	0.02	0.05	0.20
FC	0.07	0.80	0.66	0.21	0.76	0.82	0.66	0.96	0.53
FS	0.05	0.59	0.07	<0.01	0.55	0.87	<0.01	0.14	<0.01
FT	0.24	0.06	0.14	0.69	0.31	0.22	0.16	0.11	0.03
LA	0.90	0.86	<0.01	<0.01	0.36	0.30	0.03	0.04	0.06
LI	0.01	<0.01	0.80	0.62	<0.01	0.08	0.25	0.92	0.26
LL	0.20	0.07	0.94	0.56	0.03	0.18	0.08	0.11	0.24
LN	0.92	0.52	<0.01	0.07	0.49	0.08	<0.01	<0.01	<0.01
LS	0.15	0.17	0.97	0.43	0.42	0.68	0.57	0.53	0.80
LTr	0.12	<0.01	0.23	0.32	0.22	0.59	0.10	0.31	0.26
LWi	0.04	<0.01	0.98	0.90	0.08	0.28	0.91	0.16	0.48
Lco	0.71	0.39	0.90	0.82	0.34	0.37	0.53	0.57	0.92
Les	0.03	0.03	0.05	0.39	0.23	0.05	0.57	0.73	0.86
Lti	0.85	0.64	0.55	0.81	0.56	0.21	0.37	0.77	0.13
Lwe	0.74	0.74	0.88	0.23	0.37	0.69	0.47	0.69	0.03
PW	0.17	0.10	0.13	0.38	0.67	0.11	0.78	0.60	0.85
TCl	*	<0.01	0.71	0.89	0.08	0.10	0.73	0.32	0.59
TCu	0.81	*	0.32	0.51	<0.01	<0.01	0.60	0.82	0.92
TD	0.05	0.12	*	<0.01	0.41	<0.01	<0.01	<0.01	<0.01
TSh	-0.02	-0.08	-0.51	*	0.29	0.16	<0.01	<0.01	<0.01
TSs	-0.22	-0.41	-0.10	0.13	*	0.04	<0.01	0.22	<0.01
Tso	-0.21	-0.36	-0.32	0.17	0.25	*	<0.01	0.03	0.08
Twe	-0.04	0.07	0.47	-0.45	-0.41	-0.32	*	<0.01	<0.01
Twi	-0.12	-0.03	0.49	-0.48	-0.15	-0.26	0.70	*	<0.01
root_shoot_ratio	-0.07	-0.01	0.48	-0.55	-0.33	-0.22	0.85	0.64	*

correlation between turnip traits
correlation between flower and turnip traits
correlation between leaf and turnip traits

Appendix IX: Table with all marker-trait associations

Table 14. Overview of all the significant marker-trait associations per allele of each marker (P<0.01) for the two different models used.

		Traits significantly associated with allele	
marker	allele*	GLM	MLM
Na12A01m	118	LL_0, LL_8, LS_8a	
	121	Les_4, Les_8, LL_0, LTr_8, Tcu_4, Tcu_8	
	122	LL_0, LL_8	
BRMS-051t	252	PW_4	
	253	LL_8, LTr_8	
	266	LN_4	
BRMS-042-2t	214	LA_4	
	218	LL_4, LL_8, LS_8	
	229	LN_0, TSs_0, Twe_0, Twi_0	
	231	LN_4, PW_0	PW_0
	236	FT_4, Lwe_4, Twe_8, Twi_8	FT_4, Lwe_4, Twe_8, Twi_8
F3H-SSR2t	283	LN_0	
chsssr13	360	TD_0, TSs_0	
	368	FS_4	
BRMS-054	81	LA_4, LN_0	
	83	Tso_4	
	A	TCl_0	
Na10A08	151	LN_0	
EJU6R10	390	Les_8, Lti_4	
ENA23h	186	TCl_8	
	215	FS_8, LW_4	
	219	LN_8	LN_8
GOL3	259	Twe_0, Twe_4, Twe_8, Twi_0, Twi_8	
	261	PW_0	
	262	LW_8	
KBRH143D22	H	Tso_4	Tso_4
BRMS-043t	F	TCl_0, TCl_4, TCl_8, Tcu_0, Tcu_4, Tcu_8	
nw_60	379	FB_8	
	385	LN_0, Tcu_4	
	387	LA_8, Twe_0, Twe_4, Twi_0	
BRMS-040	188	FT_4	FT_4
	205	FC_8	FC_8
Ra2E12t	129	LL_0, LL_4, LW_0, LW_4	LW_4
	161	LN_4	
	163	LN_4, TSh_8, Twe_8, Twi_8	TSh_8, Twe_8
Ra2A01	111		FC_4
	125	LL_8, TD_4 , TSh_8	
	127	Lco_8, LL_0, LL_4, LW_0	
BRH80A08flc1t	182	LS_4	

		Traits significantly associated with allele	
marker	allele*	GLM	MLM
BRH80C09flc3	306	TCl_0, TCl_4, TCl_8	
	309	LW_0	
	322		Les_8
	333	Lti_8, LW_8, TCl_8	Lti_8
	336	LA_4, Lti_8, LW_8	Lti_8
	339	Lti_8	Lti_8
Br326t	709	FS_4, Twe_0	
	719	TCu_4, TCu_8, LTr_8	Les_8
Br308t	224	Les_0, Les_8	
	226		Les_4
	236	LW_4, Lwe_8	
	238	LW_4, Lwe_8	
BR343t	361	Tso_0, Tso_4, Tso_8	Les_8, Tso_8
Br340t	483	FS_8, LA_0	
BR356t	189	TCu_0	
	196	Lco_4	
	205	FC_8	FC_8
BR378t	306	Lwe_4	
	310	Lwe_4	Lwe_4
nw_51	286	FT_8	FT_8
BRMS-050	167	Lwe_4, TD_8	
	180	FS_4, Twe_0, Twe_4, Twi_0, Twi_4	Twe_0, Twi_0, Twi_4
	182	Les_0, Les_4, PW_0	PW_0
BC7t	154		LTr_0
	155	TCl_4, TCu_4	
	165		TD_4
	178		FT_0
BR319t	450	LS_0	
	455	Lti_4, Tso_8	LTr_0, Tso_8
	458	LN_8	
Br360	265	TCl_8	
	267	LS_4	
BRMS-034t	129	Les_0, Les_8	
	134	LW_8	
	149	FT_0, LL_4, TSh_0	TSh_0
BR321	148	Lco_8	
	152	Les_0, TCu_0	
Na12H07t	146	TCl_8, TCu_4, TCu_8	LTr_0
	147	TSs_4	
BRH04D11flc2t	200	LA_8	LA_8
	202	LTr_0	
	204	FT_0, LN_0	
	208	TD_4, TSh_4, Twi_4	

		Traits significantly associated with allele	
marker	allele*	GLM	MLM
	211	FT_8	
	215	FB_4, Lwe_4	
	219		Tso_4
	244	TCl_0, TCl_4, TCu_4, TCu_8, Tso_8	
BR384t	290	LN_0	
	295	Tso_0, Tso_4, Twi_0	Tso_0, Tso_4
	297	FB_8	

* If a numbers were used, this is the size (bp) of the amplification product. For some markers, the size was not detected and the allele were named with a character.