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Genotypic and phenotypic characterization of winter hardiness in Beta species

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Abbreviations (i)

ABA	Abscisic Acid
ABRE	Abscisic Acid responsive element
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
AP1	*APETALA1*
B	Bolting Locus
B.C.	Before Christ
BAC	Bacterial artificial chromosome
BCTV	Beet curly top virus
BLAST	Basic local alignment search tool
BLUP	Best Linear Unbiased Predictor
BNYVV	Beet necrotic yellow vein virus
bp	Basepair(s)
BR	Bolting Rate
BTC1	*BOLTING TIME CONTROL1*
Bv	*Beta vulgaris*
BVM	*Beta vulgaris* subspecies *maritima*
°C	Degree Celsius
°Cd	Degree days
C	Cytosine
Ca	Calcium
CAPS	Cleaved amplified polymorphic sequence
CBF1	*C-REPEAT BINDING FACTOR1*
CBF2	*C-REPEAT BINDING FACTOR2*
CBF3	*C-REPEAT BINDING FACTOR3*
CCA	*CIRCADIAN CLOCK-ASSOCIATED1*
CCE	Crude Celery Extract
CG	Candidate gene
Cl	Chlor
cM	Centimorgan
cm	Centimeter
CMS	Cytoplasmatic male sterility
CO	*CONSTANS*
COL1	*CONSTANS-LIKE1*
COR	cold responsive
CRT/DRE	C-repeat/drought-responsive element
CTAB	Cetyltrimethylammoniumbromid
DH	Double Haploid
DNA	Deoxyribonucleic acid
dNTP	2´-deoxyribonucleotide-5´triphosphate
eQTL	Expression QTL
E-type	Ertragstyp (yield type)
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia*
EMS	Ethyl methanesulfonate
esk1	*ESKIMO1*
EST	Expressed Sequence Tag

Abbreviations (ii)

F_1	First generation
F_2	Second generation
F_3	Third generation
FCA	*FLOWERING LOCUS CA*
FD	*FLOWERING LOCUS D*
FISH	Fluorescence in situ Hybridization
FL1	*FLOWERING LOCUS1*
FLC	*FLOWERING LOCUS C*
FLK	*FLOWERING LOCUS K*
FR-1	Frost Tolerance Locus 1 in cereals
FR-2	Frost Tolerance Locus 2 in cereals
FRI	*Frigida*
FSI	Frost Severity Index
FT	FLOWERING LOCUS T
FT1	FLOWERING LOCUS T1
FT2	FLOWERING LOCUS T2
FVE	Flowering Locus VE
G	Guanin
GA	Gibberelin
Gi	*GIGANTEA*
Gö	Göttingen
GolS3	*GALACTINOL SYNTHETASE3*
GS	Genomic Selection
GWAS	Genome Wide Association Study
h	Hour
h^2	Heritability
H	Haplotype
ha	Hectare
Hos1	*HIGH EXPRESSION OF OSMOTIC RESPONSIVE 1*
Ht	Gene diversity index (heterozygocity)
JA	Jasmonic Acid
K	Kilo =1000
Kb	Kilobase pairs = 1000 bp
Ki	Kiel
LD	Linkage Disequilibrium
LG	Linkage Group
LHP1	*LIKE HETEROCHROMATIN 1*
LHY	LATE ELONGATED HYPOCOTYL
LMA	Linkage map based on all mapable co-dominant and dominant markers
LOD	Logarithm of odds
LOV1	*LONG VEGETATIVE PHASE 1*
LT_{50}	Lethal temperature for 50% of the tested individuals
µl	Microlitre
mA	Milliampere
MAS	Marker Assisted Selection
Mb	Megabases
mg	Milligram
Mg	Magnesium
Mi	Minsk

Abbreviations (iii)

ml	Millilitre
mM	Millimolar
n	Haploid set of chromosomes
N-type	Normaltyp (normal type)
na	Not available
Nes	Nesvizh
ng	Nanogram
NGS	Next Generation Sequencing
NHF	Non-reference haplotype frequency
NNF	Non reference nucleotide frequency
O-type	Maintainer of cytoplasmic male sterility in sugar beet
P	Error Probability
PAR	Photosynthetic Active Radiation
PCR	Polymerase Chain Reaction
phyB	*PHYTOCHROME B*
phyD	*PHYTOCHROME D*
phyE	*PHYTOCHROME E*
PRR	*PSEUDO RESPONSE REGULATOR*
QTL	Quantitative Trait Locus
R	Locus for red hypocotyl color in sugar beet
r^2	Coefficient of determination
RAPD	Random Amplified Polymorphic Deoxyribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
RIL	Recombinant Inbred Line
RLP	Rate of living Plants
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
Rz1	*RHIZOMANIA RESISTANCE GENE 1*
SA	Salicylic Acid
SBEBM	Sugar Beet Elite Breeding Material
SBGP	Sugar beet germplasm
SC2	Strube component 2 (Parent 1 of mapping population)
SCK	Strube component K (Parent 2 of mapping population)
SE	Standard error
sec	Second
SD	Short day
SNP	Single Nucleotide Polymorphism
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
Sö	Söllingen
SR	Survival Rate
ssp	Subspecies
SSR	Simple Sequence Repeat
SVP	*SHORT VEGETATIVE PHASE*
TILLING	Targeting Induced Local Lesions In Genomes
UTR	Untranslated region
V	Volt
VIN3	*VERNALIZATION INSENSITIVE 3*
VRN1	*VERNALIZATION1*
VRN2	*VERNALIZATION2*

Abbreviations (iv)

W	Watt
X	Number of homologous chromosomes
Y	Locus for yellow root color in sugar beet
YAC	Yeast Artificial Chromosome
Z-type	Zuckertyp (sugar type)

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1 General Introduction

1.1 Wild and cultivated species of the genus Beta

1.1.1 Systematics of Beta species

Sugar beets are dicotyledonous biennial plants forming a large taproot and a leaf rosette in the first year. In the second year sugar beets develop reproductive organs induced by long day conditions after exposure to low, but non freezing temperatures, called vernalization. The DNA content of sugar beet was estimated 758 million base pairs per haploid genome (n = x = 9) (Arumuganathan and Earle, 1991) and most cultivars are diploid (2n = 2x = 18), although many sugar beet varieties are triploid hybrids (Bosemark, 1993).

The natural gene pool of sugar beets includes the genera *Beta* and *Patellifolia*, both members of the *Chenopodiaceae* family (Kadereit et al. 2006). The Beta genus is native to Europe and adjacent countries (Frese et al. 2001) and includes the sections Beta and Corollinae (Kadereit et al. 2006). The section Beta represents the primary gene pool of the sugar beet and comprises the species *B. vulgaris*, *B. macrocarpa* (Guss.) and *B. patula* (Ailton). The highest relevance for agriculture has the species *B. vulgaris*, which is subdivided into the *B. vulgaris* L. ssp. v*ulgaris*, *B. vulgaris* ssp. *maritima* (Arcang.) and *Beta vulgaris* L. ssp. *adanensis* (Pamuk.). The subspecies of *B. vulgaris* are easily crossable among each other and especially one family member, *B. v. maritima*, has been used as the major source to obtain of resistance and tolerance genes against biotic and abiotic stresses (Frese et al., 2001; Panella and Lewellen, 2007).

1.1.2 Domestication and breeding

B. vulgaris L. ssp. v*ulgaris* includes the four morphologically defined cultivar groups leaf beet, garden beet, fodder beet and sugar beet (Letschert et al., 1994). All these cultivar groups are derived from the sea beet *B. vulgaris L. ssp. maritima*. It is estimated that the annual sea beet was domesticated about 12,000 years ago around the Persian Gulf (Simmonds, 1976). The earliest references about beets being used as a leaf vegetable were assigned to the Greeks Aristophanes (445-385 B.C.) and Euripides (480-406 B.C.). With the climax of the Roman Empire the beets spread all over Europe and Pliny the Elder was the first who reported about the ancestors of garden beets being used as root vegetables. During the Middle Ages beets were selected in Spain or France as suitable winter feed for cattle. The forth, and nowadays most important use of beets is based on the finding of Andreas S. Marggraf in 1747, that the sugar extracted from garden and leaf beets is identical to cane sugar. Based on this knowledge Franz C. Achard (1806) applied mass selection in fodder beets to develop the "White Silesian", the ancestor of all modern sugar beet cultivars (Biancardi et al., 2005; Fischer, 1989).

Historically, right from the start sugar beet cultivation was linked to milestones in breeding development promoted by supporting politicians. Napoleon for example has increased sugar beet production enormously due to the need of a cheaper alternative to imported cane sugar for France (1806-1814). At the end of the 19th century sugar beet cultivation had spread over Europe reinforced by improved sugar factories as well as increased root yield and sugar content of the sugar beets. About 100 years later selection methods based on progeny tests rapidly increased root and sugar yield further. In the 1940s the discovery of cytoplasmic male sterility (CMS) opened the gate towards hybrid breeding in sugar beets (Owen, 1945). A further milestone for the development of modern elite sugar beet varieties and the mechanization of sugar beet cultivation was the introduction of genetic monogermity (Savitsky, 1952).

The elite sugar beet gene pool is considered to be of low genetic diversity for two reasons: i. a founder effect, which is caused by the fact that most of the sugar beet germplasm is derived from progenies of the open pollinated "White Silesian" population, (Fischer, 1989) and ii. a genetic bottleneck, due to introduction of male sterility (Bosemark, 1979).

However, breeders require genetic variation to continuously improve sugar beets by recombining favorable alleles in new varieties. Crosses performed in a genetically narrow background decrease the probability of obtaining superior germplasm. One strategy to broaden the genetic diversity in breeding elite material is the introgression of traits of interest from a natural gene pool. Important sources for genetic variation are the more than 10.000 Beta accessions maintained in gene banks worldwide. To facilitate the utilization of this resource the concept of a synthetic Beta core collection was established (Frese, 2000). Based on molecular markers, morphology, yield and quality about 805 accessions were selected that represent a maximum of genetic diversity. With a total of 586 accessions *B. vulgaris* accounts for the species with the highest genetic diversity in the core collection (Frese, 2000). Among subspecies *B. vulgaris* ssp. *maritima* shows the highest genetic diversity with 204 accessions present in the core collection of Beta.

Even though many resources are available to enlarge the genetic basis of sugar beet germplasm, commercial breeders rarely use distantly related resources in order to avoid introgressions of undesired traits. To overcome these genetic disadvantages for germplasm improvement Panella and Lewellen (2007) proposed an intensive cooperation between public resources and commercial breeders. By optimizing their cooperation with public breeding and gene banks commercial breeders could minimize expenses caused by genetic introgression using genetically distinct material. Publicly funded institutions should emphasize on a process called prebreeding, which includes the identification of genes in genetic resources that underlie agronomic traits and their introgression into elite germplasm, which is then released as a registered germplasm line. This material can then be used for cultivar development by commercial breeders.

In any case, a new sugar beet variety has to meet the benchmark criteria sugar yield which depends on root yield and sugar concentration. Since both criteria are negatively correlated, sugar beet varieties are classified into groups depending on their root yield and sugar content. High root yielding varieties with low sugar content are designated E-types (from German "Ertrag" (yield)), varieties with high sugar content and low root yield are classified as Z-types (from German "Zucker" (sugar)) and intermediate varieties belong to the N-types (from German "normal" (normal)) (Biancardi et al., 2005). However, crucial for sugar production is not the gross sugar yield but the extractable white sugar yield, which is decreased by molasses forming components like potassium, sodium, and α-amino nitrogen (Biancardi et al., 2005). Both, the sugar content and the non-sucrose components are highly affected by environmental factors and selection for higher root yield. So for new varieties it is more promising to improve sugar yield than the selection of increased sugar content (McGrath and Trebbi, 2007).

Of major importance for stable root yield of sugar beet has been a strong selection against premature bolting. Premature bolting can be promoted by drilling too early in spring and therefore exposing the young plant to cold temperatures that meet the vernalization requirements for bolting. Also, premature bolting can be caused by accidental introgression of the dominant allele of the bolting gene *BOLTING TIME CONTROL1* (*BTC1*) during seed production. The dominant *BTC1* allele overcomes the vernalization requirement and causes annuality under long day conditions (Abegg, 1936; Munerati, 1931; Pin et al., 2012).

A precondition for hybrid breeding and therewith the exploitation of heterosis was the discovery of cytoplasmic male sterility (Owen, 1945). Together with the introgression of genetic monogermity (Savitsky, 1952), this finding was a milestone with enormous consequences for the production of monogerm hybrid varieties.

Hybrid breeding in general is based on the reciprocal selection of seed parent and pollinator lines for combining ability and the synthesis of the best parental lines to hybrid varieties. A substantial part of sugar beet hybrid breeding is the selection and maintenance of seed parents. The seed parents of hybrid varieties not only have to carry the sterile cytoplasm but moreover they have to show monogermity, which is inherited only from the seed parent. CMS plants can only be maintained if they are pollinated by corresponding isogenic maintainer genotypes (O-types). The identification of plants with the O-type character is very tedious as they occur at very low frequencies and test crosses have to be performed to ensure that the plants carry the required homozygous recessive alleles at the pertinent nuclear genes. In practice, seed parents are usually developed by repeated backcrossing of an O-type genotype with a CMS donor. Compared to the development of the seed parent the development of pollinators appears to be straightforward because the pollinators do not require the introgression of monogermity or CMS. On the other hand the pool of pollinators has to fulfil the balancing act of providing high genetic diversity to react fast on changing demands and a high combining ability (Biancardi et al., 2005).

In recent years rhizomania became an increasing problem for sugar beet cultivation all over Europe after spreading from the Mediterranean northwards. Rhizomania causes root yield losses of more than 50% and reduced sugar content (Scholten and Lange, 2000). Responsible for rhizomania symptoms is the soil borne Beet Necrotic Yellow Vein Virus (BNYVV), which cannot be controlled by pesticides. The only strategy to avoid yield losses on infested soils is the cultivation of resistant cultivars. A major breakthrough for resistance breeding against rhizomania was the identification of the “Holly” resistance conferred by the gene *Rz1* (Lewellen et al., 1987).

The sugar beet cyst nematode (*Heterodera schachtii*) is widely distributed in sugar beet cultivation areas and causes yield losses of up to 50% by damaging the primary root system (Agrios, 2005). The impact of *H. schachtii* on the cultivation of sugar beets in Germany can be inferred from the fact that the descriptive variety list of the federal variety office contains a separate chapter for cultivars with tolerance or resistance against *H. schachtii* (http://www.bundessortenamt.de). Due to its wide host range and the restrictions on nematicide utilization, choice of resistant and tolerant varieties are the most effective method to limit yield losses besides the cultivation of catch crops (Biancardi et al., 2005).

Cercospora beticola leaf spot is the most important fungal disease in humid temperate areas and has spread like rhizomania into northern production areas (Holtschulte, 2000). To control *C. beticola* an integrated management system is recommended, combining both fungicides and resistant varieties (Miller et al., 1994). The only source of resistance with agronomic importance is a quantitative and only partial resistance which was introgressed from *B. vulgaris* ssp. *maritima* into sugar beets by Munerati around 1915 (Biancardi et al., 2005). This resistance is controlled by at least four genes and shows a negative correlation with sugar yield (Smith and Campbell, 1996). A further drawback is that the seed parents of monogerm hybrids display low levels of resistance and almost no variation. This results in hybrids with lower resistance levels than the parental pollinators (Skaracis and Biancardi, 2000).

In the 1920s sugar beet cultivation almost stopped west of the Rocky Mountains due to yield losses caused by Beet Curly Top Virus (BCTV). To counteract BCTV, which is a major threat

for sugar beet production under semi-arid conditions, the cultivation of available resistant varieties is most successful (Kaffka et al., 2002). Even though a partially dominant resistance factor was shown to be linked to red hypocotyl (Murphy and Savitsky, 1952) resistance against BVTV is thought to be at least oligogenic (Murphy and Savitsky, 1954). An overview about genetic analyses of biotic stress tolerance is given in Supplementary Table 1.

Sugar beets often cannot tap their full yield potential due to adverse environmental conditions causing abiotic stress (Jaggard et al., 1998; Kenter et al., 2006). Therefore, breeders aim to develop sugar beet varieties with high yield stability even under suboptimal conditions. Due to changing climate conditions sugar beets will be exposed to an increased risk of heat stress and therefore heat tolerant varieties have to be selected. As shown by Srivastava (1996) sugar beets display sufficient variation of heat tolerance and Clarke (1995) suggested measuring chlorophyll fluorescence to identify heat stress tolerant genotypes.

In most production areas sugar beet yield is constrained by the availability of water (Jaggard et al., 1998; Kenter et al., 2006). Therefore, breeders intent to improve drought tolerance and genetic variation for seedling and adult plant drought tolerance (Ober and Luterbacher, 2002; Sadeghian and Yavari, 2004; Sadeghian et al., 2000). To select for drought tolerant genotypes, phenotypic analyses must be practicable in large scale field experiments. Even in arid areas this is difficult possibly due to occasionally rain fall and the use of rain shelters is too expensive on a regular basis in breeding programs (Ober et al., 2004). Therefore, Ober (2005) suggested several physiological traits including water use efficiency, a threshold index for wilting and succulence as indirect selection criteria.

Sugar beets can suffer from cold stress at distinct developmental stages of the lifecycle, which differ in their ability to withstand frost. The most common cold stress occurs during germination and seedling development due to early seeding in spring. Moreover, cold tolerance is required in nurseries for seed production where stecklings overwinter in the field. Further, mature plants frequently have to withstand subzero degrees in the field before they are harvested. Biancardi (2005) reported that frost tolerance in young sugar beets increases as the plants develops. However, A. Schechert (pers. communication) observed an optimal growth stage for a maximum level of winter-hardiness, as plants which have developed a tap root more than approximately 2.5 cm in diameter displayed reduced survival rates after winter.

1.1.3 Genetic analysis of sugar beet

Long time before the first molecular markers were available the first linkage group in sugar beet had been published by Abegg using morphological markers. The *Y-R-B* linkage group comprises the loci *Y* (yellow root), *R* (red hypocotyl) and *B* (bolting locus *B*) (Abegg, 1936). In the 1980s several monogenic traits of economic importance could be mapped based on isoenzyme markers (Abe et al., 1992; Jung et al., 1986; Wagner et al., 1992). Due to the development of DNA based restriction fragment length polymorphisms (RFLPs) procedure the number of available markers increased tremendously and allowed the construction of the first linkage maps of the whole sugar beet genome (Barzen et al., 1992; Pillen et al., 1992). Using PCR-based analyses, including random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs), the availability of molecular markers for sugar beet analyses has increased substantially (Barzen et al., 1995; Schäfer-Pregl et al., 1999; Schondelmaier et al., 1996; Uphoff and Wricke, 1995). With the ability to identify single nucleotide polymorphisms (SNPs) the whole sequence variation of the genome can be exploited for marker analysis in sugar beets (Möhring et al., 2005; Schneider et al., 2001; Schneider et al., 2007). The presence of millions of potential

markers increases the demand for multiplex and high throughput technologies to identify these polymorphisms. Recently, Lange et al. (2010) presented a high-throughput microarray based approach which led to the identification of hundreds of new markers.

The development of DNA based markers and therefore the scale of available polymorphisms is now sufficient to perform genome wide analyses for complete regions which affect polygenic traits (Kearsey and Farquhar, 1998). Even though the theoretical background for genetic mapping was paved for decades, a major breakthrough was based on linkage of molecular markers with genetic factors controlling the trait of interest that is segregating in a mapping population. With suited statistical methods these markers can be associated to quantitative trait loci (QTL) affecting the trait of interest. Ideally, the phenotype of a given individual can be predicted by a marker within or adjacent to the QTL.

For linkage mapping, populations are ideally derived from a bi-parental cross of homozygous parents. These populations have undergone only a limited number of recombination events and large DNA regions with high linkage disequilibrium (LD) are conserved. Under these circumstances, linkage mapping is very successful to identify major QTL with a limited number of markers. Consequently, mostly major QTL could be mapped in sugar beet and the number of QTL was rather limited. The downside of linkage mapping is that due to large LD blocks the confidence interval of a QTL might span hundreds of genes, which makes the identification of the genes of interest a tedious task (Ingvarsson and Street, 2011; Mackay et al., 2009). Over the past 20 years more than 40 linkage analyses have been performed in sugar beet. The average genetic map size across these studies was 645 centiMorgan (cM). The major targets of these studies were resistances, focusing on rhizomania and nematodes, yield related traits and fertility related traits, including monogermity and the bolting behavior. An overview about linkage studies in sugar beet is given in Supplementary Table 2. Compared to linkage mapping, association mapping does not require specifically designed mapping populations. Hence association studies are suited for mapping in breeding or gene bank material without the need to develop bi-parental populations for the mere purpose of mapping. In contrast to linkage mapping, association mapping utilizes not only the recombination that occurred in a limited number of generations after a bi-parental cross but employs historic recombination events that took place over many generations (Flint-Garcia et al., 2003; Mackay et al., 2009). This leads to smaller and more numerous LD blocks under *ceteris paribus* conditions resulting in a higher mapping resolution. However, if the marker density is not sufficient to cover most LD blocks, QTL might not be detected. Moreover, in contrast to linkage mapping, a sufficient power to detect QTL requires a lot more individuals in the mapping population. Most important, a pitfall of LD mapping might be the confounding effect of unconsidered population structure which inflates the number of false positive associations (Ingvarsson and Street, 2011). To minimize the risk of false positives, markers inherited independently of the QTL can help to control population structure. Also, candidate gene based LD mapping is less affected by population structure and has been shown to be suited to map QTL for highly quantitative yield components in sugar beet such as root yield (Stich et al., 2008). Notably, even though based on a limited number of markers, the first genome wide association study (GWAS) in plants was conducted in *B. vulgaris* ssp. *maritima* to map the bolting locus *B* (Hansen et al., 2001). The success of such genome wide approach highly depends on the available marker density and LD decay (Hirschhorn and Daly, 2005; Ingvarsson and Street, 2011). More recent genome wide association studies in sugar beets were still based on a limited number of markers (Würschum et al., 2011a; Würschum et al., 2011b). However, in the future the available sugar beet genome sequence should enable higher marker densities required to cover the entire sugar beet genome in order to detect minor QTL or the effect of rare alleles (Li et al., 2011a).

1.1.4 The genome of *Beta vulgaris*

In the last 20 years physical maps of several sugar beet genotypes have been constructed. In contrast to genetic maps, physical maps are not based on genetic distances in terms of recombination frequency but on physical distances on a given chromosome or DNA fragment. The first physical maps were cytogenetic maps which were based on visual analysis of stained chromosomes (Gall and Pardue, 1969). With a resolution of 2Mb to 10 Mb the resulting physical maps were rather imprecise (Lange, 2010). A major improvement for cytogenetic analysis was the development of high resolution fluorescence in situ hybridization (FISH) resolving down to 3 kb of the sugar beet genome (Dechyeva, 2008). The mapping resolution increased substantially with the introduction of large insert clone libraries. To construct maps the cloned DNA inserts were sorted and aligned by the overlapping regions of different DNA inserts. The first clone libraries in sugar beet were based on yeast artificial chromosomes (YAC) (Eyers et al., 1992; Kleine et al., 1995), but due to vector instabilities they were later substituted by bacterial artificial chromosome (BAC) libraries (Gindullis et al., 2001; Hagihara et al., 2005; Hohmann et al., 2003; McGrath et al., 2004; Schulte et al., 2006).

To identify and analyse candidate genes, expressed sequence tags (EST) are a powerful resource. A sugar beet EST database has been provided and maintained by the Michigan State University (http://genomics.msu.edu/sugarbeet/index.html) encompassing 29,830 *B. vulgaris* ESTs that are listed in Genbank (NCBI ESTs). Approximately 1% of the available ESTs have been integrated into the genetic sugar beet map of Schneider (2007) and roughly 10% of the ESTs were screened by macroarray expression analysis (Pestsova et al., 2008). To enable high-throughput marker development in a mapping population a 15 K oligonucleotide microarray was established based on BAC-end and EST derived sequences (Lange et al., 2010). Fundamental studies about the identification and analysis of the sugar beet genome were performed in 2004, when the sugar beet mapping and sequencing consortium was founded (GABI – The German Plant Genome Research Program Progress http://www.gabi.de/progrep_ii_web.pdf). Approximately 28,000 genes have been tentatively annotated (Weisshaar et al., 2011) and a first draft of the physical map of the sugar beet genome has been published recently (Dohm et al., 2011).

Second generation sequencing technologies allow the analysis of millions of DNA molecules at a time, making SNP discovery on a genome wide scale affordable even for whole mapping populations (Nordborg and Weigel, 2008). On a medium term scale the *ultima ratio* for genotyping sugar beet populations will be whole genome re-sequencing as applied in maize, rice, soybean and *A. thaliana* accessions to generate whole genome haplotype maps (Cao et al., 2011; Gore et al., 2009; Huang et al., 2012; Huang et al., 2010; Lam et al., 2010).

To transfer the advanced knowledge of genes, their functions and regulatory networks from model organisms to non-model organisms, candidate gene (CG) approaches have been developed. In a first step genes already identified in model organisms are selected based on their function, expression level or other criteria indicating their role in the trait of interest. Subsequently CGs are screened to identify a CG marker that either co-segregates with QTL controlling the trait of interest, or shows a statistical association with the trait variation. In a third step the CG has to be validated (Pflieger et al., 2001).

In sugar beet research, EST derived CG identification has been successfully applied to identify a number of genes conveying resistance, yield, and flowering time (Abou-Elwafa et al., 2010; Büttner et al., 2010; Chia et al., 2008; Reeves et al., 2007; Schneider et al., 1999). In the near future the identification and characterization of such candidate genes will strongly benefit from the publication of the sugar beet genome sequence.

CG sequences can then be used to develop molecular markers for integration in genetic maps (Bellin, 2003). In case an identified CG marker co-localizes with a QTL it suggests a functional role of this candidate gene for the trait under investigation. This approach can be applied to linkage mapping as well as association mapping. Applying an association approach Stich and coworkers (2008) identified in total six marker-trait associations for sugar content and root yield in elite sugar beet germplasm, while Bellin (2003) used a linkage approach to integrate 35 developmental and metabolism related candidate genes into the sugar beet linkage maps of Schäfer-Pregl et al. (1999) and Schneider et al. (1999).

However, to proof function of candidate genes, the *ultima ratio* is complementation or RNA interference (RNAi) knockout by generated genetic transformation. Unfortunately as to this date no transformation protocol is available for sugar beet. Most complementation or knockouts for sugar beet candidate genes have been done in hairy root culture of sugar beet or in *A. thaliana* (Abou-Elwafa et al., 2010; Cai et al., 1997; Chia et al., 2008; Ehlers et al., 1991; Kifle et al., 1999). So far, only Pin et al. (2010) published results of sugar beet transformants in which either *FLOWERING LOCUS T2* (*BvFT2*) was knocked out via RNA interference (RNAi) or *BvFT1* was overexpressed.

1.2 Cold stress response in plants

The term cold tolerance is rather arbitrarily used in the literature. In a broad sense, cold tolerance refers to the performance at temperatures below the optimum for growth (Saulescu and Braun, 2001). In a more narrow sense cold tolerance is referred to the ability to withstand temperatures below the minimal growth temperature. However, most often the term cold tolerance is used to describe a plant's frost tolerance (Saulescu and Braun, 2001).

More specific is the term winter-hardiness, which is the ability to survive during winter time under field conditions. The determinants of winter-hardiness are tolerances against biotic and abiotic stresses, including frost tolerance as the major component (Arbaoui, 2007; Chinnusamy et al., 2010; Lejeune-Henaut et al., 2008; Pan et al., 1994). In many plants the exposure to cold, but non-freezing temperatures, increases frost tolerance and thereby winter-hardiness. This adaptation is known as cold acclimation (Thomashow, 1999). Because of the complexity of winter hardiness most research is focused on frost tolerance *per se*.

Methods to evaluate cold tolerance can be classified into indirect and direct approaches (Table 1). Indirect methods rely on the prediction of winter-hardiness by evaluating correlated traits without exposing the plants to frost (Saulescu and Braun, 2001). An ideal indirect method shows high correlations with field data and is highly repeatable, rapid, inexpensive as well as non-destructive (Fowler et al., 1981). Such methods include tissue water content (Fowler et al., 1981), free proline content (Dörffling et al., 1990) and the analysis of molecular markers which are linked to genes affecting winter-hardiness (Houde et al., 1992; Thomashow, 1999).

In direct frost tests plants are cold hardened and subsequently exposed to frost. Traits of interest for direct tests are plant survival (Fowler et al., 1981), leaf damage (Fuller and Eagles, 1978), root regrowth (Sutka, 1981), cell damage (Steponkus, 1983), electrolyte leakage (Hincha, 1994), fluorescence (Smillie and Hetherington, 1983), and electrical conductivity (Dexter et al., 1932). Direct tests can be conducted under controlled (e.g. climate chambers), semi-controlled (e.g. greenhouse), and field conditions.

Over the years a number of crops have been studied for winter hardiness in field trials including cereals, legumes, grasses, and oilseed rape (*Brassica napus* L.), (Alm et al., 2011; Arbaoui, 2007; Chawade et al., 2012; Dumont et al., 2009; Fowler and Gusta, 1979; Hayes et

al., 1993; Kahraman et al., 2004; Kosmala et al., 2007; Lejeune-Henaut et al., 2008; Li et al., 2011b; Rapacz and Markowski, 1999). While overwintering field trials are affected by multiple environmental factors, testing for frost tolerance under artificial conditions allows reduction of the experimental complexity. Most data for plant response to cold, including the genetics of frost tolerance, were derived from experiments in controlled environments. Beside *A. thaliana*, barley (*Hordeum vulgare* L.) has been extensively studied in frost chamber experiments. Moreover, a wide range of crops was analyzed for frost tolerance including wheat (*Triticum aestivum* L.), oilseed rape, pea (*Pisum sativum*), fodder grasses (*Festuca pratensis* Huds.) and rye (*Secale cereale* L.) (Alm et al., 2011; Dumont et al., 2009; Li et al., 2011b; Sutka, 1981; Waalen et al., 2011).

Until now, no studies on the genetics of winter-hardiness and frost tolerance have been performed in sugar beets. However, overwintering field trials were performed in the context of the risk assessment of genetic modified sugar beets and to elucidate physiological traits of overwintering sugar beets (Hoffmann and Kluge-Severin, 2010; Hoffmann and Kluge-Severin, 2011; Pohl-Orf et al., 1999).

1.2.1 Cold stress research in model plants

The cold response of plants starts immediately after exposure to cold temperatures and includes severe changes in genetic, metabolic and developmental networks (Chinnusamy and Zhu, 2009; Guy et al., 2007; Stitt and Hurry, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Analysis of the transcriptome and metabolome of *A. thaliana* after cold treatment revealed that hundreds of metabolites were affected by cold stress (Cook et al., 2004; Kaplan et al., 2004; Maruyama et al., 2009). The most prominent changes were observed in the contents of soluble sugars (e.g. sucrose, glucose and fructose) and amines (e.g. proline and alanine) (Cook et al., 2004; Guy et al., 1992; Kaplan et al., 2004; Maruyama et al., 2009).

Upon cold stress the membrane fluidity is reduced. Due to the changes of the membrane the cytoskeleton is remodeled, Ca^{2+} channels are activated and thereby cytosolic Ca^{2+} is increased (Carpaneto et al., 2007; Sangwan et al., 2001; Vaultier et al., 2006). Such changes in cytosolic calcium can be measured within seconds after cold exposure and are associated with membrane depolarization (Knight et al., 1991). The intensity of the calcium increase depends on the magnitude of the temperature shift, the absolute temperature, and the frequency of cold exposure (Knight et al., 1996; Plieth et al., 1999). The change in calcium levels is required for the full induction of cold responsive (*COR*) genes (Sangwan et al., 2001; Tähtiharju et al., 1997). The fast induction of *COR* genes are therefore part of the major genetic reprogramming upon exposure to cold stress.

Table 1: Overview of methods to assess frost tolerance.

frost exposure				
environment	**test type**	**cold tolerance assessment**	**examples**	**Reference**
		direct methods		
natural winter conditions	plants in boxes, pots	Survival rate (Field survival index)	Selection for winter-hardiness in Wheat	Fowler et al., 1981
modified field conditions (snow removal, boxes above ground)	plants transplanted from field	Survival rate	Identification of *ICE1* in Arabidopsis	Chinnusamy et al., 2003
frost chamber	seedlings	LT50	Mapping of frost tolerance in Barley	Hayes et al., 1993
dipped in refrigerated solution	leaves discs	leaf damage	Association mapping of winter-hardiness in semi-controlled environment in rye	Li et al., 2011
		root regrowth	Assessment of freezing tolerance in oilseed rape	Waalen et al., 2011
		electrolyte leakage	Study of natural genetic variation of freezing tolerance in Arabidopsis	Hannah et al., 2006
		fluorescence	Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis	Ishitani et al., 1997
		electrical conductivity	Analysis of cold tolerance in tomato	Kim et al., 2002

	frost exposure			
environment	**test type**	**cold tolerance assessment**	**examples**	**Reference**
		indirect methods		
-	-	tissue water content	Selection for winter-hardiness in Wheat	Fowler et al., 1981
-	-	free proline content	improvement of frost tolerance in winter wheat by proline over-accumulating mutants	Dörffling et al., 1990
-	-	electrical resistance	Evaluation of cold and frost tolerance in olives	Manusco et al., 2000
-	-	Molecular markers	WSC120 protein as a parameter for cold acclimation in wheat	Houde et al., 1992

Depending on the study, between 939 and more than 3.300 genes have been differentially expressed in *A. thaliana* in response to cold (Hannah et al., 2005; Lee et al., 2005; Matsui et al., 2008; Zeller et al., 2009). The most prominent cold responsive pathway is the *CBF*-regulon controlled by the transcription factors *C-repeat binding factors* (Thomashow, 1999). At least parts of the CBF pathway are expressed in monocots and dicots including cold sensitive and cold tolerant plant species (Choi et al., 2002; Dubouzet et al., 2003; Jaglo et al., 2001; Qin et al., 2004; Savitch et al., 2005; Welling and Palva, 2008; Zhang et al., 2004). The three *C-REPEAT BINDING FACTORs CBF1, CBF2* and *CBF3* in *A. thaliana* affect the expression of several hundred target genes (Maruyama et al., 2004; Maruyama et al., 2009; Vogel et al., 2005). The transgenic down regulation of *CBF1* and *CBF3* results in 25% to 50% reduced frost tolerance in Arabidopsis (Novillo et al., 2007). Moreover, a major QTL detected in *A. thaliana* explaining 20% of the observed variation in freezing tolerance co-localized with a CBF locus (Alonso-Blanco et al., 2005). Shortly after exposure to cold stress *CBFs* interact with the CRT/DRE elements in the promotor region of *COR* genes and induce their expression (Liu et al., 1998; Stockinger et al., 1997).

Phytohormones play an essential role for growth adaption in response to biotic and abiotic stressors. In the context of cold stress it has been shown that plants exposed to cold stress display differential growth and developmental patterns which can be at least partially related to altered hormone status (Choi et al., 2000). Even though several *COR* genes carry an ABA responsive element (ABRE) in promotor region and respond to exogenous ABA application, the role of ABA in cold response is ambiguous (Choi et al., 2000; Kang et al., 2002; Thomashow, 1999). Beyond controversy is, that plants with impeded ABA synthesis are unable to cold acclimate (Gilmour and Thomashow, 1991; Lang et al., 1994). However, ABA deficit plants did not show altered expression of several *COR* genes (Gilmour and Thomashow, 1991) and microarray data revealed that ABA synthesis genes are not regulated in response to cold (Lee et al., 2005). Moreover, Liu et al. (1998) concluded from analysis of the CRT/DRE element in the promotor of *COR* genes, that the *CBF* pathway is ABA independent. From these results together with the observation that ABA does not accumulate in response to cold Shinozaki and Yamaguchi-Shinozaki (2000) concluded that ABA is not required for low temperature signaling. Surprisingly Liu (1998) and Knight (2004) reported ABA dependent signaling through the CRT/DRE elements in *COR* promotors. Moreover, these authors conclude that the ABA induced accumulation of CBF proteins is at least partially responsible for the activation of COR genes through the CRT/DRE element. Further experiments will be necessary to clarify these contrary results.

Another stress induced phytohormone that has been shown to be upregulated upon n cold exposure is salicylic acid (SA), which accumulates shortly after cold exposure and leads to reduced growth under cold stress (Kaplan et al., 2004; Scott et al., 2004). Relatively little is known about the effects of cold stress on jasmonic ascid (JA) synthesis but it has been proposed that JA only plays a role in membrane damaging stresses (Blechert et al., 1995) which includes frost. Consistently with this theory, plants which were exposed to cold for 10 days displayed a 19-fold increase of JA precursor in the stroma of leaves (Goulas et al., 2006).

A central role in the regulation of plant growth in response to stress play gibberellin (GA)-regulated DELLA proteins. In 2008 Achard and coworkers described that cold induces the expression of *CBF1,* provokes the accumulation of DELLA proteins (Achard et al., 2008), and causes growth retardations (Jaglo-Ottosen et al., 1998). Both, the reduced growth and the increased frost tolerance of *CBF1* expressing plants were at least partially reversed in mutants lacking two genes required for DELLA accumulation (Achard et al., 2008). Moreover, after analyzing DELLAs under salt stress Achard et al. (2006) speculated that these proteins play a vital role in plants growth. Furthermore, Achard et al. (2006) assumed that this growth retardations releases resources to survive under stress conditions.

For the reproductive success of plants it is pivotal to avoid the transition to the cold sensitive flowering stage shortly before or during winter. As a consequence many plants have developed an obligate or facultative vernalization requirement. These plants either do not flower or they flower delayed without vernalization. Consistently with the natural winter conditions, *A. thaliana* ecotypes differ in their vernalization requirement and flowering time following latitudinal and altitudinal clines, indicating the importance of temperature for the timing of flowering (Caicedo et al., 2004; Mendez-Vigo et al., 2011; Shindo et al., 2006). Moreover, a correlation between decreasing temperatures and delayed flowering was observed (Mendez-Vigo et al., 2011). Genetic analyses have revealed that most of the above mentioned latitudinal and altitudinal differences in flowering time and vernalization response can be assigned to the major determinants of vernalization requirement, *Flowering Locus C* (*FLC*) and its regulator *FRIGIDA* (*FRI*) (Caicedo et al., 2004; Clarke and Dean, 1994; Johanson et al., 2000; Li et al., 2010; Mendez-Vigo et al., 2011; Michaels and Amasino, 2000; Shindo et al., 2006). However, *FLC* is not only a major determinant of vernalization response, but plays a dual role in flowering time regulation and cold stress response. Cold stress induces CBF expression and causes late flowering via increased *FLC* expression in *A. thaliana* (Seo et al., 2009) and recently it has been shown that *FLC* in turn regulates the expression of *CBFs* (Deng et al., 2011). Results of both studies indicate a cold stress and temperature dependent feedback loop of flowering time mediated by *FLC*. However, the interaction between vernalization response and cold response is limited not only to *FLC*, but also to *HIGH EXPRESSION OF OSMOTIC RESPONSIVE 1* (*HOS1*), that regulates both, the CBF regulon and flowering time via *FLC* induction (Ishitani et al., 1998; Lee et al., 2001).

The autonomous flowering pathway genes *FLOWERING LOCUS CA* (*FCA)* and FLOWERING LOCUS VE (*FVE)* in Arabidopsis have been shown to act in flowering regulation and temperature sensing. Mutations in *FVE* not only lead to expression of *FLC* and repression of the floral integrators *FLOWERING LOCUS T* (*FT)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* 1 (*SOC1*), but also to enhanced expression of *COR* genes and increased freezing tolerance (Blazquez et al., 2003; Kim et al., 2004).

Another gene in the cross talk between temperature sensing and flowering regulation in *A. thaliana* is the floral repressor *SHORT VEGETATIVE PHASE* (*SVP*). Loss of *SVP* function causes insensitivity to ambient temperature changes and abolishes the late flowering phenotype observed in *FCA* and *FVE* mutants (Lee et al., 2007). The expression of cold inducible *CBF*- and *COR* genes is negatively regulated by the floral activator *SOC1* (Seo et al., 2009).

The regulation of flowering and cold stress response depends on both light quality and the circadian clock. The importance of the circadian clock for frost tolerance has been shown by Fowler et al. (2005), who demonstrated that the expression of *CBF1*, *CBF2* and *CBF3* is gated by the circadian clock. Moreover, Bieniawska et al. (2008) has shown that the disruption of the circadian clock is causative for a wide range of transcriptional changes upon cold stress. Several circadian clock genes have been shown to affect the expression of *CBF* pathway genes. In this context Dong et al. (2011) demonstrated that the core components of the circadian clock, *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) reduce the former cold induced expression of *CBF* genes and disrupted the circadian oscillation. Moreover, Nakamichi (2009) reported effects on cold stress response by *PSEUDO RESPONSE REGULATOR* (*PRR)* genes, which are essential components of the circadian clock. Mutations in three *PRR* genes, including the *BvBTC1* homologue *PRR7* increased the expression of *CBF1* and enhanced frost tolerance. Further evidence for the interaction of the circadian clock and frost tolerance was given by Cao et al. (2005) who has shown that the circadian clock gene *GIGANTEA* (*GI*) is induced by cold stress and that *gi* mutants are frost sensitive. However, the GI dependent frost tolerance is independent of the

CBF regulon. The circadian clock and the photoperiod response are integrated by *CONSTANS* (*CO*) that negatively affects frost tolerance through the effect of *LONG VEGETATIVE PHASE 1* (*LOV1*). *LOV1* acts as a floral inhibitor under long day conditions and plants deficit for *LOV1* are hypersensitive to frost (Yoo et al., 2007).

The photoperiodic pathway genes *PHYTOCHROME B* (*phyB*), *PHYTOCHROME D* (*phyD*) and *PHYTOCHROME E* (*phyE*) play a major role for sensing light quality. Plants mutated in *phyB* and *phyD* not only show an early flowering phenotype but also react with decreased expression of *COR* genes (Devlin et al., 1999; Franklin and Whitelam, 2007).

1.2.2 Cold stress research in crop plants

Under low temperate conditions cold stress tolerance has a major impact on crop yield. Therefore, many crop species have been intensively investigated for cold tolerance. Crops can be exposed to cold stress by early sowing in spring or overwintering in the field. An excellent example for cold tolerance in a spring sown crop is the adaptation of maize to cold temperate conditions. The tremendous expansion of maize production across Europe and northern regions of the USA over the last thirty years is at least partially driven by major improvements in cold tolerance. Over the years many physiological effects of cold stress on total growth, photosynthesis, and seedling-, leaf- and root development have been elucidated (Beauchamp and Lathwell, 1967; Fryer et al., 1998; Hund et al., 2008; Hund et al., 2004; Stamp, 1984; Verheul et al., 1996). In genetic studies several QTL affecting cold tolerance of maize seedlings have been identified (Jompuk et al., 2005; Presterl et al., 2007). Moreover, the identification of two *CBF* transcription factors and the involvement of phytohormones like SA, JA and ABA in the cold response of maize indicate that at least parts of the cold response are conserved between maize and *A. thaliana* (Janda et al., 1999; Qin et al., 2004; Wang et al., 2008) (Anderson et al., 1994; Lee et al., 1996).

Wheat and barley are the most important small kernel grains in Europe. However, in Northern and Eastern European areas cultivation of high yielding autumn sown varieties are limited due to insufficient winter-hardiness (Galiba et al., 2009). To gain yield stability and expand the cultivation area improvement of winter-hardiness and cold tolerance has been a long-term research focus in cereals. Winter-hardiness in cereals is determined by the three factors vernalization response, low temperature tolerance, and photoperiod sensitivity (Pan et al., 1994). Several loci affecting low temperature tolerance have been identified by QTL and association mapping studies (Laurie et al., 1995; Pan et al., 1994; von Zitzewitz, 2010). A major QTL for frost tolerance in barley, called FR-2, co-localizes with a cluster of CBF genes (Francia et al., 2004; Galiba et al., 2009; Skinner et al., 2005). FR-1, a second QTL for frost tolerance co-localizes with the previously identified locus for vernalization sensitivity *VERNALIZATION1* (*VRN1*) (Fu et al., 2005; von Zitzewitz et al., 2005). Vernalization leads to a continuous up-regulation of *VRN1,* which shares similarities with the *A. thaliana* floral meristem identity gene *APETALA*1 (*AP1*) (Yan et al., 2003). Observing a 2.4 fold higher low temperature tolerance in plants carrying the recessive winter *vrn1* allele Limin and Fowler (2006) suggested a model in which the vernalization dependent activation of *VRN1* in wheat down regulates the *CBF* pathway and thereby decreases frost tolerance. This could explain why plants which have entered a reproductive state show reduced frost tolerance (Galiba et al., 2009). Moreover, Dhillon (2010) proposed that the locus for frost tolerance on chromosome 5 is a pleiotropic effect of *VRN1* and it can be speculated that activation of *VRN1* causes the loss of frost tolerance in plants which are fully vernalized (Trevaskis, 2010). Interestingly, co-localization of QTL for flowering time and cold tolerance has not only been reported from cereals and *A. thaliana*, but also in legumes (Lejeune-Henaut et al., 2008). The identification of *CBF* transcription factors in a wide range of frost tolerant and frost sensitive crop species, including rice, oilseed rape, tomato, strawberry and birch drove Jaglo (2001) to the conclusion that at least components of the *CBF* regulon are highly conserved in flowering

plants (Dubouzet et al., 2003; Jaglo et al., 2001; Owens et al., 2002; Qin et al., 2004; Skinner et al., 2005).

Even though the mechanisms of frost tolerance and winter-hardiness have been investigated in a wide range of crop species, it has to be elucidated, whether the *CBF* regulon and other cold responsive elements are conserved in sugar beet. Moreover, little is known about frost tolerance and winter-hardiness in sugar beets, even though from the very beginning of sugar beet cultivation it was assumed that autumn sown sugar beets are capable of surviving winters under central European conditions (Achard 1806). The aim to develop these winter sugar beets is driven by the fact that under cool-temperate conditions yield of spring sown sugar beets is limited by slow seedling development and insufficient canopy in spring (Kenter et al., 2006). An expansion of the vegetation period by sowing winter hard sugar beets in autumn would overcome these limitations and in theory yield increases of up to 26% were calculated (Hoffmann and Kluge-Severin, 2010; Jaggard and Werker, 1999). Even though this yield gain is hypothetical and was not known 100 years ago the first breeding activities to develop winter sugar beets started at the beginning of the 20th century. As reported by Bauer in 1932 breeding activities in Hungary had started in the 1910s resulting in winter beets with increased dry matter and sugar content. Moreover, he pointed out that "the winter beets of Professor Nemeth have succeeded in combing a) bolting resistance under maintenance of a high sugar content, and b) a high vigour, caused by fast development in spring and summer, preventing the beets from pests and negative weather conditions. In 1935 Schneider doubted the reports of Bauer about a yield gain of more than 100% and complete bolting resistance, but expected a yield increase of the magnitude of the yield difference between winter- and spring types in cereals (Schneider 1935). In 1928, Hall (1928) published results of experiments showing the strong effect of vernalization on bolting in leaf beets, garden beets and sugar beets. Moreover, he published results demonstrating the success of mass selection on bolting rates by reducing bolting rates within four generations from up to 75% to 0% in response to early sowing. In 1948 McFarlane (1948) reported about sugar beet germplasm that was highly bolting resistant when sown in August in Utah. In two breeding populations a bolting rate below 2% was observed in mid-April. At the beginning of the 1950s two aims of a winter sugar beet breeding program in Germany were defined as: 1. Breeding of a mid-summer sowable sugar beet, with a superior yield compared to spring sowing. 2. Increase of frost tolerance in seedlings, combined with high bolting resistance. Compared to regular spring types, winter beets were selected that showed a significant yield advantage of up to 99% in all five test environments in Germany when sown in August. Eichholz and Röstel (1962) concluded from the results of a comparison of sugar and non-sugar components between winter beet and spring sown beets that the physiological maturity of mid-summer sown beets is already reached in September of the second year. Interestingly, across three years, the most bolting resistant winter beet population showed bolting rates of 3%, 15% and 0.5 %, respectively. In the 1970s, Wood and Scott (1975) investigated the possibility of extending the growing season of sugar beet by sowing in autumn. Until mid-June, September sown beets intercepted fourfold more light and estimated to be 20-fold heavier compared to April sown beets. However, these advantages were progressively lost with the onset of bolting.

Despite all efforts to develop a winter beet with sufficient winter-hardiness and an applicable system for bolting control no winter sugar beet variety has ever been introduced into markets. The main obstacle in breeding winter beets was the requirement to combine two contrary breeding goals: i. winter beets have to stay in the vegetative growth stage after exposure to cold but non-freezing conditions, which requires bolting oppression, and ii. seeds are required to propagate and cultivate these beets, which requires bolting. Therefore, a system for bolting control has to be developed. Recently, Jung and Müller (2009) proposed a hybrid approach to overcome this challenge. Their model is based on genetic modification of two hybrid

components with inactive bolting suppressors. While the hybrid components are still bolting after vernalization, the bolting oppressing transgene becomes active only in the resulting hybrid variety. With the functional analysis and cloning of several flowering time and bolting genes in sugar beets the required bolting control for breeding winter beets will be fulfilled in the near future and attention can be focused on frost tolerance and winter-hardiness.

1.3 Objectives and scientific hypothesis

Autumn sown sugar beets are expected to increase sugar beet yield. Under Central European conditions sugar beets overwintering in the field require winter-hardiness and frost tolerance to meet the expected yield gains. Before this study little information was available about the capability of *B. vulgaris* accessions to survive cold winters. A graphical presentation of the concepts to gain knowledge on winter-hardiness and frost tolerance in *B. vulgaris* is given in Figure 1.

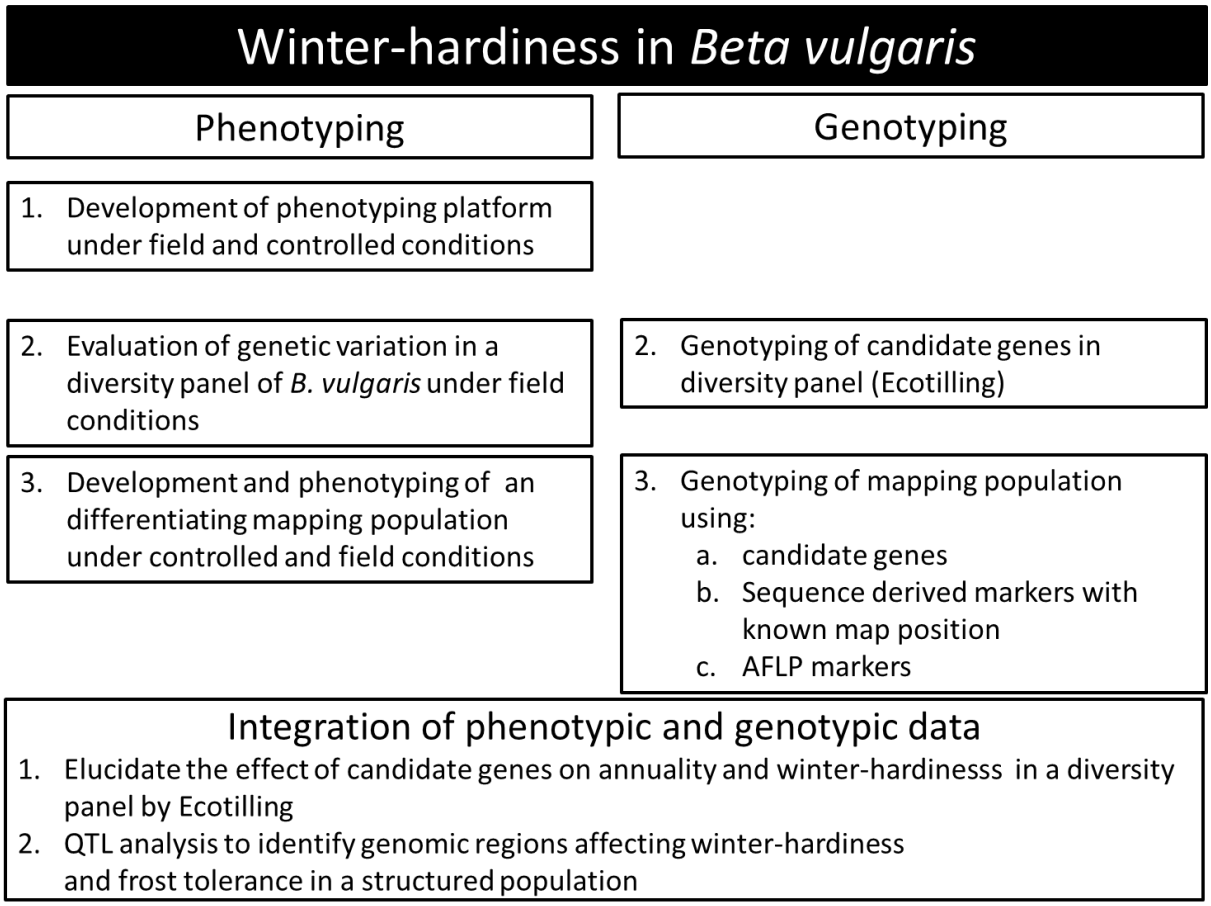

Figure 1: Overview of the strategies applied to open the phenotypic and genotypic "black box" of winter-hardiness in *Beta vulgaris.*

This study was driven by 5 hypotheses:

I. *Beta vulgaris* accessions differ in their level of winter-hardiness and frost tolerance.
II. *Beta vulgaris* and *A. thaliana* share similar mechanisms of cold stress response and therefore *A. thaliana gene* homologues exist in *B. vulgaris.*
III. The differences in winter-hardiness and frost tolerance are controlled by quantitative genetic factors.
IV. The proportion of the phenotypic variance that can be explained by genetic factors is sufficient to identity regions of the *B. vulgaris* genome that affect winter-hardiness and frost tolerance.

V. The genetic regulatory networks controlling winter-hardiness and frost tolerance interact with flowering time genes of *B. vulgaris.*

To test these hypotheses this study aims to:

I. Access the genetic variation of winter-hardiness of *B. vulgaris* in multi-environment field trials.

II. Identify candidate genes with putative effect on winter-hardiness and frost tolerance in *B. vulgaris* by sequence comparisons with known cold regulated genes in *A. thaliana*.

III. Perform a QTL analysis for winter-hardiness and frost tolerance with phenotypic data collected under natural and artificial conditions.

IV. Estimate the effect of major flowering time regulators of *B. vulgaris* on winter-hardiness via Ecotilling.

2 High degree of genetic variation of winter hardiness in a panel of *Beta vulgaris* L.

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2.1 Abstract

Growing sugar beet [*Beta vulgaris* L. ssp. *vulgaris* (sugar beet group)] as a winter crop requires the development of a winter sugar beet with controlled bolting and sufficient winter hardiness. To evaluate the genetic variation for winter hardiness in *B. vulgaris* L., we determined the survival rate (SR) in a panel of 396 accessions tested in eight overwintering field trials in Germany and Belarus. The panel included the cultivar groups sugar beet, fodder beet, garden beet and leaf beet as well as the wild beet *B. vulgaris* ssp. *maritima* (L.) Arcang. (BVM). Across all environments the effects of accession, environment and accession by environment interaction were highly significant. Despite the complexity of the trait, the heritability for SR was estimated as h^2=0.81 reflecting a large genetic variation in the panel. Environmental SRs ranged from 0.7% to 86.3% with a grand mean of 28.6%. In all environments at least one accession completely died while the maximum SR ranged from 39.9% to 100%. On average, sugar beet accessions performed best while accessions with the highest SR were among BVMs and leaf beets. The largest variation for SR was found in BVMs followed by the leaf beets whereas sugar beets showed the smallest variation. Our results suggest that winter hardiness in sugar beet is sufficient to survive mild winters but needs to be improved for continental climates with colder winters. Whether the limited variation in sugar beet is sufficient for this has to be further investigated.

2.2 Introduction

Under cool-temperate conditions, sugar beets [*Beta vulgaris* ssp. *vulgaris* cult. sugar beet (Lange et al., 1999)] are cultivated as a spring crop. Like in many other spring sown crops yield is limited by slow seedling development and insufficient canopy at the beginning of the vegetation period. A spring sown sugar beet does not develop full canopy cover before the middle of June (Kenter, 2003). Therefore, from April to June, only part of the photosynthetic active radiation (PAR) is captured by young plants (Röver, 1995). An increased leaf area index in early spring would improve the light absorption and thereby raise photosynthesis and consequently yield (Röver, 1995). One strategy to overcome the asynchrony of leaf development and PAR is a sugar beet sown in autumn and harvested in the following year (Eichholz and Röstel, 1962). This so-called winter beet is expected to have a faster canopy development in spring and therefore the ability to capture a higher portion of PAR available before the middle of June. However, such a winter sugar beet requires two properties: i) sufficient winter hardiness and ii) bolting control after winter.

First breeding activities for a winter sugar beet started in the early 20th century. Bauer (1932) reported breeding activities in Hungary in the 1910s: "the winter beets of Prof. Nemeth have succeeded in combining i) bolting resistance under maintenance of a high sugar content with ii) a high vigour, caused by fast development in spring and summer that prevents the beets from pests and negative weather conditions." Bauer reported yield gain of more than 100% and complete bolting resistance after winter. To our knowledge, however, these early breeding efforts did not lead to the release of a winter sugar beet cultivar and it was Schneider (1935) who critically questioned Bauer´s report on such increase in yield. Schneider expected a moderate yield increase in the magnitude of the yield difference between winter and spring types in cereals. Nevertheless, breeding efforts continued and at the beginning of the 1950s

two aims of a winter sugar beet breeding program in Germany were defined (Eichholz and Röstel, 1962) as: i. Breeding of a mid-summer sown winter sugar beet with a superior yield compared to regular spring type sugar beets and ii. Improvement of frost tolerance, combined with high bolting resistance. Eichholz and Röstel (1962) observed a yield increase of 38% (ranging from 13% to 99%) in winter beets and selected populations with bolting rates reduced to 0.5%. To our knowledge, however, the results of Eichholz and Röstel never led to the production of winter sugar beets or any variety with increased winter hardiness. We do not have any evidence that the work of Eichholz and Röstel was continued or the genetic resources conserved in any way. Assumingly, all efforts to develop a winter sugar beet eventually failed with lacking a system for bolting control that allows bolting for seed production while suppressing bolting in crop production. Despite this, further investigations were conducted in the 1970s by Wood and Scott (1975) in the U.K. with sugar beet sown as a winter crop in September. These beets, when harvested before the onset of bolting in mid-June of the following year, were reported to have an astonishingly 20-fold higher biomass compared to spring sown non vernalized beets harvested at the same time. However, this lead was progressively lost with the onset of bolting later in season.
As bolting control is likely to be achieved by genetic engineering (Jung and Müller, 2009) the development of a winter sugar beet gained new momentum in recent years, not only as a sugar crop but also as an energy crop. Besides tackling bolting control, focus needs to be put on the assessment and improvement of winter hardiness. Little work has been done on winter hardiness for the past 50 years except a study in the scope of risk assessment of transgenic sugar beet (Pohl-Orf et al., 1999). For this, SRs of a limited number of breeding lines, cultivars and transgenic varieties were evaluated in 12 environments from 1994 to 1998. The average SRs in the different environments ranged from 0% to 87.4%, indicating insufficient winter hardiness in sugar beet.
The ability of crops to survive winter depends on i. frost tolerance, ii. tolerance against other abiotic winter stresses, and iii. resistance against biotic stressors (Arbaoui et al., 2008).

While some components of winter hardiness such as frost tolerance can be evaluated in laboratory or climate chamber experiments, the whole complexity of this trait is best captured in overwintering experiments under field conditions. These experiments have to be performed in various environments because winter conditions extremely vary with geographic location and yearly weather conditions (Pohl-Orf et al. 1999).

To improve winter hardiness in sugar beet the use of genetic resources might play a key role, especially the primary gene pool which is formed by the section Beta in the genus Beta (Letschert et al., 1994). Section Beta includes the species *B. vulgaris* L., *B. macrocarpa* Guss. and *B. patula* Ait., of which *B. vulgaris* L. comprises besides sugar beet the other cultivar groups *B. vulgaris* ssp. *vulgaris* cult. leaf beet (leaf beet), *B. vulgaris* ssp. *vulgaris* cult. garden beet (garden beet) and B. *vulgaris* ssp. *vulgaris* cult. fodder beet (fodder beet) as well as the sea beet *B. vulgaris*. ssp. *maritima* (L.) Arcang. (BVM) (Lange et al., 1999; Letschert, 1993). The latter is a wild relative of sugar beet, can be easily crossed and is a major source of resistance genes against biotic and abiotic stresses (Biancardi et al., 2002; Frese et al., 2001; Panella and Lewellen, 2007; Scholten et al., 1999). To facilitate the utilization of the more than 10,000 Beta accessions stored in gene banks worldwide a Beta core collection was compiled by Frese (2000) to cover a maximum genetic diversity with a limited number of accessions. This collection includes 696 *B. vulgaris* accessions. Using this resource as well as breeding germplasm we conducted overwintering field trials to evaluate the *B.vulgaris* gene pool as a genetic resource for the improvement of winter hardiness in sugar beet. The objectives of the presented work were i) to assess winter hardiness in a panel of 396 *B. vulgaris* accessions in field experiments across eight environments and ii) to describe the genetic variation for winter hardiness between and within various *B. vulgaris* cultivar groups and BVM.

2.3 Materials and Methods

2.3.1 Plant material

A representative panel of *B. vulgaris* germplasm was investigated for winter hardiness. The 396 accessions of the test panel include the four cultivar groups fodder beet (61), leaf beet (62), garden beet (90), and sugar beet (100) as well as 56 BVMs and 27 uncharacterized *B. vulgaris* accessions.

The material was provided by various gene banks and breeders as shown in Table 2. More than two thirds (278) of the accessions are part of the Beta core collection described by Frese (2000). Thus the tested accessions cover a wide range of genetic diversity. In 2009/10 the number of tested accessions was reduced to 268 due to limited seed availability.

2.3.2 Field trials

To determine the genotypic variation for winter hardiness, the panel was evaluated in a field experiment in eight environments at five different locations (Table 3). The environments cover a wide range of climate conditions ranging from maritime in Kiel (Ki, Germany) to continental in Minsk (Mi, Belarus) and Nesvizh (Nes, Belarus). The experiments were designed as a randomized complete block design. Experimental units were 2.1 m long single rows with a between row distance of 45 cm. Between 5 Aug. and 30 Sept. 2008 as well as 22 July and 14 Aug. 2009 30 seeds were sown per row. The number of plants was thinned to 15 per row before winter. In Ki 08/09 seeds were sown in the greenhouse and transplanted after 4 weeks into the field. The leaf biomass before winter in Ki 08/09 and Göttingen (Gö, Germany) 08/09 was visually scored as a leaf biomass index ranging from 1 = very small to 9 = very large. The SR of the accessions was determined as surviving plants per row after winter divided by plants per row before winter. Surviving plants were recorded between 15 April and 15 May depending on the weather conditions of the field environment. An environmental SR was estimated as the grand mean of all accessions in a given environment.

Daily temperatures taken 2m above ground and precipitation were either recorded at meteorological field stations (Kiel, Söllingen, Göttingen) or provided by the closest available meteorological station (Minsk and Nesvizh). The snow heights were recorded by the closest available meteorological station. Temperature sums (thermal time) were calculated as the cumulated average daily temperature above a base temperature of 4°C to obtain a measure for pre-winter development before winter. As a measure of frost severity cold sums were calculated for each environment, defined as the minimal daily temperature below 0°C. Further, to take snow insulation into account, we calculated the ratio of days with snow coverage for the days with sub-zero temperatures.

Table 2: Source of *Beta vulgaris* accessions evaluated in field trials for winter hardiness

Seed provider	Location	Accessions provided
Agroscope Changins-Wädenswil ACW	Wädenswil, Switzerland	14
Christian Albrechts University Kiel	Kiel, Germany	10
Institute for Agricultural and Fisheries Research, PLANT	Melle, Belgium	11
KWS Saat AG	Einbeck, Germany	2
Leibniz Institute of Plant Genetics and Crop Plant Research	Gatersleben, Germany	140
National Academy of Sciences of Belarus, Institute of Genetics and Cytology	Minsk, Belarus	1
Nordic Genetic Resource Center	Alnarp, Sweden	1
Strube Research GmbH & Co. KG	Söllingen, Germany	52
US Department of Agriculture, Sugar beet Research Unit	Fort Collins, CO,USA	109
University of Warwick-Genetic Resources	Warwick, Great Britain	11
Vavilov Institute of Plant Industry	St. Petersburg, Russia	44

Table 3: Specifications for test environments of overwintering field trials.

Environment	Year	Location	Coordinates	height above sea level [m]	Acc	Rep	Sowing Date
Gö 08/09	2008/09	Göttingen	51.53, 9.74	290	391	2	6 Aug. 2008
Gö 09/10	2009/10	Göttingen	51.47, 9.92	159	268	2	5 Aug. 2009
Ki 08/09	2008/09	Kiel	54.35, 10.11	22	391	1	9 Sept. 2008†
Ki 09/10	2009/10	Kiel	54.31, 9.99	39	268	2	14 Aug. 2009
Mi 08/09	2008/09	Minsk	53.88, 27.63	280	391	1	5 Aug. 2008
Mi 09/10	2009/10	Minsk	53.88, 27.63	280	268	2	22 July 2009
Nes 08/09	2008/09	Nesvizh	53.23, 26.65	180	391	2	5/30 Sept. 2008‡
Sö 09/10	2009/10	Söllingen	52.20,10.90	142	268	2	13 Aug. 2009

Acc: Accessions. Rep: Replication.

† Four week old pre-cultivated plants were transplanted to the field.

‡ first and second replication were sown on 5 Sep. 2009 and 30 Sep. 2009, respectively.

2.3.3 Statistical Analysis

An analysis of variance (ANOVA) for SR was performed across all eight environments using SAS PROC GLM (SAS Institute Inc., 2009, Cary, USA, Version 9.2). All factors were treated as random effects using the model:

$$y_{ijkl} = \mu + l_i + b(l)_{ij} + g_k + gl_{ik} + e_{ijkl}$$

where y is the SR of the k^{th} accession tested in the j^{th} block of the i^{th} environment, μ is the overall mean, l_i is the effects of the i^{th} environment, $b(l)_{ij}$ is the effect of the j^{th} block nested in the i^{th} environment, g_k is the genetic effect of the k^{th} accession, gl_{ik} is the interaction effect of the k^{th} accession and i^{th} environment, and e_{ijkl} is the random experimental error. In SAS PROC MIXED (SAS Institute Inc., 2009, Cary, USA, Version 9.2) variance components were estimated based on the same statistical model and used to estimate the broad sense heritability as

$$h^2 = \frac{V_g}{V_g + V_{gl}/l + V_e/R}$$

where h^2 is the broad sense heritability, V_g is the genetic variance of the test panel, V_{gl}/l is the variance of the genotype by environment interaction divided by the number of environments and V_e/R is the residual variance divided by the total number of replications. Best linear unbiased predictors (BLUPs) were estimated for the mean SRs of each accession, for the mean SRs of each *B. vulgaris* cultivar group and BVM and for the mean SRs of each environment. We decided to use BLUPs because the number of accessions tested across environments was not balanced. Further, five preplanned contrasts were calculated across all environments to compare the SR of the group of sugar beet accessions with each of the three other cultivar groups fodder beet, leaf beet and garden beet as well as BVM.

The variance for leaf biomass index in Ki 08/09 and Gö 08/09 was analyzed using the above model except that accession by environment interaction was dropped out. This was done because the experiment in Ki 08/09 had only one replication. Best linear unbiased predictors were estimated for the mean leaf biomass index of each accession.

A regression analysis was performed to estimate the influence of leaf biomass before winter on SR using SAS PROC GLM. For this the SRs estimated across all environments were regressed on the leaf biomass indices estimated across Ki 08/09 and Gö 08/09 assuming no accession by environment interaction for leaf development. This regression was done separately within each cultivar group as well as within the BVMs. Further, a regression analysis was performed for environmental SRs on minimal temperature, cold sums and snow covered days, where an environmental SR was estimated as the grand mean of all accessions in a given environment. R2.12 (R Development Core Team, 2010) was used to design box plots.

2.4 Results

We observed a large variation for SR in the overwintering experiment with 396 *B. vulgaris* accessions across eight field environments (Figure 2, Supplementary Table 3). In the ANOVA for SR the factors environment, accession as well as the accession by environment interaction were tested as highly significant with $p < 0.001$. The broad sense heritability was estimated as $h^2 = 0.81$. The grand mean of SRs over all environments was 28.4%.

The pre-winter conditions varied largely among environments. The best pre-winter development was observed in Gö 09/10 where the canopy was almost closed between rows

followed by Gö 08/09 and Min 09/10 with moderate pre-winter development. Plants in Mi 08/09, Ki 09/10 and Söllingen (Sö, Germany) 09/10 were clearly behind in their development before winter while the poorest plant growth before winter was observed in Nes 08/09, where a large portion of plants did not even develop beyond seedling stage.

The different environments displayed distinct weather conditions in the field trials. Average temperatures from the day of sowing until 1 Nov. ranged from 9.4°C in Nes 08/09 to 13.3°C in Gö 09/10. The lowest overall temperatures were recorded in Mi 09/10 (-23.7°C) and Nes 08/09 (-22.8°C) while the winters in Ki 08/09 and Ki 09/10 were mildest with minimal temperatures up to 14.6°C higher than in the other environments. The average temperature from 1 Nov. until 15 Apr. ranged from 3.2°C in Ki 08/09 to -2.2°C in Mi 09/10. For more details see Table 4.

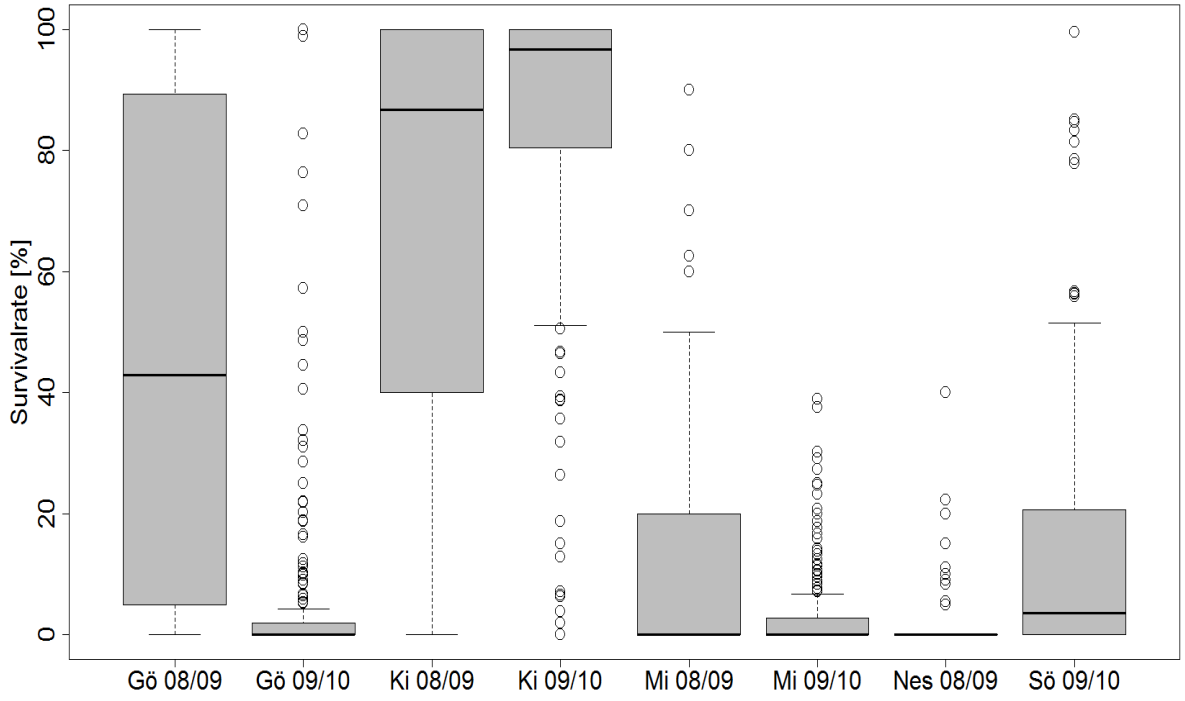

Figure 2: Boxplot of the survival rates [%] for 396 *B. vulgaris* accessions tested in eight different overwintering field trials in Germany and Belarus.

The SRs across the test locations showed a wide range of variation (Figure 2) Average SRs in each environment ranged from 0.7% (Nes 08/09) to 86.3% (Ki 09/10). The highest SRs in both years were observed at Kiel with 70.6% and 86.3%, respectively.

In a regression analysis 81% of the variation for environmental SR could be explained by the minimal temperature in each environment (P=0.0023) with an intercept of 138.2 and a slope of 6.028 (Figure 3). Regression of the cold sum or the snow covered days on the SR were not significant (alpha = 0.05).

Table 4: Meteorological data of the field trial locations between sowing and 15 Apr. of the following year.

Environment		Average daily temperature before 1 Nov. [°C]	Thermal Time before 1 Nov. [°Cd]	Precipitation before 1 Nov. [mm]	Average daily temperature from 1 Nov. till 15 April [°C]	Average minimal daily temperature from 1 Nov. till 15 April [°C]	Minimal temperature[°C]	Cold sum of daily minimal temperatures below 0°C [°Cd]	Days with snow	Frost days (with snow[%])
Gö	08/09	12.1	696.8	191.7	2.3	-0.3	-18.8	-345.4	33	82 (39.0)
Gö	09/10	13.3	817.0	164.9	2.4	-0.8	-19.3	-473.6	63	74 (85.1)
Ki	08/09†	10.1	335.3	147.7	3.2	1.1	-9.1	-155.5	17	68 (25.0)
Ki	09/10	12.4	661.6	127.1	1.1	-0.6	-11.8	-420.8	69	87 (79.3)
Mi	08/09	9.8	324.5	116.9	-0.6	-2.8	-18.8	-348.1	71	86 (82.6)
Mi	09/10	12.6	883.9	295.3	-2.2	-4.5	-23.7	-718.3	85	93 (91.4)
Nes	08/09‡	9.4	75.6	67.6	-1.5	-4.0	-22.8	-230.8	86	103 (83.3)
Sö	09/10	13.2	742.1	170.5	1.8	-0.3	-20.5	-433.0	58	77 (75.3)

† recordings of meteorological data started on the day of transplanting plants into the field.

‡ Recordings started with the sowing of the first replication. Thermal Time: cumulated average daily temperature above a base temperature of 4°C.

The ANOVA for leaf biomass index taken before winter in two environments revealed significant genotypic variation ($P < 0.0001$). As displayed by the box plots in Figure 4 leaf biomass before winter appeared to be best developed in fodder beets and leaf beets and least in BVMs. The distinct *B. vulgaris* cultivar groups and BVMs differed in their ability to survive winter (Figure 5, Supplementary Table 4). The largest variation of SRs was observed within the BVMs with a range in SR of 56.8%, and a mean of 35.3%. The BVM SR range was most similar to leaf beets with a range of 49.8%. Fodder beets, uncharacterized *B. vulgaris* and garden beets displayed similar SR ranges with the highest variation in the uncharacterized beets and the lowest in garden beets. With a range of 23.9% the lowest variation was observed in sugar beet. Sugar beet was most winter hardy with an SR of 39.7% across all environments, followed by the BVMs with 35.3%. Closest to the overall average SR of 28.4% were leaf beets (27.3%), followed by uncharacterized *B. vulgaris* accessions (24.9%) and the fodder beets (24.3%). The lowest SRs were observed in garden beets with 19.5%. All linear contrasts for comparing the SR of sugar beets with each of the other *B. vulgaris* cultivar groups and BVMs were highly significant ($P>0.001$).

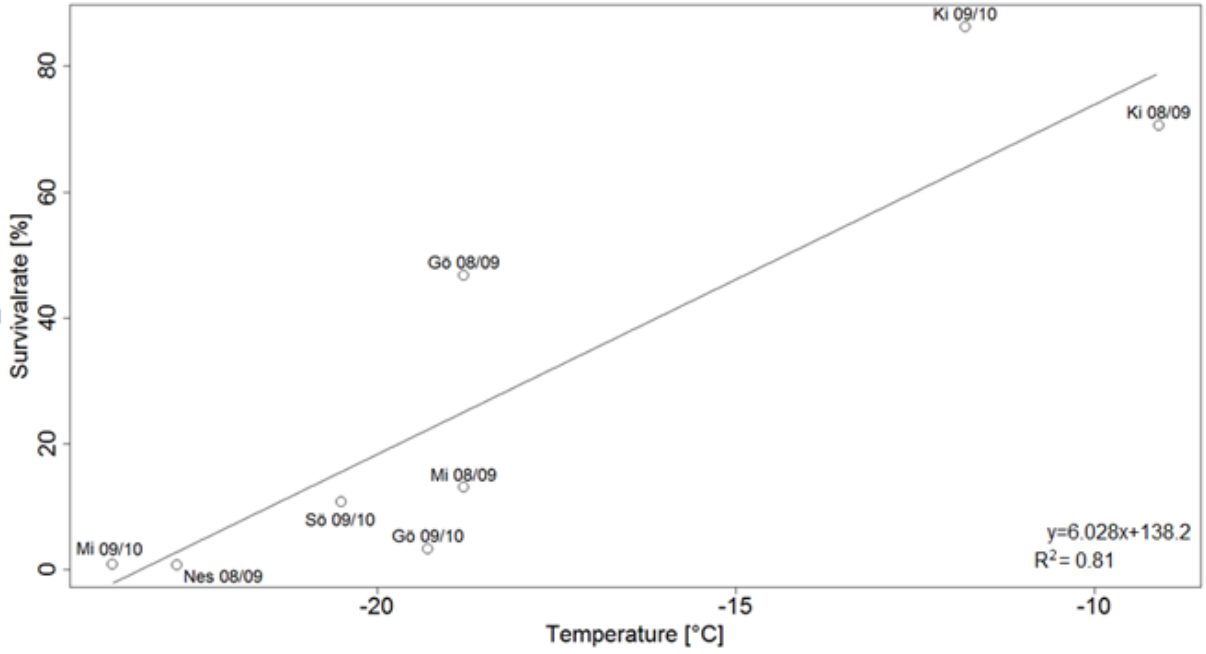

Figure 3: Regression of survival rate of a *B. vulgaris* panel on minimal temperature in eight environments.

Regression analysis revealed a negative dependency of SR on the leaf biomass index in fodder beets and leaf beets (alpha = 0.05), but this effect was not significant in the other cultivar groups and BVM (Table 5).

The most winter hardy accessions tested in all environments (top 5%) included 13 BVMs, one sugar beet and one leaf beet. Five of the 13 BVMs originated from Denmark, two from the Netherlands and the remaining four from France, Great Britain, Greece and Sweden. The least winter hardy accessions (bottom 5%) comprised seven leaf beets, three garden beets, two BVMs and two uncharacterized *B. vulgaris* accessions.

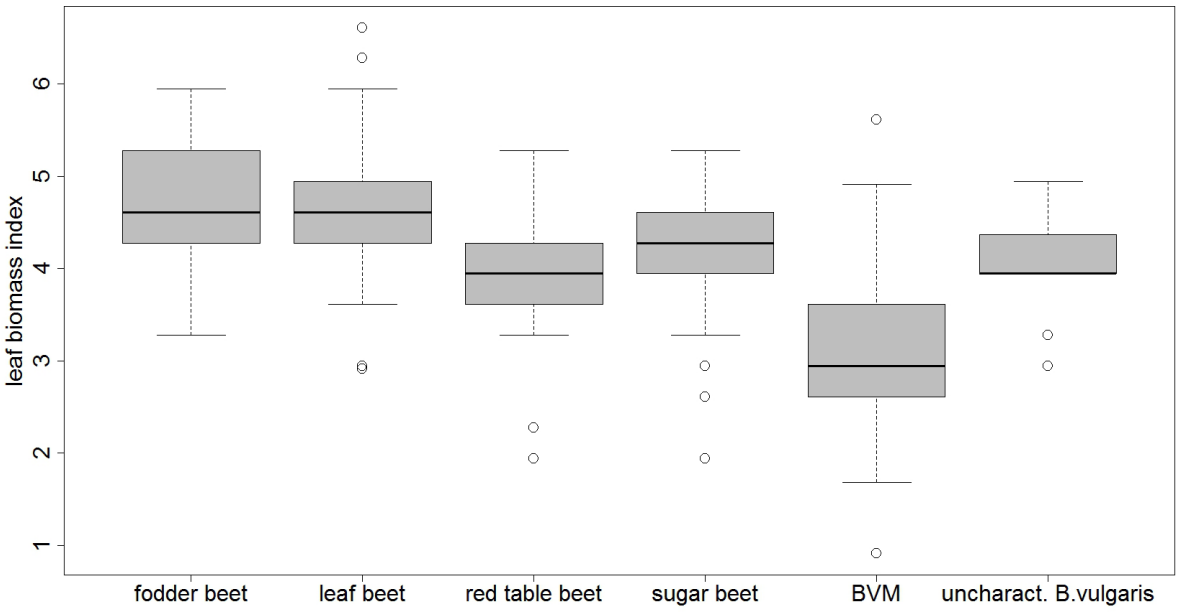

Figure 4: Boxplot of leaf biomass index of different *B. vulgaris* cultivar groups and *B. vulgaris* ssp. *maritima* (BVM) before winter.

2.5 Discussion

We detected a wide range of genetic variation for winter hardiness within and between *B. vulgaris* cultivar groups and BVM observed in an overwintering field experiment across a contrasting set of environments. While in milder environments a large part of the accessions completely survived the winter, SRs in Nes 08/09 and Mi 09/10 (colder environments) did not exceed 40% indicating insufficient winter hardiness for harsh environments.

To our knowledge this is the first report about genetic diversity of this trait in a test panel representing the *B. vulgaris* gene pool. Winter hardiness in *B. vulgaris* was studied before, however mostly limited to sugar beet germplasm and only a small number of genotypes. The purpose of most of the earlier experiments was not necessarily to describe the genetic variation for winter hardiness but the selection of winter hardy and bolting resistant sugar beet genotypes (Bauer, 1932; Eichholz and Röstel, 1962; Schneider, 1935). Large differences in the average SR were observed between the different environments. These differences were due to test locations but also to the effect of test years. This was most prominent in Göttingen with a mean SR of 46.8% and 3.3% in 2008/09 and 2009/10, respectively. Interestingly, in Kiel the mean SR was lower in 2008/09 (70.6%) compared to 2009/10 (86.3%) indicating a year by location interaction. Such year effects are in congruence with results of Pohl-Orf et al. (1999) who evaluated the overwintering of sugar beets in four subsequent winters in Aachen, Germany. They observed environmental SRs that ranged from 0% to 70%. Such large variation between environments can be explained to a great extent by pre-winter development and winter conditions.

Although we did not record the growth stage before winter, we observed a distinct pre-winter development in each environment. Apart from the sowing date, pre-winter development is affected by pre-winter temperature, soil conditions and soil moisture (Hoffmann and Kluge-Severin, 2011). Studying juvenile sugar beets sown in spring, Milford et al. (1985) and Kenter et al. (2006) could show that the increase of dry matter is proportional to the temperature sum until soil moisture becomes the limiting factor in summer. Likewise, in autumn sown sugar beets it was shown recently that both leaf and beet yield are best described by thermal time

(Hoffmann and Kluge-Severin, 2011), which depends on the weather condition as well as on the sowing date. Indicated by a rapid pre-winter development the best combination of these factors was probably given in Gö 08/09, Gö09/10 and Mi 09/10. In comparison, pre-winter development in Sö 09/10 and Ki 09/10 was delayed, probably as a consequence of drought at sowing, followed by cold and wet conditions in autumn.

In which way the pre-winter development affects winter hardiness cannot be concluded from our data. To address this, overwintering experiments with different sowing dates in the same test environment are required. Nevertheless, Schneider (1935) reported, that sowing after mid-September led to a complete loss of the stand. Therefore, he recommended sowing of winter beets before 1 Sept. On the other hand it is well known from steckling cultivation for seed production, that too early sowing dates negatively affect the SR of the stecklings. Hence, there appears to be an optimum growth stage to survive sub-zero temperatures that is best realized by sowing from beginning to mid-August depending on environment (Axel Schechert, unpublished data). This is also reflected in our experiments, in which we observed the lowest SRs in Nes 08/09, where plants were least developed, followed by Mi09/10 and Gö 09/10 where plants were most developed.

While we assume that plants with little development lack sufficient vigor to tolerate winter stress, more developed plants might suffer damage from exposure of frost sensitive organs. This seems to be mostly the case for the beet forming *B. vulgaris* cultivar groups, where the hypocotyl is increasingly elevated above ground therefore exposing the meristem to frost. Interestingly, in the leaf beets, which do not form a storage root, too far developed plants also appear to be more frost sensitive as shown by the negative regression of SR on the leaf biomass index. This is surprising, as a more developed leaf apparatus should be able to shelter the meristematic shoot tissue to a certain degree from frost. The increased frost sensitivity of larger plants of leaf beets might be caused by physiological changes during plant growth.

The winter conditions varied largely between locations and years. Both in Germany and in Belarus the minimal and the average temperatures were lowest in 2009/10. In Nes 08/09, Mi 09/10 and Sö 09/10 temperatures dropped below -20°C and in Gö 09/10 below -19°C. This had direct impact on the mean SR of each environment as shown by regression analysis (Figure 3). However, with R^2=81% minimal temperature alone does not explain the total variation of SRs between environments as the SRs were higher in the colder winter of 2009/10 in Kiel compared to 2008/09. Moreover, a comparison of SR and minimal temperatures reveals a more than threefold higher SR in Gö 08/09 compared to Mi 08/09 even though minimal winter temperatures were the same.

The comparison of temperature and snow cover revealed that whenever temperatures dropped below -6°C in Ki 09/10 the plants had been covered by at least 8cm of snow. In contrast in Gö 09/10, Mi 09/10 and Sö 09/10 plants had to withstand temperatures below -18°C with less than 5 cm of snow. These observations indicate the positive effect of an insulating snow cover on the SR of *B. vulgaris*.

Whether an environment is suited for assessing winter hardiness in *B. vulgaris* depends very much on what particular research objective is being addressed. If the major goal is to select the most winter hardy accessions the continental environments in Belarus are ideal. On the other hand, if focus is put on evaluating the genetic variation present in *B. vulgaris* Gö 08/09 turned out to be best suited because in this environment we observed the best differentiation between and within different *B. vulgaris* cultivar groups and the BVMs (Figure 2). However, as winter conditions differed between years, a good differentiation could not be observed in Gö 09/10.

The results presented here display a wide variation of winter hardiness observed in a *B. vulgaris* panel in field experiments under central and eastern European conditions. Across all environments the SRs ranged from 6.9 to 65.6% with a mean of 28.4%, indicating a good differentiation among the tested accessions. Garden beets and fodder beets showed the lowest mean SR with 19.5% and 24.3, respectively. This is probably a consequence of plant architecture as in these cultivar groups the shoot meristem is most exposed to frost due to a large hypocotyl. Next in terms of SR are the leaf beets with a SR of 27.3%. Interestingly, the leaf beets showed a large range of SR from 6.9% to 56.7%. This large variation for SR can be partially explained by the size of the leaf apparatus developed before winter (R^2=20%), whereby a large leaf apparatus has a negative impact on SR (Table 5).

Table 5: Regression analysis of the survival rate on leaf biomass index for different *B. vulgaris* cultivar groups and *B. vulgaris* ssp. *maritima*. For each *B. vulgaris* accession the survival rate was estimated across eight environments while biomass index was determined only in the environments Ki 08/09 and Gö 2008/09.

	R^2	P	slope	intercept	sample size
Fodder Beet	0.13	0.0042	-6.83	52.16	61
Leaf Beet	0.20	0.0003	-13.71	83.48	61
Garden Beet	0.02	0.2514	-2.42	29.49	90
Sugar Beet	0.08	0.8545	0.24	38.28	99
Beta vulgaris ssp. *maritima*	0.06	0.1013	-7.29	68.4	44

R^2: coefficent of determination. P: error probability.

Of all the cultivar groups, sugar beets displayed the highest SR and the smallest range. Both may indicate direct or indirect selection for winter hardiness. This might have occurred by selection of sugar beet genotypes with tolerance to early sowing, which requires cold tolerance at the seedling stage (Wood 1952). Moreover, sugar beets may have been indirectly selected for cold tolerance during seed production which usually requires overwintering of the sugar beet stecklings. In addition, morphological as well as physiological characteristics of sugar beet might explain the high SR. In comparison to garden beet and fodder beet, the beets of sugar beet are mainly composed of the root growing below the soil surface and only a small part of the hypocotyl that is less exposed to frost. Furthermore, the high sugar concentration of sugar beet may also contribute to winter hardiness. It is well known that sugars increase the osmotic pressure of the vacuole and therefore play a major role in cryo-protection of plants (Hincha et al., 2000; Olien and Lester, 1985). In particular, it was shown that the sugar content affects *in-vitro* frost tolerance of sugar beet shoots (Dix et al., 1994).

Our observed SRs were much lower compared to the SRs of 50% to 92% observed in pre-selected sugar beet breeding families by Bauer (1932). Bauer reported temperatures below -20°C from 1 to 25 Feb. for his overwintering trials, in which snow was partially removed to expose the beets to frost. Moreover, Schneider (1935) reported about sugar beets which completely survived in all tested years, even in cases in which severe frosts occurred without any snow protection after the plants re-started growing in spring. From these observations Schneider concluded that “winter hardiness is a common feature of sugar beets sown at an appropriate sowing date”. More than 70 years ago these authors were rather optimistic about reaching the goal of a winter hardy and bolting tolerant winter sugar beet. However, we cannot confirm their findings in presently available sugar beet germplasm. Nor could Pohl-Orf et al. (1999) confirm sufficient winter hardiness of modern sugar beet germplasm for continental climates in their overwintering experiments. They reported mean SRs below 25% for six out of twelve environments.

With a mean SR of 34.9% the BVMs ranked second after the sugar beets. Moreover, BVMs showed the widest range of SRs from 8.8% to 65.6% including the 5 most winter hardy *B. vulgaris* accessions. This large variation in phenotype corresponds to the variation of climate conditions in the natural habitats of BVMs (Hautekèete et al., 2002). Depending on the ecological conditions, BVMs have an extremely variable lifespan (Hautekèete et al., 2002; Letschert, 1993). While it can be assumed that in southern habitats annual life forms have evolved to escape the ecological disturbance of drought, in northern habitats biennial and perennial life forms are predominant that have developed sufficient winter hardiness to survive winters. In correspondence with this the most winter hardy BVMs in our experiments were originally collected in the northern habitats.

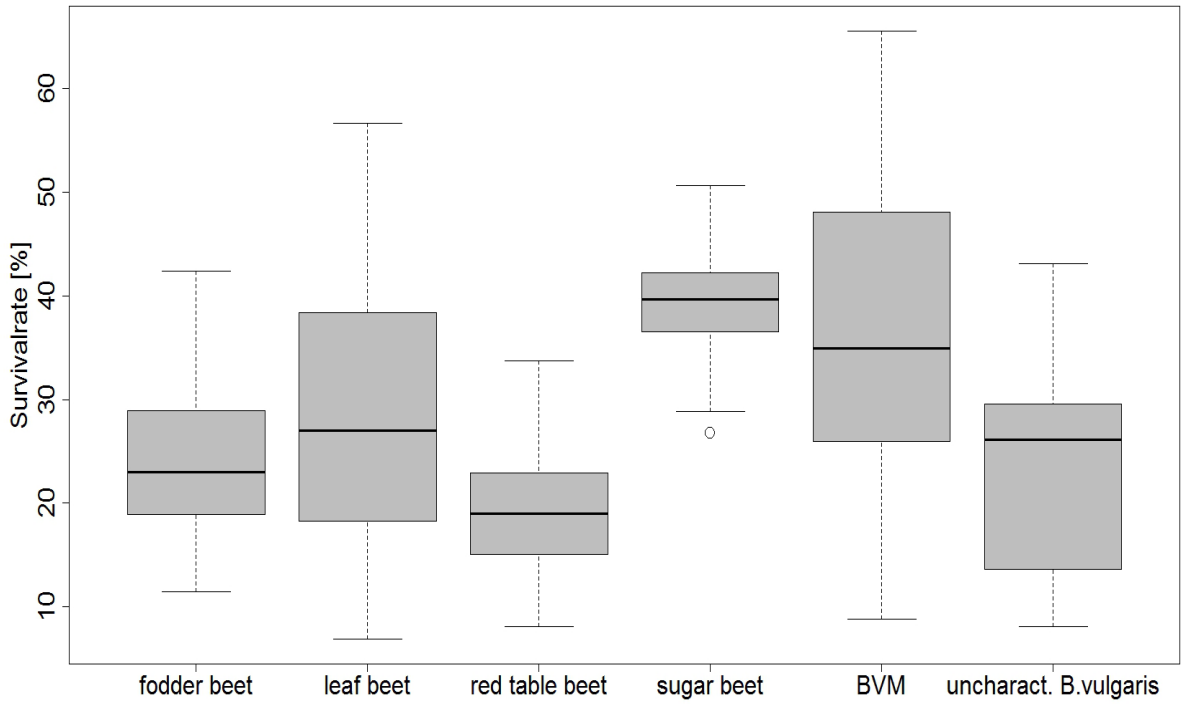

Figure 5: Boxplot of the survival rates for different *B. vulgaris* cultivar groups and *B. vulgaris* ssp. *maritima* (BVM).

As expected for a complex trait affected by a number of factors we detected a significant genotype by environment interaction for winter hardiness. This affects the selection for the most winter hardy accessions. Within the 5% most winter hardy accessions based on SRs across all environments none was among the top 10% in each of the eight environments. The environmentally most stable accessions for SR performed among the top 10% only in up to seven environments. One important factor for this interaction might be the requirements of the plants to reach an optimal developmental stage for overwintering (see above). Accessions needing a higher thermal time for juvenile growth might not have reached the optimum stage in cold environments. In contrast, accessions with a rapid juvenile growth might have reached that stage even in colder environments but have already passed it in warmer environments. Another factor causing interaction might be the different snow heights in different environments. We might assume that the morphology of accessions interacts with the insulating effect of a snow cover. Prostrate plants with the meristematic tissue close to the soil surface might require less snow to reach the same level of insulation compared to more erect plants. Therefore, two accessions with different morphology might have a similar SR in an environment with sufficient snow coverage. In an environment with limited snow coverage, however, an accession with prostrate growth might be damaged by frost much less than an

accession with erect growth that is sticking out of the snow. To further study frost tolerance independent from morphology, frost experiments in a cryochamber are currently being conducted. Despite the complexity of the trait winter hardiness and the detected genotype by environment interaction, the heritability of SR was estimated as h^2=0.81. The main reason for this high heritability is assumingly the high genotypic variation present in the test panel.

2.6 Conclusions

In our experiments we detected a large variation for winter hardiness in the *B. vulgaris* gene pool. The winter hardiness observed in the sugar beet accessions was sufficient for winter conditions in Kiel 08/09 and Gö 08/09. However, under winter conditions in Mi 08/09, Mi 09/10, Nes 08/09, Sö 09/10 and Gö 09/10, winter hardiness was by far not sufficient for growing sugar beet as a winter crop. Therefore, it is of uppermost importance to improve winter hardiness, if the winter sugar beet is to be grown not only in maritime climates with milder winters but also in continental climate with harsh winters.

In order to improve winter hardiness in sugar beet, exploiting the genetic variation within sugar beet appears to be most successful at least on a short term scale. The most straightforward approach is to cross the most winter hardy sugar beet genotypes and select transgressive segregants in the offspring. In how far recurrent selection within sugar beet will further improve this trait on a long term scale, cannot be predicted. Therefore, focus needs to be put also beyond immediate selection progress by considering non sugar beet genetic resources. Most promising among the other *B. vulgaris* cultivar groups are the leaf beets, of which one accession performed better than any sugar beet in our test, although statistically not significant. Even winter hardier were some of the BVM accessions that should be considered an additional resource for improving winter hardiness on a long term scale. Even though crosses of sugar beets with wild beets or leaf beets can be easily performed, the genetic burden carried by wild beets and to less extend by leaf beets is immense including low root yield and quality as well as insufficient sugar yield (Frese et al., 2001; Van Geyt et al., 1990). In the worst case, genes for winter hardiness might be linked with or even pleiotropic to undesired traits. A dissection of winter hardiness via QTL mapping will provide more information on that as well as markers useful for marker assisted selection for rapid incorporation of the trait into elite sugar beet germplasm.

Apart from choice of germplasm it is critical to have optimal test conditions for selection of superior genotypes. Most important are test environments with winter conditions harsh enough to discriminate among the best performing genotypes. In our study, such conditions were met in the continental environments in Belarus or the environments in Germany, where snow coverage was not sufficient to protect the plants from strong frost. However, as winter conditions cannot be precisely predicted, a part of test environments will likely turn out to be too mild or even too harsh killing the complete test. To compensate for this, we recommend to test across a number of different locations, which also accounts for the strong genotype by environment interaction we observed. Due to limited seed availability, we chose a plot size of only 15 plants and two replications per environment. While this was sufficient for the objectives of our study, these test dimensions clearly lack the statistical power to discriminate among the best genotypes. Therefore, for cultivar improvement we strongly recommend to increase plot size as well as the number of replications.

2.7 Acknowledgements

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3 Genetic dissection of frost tolerance in sugar beets (*B. vulgaris* L.): climate chamber trials

3.1 Abstract

A prerequisite for the development of autumn sown winter-sugar beets (*Beta vulgaris* L.) in temperate climates is an understanding of the genetic mechanisms controlling frost tolerance. The aim of this study was the dissection of genetic factors controlling frost tolerance in sugar beet by QTL analysis under consideration of cold regulated candidate genes. A population of 226 $F_{2:3}$ families derived from crossing the frost sensitive inbred line SC2 and the frost tolerant inbred line SCK was phenotyped for frost symptoms and SRs in a frost chamber. The population was genotyped with AFLP, SSR, SNP and CAPs markers. To integrate candidate genes into the linkage maps 37 EST derived homologous sugar beet sequences of *A. thaliana* genes involved in cold stress were screened for polymorphisms and eight candidates showed polymorphisms that could be analyzed by CAPS markers. Although no candidate gene could be associated with frost tolerance, mapping resulted in three QTL for frost symptoms on chromosome 1, 3 and 8 which explained 17.3% of the total phenotypic variance. The QTL on chromosome 3 and 8 co-segregated with two QTL for SRs that explained 13.1 % of the phenotypic variation. To validate the contribution of the detected QTL to the development of autumn sown winter-sugar beets the results of the current overwintering field tests of the mapping population are required.

3.2 Introduction

Under cool-temperate conditions yield of sugar beet (*Beta vulgaris* ssp. *vulgaris* cult. sugar beet) is limited by slow seedling development and insufficient canopy in spring. One long discussed strategy to prolong the vegetation period is an autumn sown sugar beet that is harvested in the following year (Eichholz and Röstel, 1962). Even at the very beginning of sugar beet cultivation Achard (1806) assumed that beets are capable of surviving winters under central European conditions and several attempts were taken to develop autumn sown sugar beets (Bauer, 1932; Eichholz and Röstel, 1962; McFarlane et al., 1948). However, autumn sown winter beets exposed to winter temperatures require a system for bolting control, and they must have sufficient winter hardiness to survive winters. A system for bolting control is required because sugar beets which have been exposed to winter temperatures are vernalized. Vernalization causes the transition to flowering and the elongation of the shoot. This process is called bolting and goes along with altered sink source capacities leading to reduced root and sugar yield (Jaggard et al., 1983). Therefore bolting is highly undesired for crop production. On the other hand, breeders have a demand for high seed yields to propagate beets. Presumably due to difficulties in combining these contrasting breeding goals no autumn sown sugar beet has ever been introduced into cold-temperate markets. However, with a hybrid approach proposed by Jung and Müller (2009) bolting control in sugar beets comes into perspective. The proposed model is based on genetic modification of two hybrid components. While the components are still bolting after vernalization, the bolting oppressing transgene becomes active only in the resulting hybrid cultivar.

With the expected system for bolting control, attention can be paid to characterize winter hardiness in sugar beets and identify genetic factors for selection of frost tolerant genotypes. Winter hardiness depends on the integrated response on biotic and abiotic winter stresses

including frost tolerance as a major component of winter-hardiness (Arbaoui, 2007; Chinnusamy et al., 2010; Lejeune-Henaut et al., 2008; Pan et al., 1994). Winter hardiness can therefore only be assessed under field conditions. However, multi-location field experiments are biased by multiple environmental factors and the uncertainty of selective conditions have led to the development of artificial frost tests under controlled conditions (Arbaoui, 2007; Fowler, 1979; Saulescu and Braun, 2001). Even though artificial tests are unable to cover the wide range of winter stresses, the major component of winter-hardiness, namely frost tolerance, can be quantified. Further, artificial frost tests allow season independent testing with limited environmental factors as error sources (Saulescu and Braun, 2001; Sutka, 1981). To identify the genetic factors controlling frost tolerance under controlled conditions linkage mapping has been successfully applied in wide range of crop species over the last 20 years. In cereals, frost tolerance has been studied intensively in frost chamber experiments and several quantitative trait loci (QTL) have been identified (Blake et al., 1993; Francia et al., 2004; Galiba et al., 1995). In recent years, several genetic studies on frost tolerance have been performed in crops that were traditionally cultivated as spring crops in cool climates. In all cases quantitative inheritance of frost tolerance was observed. In faba beans (*Vicia faba* L.) 4 QTL explaining in total 18.9% of the phenotypic variance were detected on linkage groups 6, 9, 10 and 11 (Arbaoui, 2007). Moreover, a study on winter-hardiness and frost tolerance in peas (*Pisum sativum* L.) revealed multiple QTL on six linkage groups explaining up to 46,5% of the observed phenotypic variation (Dumont et al., 2009),

Candidate genes (CG) enable the transfer of advanced knowledge of genes, their functions and regulatory networks from model organisms to non-model organisms, like sugar beets. In sugar beets a number of resistance, yield-related and flowering time genes have been identified using CG approaches (Abou-Elwafa et al., 2010; Chia et al., 2008; Lein et al., 2007; Reeves et al., 2007; Schneider et al., 1999). Until now, no data on the genetics of frost tolerance in sugar beet have been published. However, model organisms, like *A. thaliana* have been extensively studied for the genetic mechanisms controlling frost tolerance under artificial conditions.

The best understood cold responsive pathway in *A. thaliana* is controlled by the transcription factors *C-repeat binding factor1 (CBF1), CBF2* and *CBF3.* These genes are affecting the expression of several hundred genes in *A. thaliana* (Maruyama et al., 2004; Maruyama et al., 2009; Vogel et al., 2005). Consistently, knock out mutants of *CBF1* and *CBF3* react with 25% to 50% decrease in frost tolerance (Novillo et al., 2007). Moreover, a QTL study in *A. thaliana* revealed that about 20% of the observed variation in freezing tolerance can be explained by a QTL which co-localizes with the CBF locus (Alonso-Blanco et al., 2005). Shortly after exposure to cold stress *CBFs* interact with the CRT/DRE elements in the promoter region of cold responsive (*COR)* genes and induce their expression to trigger the cold response. (Liu et al., 1998; Stockinger et al., 1997). However, the *CBF* pathway is insufficient to explain the total variation for cold response (Alonso-Blanco et al., 2005). Consistently, several genes have been identified that affect frost tolerance in *A. thaliana* independently of the *CBF* pathway such as *LONG VEGETATIVE PHASE1* (LOV1), *HIGH EXPRESSION OF OSMOTIC RESPONSIVE15 (HOS15), HOS9* and *ESKIMO1* (*esk1*) (Xin and Browse, 1998; Yoo et al., 2007; Zhu et al., 2008; Zhu et al., 2004).

A crosstalk between the regulation of flowering time and cold response has been well established for *A. thaliana* and cereals. One of the major QTL controlling vernalization (*VRN1*) in cereals co-localizes with a major QTL for low temperature tolerance and it has been shown that allelic

variation in *VRN1* affects frost tolerance (Dhillon et al., 2010; Francia et al., 2004). In Arabidopsis it has been shown that genes of the autonomous flowering pathway (*FVE*), the vernalization pathway (*FLC*) and the photoperiodic pathway (*LHY*, *CCA1*, *PRR5*, *PRR7*, *PRR9*) affect the expression of *CBF* and several *COR* genes (Deng et al., 2011; Kim et al., 2004; Nakamichi et al., 2009; Seo et al., 2009; Yoo et al., 2007).

This study aims (i) to identify *in silico* candidate sequences for homologs to cold regulated *A. thaliana* genes and (ii) to map genetic factors controlling frost tolerance in sugar beet using a segregating population of 226 $F_{2:3}$ families tested in a frost chamber.

3.3 Materials and Methods

3.3.1 Plant material

Twohundredandtwentysix sugar beet $F_{2:3}$ families were phenotyped for their frost tolerance under artificial conditions. The $F_{2:3}$ families were based on two F_1 full sibs derived from a cross of the frost tolerant inbred line Strube component (SCK) and the susceptible inbred Strube component 2 "SC2", which were provided by Strube research (Söllingen, Germany)(Table 6; electronic supplement 1). Field trials test had shown a differentiation between SCK and SC2 in their ability to survive winters and differentiaton in frost tolerance was revealed under artificial conditions. As standards the sugar beet variety "Theresa" (KWS, Einbeck, Germany) the biennial sugar beet "93161P" and the annual *B. vulgaris* "930190" were included in the test.

Table 6: Plant material used for genetic dissection of frost tolerance in sugar beets.

Seed code	name	genotype	type	phenotype
090014[a)]	SCK	Inbred line	Sugar beet	biennial, frost tolerant, late flowering
090019 [a)]	SC2	Inbred line	Sugar beet	biennial, susceptible to frost, early flowering
F09Z2434^145 [b)]	F_1	F_1 progeny derived from crossing of 090014 and 090019	Sugar beet	biennial, no phenotyping for frost tolerance or winter-hardiness
F09Z2434^146 [b)]	F_1	F_1 progeny derived from crossing of 090014 and 090019	Sugar beet	biennial, no phenotyping for frost tolerance or winter-hardiness
F09Z2434^145.256 to F09Z2434^145.379 [b)]	F_2	F_2 progenies derived from crossing of 090014 and 090019	Sugar beet	biennial, segregating for frost tolerance
F09Z2434^146.380 to F09Z2434^146.503 [b)]	F_2	F_2 progenies derived from crossing of 090014 and 090019	Sugar beet	biennial, segregating for frost tolerance
111427 to 111653 [a)]	$F_{2:3}$ families	$F_{2:3}$ families derived from F2 progenies of crossing of 090014 and 090019	Sugar beet	biennial, segregating for frost tolerance

a) Kiel Code. B) Strube Code

Table 7: Criteria for scoring of plant development before frost and after frost treatment

Score	Phenotype	Remarks
Before cold acclimation		
1	BBCH 11	First pair of leaves visible
2	BBCH 12	First pair of leaves unfolded
3	BBCH 13	3 leaves unfolded
4	BBCH 14	4 leaves unfolded
5	BBCH 15	5 leaves unfolded
6	BBCH 16	6 leaves unfolded
7	BBCH 17	7 leaves unfolded
8	BBCH 18	8 leaves unfolded
9	BBCH 19	9 leaves unfolded
After frost treatment		
1	no visual frost damage	extraordinary high vigour, unaffected plant
2	minimal frost symptoms	e.g. parts of leaves show minor frost symptoms
3	more than two large intact leaves	minor leaf losses, however high vigour
4	two large intact leaves	substantial leaf losses, however high vigour
5	two medium sized intact leaves	centre of plant with high vigour, however substantial leaf losses
6	two small sized intact leaves	high leaf losses, moderate vigour
7	one small leaf with minor frost damages	plant severely damaged, limited vigour
8	severe frost damages, minimal green leaf area left,	low vigour
9	plant is dead	soft hypocotyl; beginning of rot; no anchoring in soil

3.3.2 Artificial frost test

Plants were cultivated in Göttinger plant pots 13cm*13cm*13cm (Meyer, Germany) filled with "Einheitserde classic" soil provided by the Einheitserdewerk Uetersen (Uetersen, Germany) with one plant per pot. Plants were grown for six weeks at 20°C under 16 h supplementary light with

900 µmol/m^2s (Son-T Agro 400W (Philips Electronics N.V., Eindhoven, The Netherlands) in the greenhouse. To account for positional effects in the greenhouse pots were randomly rearranged once a week. Plants were then transferred to a frost chamber "Tecto Spezial Kühlzelle 100" (Viessmann, Allendorf, Germany) and cold acclimated for two weeks at 4°C with 16 h light with 200 µmol/m^2s provided by fluorescence tubes (Lumimax 58W (Osram AG, München, Germany). After cold acclimation the temperature was dropped down to -7°C for 48h. This temperature had been reported by Geisler (1988) as critical for field survival of spring sown sugar beets and had been used in precursor experiments to determine the contrasting frost tolerance phenotypes of SCK and SC2 by screening more than 100 plants per parental line. For regeneration after frost treatment plants were kept for five days at 4°C and subsequently transferred to 20°C in the greenhouse (Table 8).

Table 8: Growth conditions for artificial frost tests.

	Pre-cultivation	**Cold acclimation**	**Frost**	**Recovery cool**	**Recovery warm**
Number of days	42	14	2	5	21
Day length artificial light [h]	16*	16	16	16	16*
Temperature[°C]	20	4	-7	4	20
Light intensity [µmol/m^2s]	900*	200	200	200	900*

* additionally natural light

An experimental unit consisted of three plants per genotype and due to limited space in the frost chamber the experiment was divided into six batches, each consisting of one complete replication of all 226 $F_{2:3}$ families (3 plants/family) and two replications of the parental lines and standards. Experiments were conducted between July 2011 and April 2012. A timeline for the frost chamber experiments is given in Supplementary table 5. To be able to account for developmental differences plants were scored from 1 (extremely poorly developed) to 9 (extraordinary well developed) for their development before cold acclimation (Table 7, Figure 6). After frost treatment plants were evaluated once a week over four weeks for frost symptoms from 1 (no symptoms) to 9 (dead) (Table 7; Figure 6). Twenty-six days after frost treatment final frost symptoms were evaluated and a frost severity index (FSI) was determined as the mean frost symptoms at a given date across one genotype. The survival rate (SR) 26 days after frost treatment was calculated as the number of surviving plants within a genotype after 26 days divided by the number of plants before frost.

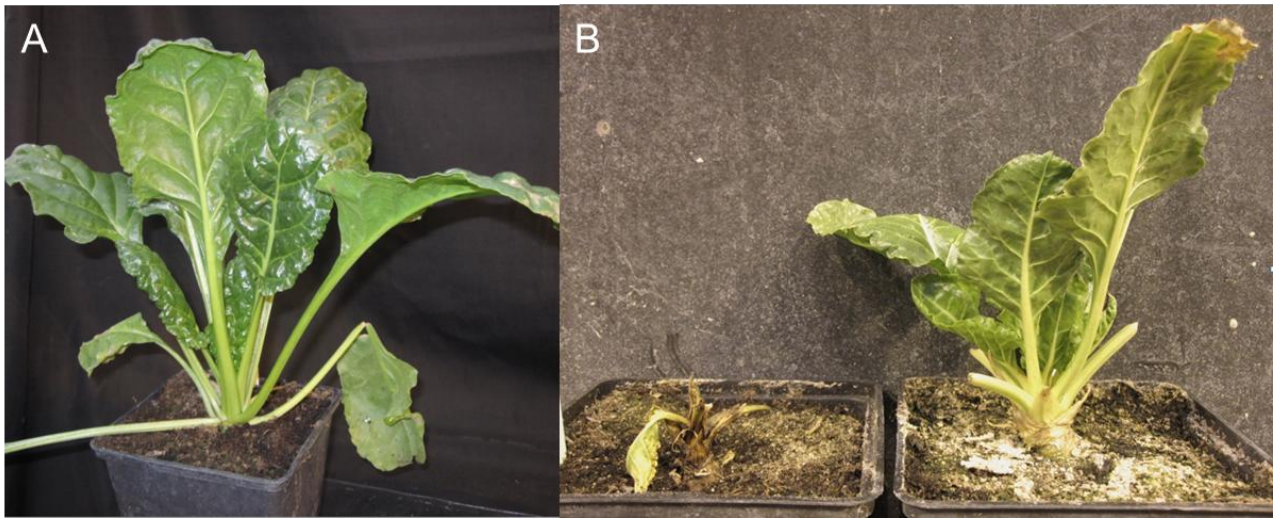

Figure 6: A) The frost tolerant parental line SCK of the mapping population before frost treatment; B) Differences in frost tolerance six weeks after frost treatment. The lines SC2 (left) and SCK (right) were used to establish the mapping population.

3.3.3 DNA extraction and marker development

For molecular analyses leaves of the F_2 plants and the parents SCK and SC2 were harvested and freeze dried. Genomic DNA was extracted using the CTAB protocol according to Rogers and Bendich (1985) and adjusted to 5ng/µl.

The mapping population was genotyped with 28 PCR based markers. These markers were either cleaved amplified polymorphic sites (CAPS) or simple sequence repeat (SSR) markers. Additionally, 33 SNP based anchor markers were provided by Strube research (Supplementary table 6). Primers were designed based on previously published *B. vulgaris* sequence information (Dohm et al., 2011; Lange et al., 2010; Laurent et al., 2007; McGrath et al., 2007; Schneider et al., 2002; Schneider et al., 2007). Properties of all PCR primers were checked with the software Oligocalc (Kibbe, 2007). Standard PCR conditions were as follows: Reactions were incubated for 3 min at 95°C, followed by 30 cycles of 94°C for 30 s, 54-61°C according to Oligocalc for 30 s, 72°C for 30 s, and a final extension of 72°C for 3 min. Touchdown PCR conditions were as follows: Reactions were incubated for 1.5 min at 95°C, followed by 13 cycles with subtracting 0.8°C per cycle from a primary annealing temperature of 58°C followed by an elongation of 1 min at 72°C and subsequently 31 cycles as described above with the exception of 30 sec. annealing at 47°C. Additionally, 26 primer combinations were screened for amplified fragment length polymorphisms (AFLP), resulting in 124 polymorphic AFLP markers which were used to enrich the genetic map. AFLP markers were analyzed essentially as described by Vos (1995) and Büttner (Büttner, 2010). In brief, genomic DNA of the parental lines and the F_2 plants were digested with *Pst*I and *Mse*I (Fermentas, St. Leon-Roth, Germany) and subsequently *Pst*I and *Mse*I adapters were ligated to the digested DNA. Pre-amplification was performed using the primers P01 and M01 and main-amplification with the primers listed in supplementary table 7. Polymorphic fragments were named according to Büttner (2010) with the following modification: the parents carrying the dominant fragment allele were abbreviated either with T (SCK) or S (SC2). AFLP fragment sizes were determined by comparison with the LI-COR size standard (LI-COR, LICOR Biosciences, Lincoln, USA) and gels were evaluated by using the software

GelBuddy (Zerr and Henikoff, 2005). On each LI-COR gel the parents SC2 and SCK were loaded as controls.

3.3.4 Identification and mapping of candidate genes

One hundred sixty *A. thaliana* genes were selected from the literature based on their function as cold responsive genes. The sequences of these genes were submitted to tBLASTn analysis against publicly available as well as previously unpublished sugar beet ESTs (A. Müller, unpublished data) and the best putative sugar beet homologs were selected. The nucleotide sequences of the candidate genes were identified by aligning the ESTs against the draft sugar beet genome sequence (version 0.9) (Dohm et al., 2011). A total of 85 specific PCR primers pairs were derived from genomic sequences of 38 CGs covering at least parts of the ESTs to amplify fragments of 450 bp to 1000 bp length (Supplementary table 8, electronic supplement 2). For primer design, the software Oligocalc was used (Kibbe, 2007). The reactions were incubated for 3 min at 95°C, followed by 30 cycles of 94°C for 30 s, 54-61°C according to Oligocalc for 30 s, 72°C for 30 s, and a final extension of 72°C for 3 min. Fragments were checked via gel electrophoresis in the parental lines and 6 arbitrarily chosen F_2 individuals. Subsequently, PCR products of the SC2 and SCK were sequenced to identify polymorphisms and verify the CG sequences. The raw sequences were assembled with CLC Main Workbench 6.0.1 (CLC bio, Arhus, Denmark) and compared with the previously mentioned ESTs and the *B. vulgaris* genome sequence for verification of correct amplification. If sequencing revealed cleavable polymorphisms between SC2 and SCK, CAPS markers were designed. Enzymes for DNA digestion were used as recommended by the manufacturer (Fermentas, St. Leon-Roth, Germany).

3.3.5 Statistical analysis

Analyses of covariances (ANCOVA) were performed with the software R (R Development Core Team, 2010) to test the fixed effect of $F_{2:3}$ families on FSI or SR. The effects of run and the run by development before frost interaction were considered as random and development before frost was used as co-factor. Broad sense heritabilities across runs were determined according to Hallauer and Miranda (1988). Differences between parents were tested at a comparison wise error rate of $\alpha_C = 0.05$. To identify families with improved frost tolerance, one-sided Dunnett comparisons (Dunnett, 1955) of all families with the parent SCK at an experiment-wise error rate of $\alpha_E = 0.05$ were conducted for FSI and SR.

3.3.6 Linkage Map Construction and QTL Mapping

To construct the genetic map the F_2 plants were genotyped at the polymorphic sites and the genetic map was enriched with AFLP markers.The linkage map was constructed using the Kosambi mapping function (Kosambi, 1943) in JoinMap 3.1 (Van Ooijen, 2006) applying a LOD threshold of 2.5 and a maximum recombination frequency of R = 0.4 Quantitative trait loci (QTL) for FSI and SR were mapped with PLABQTL v 1.2 by composite interval mapping with a LOD threshold of 2.5 (Utz and Melchinger, 1996).

3.4 Results

3.4.1 Artificial frost test

Twohundredandtwentysix sugar beet $F_{2:3}$ families, the parental lines and three additional controls were phenotyped for frost symptoms and SRs under artificial conditions in six runs. Effects of $F_{2:3}$ families as well as the development before frost were significant on FSI ($\alpha = 0.05$). Parental FSI means were 5.19 for SCK and 6.78 for SC2, while the overall mean FSI was 6.41 Figure 7,Table 9).

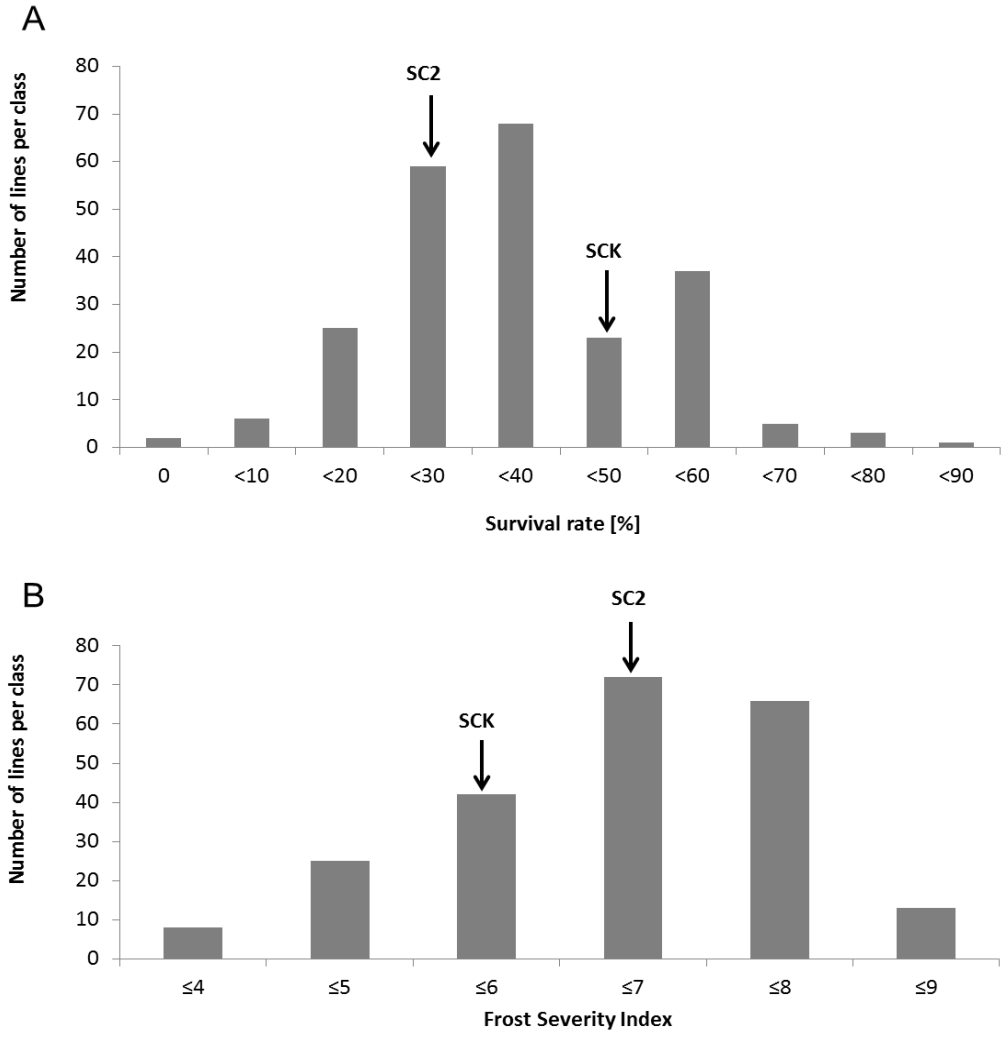

Figure 7: Histogram for 226 sugar beet $F_{2:3}$ families segregating for A) frost severity index (FSI) and B) survival rates (SR) after artificial frost treatment. The population was developed from crossing inbred lines SCK and SC2.

The difference between parents was not significant ($\alpha = 0.05$). The overall mean SR was 34.24 %. In all runs SR ranged from 0% to 80% across all families, however in precursor experiments SCK displayed a 12% higher SR compared to SC2. In each run, SRs among the controls were highest in SCK, while the lowest SR was observed in the annual 930190. Average SRs in the controls

ranged from 2.78 % in 090190 to 50 % in SCK and 27.77% of the parent SC2 survived (Figure 7, Table 9). However, both the differences of SRs between parents and between $F_{2:3}$ families were not significant ($\alpha = 0.05$). No significant effects of the development before frost on the SR or FSI were observed across experiments.

Table 9: Range, means and broad sense heritability (h^2) estimates for Survival rates (SR) and frost severity index of 226 $F_{2:3}$ families and their parents.

	SR [%]	**FSI**
Parents		
SCK	50	5.2
SC2	27.8	6.8
Population		
Range	0-80	3.2-9
Mean	34.2	6.4
h^2	12.5	17.02

3.4.2 Linkage map and QTL analysis

The population was genotyped with 124 AFLP, 20 CAPS, 9 SSR and 33 SNP markers covering all chromosomes of the sugar beet genome. 127 markers grouped into nine LGs with a total map size of 674 cM, resulting in an average marker interval of 5 cM. The size of the linkage groups ranged from 52 to 135 cM (Figure 8). Sixty markers remained unlinked, including 52 AFLPs (Supplementary table 7). Three gaps of the linkage map exceeded 15cM, with the largest gap on chromosome 5, spanning 21cM (Table 10). QTL mapping revealed three significant QTL with LOD >2.5 for FSI and two for SR, (Table 11). The three detected QTL explained 17.3% of the total phenotypic variance and were mapped to chromosome 1 (FSI_1a ; LOD= 2.83), 3 (FSI_3a , LOD= 2.96), and 8 (FSI 8.a LOD= 2.91). For FSI_1a and FSI_3a the allele conferring frost tolerance was inherited from the frost susceptible parent SC2. In contrast, the desired allele of FSI 8.a was inherited by the tolerant parent SCK. In congruence with the QTL for FSI two QTL for SR were mapped to chromosome 3 (LOD = 4.02) and 8 (LOD = 2.58), explaining 8.0 % and 5.1% of the phenotypic variance, respectively.

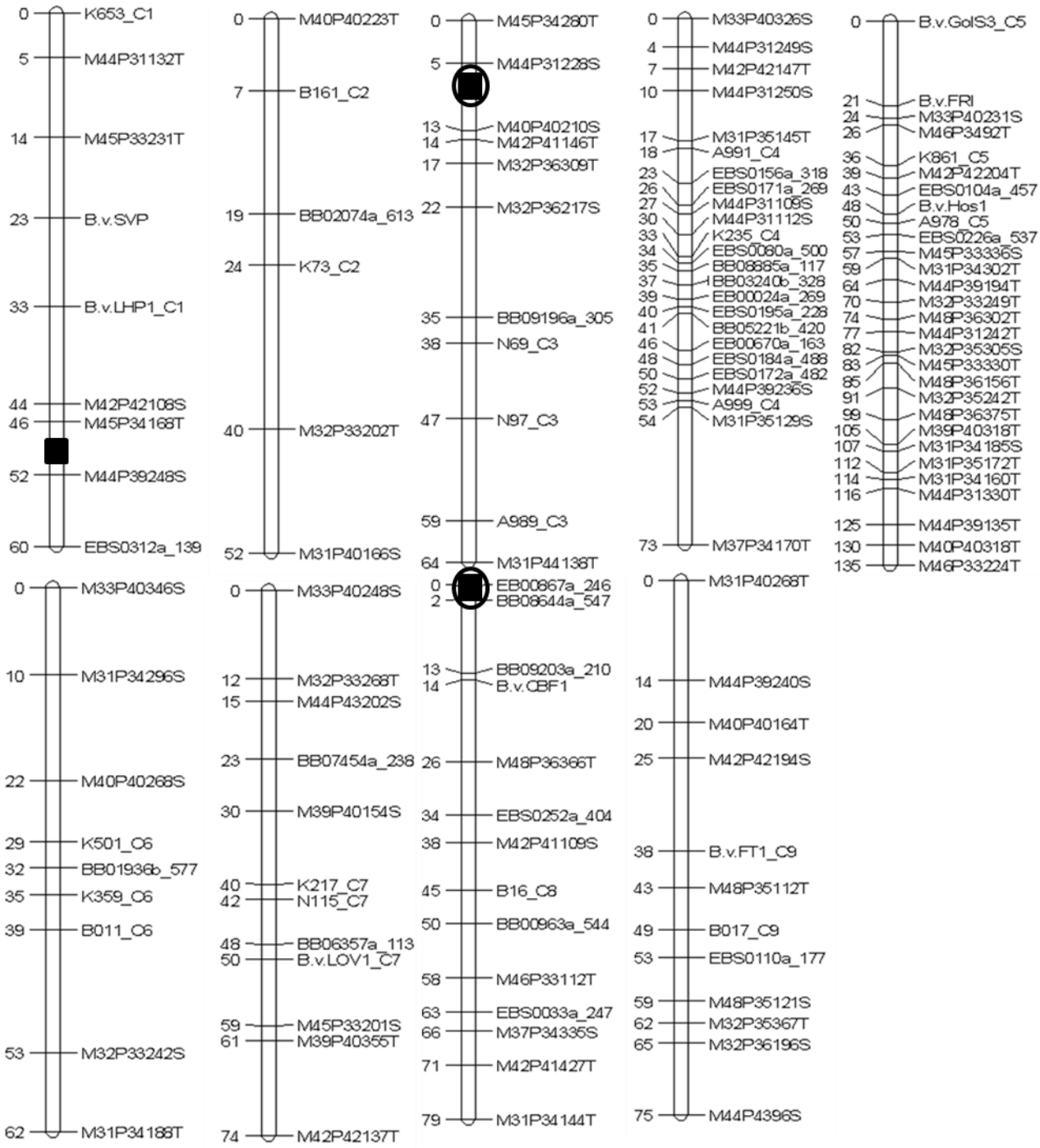

Figure 8: A sugar beet linkage map developed in F_2 progenies derived from a cross of the two inbred lines SC2 and SCK covering 674 centiMorgan (cM) with 127 markers. Marker names ending with T indicate AFLP marker dominant for the allele coming from parent SCK while marker names ending with S represent AFLP marker dominant for the allele coming from SC2. Linkage groups (LG) were named according to Schondelmaier and Jung (1996). Black filled boxes indicate the QTL position mapped for FSI while black circles illustrate the QTL positions mapped for SR.

Table 10: Characteristics of theinkage map (LMA). The map is based on 127 polymorphic markers**.**

Chromosome	Size LMA [cM]	Markers in LMA	Number of Gaps >15cM	Gap length > 15cM in cM	Gaps in % of Chromosome
1	60	9	0	0	0
2	52	6	1	1	1.9
3	64	11	0	0	0
4	73	25	1	4	5.5
5	135	29	1	6	4.4
6	62	9	0	0	0
7	74	12	0	0	0
8	79	14	0	0	0
9	75	12	0	0	0
Total	674	127	3	11	1.6

3.4.3 Identification and mapping of candidate genes

Based on a literature review 160 *A. thaliana* cold responsive genes were selected to search for homologous *B. vulgaris* ESTs. Sequence analysis revealed 92 homologous sugar beet ESTs of which 38 were selected for further investigation. The 38 ESTs were aligned against the genome sequence of *B. vulgaris* and PCR primers were designed resulting in successful amplification of 37 candidate sequences. The amplicons were verified by sequencing for correct amplification of the target regions. The 37 corresponding ESTs show homology to known *A. thaliana* genes representing 22 *CBF* pathway genes including two sequences that show homologies with *CBF1* and *CBF2*. Additionally, twelve flowering time genes with known function in temperature response were analysed. Five of these genes have been previously shown to play a dual role in the *CBF* pathway and flowering time in *A. thaliana*. Additionally a homologous sequence to the flowering time gene *LOV1* was identified, which affects frost tolerance in a *CBF* independent manner. Beside *LOV1* three additional homologues of *A. thaliana* genes with a *CBF* independent effect on frost tolerance were identified. Moreover *B. vulgaris* sequences were identified showing homology with three heat stress genes, and two genes responsible for physiological adaptations upon cold stress in *A. thaliana* (Supplementary table 9). Sequence comparisons of the amplicons in SC2 and SCK revealed polymorphisms in 17 candidate sequences (Supplementary table 9) and eight candidate sequences could be integrated into the linkage map (Supplementary table 10,Table 12, Figure 8). Homologous sequences of *FRIGIDA*, *GALACTINOL SYNTHETASE3* (*GolS*) and *HOS1* were mapped on chromosome 5, while candidates for *LHP1* and *SVP1* were mapped on chromosome 1. In addition a homologous sequence of *LOV1* was mapped on chromosome 7. Moreover, on chromosome 8 a homologous sequence of the major determinant of acclimation response and frost tolerance in *A. thaliana*, *CBF1* was mapped. In accordance with Pin et al. (2010) *FLOWERING LOCUS T1* (*BvFT1*) was mapped to chromosome 9, (Figure 8).

3.5 Discussion

3.5.1 Artificial frost test

Here I present the first QTL for frost tolerance detected in sugar beets. These QTL were detected by phenotyping a segregating sugar beet population of 226 $F_{2:3}$ families in an artificial frost test by determining a frost severity index and the SR. Moreover, 37 candidate sequences in *B. vulgaris* were identified with homologies to *A. thaliana* genes that have been reported to be cold regulated. However, none of the candidate sequences co-localized with the identified QTL.

The artificial frost test was suitable to uncover genotypic variation for FSI among the $F_{2:3}$ families. However, no significant genotypic effects were observed for SR. Even though the parental lines differed in their SR by 22.70 %, the genetic effect of the $F_{2:3}$ families on SR was not significant. Therefore, the question has to be raised, why no genotypic effects for SR were observed. To dissect the reasons two factors have to be addressed: i. unaccounted variation caused by the artificial frost test resulting in experimental error and ii. the genetic variation for SR within and between $F_{2:3}$ families.

During pre-cultivation plants were exposed to 16h of artificial light and additional natural light shining into the greenhouse. To limit positional effects, plants were randomized once a week. However, plants displayed developmental differences such as phenological growth stages within and across $F_{2:3}$ families as well as across runs, although no significant effects on the SRs were

detected. Nevertheless, this variation might have contributed to the experimental error. One reason for differences in the development might have been varying light conditions. Plants tested in the first run of the frost test were sown at the beginning of September while plants of the last run were sown in the middle of December. Therefore, changing natural light conditions such as day length and light intensity/quality might have affected both, plant growth and frost tolerance (Franklin and Whitelam, 2007; Gusta and Wisniewski, 2012). While elongated twilight periods in autumn might have contributed to frost tolerance, decreasing day length might have a negative effect on plant growth (Franklin and Whitelam, 2007; Wisniewski et al., 2006). Moreover, the effects of divergent light conditions on plant development and cold acclimation might have interacted with genotypic effects including germination capacity and vigour. To reduce environmental variation including light effects one strategy would be the conduction of the whole experiments in a climate chamber. However, this would decrease the experimental through-put and increase the technical requirements of the climate chamber.

During the actual frost test air flow effects might have caused irregular temperature gradients in the frost chamber which can hardly be eliminated by the experimental design.

Besides environmental variation genetic variation within $F_{2:3}$ families very likely contributed to error variation. As $F_{2:3}$ families are derived from F_2 plants, I expect that only 50% of the total additive genetic variance is present between families. Consequently, the remaining 50% is still present within families leading to a large sampling error for a sampling size of only 3 plants per experimental unit and consequently to low power for QTL detection. Due to space constraints experimental units could not be increased under the premise of complete replications. To reduce or even avoid sampling errors within lines and to increase the precision of phenotyping, previous QTL studies on frost tolerance have either used recombinant inbred line (RIL) populations (Arbaoui, 2007; Dumont et al., 2009; Gery et al., 2011) or DH lines. Therefore, reducing the variation within lines will increase the power of genetic studies. However, the development of RILs is very time consuming and the development of sugar beet DH-lines is constrained by the high costs and technical limitations. Therefore, if neither is feasible, the experimental size has to be increased to allow experimental units of at least 10 plants and even replications within runs. This would drastically increase the power to detect QTL. One strategy to increase the test capacity offers a seedling test. With increased number of plants tested sampling effects could be reduced and the experimental precision increased while replicated experimental units would increase the statistical power. However, sugar beets tested in a seedling stage are less developed compared to our study and therefore expected to be more frost sensitive (Kirchhoff et al., 2012). Therefore, even though the precision of phenotyping would be increased by a seedling test it is most likely that the frost tolerance that would be characterized with increased precision shows lower correlations with field survival compared to our study and the practical relevance of such a test has to be questioned.

Artificial frost tests aim to predict overwintering behaviour under field conditions and to limit environmental complexity. However, frost tolerance and winter-hardiness are the integrative responses to complex environmental cues. Reducing complexity of artificial frost tests increases the risk to evaluate rather artificial environmental effects than “true” frost tolerance (Gusta and Wisniewski, 2012). Several studies which have compared the effects of natural and artificial acclimation on plants have revealed major transcriptional differences with effect on frost tolerance (Dhanaraj et al., 2007; Robertson et al., 1994; Wisniewski et al., 2006). Driven by these and further observations Gusta and Wisniewski (2012) concluded that these environmental

differences between artificial and natural conditions, including light, wind and temperature oscillation and their interactions impede at least partially the improvement of frost tolerance in crop species. To be able to estimate the correlation of field survival and artificial frost tolerance the mapping population is phenotyped under field conditions (Chapter 4). The major challenge for the future will be the fine tuning of the artificial frost test for accurate prediction of survival in the field and efficiency to be manageable for breeding applications.

3.5.2 Linkage Mapping and QTL Analysis

The linkage map presented in this study is based on 248 F_2 individuals and 127 markers covering 674 cM across all nine chromosomes. This corresponds to previously published maps in *B. vulgaris* (Grimmer et al., 2007; Halldén et al., 1996; Laurent et al., 2007; Schneider et al., 2007; Weber et al., 2000). In total mapping of FSI and SR revealed five QTLs, of which two where mapped to identical map positions on chromosome 3 and 8. Additionally, a QTL for FSI was mapped to chromosome 1 without a co-localizing QTL for SR. This difference might be a consequence of the higher phenotypic information content of FSI, as SRs only give information about the proportion of living plants and not about the intensity of frost damage on plants that survived the artificial frost test. However, the co-localization of FSI_3a and FSI_8a with SR_3a and SR8a is not surprising as genotypes with a low frost tolerance displayed a higher level of frost damages and lower SRs. Therefore it is most likely that the QTLs for FSI and SR detected on chromosome 3 and 8 were caused by one genetic factor, affecting both traits. In consistence with the observed transgressive variation (Figure 7) both parents contributed to FSI and SR.

Table 11: Summary of the QTL detected for Frost Severity Index and Survival Rate in 226 $F_{2:3}$ families derived from a cross of the inbred lines SC2 and SCK.

Chr.	QTL name	Left marker flanking the QTL peak	Right marker flanking the QTL peak	Position of QTL Peak [cM]	LOD	R^2	additive effect of SC 2
1	FSI 1.a	M45P34168	M44P31228S	48	2.83	5.6	-0.18
3	FSI 3.a	M44P31228	M44P31112S	6	2.96	5.9	-0.52
8	FSI 8.a	EB00867a_246	M44P31228S	1	3.39	5.8	0.20
3	SR 3.a	M44P31228	M44P31112S	6	4.02	8	10.75
8	SR 8.a	EB00867a_246	M44P31228S	1	2.58	5.1	-1.52

Chr.= Chromosome.

As only a limited proportion of the phenotypic variation could be explained by the detected QTL the probability to detect QTL has to be discussed: i. the genetic variation in the mapping population; ii. the genetic architecture of the traits under investigation, iii. the ability for accurate phenotyping of these traits (discussed in 4.1) and iv. the mapping resolution. Sufficient genetic variation between the phenotyped families is fundamental for QTL mapping. In this study a population based on $F_{2:3}$ families was phenotyped. As mentioned above 50% of the total additive genetic variation has been released between families. In addition, 25% of the total additive

variation is segregating within families (Liu, 1998). Moreover, both parents are elite breeding material from the same breeder and therefore, the genetic distance is expected to be limited. Congruently, no significant differences between the parents SC2 and SCK2 for SR and FSI were observed in this study. However, SC2 and SCK were chosen for the development of the mapping population due to clear differences in their field survival. Moreover, in previous artificial frost tests significant genotypic effects of $F_{2:3}$ families on FSI were observed. This, together with the QTL detected indicates that the genetic variation in the F_2 population derived from SC2 and SCK was sufficient for genetic mapping.

Beside the genetic variation in a population, the genetic architecture of the trait under investigation has a major impact on the ability to detect QTL. Analyses of the *A. thaliana* transcriptome have revealed that cold stress affects the expression of hundreds of genes (Maruyama et al., 2004; Maruyama et al., 2009; Vogel et al., 2005). These results together with previous QTL studies in various crops indicate a complex mode of inheritance of frost tolerance in *B. vulgaris*, too. However, experimental designs similar to the one applied in this study are suitable to detect only a limited number of major QTL. This has been demonstrated in previous QTL studies of frost tolerance in barley (Francia et al., 2004), wheat (Galiba et al., 1995), beans (Arbaoui, 2007), pea (Dumont et al., 2009) and *A. thaliana* (Gery et al., 2011) where between one and six QTL with effects on frost tolerance were detected. Consistent with similar mapping studies I have detected three QTL for FSI and one for SRs. In previous studies the detected QTLs for frost tolerance varied largely in their contribution to the phenotypic variance. A study in barley was able to explain 61.3% of the observed variation with two QTL (Francia et al., 2004), while two QTL in beans explained only 8.2% of the variance for frost tolerance (Arbaoui, 2007). Moreover, frost chamber experiments in *A. thaliana* and pea identified QTL effects on frost tolerance ranging from 7.6% to 47% (Dumont et al., 2009; Gery et al., 2011). With a total of 17.3% and 13.1% the QTL detected in this study explain only a small proportion of the observed phenotypic variation. This can either be an effect of the polygenic character of winter-hardiness in beets, which would result in many small QTL that remained undetected or by the relatively low heritability of the trait.

The power to detect and localize QTL depends on the ability to disclose recombination events, which is a function of the number of markers and individuals analyzed. The smaller the expected QTL effects, the higher is the number of detected recombination events required to provide the statistical power sufficient for QTL detection (Doerge, 2002). Compared with previous studies the number of markers in the linkage maps of this study is relative low (Bellin, 2003; Hunger et al., 2003; Schneider et al., 2007; Weber et al., 1999). However, even though the linkage map represents a limited number of markers, the average marker interval of 5 cM has been demonstrated to be sufficient for the detection of QTL (Bernardo, 2008). Moreover, it has been simulated that increasing the marker density beyond an average marker interval of 10 to 15 cM does not increase the power to detect QTL significantly (Bernardo, 2008; Darvasi et al., 1993). However, the linkage map has three gaps larger than 15 cM, representing 1.6% of total map size. Therefore, it cannot be excluded that QTL are not detected due to insufficient marker density. The focus for the proximate future should therefore be laid on the improvement of marker density to close these gaps.

Beside the number of markers the inheritance mode of markers highly affects the power to dissect genotypic differences in a F_2 population. In this study AFLP, CAPS and SSR markers were used for creating the genetic map. AFLP markers offer a relatively cost effective approach to achieve

genome wide marker saturations and have been used in multiple QTL studies in sugar beet (Schäfer-Pregl et al. 1999; Nilsson et al. 1999; Setiwan et al., 2000; Weber et al., 2000, Gidner et al. 2005; Grimmer at al., 2007). Even though there are cases where AFLP markers have been interpreted as co-dominant markers (Castiglioni et al., 1999; Jansen et al., 2001; Piepho and Koch, 2000), they are predominantly considered as a dominant marker system, because marker genotypes that are homozygous for the dominant allele can hardly be distinguished from heterozygous ones. Therefore, AFLPs are expected to have only limited information content compared to co-dominant marker systems. In a F_2 population 50% of the individuals are expected to be heterozygous at a given locus and therefore, the information content of AFLPs is low. Further problems arise if dominant markers are linked in repulsion phase as recombination frequencies and marker orders are often misestimated (Knapp et al., 1995). This might be the reason for the relative low proportion of AFLP markers that could be integrated into the linkage map.

Representing 226 $F_{2:3}$ families, the population size of this study was relatively large compared with previous QTL studies in sugar beet of Laurent (2007), Grimmer (2007) and Schneider (2007) who have tested less than 200 families per population. This is of major importance as the population size has a larger effect on the mapping resolution than the number and type of markers under investigation (Tanksley, 1993; Xu, 2003). Therefore, after the gaps in the linkage map have been closed, increasing the population size is the most efficient way to increase the number of recombination events which are required for the detection of minor QTL and the most promising way to increase the precision of a mapping study (Kearsey and Farquhar, 1998).

3.5.3 Identification and mapping of candidate genes

Even though *A. thaliana* and *B. vulgaris* have diverged about 120 million years ago candidate approaches using knowledge of *A. thaliana* have been successfully applied to identify homologous genes in *B. vulgaris* (Abou-Elwafa et al., 2010; Chia et al., 2008; Davies et al., 2004; Reeves et al., 2007). Based on a literature survey I used cold regulated *A. thaliana* genes for *in silico* identification of homologous sugar beet candidate sequences. Thirty-seven of these candidate sequences were successfully amplified in SC2 and SCK and subsequently identity of the candidate sequences was confirmed by alignments with the corresponding sugar beet ESTs and the genomic scaffolds.

In this study 37 homologous genes of known cold responsive genes representing different cold regulated pathways in *A. thaliana* have been identified in *B. vulgaris*. However, only eight candidates could be integrated into the linkage map, indicating low genetic diversity in these candidate sequences in the mapping population (Table 13). Twenty-two of the identified candidate sequences in *B. vulgaris* show homologies to genes of the *CBF* pathway, which is the major cold stress response pathway in a wide range of plants, in both frost tolerant and sensitive species (Choi et al., 2002; Jaglo et al., 2001; Savitch et al., 2005; Wang et al., 2008).

A homolog of the *CBF* pathway gene *HOS1* was mapped to sugar beet chromosome 5 in this study. In previous studies *HOS1* has been reported to have a dual function by affecting frost tolerance via negative regulation of *CBFs* and downstream *COR* genes as well as causing early flowering via reduced *FLC* expression (Dong et al., 2006; Ishitani et al., 1998; Lee et al., 2001). In recent years, results from *A. thaliana* have elucidated the prominent role of *FLC* for developmental processes and adaptations to environmental cues (Deng et al., 2011; Mendez-Vigo

et al., 2011). Consistently, expression analyses of *FLC* and *CBF* in *A. thaliana* indicate a feedback-loop that prevents early flowering under cold stress conditions (Deng et al., 2011; Seo et al., 2009). Moreover, it was shown that *FLC* together with *FRIGIDA* (*FRI*) determine the winter growth habit in *A. thaliana* and positively affect SRs under field conditions (Johanson et al., 2000; Korves et al., 2007; Lee et al., 2001). The *FRI* homolog was mapped to chromosome 5 while *BvFL1*, the sugar beet homolog of *FLC* could not be integrated into this linkage map due to missing polymorphisms. However, *BvFL1* was previously mapped to chromosome 4, where no QTL effects were detected. This is in congruence with the results of a field trial where *FL1* affected the SRs in the wild beet *B. vulgaris* ssp. *maritima* as well as in garden beets but not in sugar beets (Chapter 5).

On chromosome 1 two homologs of genes required for *FLC* mediated flowering delay in *A. thaliana* have been mapped: *LIKE HETEROCHROMATIN PROTEIN1* (*LHP1*) and *SHORT VEGETATIVE PHASE* (*SVP*). While *LHP*1 mediates epigenetic silencing of *FLC*, *SVP* forms a physical complex with *FLC* and is required for ambient temperature sensing (Lee et al., 2007; Mylne et al., 2006). However, it is highly unlikely that the candidate sequences for *LHP1* and *SVP* are the causative gene responsible for FSI_1a as multiple recombination events between the flanking markers of FSI_1a and the candidate genes were observed.

On chromosome 7 a homolog to *LONG VEGETATIVE PHASE1* (*LOV1*) was mapped. *A. thaliana* plants that overexpress *LOV1* displayed a late flowering phenotype caused by reduced expression of the floral promotor *CONSTANS*. Moreover, a positive effect of *LOV1* on frost tolerance has been shown without affecting the CBF transcription factor expression (Yoo et al., 2007). Therefore, Yoo (2007) suggested that *LOV1* is part of an overlapping pathway affecting flowering time and cold response.

A homologous sequence of *CBF1* was integrated into the linkage map and mapped to chromosome 8. It has been shown that the three *CBF* transcription factors are major determinants of acclimation response and frost tolerance. Knock out mutants of *CBF1* and *CBF3* displayed a 25 % to 50% reduction in frost tolerance and a QTL study mapped a locus in the vicinity of the three *CBF* transcription factors that explained about 20% of the variation for frost tolerance (Alonso-Blanco et al., 2005; Novillo et al., 2007). Like in *A. thaliana*, *CBF* genes are associated to major QTL for frost tolerance and winter-hardiness in cereals (Francia et al., 2004; Li et al., 2011; Skinner et al., 2005; Vágújfalvi et al., 2003). In contrast to the *CBF* effects on frost tolerance in *A. thaliana* and cereals the *B. vulgaris CBF* candidate sequence was not mapped in the support interval of the QTL detected on chromosome 8. However, only 4 cM proximal of the support interval of FSI_8a /SR_8a the candidate sequence of *CBF1* was mapped. Moreover, like in previous studies where CBF genes were identified as causative genes underlying QTL the frost tolerant parent contributed the desired allele of FSI_8a /SR_8a. Therefore, future studies have to elucidate whether the *CBF* candidate sequence is functional and contributes to frost tolerance in our mapping population.

Studies in cereals and *A. thaliana* have revealed that soluble sugars like raffinose contribute to frost tolerance due to their osmoprotective function (Olien and Clark, 1993; Zuther et al., 2004). One gene that has been shown to be essential for the accumulation of raffinose in response to cold stress in *A. thaliana* is *GALACTINOL SYNTHETASE3* (*GOLS3*) (Taji et al., 2002). Whether raffinose accumulation can contribute to frost tolerance and winter-hardiness in sugar beets,

which accumulate high levels of sucrose has to be elucidated. The homolog of *GOLS*3 was mapped on chromosome 5 of the sugar beet linkage map.

In this study no co-segregation of the identified QTL with any mapped candidate sequences was observed. Therefore, futures studies have to determine whether the identified candidate sequences are functional. Moreover, additional studies are needed to determine the contribution of the candidate genes to functional variation within the mapping population and the *B. vulgaris* genepool.

The aim of the candidate gene approach was the integration of candidate sequences into the linkage map to give first evidence for genes causative for quantitative variation. However, only a limited number of candidates could be integrated into the linkage map. Moreover, none of the mapped candidates were tightly linked to the identified QTL. This failure might have either been the consequence of insufficient genetic variance in the analysed genetic background or the statistical power of this study was too low to detect small effects.

As an alternative approach that overcomes the limited genetic variation of a population derived from a bi-parental cross, candidate gene association mapping has been recently used to elucidate the effect of these candidates on frost tolerance and winter-hardiness in rye (Li et al., 2011). This resulted in the identification of SNPs within 9 candidate genes that were significantly associated with frost tolerance under controlled conditions. Even more promising for a complex trait with little *a priori* information, like frost tolerance and winter-hardiness in sugar beet, is genome wide association mapping, which has recently been applied for the first time for traits of agronomic importance in sugar beet (Würschum et al., 2011b).

Table 12: Map position of eight candidate genes for cold response integrated into the linkage map of *B. vulgaris.*

Putative *B. vulgaris* gene	Sugar beet EST	Sugar beet scaffold	Marker	Sugar beet chromosome	Map position [cM]
CBF1	GBQ88_GBQ42_CauKiel_c1913	Scaffold00066	*B.v.CBF1*	8	14
FRI	GBQ88_GBQ42_CauKiel_c2059	scaffold00012	*B.v.FRI*	5	21
GolS3	GBQ88_GBQ42_CauKiel_c7651	scaffold00308	*B.v.GolS3*_C5	5	0
HOS1	GBQ88_GBQ42_CauKiel_c6314	scaffold00432	*B.v.Hos1*	5	48
LHP1	GBQ88_GBQ42_CauKiel_c16987	scaffold00062	*LHP1*_C1	1	33
LOV1	GBQ88_GBQ42_CauKiel_c16210	scaffold00183	*B.v.Lov1*_C7	7	50
SVP	GBQ88_GBQ42_CauKiel_c7919	scaffold00041	*B.v.SVP*	1	23
FT1	-	-	*B.v.FT1*_C9	9	38

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4 Mapping of quantitative trait loci for winter-hardiness in sugar beets (*B. vulgaris* L.): field trials

4.1 Abstract

Breeding autumn sown winter-sugar beets (*Beta vulgaris* L.) in temperate climates requires an understanding of the genetic mechanisms controlling winter-hardiness. The aim of this study was the dissection of genetic factors affecting winter-hardiness in sugar beet by QTL analysis under consideration of candidate genes. A population of 238 $F_{2:3}$ families derived from the sensitive inbred line SC2 and the winter-hard inbred line SCK was evaluated for overwintering behaviour in Göttingen and Söllingen, Germany as rate of living plants (RLP) three weeks after the last frost day and as survival rates (SR) after seven weeks of a recovery phase following the last frost day. Due to heavy rainfalls in autumn and harsh winter conditions data from Söllingen were excluded from the analysis. In Göttingen severe frost without snow cover was causative for a mean SR of 0.2% across $F_{2:3}$ families and lacking variation recorded on April 6. In contrast, for the rate of living plants (RLP) recorded on March 5 transgressive segregation was observed and RLP ranged from 0% to 82.7 %. Although no candidate gene could be associated with winter-hardiness two QTL affecting RLPs in Göttingen were mapped to chromosome 4 and 8. The QTL explained 5.1% and 6.6% of the observed phenotypic variance. To validate the contribution of the detected QTL to the development of autumn sown winter-sugar beets additional multi-environment field trials are required.

4.2 Introduction

A major limiting factor for sugar beet yield under cool-temperate conditions is the insufficient canopy to capture much of the solar radiation from April to June (Röver, 1995). Therefore, an extension of the vegetation period increases yield as a consequence of improved canopy in spring (Hull and Webb, 1970; Jaggard et al., 1983). A promising strategy to prolong the vegetation period and therewith increase yield is a sugar beet sown in autumn and harvested in the following year. Yield simulations have calculated a hypothetical yield increase of autumn sown winter sugar beets based on the improved synchronization of canopy and solar radiation of up to 26% (Hoffmann and Kluge-Severin, 2010; Jaggard and Werker, 1999). Even though the idea of improving sugar beet yield by cultivating winter beets is more than 200 years old (Achard, 1806) and several studies (Bauer, 1932; Eichholz and Röstel, 1962; Schneider, 1935) reported about intriguing yield increases no winter sugar beet variety has even been introduced into cool-temperate markets. This is most likely a consequence of the bolting behavior of sugar beets. Plants which have been exposed to vernalization are bolting. This shoot elongation goes along with altered sink source capacities causing reduced root and sugar yield (Hoffmann and Kluge-Severin, 2011; Jaggard et al., 1983). On the other hand bolting of sugar beets is required for seed production. Therefore, breeding autumn sown winter beets depends on an efficient system for bolting control. With the identification of several target genes with effects on bolting (Pin et al., 2010; Pin et al., 2012) bolting control is likely to be achieved in the near future by genetic engineering (Jung and Müller, 2009). This gain of technologies and knowledge gave new momentum to the development of a winter sugar beet, not only as a sugar crop but also as an energy crop.

With a system for bolting control coming into perspective, attention can be paid to characterize winter hardiness in sugar beets and to identify genetic factors for selection of winter-hard genotypes. Recently, Kirchhoff et al. (2012) concluded from the analysis of a diversity panel that the genepool of *B. vulgaris* displays a wide variation for winter-hardiness.

In a follow-up study Frerichmann et al. (Chapter 5) analyzed the effect of three flowering time genes on winter-hardiness. The authors revealed an effect of *BvFL1*, a homolog of *Flowering locus C* (*FLC*) in *A. thaliana* on winter-hardiness. Subsequently, a linkage mapping approach including candidate genes was applied to identify three quantitative trait loci (QTL) on chromosomes 3 and 4 affecting artificial frost tolerance, which is a major determinant of winter-hardiness. Kirchhoff et al. (Chapter 3).

Even though recent studies gained some knowledge about winter-hardiness and frost tolerance in sugar beet until now no genome wide analysis of winter-hardiness has been performed. However, several QTL for winter hardiness have been identified in a wide range of crops including cereals, legumes, oilseed rape and trees (Hayes et al., 1993; Jermstad et al., 2001; Kole et al., 2002; Lejeune-Henaut et al., 2008). Several of these QTL co-localize with *C-repeat binding factors* (*CBF*) (Francia et al., 2004; Vágújfalvi et al., 2003). *CBF* genes are highly conserved among monocots and dicots including cold sensitive and cold tolerant plant species (Jaglo et al., 2001; Owens et al., 2002; Qin et al., 2004; Savitch et al., 2005; Welling and Palva, 2008; Zhang et al., 2004). *CBF* genes and components of the *CBF* pathway have been identified as major regulators of winter-hardiness and frost tolerance in a wide range of species (Choi et al., 2002; Dubouzet et al., 2003; Gery et al., 2011; Jaglo et al., 2001; Qin et al., 2004; Savitch et al., 2005; Welling and Palva, 2008; Zhang et al., 2004)

Beside the dominant role of the *CBF* pathway, several studies have revealed a co-localization of loci affecting winter-hardiness and flowering time (Francia et al., 2004; Lejeune-Henaut et al., 2008; Vágújfalvi et al., 2003). In addition, studies in *A. thaliana* have demonstrated that genes of the autonomous flowering pathway (*FVE*), the vernalization pathway (*FLC, SOC1, FRI*) and the photoperiod pathway (*LHY, CCA1, PRR5, PRR7, PRR9*) affect the expression of *CBF* and several cold regulated (*COR*) genes (Deng et al., 2011; Kim et al., 2004; Korves et al., 2007; Nakamichi et al., 2009; Seo et al., 2009; Yoo et al., 2007).

The best investigated crops in terms of winter-hardiness are cereals. Two major QTL determining winter-hardiness (*Frost resistance 1*) *FR1* and (*Frost resistance 2*) *FR2* have been located on chromosome 5 of barley separated by approximately 30cM (Francia et al., 2004; Skinner et al., 2005; Vágújfalvi et al., 2003). While *FR1* co-localizes with the major QTL for vernalization response *VRN1*, *FR2* was mapped to a region also containing a cluster of *CBF* genes (Vágújfalvi et al., 2003).

Both in *Brassica rapa* and *Brassica napus* QTL for winter survival have been identified (Kole et al., 2002). In *Brassica rapa* two QTL on chromosomes BR3 and BR2 co-segregate with flowering time QTL, while in *Brassica napus* two QTL on chromosome N2 for winter survival co-localize two *Brassica napus* homologs of *FLC* (Kole et al., 2002; Udall 2006). Interestingly, all of these loci delayed flowering and increased winter survival (Kole et al., 2002).

In pea (*Pisum sativum* L) three consistent QTL for winter frost damages (WFD) have been mapped on linkage groups 3, 5 and 6 (Lejeune-Henaut et al., 2008). Two of these QTL co-localize with markers that are associated with flowering time traits. The QTL *WFD 3.1* co-localizes with a QTL for flowering time and the confidence interval of *WFD 5.1* includes a marker that was derived from a homologous sequence of the *A. thaliana* flowering time gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), which has been shown to affect the expression of *CBF* pathway genes in *A. thaliana* (Aubert et al., 2006; Lejeune-Henaut et al., 2008; Seo et al., 2009).

To exploit the advanced knowledge of genes controlling winter-hardiness in other crop species and decrease the work required for QTL fine mapping and subsequent cloning the integration of candidate genes (CG) has been applied successfully (Pflieger et al., 2001). The

big advantage of integrating candidate genes into a QTL map is the combination of a-priori knowledge about genes with a genome wide scan for genetic effects on the trait under investigation. If co-localization of a QTL with a candidate gene is observed, first evidence for an effect of the candidate gene on the QTL is given. However, a QTL region can span hundreds of genes and the causative effect of the candidate gene on the QTL has to be proven. The integration of candidate genes into a linkage map of sugar beet has first been applied by Schneider et al. (1999). The authors integrated into the linkage maps of three sugar beet populations 42 candidate genes, with known effects on carbohydrate and nitrogen metabolism. Beside these yield related traits, several studies co-localized QTL regions with the map position of candidates for resistance against rhizomania, cercospora and rhizoctonia (Hunger et al. 2003; Lein et al., 2007; Lein et al. 2008). In recent years *BvCOL1* and *BvFL1*, homologous genes of the major flowering time regulators *CONSTANS*, and *FLC* in *A. thaliana*, have been identified via candidate approaches and mapped to chromosome 2 and 4 (Reeves et al., 2007,Chia et al., 2008). An even bigger milestone for the understanding of flowering time in sugar beet was the map-based cloning of *BvBTC1*, the causative gene of the bolting locus *B* (Pin et al., 2012).

Here we present the first QTL analysis of winter-hardiness in sugar beet. To exploit the advanced knowledge of genes affecting winter-hardiness, frost tolerance and flowering time regulation in *A. thaliana* eight candidate genes were integrated into the linkage map used for the QTL analysis. Two QTL with opposite effects were detected explaining up to 6.9% of the phenotypic variance.

4.3 Materials and Methods

4.3.1 Plant material

Twohundredthirtyeight sugar beet $F_{2:3}$ families were phenotyped for their winter-hardiness under field conditions. The $F_{2:3}$ families were based on two F_1 full sibs derived from a cross of the inbred lines Strube component K "SCK" (Kiel Code 090014) and Strube component 2 "SC2" (Kiel Code 090019), which were provided by Strube research GmbH, (Söllingen, Germany). SCK and SC2 differed in their ability to survive winters evaluated in field trials and their frost tolerance under artificial conditions (data not shown). As additional standards the sugar beet hybrid variety "Theresa" (KWS Saat AG, Einbeck, Germany; Kiel Code 090023) and the biennial sugar beet "93161P" were tested.

4.3.2 Overwintering field trials

To determine the genotypic variation for winter hardiness field trials in three environments were planned. However, an extraordinary wet summer in 2012 impeded sowing in Hohenschulen, Germany. Therefore, the population was evaluated in a field experiment in Göttingen and Söllingen, Germany, only (Supplementary table 11). Experiments were designed as a randomized incomplete block design. Experimental units were 2.5 m long single rows with a between row distance of 45 cm. Fifty seeds were sown per row at August 29, 2012, in Göttingen and August 30, 2012, in Söllingen. The plants were thinned to 20 per row before winter. The SR of the $F_{2:3}$ families were determined as the mean of the surviving plants per row after winter divided by plants per row before winter. The rate of living plants (RLP) was recorded at March, 5, 2012, and final SRs were determined at April 6, 2012, in Göttingen and April 12, 2012, in Söllingen. An environmental SR was estimated as the grand mean of all $F_{2:3}$ families in a given environment.

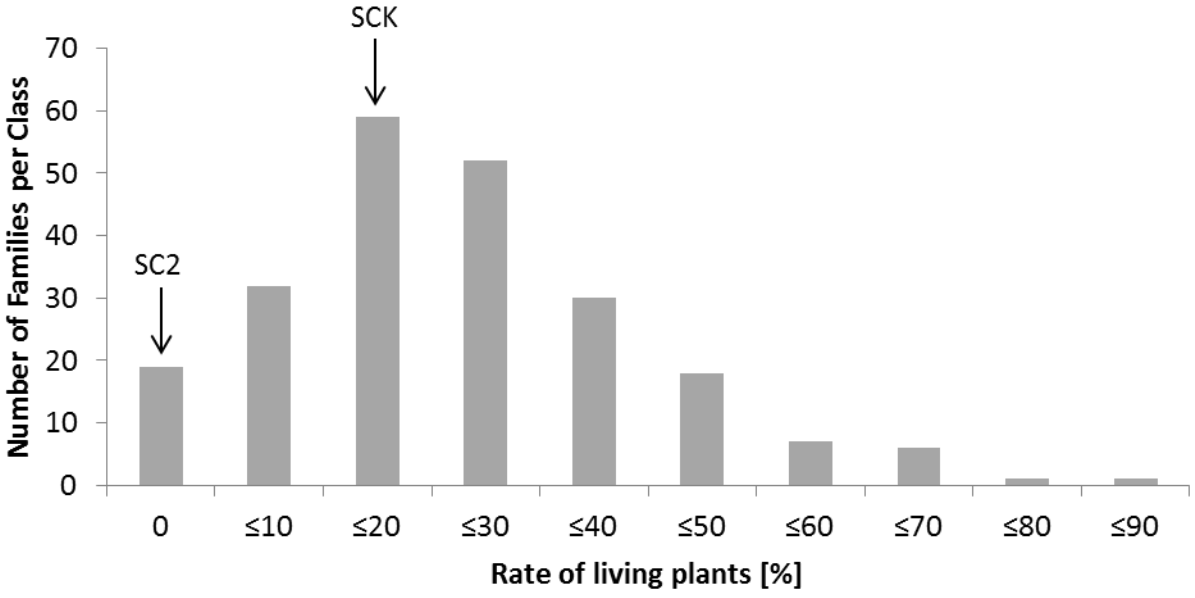
4.3.3 Statistical analysis

Analysis of covariance (ANCOVA) was performed to test the fixed effect of $F_{2:3}$ families on RLP. The effects of development before winter were considered as random and development before winter was used as co-factor. Broad sense heritabilities were estimated according to Hallauer and Miranda (1988). Differences between parents were tested at a comparisonwise error rate of $\alpha_C = 0.05$. To identify families with improved winter-hardiness, one-sided Dunnett comparisons (Dunnett, 1955) of all families with the parent SCK at an experiment wise error rate of $\alpha_E = 0.05$ were conducted. A regression analysis was performed to estimate the effect of RLP on SR of leaf biomass before winter. All statistical analyses were performed with the software R (R Development Core Team, 2010).

4.3.4 Linkage Map Construction and QTL Mapping

The F_3 families phenotyped in this field study were derived from the same F_2 plants as described in Chapter 3.3.1. Therefore the same linkage map was used for the QTL analysis of winter-hardiness. The linkage map comprises127 markers covering all 9 chromosomes with a total map size of 674 cM (Chapter 3). In the linkage map eight homologous sequences of cold regulated genes form *A. thaliana* were integrated. Quantitative trait loci (QTL) for RLP were mapped with PLABQTL v 1.2 Utz and Melchinger, 1996) by composite interval mapping at LOD ≥2.5. QTL mapping of survival rates was impossible due to insufficient variation caused by the high rates of winter killing.

Figure 9: Histogram for 238 F3 families segregating for rate of living plants at 5 March in Göttingen. The population was developed from crossing inbred lines SCK and SC2.Histogram for 238 F3 families segregating for rate of living plants at 5 March in Göttingen.



4.4 Results

4.4.1 Overwintering field trials

Twohundredthirtyeight sugar beet $F_{2:3}$ families and the parental lines were grown in the field over winter in Göttingen and Söllingen. Insufficient sowing conditions in Söllingen caused an irregular field emergence and large differences in the pre-winter-development within and across $F_{2:3}$ families. Therefore, the data from Söllingen was excluded from further analyses.

On April 6, 2012, the mean SR across $F_{2:3}$ families in Göttingen was 0.2 % and none of the parental lines had survived. These low SRs across families were insufficient for genetic dissection of winter-hardiness. Even though only little genetic variation between $F_{2:3}$ families was detected on April 6, 2012, the rate of living plants at the March 5, 2012, differed significantly ($P<0.001$) across families. Therefore, further investigations are based on the RLP scored on March, 5, 2012, in Göttingen. The relationship between SR and RLR was highly significant ($R^2 = 0.17$, $P<0.001$). The mean RLP for SC2 was 0% and 13.24 % of SCK, respectively. Across $F_{2:3}$ families RLPs ranged from 0 % to 82.69 % (Figure 9).The heritability of the RLP was 0.65 (Table 13). Comparing the RLP of the winter-tolerant parent SCK with the $F_{2:3}$ families, we identified four families with superior winter-hardiness (Kiel codes 111197, 1112666, 111298 and 111391). However, the highest overall RLPs were recorded for the hybrid variety "Theresa" with 91.17 %. Comparing the RLP of "Theresa" with the $F_{2:3}$ families under investigation revealed that 210 families deviated significantly ($P<0.001$).

Table 13: Range, means and broad sense heritability (h^2) estimates for SRs and rate of living plants (RLP) for 238 F_3-families and their parents after winter recorded in March and April 2012 in Göttingen and Söllingen.

	RLP Gö 11/12	**SR Gö 11/12**
Parents		
SCK [%]	13.23	0
SC2[%]	0	0
Population		
Range [%]	0 - 82.69	0 – 16.7
Mean [%]	21.5	0.2
h^2	0.65	0.04

4.4.2 QTL analysis

Based on the linkage map described in chapter 3 QTL mapping of the rate of living plants recorded at March, 5th 2012 revealed two QTL on chromosome 4 (RLP_4a ; LOD=2.53) and 6 (RLP_6a, LOD=3.28) (Table 14). RLP_4a accounted for 5.1% and RLP_6a for 6.6% of the observed phenotypic variation and none of the QTL was mapped in the vicinity of any of the mapped candidate genes.

4.5 Discussion

Here we report the first QTL affecting winter-hardiness under natural conditions in sugar beets. We used phenotypic data based on one location only because of inhomogeneous pre-winter development in Söllingen. We identified two regions on chromosome 4 and 6 with effect on winter-hardiness which had no effect on frost tolerance under artificial conditions (Chapter 3). All results presented here are based on one environment and can therefore only be regarded as preliminary. The results have to be validated and confirmed in multiple environment field trials.

Due to an extraordinary wet August at both locations sowing took place with a delay of three weeks compared to the date considered as ideal by Kirchhoff et al (2012). Moreover, a fast and uniform field emergence in Söllingen was impeded by bad sowing conditions. Even though 50 seeds were sown and 20 plants were desired, only 14 plants on average developed at least one extended leaf pair (BBCH 11) before winter in Söllingen. This number of plants before winter would have still been sufficient to determine reliable SRs. However, the large developmental differences observed within and across families were most likely not caused by genetic effects but by small scale differences in the seed bed quality. These small scale environmental effects could not be assessed and corrected for in the statistical analysis. Therefore, phenotypic values of the SRs of the different families are expected to be highly biased. Taking these observations together, data recorded in Söllingen were excluded from further analysis.

Table 14: Quantitative trait loci for the rate of living plants after winter detected by composite interval mapping.

chromosome	QTL name	left marker flanking the QTL peak	right marker flanking the QTL peak	LOD	QTL Peak [cM]	R^2	additive effect of SC2
4	RLP_GÖ 4a	M44P39236S		2.53	52	5.1	-0.04
6	RLP_GÖ 6a	M44P39236S		3.28	13	6.6	-0.05

In Göttingen, winter hardiness was recorded at two time points. Final SR were recorded in Göttingen at April 6, 2012, after a recovery phase of 53 days after the last frost day with an average temperature below 0°C. Preliminary data on plant survival were taken as RLP already 21 days after the last frost day while plants where still in the process to die or fully recover. With an R^2 of 0.17 correlations of RLPs and SR were low, indicating a low accuracy to predict SRs based on the observed RLPs. The low correlation of RLPs and SR gives rise to the speculation that RLPs and SR are independent traits. However, a significant correlation ($P<0.001$) between RLPs and SRs indicates that the RLPs can give a rough indication for the level of winter-hardiness. Most likely the low coefficient of determination between RLPs and SRs is a consequence of the insufficient variation of the SRs. Apparently, with temperatures down to -20°C without snow cover, winter conditions in Göttingen 2012 were too harsh to allow phenotypic differentiation for SRs. In contrast to SR, large variation was observed for RLPs recorded on March 5, 2012. As no major frost event occurred after March 5, 2012 it can be concluded that frost plants were already frost damaged on the day of recording the RLPs, even though symptoms were not developed to a full extend. It can be assumed that the degree of visible frost symptoms depends on the severity of frost damages and the physiological activity of a plant after frost which increases with increasing temperatures. Taking this into account, two explanations for the wide variation of the RLPs can be found. The RLP reflected frost damages from an earlier and less severe frost event. Taking the temperatures over winter in Göttingen into account this theory is unlikely as no temperatures occurred before the 30 January, which could have caused the selective conditions (Supplementary table 12). Therefore, it can be assumed that the speed of symptom development reflects severity of frost damage. Thus the variation of RLPs can be explained as follows: plants with inferior winter-hardiness are affected first by adverse conditions and therefore exhibit lower RLPs even at a time point when physiological activity was insufficient to assess final SRs. In contrast, genotypes with high winter-hardiness are expected to be less damaged and therefore, the RLPs decrease later compared to susceptible genotypes. However, further studies have to be conducted to explore the correlation between RLP and SR.

In previous studies SRs and over wintering behaviour differed largely depending on the genotypes analysed and environmental conditions (Pohl-Orf et al.; 1999; Drießen 2003; Hoffmann and Kluge Severin; 2010; Kirchhoff et al. 2012). Pre-winter development and winter conditions had no effects on the leaf area of sugar beet varieties in spring, unless severe frost events occur. Consistently, high SRs under mild and moderate winter conditions were reported for sugar beet varieties (Pohl-Orf et al.; 1999; Hoffmann and Kluge Severin; 2010), wild beets (Drießen 2003) and a diversity panel (Kirchhoff et al. 2012; Chapter 2). However, under more adverse conditions SRs drastically decreased (Pohl-Orf et al.; 1999; Kirchhoff et al. 2012; Chapter 2) and Kirchhoff et al. (2012) speculated that pre-winter development and winter conditions become crucial for the ability to survive under adverse conditions. Comparison of the winter conditions of this study with previous studies clearly indicates that the extremely low temperatures at the beginning of February were causative for the total loss of the plants, as in no published experiment before *B. vulgaris* plants were exposed to temperatures below -15° without snow cover. As this study could be evaluated for one environment only, the question whether genotype x environment (G x E) interactions affect the ability to survive winter in this structured population has to be investigated in further experiments. However, Kirchhoff et al. (2012) observed genotype x environment (G x E) interactions affecting winter-hardiness in a diversity panel of *B. vulgaris*.

The RLPs differed highly significantly across $F_{2:3}$ families and transgressive segregation was observed. The best families showing more than four times higher RLPs compared to the tolerant parent SCK (Figure 9). Across the $F_{2:3}$ families a high heritability for RLPs (0.65) and low heritability for SR (0.04) were observed (Table 13). The high heritability of RLPs is most

likely a consequence of the transgressive segregation while the low heritability of the SR is an effect of the insufficient variation observed. Remarkably is that the highest overall RLP was observed in the only hybrid cultivar tested, giving rise to the speculation that under the harsh winter conditions in Göttingen hybrid vigour contributed to winter-hardiness. However, further experiments are needed to elucidate the effect of heterosis on winter-hardiness.

The linkage map used in this study has been previously discussed (Chapter 3). However, it has to be recognized that the marker density of the map was relatively low. It can therefore not be excluded that QTL with small or moderate effects on RLP have been missed. The most promising strategy to improve the power of this study would be the integration of additional co-dominant markers.

The QTL analysis revealed two loci with significant effect on RLP on chromosome 4 and 6, explaining only a limited proportion of the phenotypic variation. The remaining phenotypic variation can either be caused by multiple genetic factors, each contributing, with minor effects to RLP or the phenotypic variation is not the consequence of genetic variation but environmental effects. To elucidate whether multiple minor QTL were causative for the observed variation a high density marker map would be suited to detect even smaller QTLs. To answer the question whether the variation is non-genetic, additional field trials are required.

In the future, additional multi-environment field trials will be conducted to gain additional phenotypic data. The preliminary QTL presented here can only be considered as solid if the additional experiments verify the reported results.

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5 EcoTILLING in *Beta vulgaris* reveals polymorphisms in the *FLC*-like gene *BvFL1* that are associated with annuality and winter-hardiness

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5.1 Abstract

Sugar beet (*Beta vulgaris* ssp. *vulgaris* L.) is an important crop for sugar and biomass production in temperate climate regions. Currently sugar beets are sown in spring and harvested in autumn. Autumn-sown sugar beets that are grown for a full year have been regarded as a cropping system to increase the productivity of sugar beet cultivation. However, for the development of these "winter beets" sufficient winter hardiness and a system for bolting control is needed. Both require a thorough understanding of the underlying genetics and its natural variation. We screened a diversity panel of 268 *B. vulgaris* accessions for three flowering time genes via EcoTILLING. This panel had been tested in the field for bolting behaviour and winter hardiness. EcoTILLING identified 20 silent SNPs and one non-synonymous SNP within the genes *BTC1*, *BvFL1* and *BvFT1*, resulting in 55 haplotypes. Further, we detected associations of nucleotide polymorphisms in *BvFL1* with bolting before winter as well as winter hardiness. These data provide the first genetic indication for the function of the *FLC* homolog *BvFL1* in beet. Further, it demonstrates for the first time that EcoTILLING is a powerful method for exploring genetic diversity and allele mining in *B. vulgaris*.

5.2 Introduction

EcoTILLING is a fast and easy method to detect rare SNPs or small indels in target genes in natural populations. It is an adaptation of the TILLING (Targeting Induced Local Lesions In Genomes) technique that is used to detect point mutations in mutant populations (McCallum et al., 2000). In EcoTILLING, endonucleases such as CEL I are used to cut mismatched sites in the heteroduplex DNA formed by hybridization of different genotypes in a test panel. It is a cost effective technology as sequencing is limited to individual genotypes each representing a different haplotype. EcoTILLING has been used for the characterization of the genetic variability in *Arabidopsis thaliana* (thale cress) (Comai et al., 2004), *Musa* spp. (various banana species) (Till et al., 2010), *Populus trichocarpa* (black cottonwood) (Gilchrist et al., 2006), *Phaseolus vulgaris* (common bean) (Galeano et al., 2009), and *Vigna radiata* (mung bean) (Barkley et al., 2008). Furthermore, it has been used for candidate gene-based detection of new alleles referring resistance to biotic and abiotic stress in *Hordeum vulgare* (barley) (Mejlhede et al., 2006), *Oryza sativa* (rice) (Kadaru et al., 2006; Negrao et al., 2011), *Solanum tuberosum* (potato) (Elias et al., 2009). *Cucumis* spp. (including cucumber) (Nieto et al., 2007) and *Solanum lycopersicum* (tomato) (Rigola et al., 2009). EcoTILLING has not been reported for sugar beet (*Beta vulgaris* ssp. *vulgaris* L.) which contributes to 22 % of the world production of white sugar (FAO, 2012).

Sugar beets are herbaceous, dicotyledonous plants that belong to the Amaranthaceae family (formerly Chenopodiaceae). The genus Beta is divided into the two sections *Corollinae* and *Beta*, the latter of which is further divided into cultivated beets (*B. vulgaris* ssp. *vulgaris*), wild sea beets (*B. vulgaris* ssp. *maritima* L.) and wild beets (*B. vulgaris* ssp. *adanensis*) (Kadereit et al., 2006). Within *B. vulgaris* ssp. *vulgaris*, four cultivated groups can be

distinguished: fodder beet, leaf beet, garden beet and sugar beet. While leaf beets and garden beets show an annual or biennial life cycle, sugar beets and also fodder beets are biennial plants that stay in the vegetative phase in their first year, forming a storage root with a high sucrose concentration of up to 20%. Both vernalization and long days are required for stem elongation (bolting) and flowering to occur in the second year of growth. Vernalization in sugar beet is achieved by exposure to cold temperatures for ten to 14 weeks.

Currently, sugar beets are cultivated as a spring sown crop in cool temperate climate regions. Seeds are sown in April and the roots are harvested starting in September. The late formation of a closed leaf canopy in spring is regarded as the main factor limiting beet yield (Hoffmann and Kluge-Severin, 2010). One strategy to overcome this is the production of autumn sown winter beets which develop a closed canopy earlier in spring. However, breeding of autumn sown winter beets requires sufficient winter hardiness to survive the winter and a system for bolting control which allows bolting for seed production but represses bolting after winter during crop production (Jung and Müller, 2009). With key regulators of flowering and bolting in *B. vulgaris* recently having been identified (Pin et al., 2010; Pin et al., 2012), bolting control may be achieved by genetic modification which on the one hand allows suppression of bolting after winter for cultivation of beets, but on the other hand enables bolting for seed production (Jung and Müller, 2009).

In order to avoid an untimely transition to the extremely cold-sensitive generative phase (Fowler et al., 1993) before or during winter, and to facilitate the accumulation of sufficient resources for reproduction, winter-annual and biennial plants growing in temperate zones require vernalization for induction of flowering. Cultivated beets are biennials, whereas annual beets without a requirement for vernalization are frequently observed in wild beet populations (Van Dijk and Boudry, 1991; Van Dijk et al., 1997). The vernalization response in biennial beets is mediated by the *FLOWERING LOCUS T* (*FT*) homolog *BvFT1*, which in contrast to the promotive action of *FT* in Arabidopsis functions as a repressor of flowering (Pin et al., 2010). Similar to *FLC*, *BvFT1* is gradually down-regulated during the prolonged cold of winter (Pin et al., 2010). In annual beets, *BvFT1* is not expressed even in the absence of vernalization and was shown to be negatively regulated by the pseudo-response regulator gene *BOLTING TIME CONTROL 1* (*BTC1*), formerly referred to as *BvBTC1* (Pin et al., 2012). This gene is located at the bolting locus *B* and is a major determinant of the annual growth habit in beet. The dominant *BTC1* allele promotes bolting in annuals in response to long days, whereas biennials carry a partial-loss-of-function allele which is not able to mediate the promotive effect of long days without prior vernalization (Pin et al., 2012). All cultivated (biennial) beet accessions tested were found to carry the same haplotype whereas the vast majority of wild sea beets harbour haplotypes which resemble the *BTC1* allele found in annual reference accessions (Pin et al., 2010).

Several other genes in beet have been identified on the basis of homology to floral transition genes in Arabidopsis, including the central regulator of vernalization requirement and response in this species, *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999; Sheldon et al., 1999) The *FLC-LIKE 1* gene *BvFL1* is gradually down-regulated during a prolonged exposure to cold under continuous light (Reeves et al., 2007). Constitutive expression of *BvFL1* in an *FLC* null mutant of Arabidopsis significantly delayed flowering, suggesting at least partial evolutionary conservation of function between *FLC* homologs in Arabidopsis and beet.

Interestingly, flowering time control genes also seem to affect frost tolerance, which is the most important factor contributing to winter hardiness (Bieniawska et al., 2008; Eriksson and Webb, 2011; Franklin and Whitelam, 2007). Plants can further increase their frost tolerance by a gradual adaptation of the metabolism during a hardening process that occurs at non-

freezing temperatures below 10°C. In Arabidopsis, frost tolerance is regulated by the *C-REPEAT BINDING FACTOR* (*CBF*) transcription factor family, with plants constitutively overexpressing *CBF1-3* showing an increase in frost tolerance (Gilmour et al., 2004). Plants overexpressing *CBF* genes also showed elevated levels of *FLC* expression (Seo et al., 2009). Deng et al. (2011) further reported that *FLC* is not only regulated by temperature to control flowering time, but plays a dual role in flowering time control and cold stress response. Korves et al. (2007) showed that a naturally occurring single nucleotide polymorphism (SNP) in intron 1 of *FLC* led to a modest increase in winter SRs. Interestingly, a recent study suggested that the recruitment of a repressive chromatin complex at the *FLC* locus involves the cold-induced expression of a long non-coding RNA, termed *COLDAIR*, from intron 1 of *FLC* (Heo and Sung).

In the present study, we established EcoTILLING in *B. vulgaris* to survey a large panel of cultivated and wild beets for allelic variants of candidate genes for regulators of vernalization requirement and/or winter hardiness. This panel had been phenotyped before for variation in the occurrence of bolting before winter (i.e. in the absence of vernalization) and SRs after winter (Kirchhoff et al., 2012). As candidate genes we chose (i) *BvBTC1* and (ii) *BvFT1*, because of their known functions in the regulation of vernalization requirement and response in beet, and (iii) *BvFL1*, because of the regulatory role of its homolog *FLC* in both vernalization and cold stress response in Arabidopsis. We found that haplotype variation at the *BvFL1* locus was associated with variation in bolting rate before winter and SR after winter. These data provide the first genetic indication for the function of the *FLC* homolog *BvFL1* in beet, and are relevant for sugar beet breeding and our understanding of the bolting time control network in Beta.

5.3 Methods

5.3.1 Plant material and phenotypic data

Phenotypic data for bolting before winter and winter hardiness were taken from a recent study described in detail by Kirchhoff et al. (2012). In short, a panel of 396 *B. vulgaris* accessions covering a wide range of genetic diversity was tested for winter hardiness in a replicated overwintering field experiment in eight environments at five different locations in Germany and Belarus in the winters of 2008/09 and 2009/10. Survival rates were determined as the fraction of surviving individuals among all plants of a given accession ranging from 0 to 1, where 0 means no plants survived and 1 means all plants from one accessions survived. The mean survival rates were estimated as best linear unbiased predictors (BLUPs) for each accession across all environments. Accordingly, bolting rates before winter were determined in the 2009/10 environments as the fraction of bolting individuals among all plants of a given accession ranging from 0 to 1, where 0 means none of the plants bolted and 1 means all plants of a given accession bolted. To avoid unbalanced data, we reduced the data set to a subpanel of 268 accessions that were tested in all environments. These comprise the four cultivar groups fodder beet (40), leaf beet (47), garden beet (58) and sugar beet (88), as well as 35 *B. vulgaris ssp. maritima* accessions. The 88 sugar beets can be further subdivided into 49 elite accessions (sugar beet elite breeding material, SBEBM) provided by Strube GmbH & Co. KG (Söllingen, Germany) and 39 mostly gene bank accessions of various origins (sugar beet germplasm, SBGP).

5.3.2 DNA isolation and screening for polymorphisms

DNA was isolated from freeze dried leaf samples taken from up to eight plants per accession. This was done with a NucleoSpin® 96 Plant II Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) as recommended by the manufacturer on a Tecan "Freedom Evo" Robot.

DNA concentration was measured via the Tecan Robot using a photometer and SYBR® Green (Invitrogen GmbH, Darmstadt, Germany) and normalized with DNase free water to a final concentration of 10 ng/µl in a total volume of 160 µl. The 268 DNA samples representing the 268 *B.vulgaris* accessions of the test panel were each pooled 1:1 with DNA of the biennial sugar beet 93161P as reference type and stored in 96 well plates. 93161P is an inbred line homozygous for the investigated candidate genes and was provided by Saatzucht Dieckmann.

Oligonucleotide primers amplifying conserved domains of the genes *BvFT1*, *BvFL1* and *BvBC1* were designed from genomic sequences with the software tool FastPCR (Kalendar et al., 2009). Regions were chosen after analyses of genomic DNA sequence with CODDLE (Codons Optimized to Discover Deleterious Lesions; http://www.proweb.org/coddle/). The primers were pre-screened before labelling in a so called "crash-test" adapted from Weil and Monde (2007). Forward and reverse primers were end dye labelled with Dyomics fluorescent tags DY-681 (700nm absorption) and DY-781 (800nm absorption), respectively. PCR amplification was done in a 20 µl volume containing 1 ng pooled DNA, 1x Taq buffer, 1.5 mM $MgCl_2$, 2 mM dNTPs (Invitrogen, Darmstadt, Germany), 0.2 units recombinant TAQ DNA polymerase (Invitrogen, Darmstadt, Germany) and 0.8 pmol primer (biomers.net, Ulm, Germany). The primers were used in a labeled versus unlabeled ratio of 3:2 for DY-681 and 4:1 for DY-781 according to Till et al. (2006a). PCR was performed on a DNA Engine DYAD thermal cycler (MJ Research Inc., Waltham, MA, USA). PCR steps for the amplification were as follows: an initial denaturation step at 95°C for 5 min followed by 30 cycles of a 30 sec denaturation step at 95°C, 30 sec annealing at 60°C and 60 sec elongation at 72°C. The PCR was concluded with 5 min elongation at 72°C. The crude celery extract (CCE) was extracted as described by Till et al. (2006a) with a slight modification. We did not dialyze and re-buffer our celery juice as recommended. Instead we used the crude extract for enzymatic mismatch cleavage and tested it against commercial products. The results (data not shown) were identical with those obtained by using Surveyor® endonuclease. For SNP evaluation we only used the 700 nm channel of the LI-COR 4300, because the 800 nm channel did not provide additional information. For heteroduplex formation the PCR product was denatured at 95°C for 10 min and slowly re-annealed by cooling down to 85°C by 2° per sec and further cooling down to 25°C by 0.5°C per sec. The re-annealed PCR product was digested at 42°C for 15 min with crude celery extract (CCE) containing 0.6 µl CCE and 5.4 µl CCE buffer for each 20 µl reaction. The CCE buffer was prepared according to Till et al. (2006a). The reaction was stopped with 4 µl 200 mM EDTA. PCR products were cleaned up after endonuclease digestion by Sephadex purification.

Fragment analysis was performed on a LI-COR 4300 DNA analyser using a 6.5 % KB ^{Plus} gel matrix (LI-COR®, Bad Homburg, Germany). The gel run was performed at 1,500 V, 40 mA and 40 W for 2 hours and 30 minutes. Acquired data were analysed visually using the software Gelbuddy (Zerr and Henikoff, 2005). For each gel run, an analysis window smaller than the target amplicon size was manually chosen based on image quality and the absence of PCR mispriming artefacts that can occur near the primer binding region (Till et al., 2006a). For considering gel bands as digestion fragments, bands in the 700 nm channel were scored and a binary matrix was generated reflecting the presence (1) or absence (0) of all different fragment sizes for each sample.

5.3.3 Analysis of polymorphisms and haplotypes

For simplification, each unique fragment visible after acrylamide gel electrophoresis was considered as a SNP despite the fact that fragments could also be caused by small indels. SNP densities were calculated as the number of polymorphic SNP loci divided by the total length of screened sequence in kb. Non-reference nucleotide frequencies (NNFs) were calculated for

each SNP locus as the number of accessions with the SNP allele deviating from the reference allele of 93161P divided by the number of screened accessions. Average heterozygosity Ht (i.e. gene diversity) for each SNP was calculated with the genetic distance and phylogenetic analysis package DISPAN (Ota, 1993).

Accessions with identical SNP pattern were assigned to the same haplotype. Accessions with no restriction bands on a LI-COR gel were assigned to the reference haplotype H0 (93161P). A haplotype with a frequency of less than 5% was declared rare. Non-reference haplotype frequencies (NHF) were calculated for each haplotype as the number of accessions with a haplotype deviating from the respective reference haplotype (FT1a_H0, FT1b_H0, FL1a_H0, FL1b_H0 or BTC1_H0) divided by the total number of accessions screened per cultivar form.

Selected accessions with significant haplotypes associated with BR and SR were sequenced via Sanger sequencing with the respective primer combination. To predict the functional impact of the SNPs characteristic of these haplotypes, the web based tools PARSESNP (Taylor and Greene, 2003) and SIFT (Ng and Henikoff, 2003) were used.

5.3.4 AFLP analyses and population structure analysis

The population structure of the 268 *Beta* accessions was analysed with the AFLP (amplified fragment length polymorphism) technique essentially as described by Vos et al. (1995). The following modification was applied: restriction of DNA was carried out with *PstI* instead of *EcoRI*.

Following AFLP marker analysis the population structure was calculated with the software package STRUCTURE version 2.3.4 (Falush et al., 2007; Pritchard et al., 2000). The optimum number of populations (*k*) was selected after six independent runs with a burn-in of 50,000 iterations followed by 100,000 iterations for each value of *k* (testing from $k = 1$ to $k = 8$). As program parameters for the investigation of the whole panel, the no-admixture model with the correlated allele frequency model was chosen. The most likely value for *k* was determined on the basis of the following criteria: (1) comparison of values for *L(K)* of each *k*; (2) stability of grouping patterns across five runs, and (3) value of ΔK calculated based on the second order rate of change of the likelihood ($\Delta K = m(|L''(K)|)/s[L(K)]$) (Evanno et al., 2005) by the web based interface of STRUCTRUE HARVESTER (Earl and von Holdt, 2012).

5.3.5 Statistical analysis

Association mapping was conducted using the general linear model (GLM) in TASSEL v. 3.0 (Bradbury et al., 2007). An association of a given amplicon with BR, SR, or SR with BR as cofactor was claimed at an experiment wise alpha level of 0.05 (Bonferroni correction). In case of a significant association of a given amplicon with BR or SR, a Dunnett´s *post hoc* test for multiple comparisons of all haplotypes against the reference haplotype H0 was performed. This was performed with the statistical software R (R Development Core Team, 2011) separately for each *B. vulgaris* form.

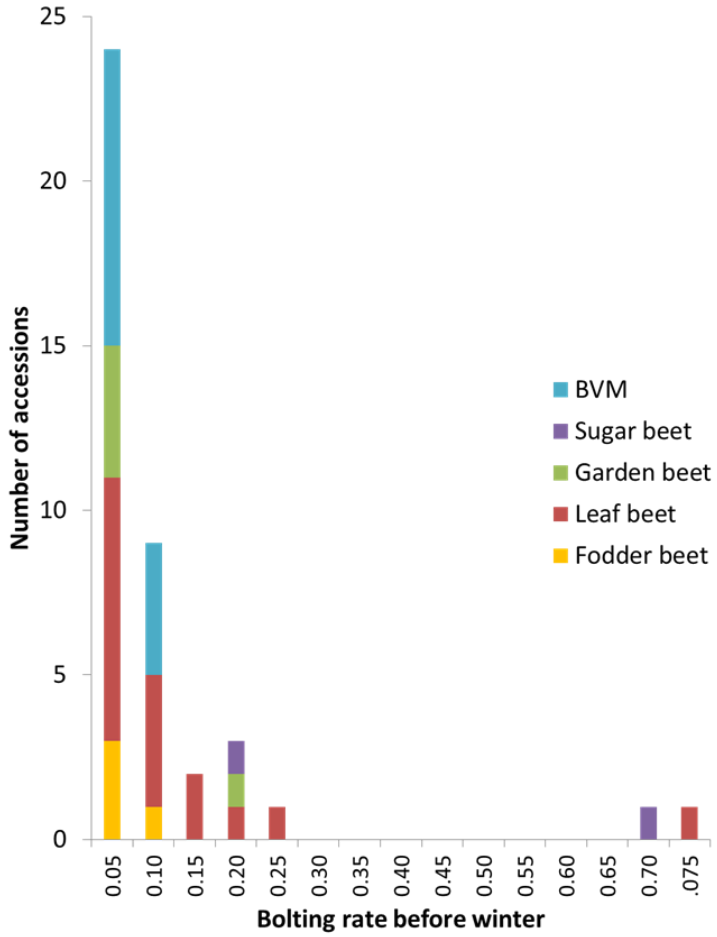

Figure 10: Distribution of bolting rate (BR) before winter among a subset of 41 *Beta vulgaris* of all accessions tested in four different environments. BVM = *Beta vulgaris* ssp. *maritima*.

5.4 Results

5.4.1 Phenotyping and model-based analyses of population structure

In fall 2009, 41 out of the 268 accessions sown in the field in July or August at four different locations, had at least one plant which had started bolting before the first frost. These accessions included two sugar beets (2.2% of accessions tested), four garden beets (6.9%), four fodder beets (10.0%), 20 leaf beets (42.3%) and eleven wild sea beets (*B. vulgaris* ssp. *maritima*) (31.4%). Across all four environments, bolting rates for the 41 accessions ranged from 0.05 to 0.75 (Figure 14). Variation for survival rate after winter across eight environments in 2008/09 and 2009/10 was described in detail by Kirchhoff et al. (2012), and ranged from 0.07 to 0.66 (Figure 15).

On average, sugar beet accessions performed best while fodder beet and garden beet performed worst. The largest variation for SR was found in BVM followed by leaf beets, whereas sugar beets showed the smallest variation. Population structure was analysed by a model-based method using the genotypic data for 40 polymorphic AFLPs detected among the 268 accessions. An independent calculation of k was repeated six times for each value of k from $k = 1$ to $k = 8$. The log probability $L(K)$ increased sharply from $k = 1$ to $k = 3$, but only slowly after $k = 3$ (Supplementary figure 1). When k is approaching a true value, $L(K)$ plateaus or continues to increase slightly (Pritchard et al., 2000). Therefore the structure analysis suggested the presence of three subgroups ($k = 3$), where most of the sugar beets fell in the first group, most of the fodder beets and garden beets in the second group, and leaf beets and BVM in the third group (Figure 11). To further increase confidence in the k value

estimate, we calculated ΔK and obtained the highest ΔK value (60.04) for *k* = 3 (Supplementary table 13).

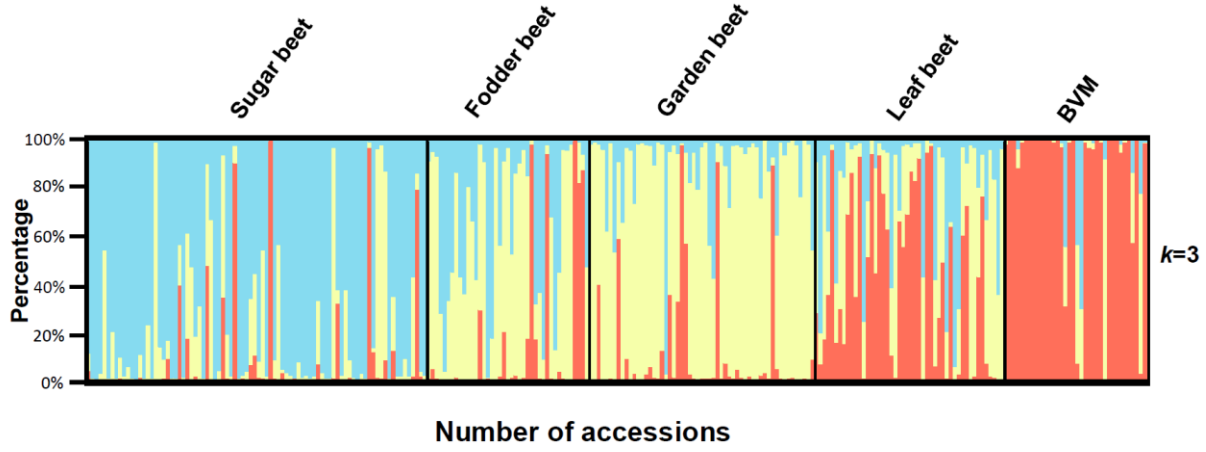

Figure 11: Population structure of 268 *B. vulgaris* accessions based on 40 AFLP markers under the assumption of k = 3 subpopulation. Each *B. vulgaris* accession is represented by one bar that is divided in up to k segments, each proportional to the inferred subpopulation. Accessions are grouped by their respective *B. vulgaris* group. BVM = *Beta vulgaris* ssp. *maritima*.

Table 15: Candidate genes investigated with EcoTILLING. The gene name, gene size in kilo base pairs (kb), protein domains, number of amplicons per gene, primer name and sequence, and the sizes of the amplicons in base pairs (bp) are given. Furthermore, the length of the investigated genomic sequence and the percentage of the open reading frame which was investigated via EcoTILLING are given.

Gene	Genomic size	Protein domain(s)	Amplicon size(s)	Primer name and sequence (5'-3')	Length and portion of genomic sequence covered by EcoTILLING	Portion of reading frame covered by EcoTILLING
BvFL1	7.45 kb	MADS box/K box	FL1a: 977 bp FL1b: 632 bp	FL1a-fw tcggactttccctataagct FL1a-rv cacgtgaatcgttacagaca FL1b-fw gctgatagtctgtccctttgtc FL1b-rv tgactccaacaccacgatgca	1,609 bp (22%)	62%
BvFT1	6.47 kb	PBP	FT1a: 916 bp FT1b: 713 bp	FT1a-fw tggtacgtgtatgaaacagaagctg FT1a-rv catcaactccatatttggggtg FT1b-fw acccatctatacttgtcgatgacc FT1b-rv caatggggaagtggttcacact	1,629 bp (14%)	93%
BvBTC1	9.68 kb	PRR	BTC1: 996 bp	BTC1-fw cagctgtaggatgttatcgtgctgag BTC1-rv agtaggtgataaggacaagacattgc	996 bp (5%)	15%

The gene names, genomic sizes in kilo base pairs (kb), protein domains, sizes of amplicons in base pairs (bp) per gene, and primer names and sequences, and the sizes of the amplicons in base pairs (bp) are given. Genomic size was defined here as the size of the genomic sequence of a gene from the start to the stop codon plus 1 kb each upstream and downstream. The genomic size given for *BvFL1* refers to the known portion of the sequence. A large part of intron 1 was not sequenced (Reeves et al., 2007) and ist not considered here. Furthermore, the length of the investigated genomic sequence and the percentage of the open reading frame which was investigated via EcoTILLING are given.

5.4.2 Amplification of candidate genes

For the three candidate genes *BvFL1*, *BvFT1* and *BTC1* we designed 24 primer pairs, of which five primer pairs passed the primer "crash-test" and therefore were suitable for EcoTILLING. The genomic sequence of *BvFL1* [GenBank: DQ189214.1 and DQ189215.1] was taken from Reeves et al. (2007) while the genomic sequence of *BvFT1* [GenBank: HM448909.1] and *BTC1* [GenBank: HQ709091.1] were taken from Pin et al. (2010, 2012). We adopted a primer pre-screen as described by Weil and Monde (2007) to test for the occurrence of unwanted amplification from single primers prior to the costly synthesis of labelled primers. Amplification from the five successfully tested primer pairs resulted in a total amplicon length of 4,234 bp (Table 15). The remaining primer combinations were not suitable for EcoTILLING due to miss-priming and single primer amplification revealed in the primer crash-test (data not shown).

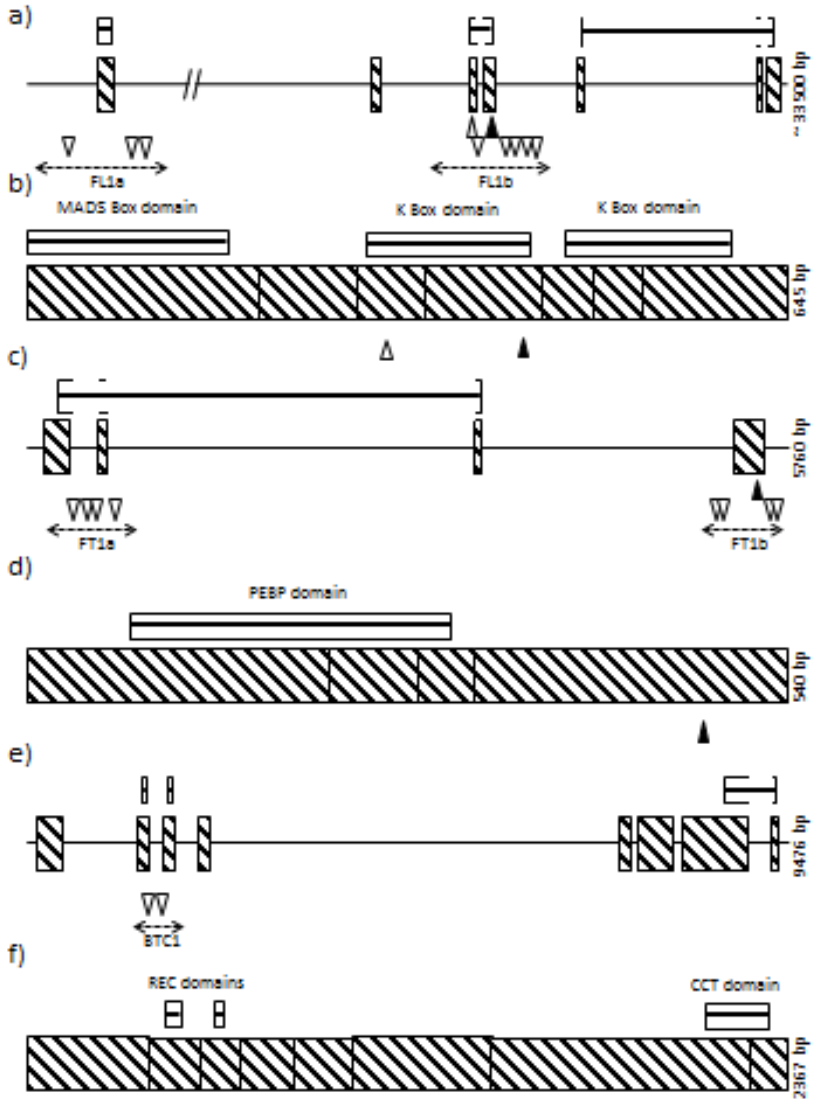

Figure 12: Flowering time genes *BvFL1*, *BvFT1* and *BTC1* and distribution of polymorphisms. Shown are graphical outputs from PARSESNP. a), c), e) Genomic sequences with intron/exon structure. b), d), f) Coding sequences. Dotted arrowed lines indicate the amplicons investigated by EcoTILLING. Striped boxes indicate exons. White arrowheads that point downwards indicate changes in non-coding regions. Black arrowheads that point upwards indicate changes that induce missense mutations in the protein products. White arrowheads that point upwards indicate silent changes.

For *BvFL1* we could amplify two regions (Figure 12). The first amplicon ('FL1a') covered 977 bp of the promoter, exon 1 (including the 5`-UTR and the 5' region of the coding sequence), and parts of intron 1. The second amplicon ('FL1b') spanned a region of 632 bp in exons 3 and 4 and the intervening intron. These regions were chosen because they contain the promoter region, the region encoding the MADS box domain as well as a TGTGAT sequence motif (K box) which is associated with transcription factor binding activity. Thus, 62% of the BvFL1 ORF was covered. Furthermore, we targeted the first intron, which is known to include a number of regulatory regions in Arabidopsis (Schmitz and Amasino, 2007). For BvFT1 we amplified a 916 bp region ('FT1a') that extends from the 5′-UTR to the 5′ region of the coding sequence in exon 1. A second amplicon ('FT1b') spanned 713 bp and was located in exon 4 and the 3′-UTR (Figure 12). Both regions together cover 62 % of the ORF and were chosen because they contain parts of the promoter and the PEB domain (Table 15). In *BvBTC1* we amplified a 996 bp fragment ('BTC1') of the conserved response regulator receiver (REC) domain region (Figure 12), which covered 15 % of the ORF (Table 15).

5.4.3 Genetic diversity, SNP densities and haplotype frequencies

Across the five amplicons (FL1a, FL1b, FT1a, FT1b and BTC1) a total of 21 SNPs were identified among the 268 accessions tested by LI-COR analyses. Nineteen SNPs were located in introns, while the remaining two were located in exon 3 and exon 4 of *BvFL1*, the latter of which was non-synonymous. The number of polymorphisms varied from gene to gene and had an overall density of 5.3 SNP/kb. The lowest SNP density was found in *BvBTC1* (2.01 SNP/kb), whereas the highest SNP density occurred in FL1b (9.82 SNP/kb) (Supplementary Table 14). The SNP allocations and gene structures are shown in Figure 12.

To evaluate the efficiency of EcoTILLING in *B. vulgaris* we estimated the rate of false negatives by sequencing all amplicons in four selected accessions, which resulted in a false negative rate of 5%. Sequencing of FT1a, FT1b, FL1a and BTC1 did not reveal additional SNPs which had not been already identified by LI-COR analyses. For the amplicon FL1b one additional SNP was identified after sequencing in a single accession. This SNP was not detected for any of the 268 accession by EcoTILLING on the LI-COR.

The mean non-reference nucleotide frequencies (NNFs; s. Materials and Methods) for *BvFL1* (0.18) and *BvFT1* (0.17) are similar across all accessions tested, but varied between individual *B. vulgaris* forms, ranging from 0.12 in garden beets to 0.23 in *B. vulgaris* ssp. *maritima* for *BvFT1*, and from 0.04 in garden beets to 0.55 in *B. vulgaris* ssp. *maritima* for *BvFL1* (Supplementary Table 15). The mean NNF for *BTC1* over all *B. vulgaris* forms was 0.07, and ranged from 0.03 in fodder beets to 0.30 in *B. vulgaris* ssp. *maritima*. We identified 55 haplotypes across all five amplified candidate regions. The numbers of haplotypes ranged from four in amplicon BTC1 to 18 in amplicon FL1b. Sixty per cent of the detected haplotypes were rare, occurring at frequencies below 0.05. The reference haplotype (H0) was the most common in each amplicon, with frequencies ranging from 0.49 for FT1b_H0 to 0.87 for BTC1_H0 (Figure 13). The non-reference haplotype frequencies (NHF) ranged from 0.01 to 0.38 across the amplicons within the distinct *B. vulgaris* forms (Figure 13). The highest NHF was observed in *B. vulgaris* ssp. *maritima*, while garden and sugar beets had the lowest NHF. Gene diversity (Ht) for each amplicon and within each cultivar group is displayed in Supplementary Table 16.

Ht range was lowest in the amplicon FT1b (0.16 to 0.30) and highest in the amplicon FL1a (0.02 to 0.51). The highest and lowest diversity for the *B. vulgaris* forms was observed in amplicon FL1a for *B. vulgaris* ssp. *maritima* (0.51) and garden beets (0.02), respectively. Subdividing the 88 investigated sugar beet accessions into 49 accessions of elite breeding material (SBEBM) provided by Strube GmbH & Co. KG (Söllingen, Germany) and the

remaining 39 accessions of sugar beet germplasm (SBGP, mostly composed of various gene bank accessions) revealed a trend towards lower diversity in the amplicons FL1a, FL1b and BTC1, while the diversity increased in FT1a and FT1b (Figure 13).

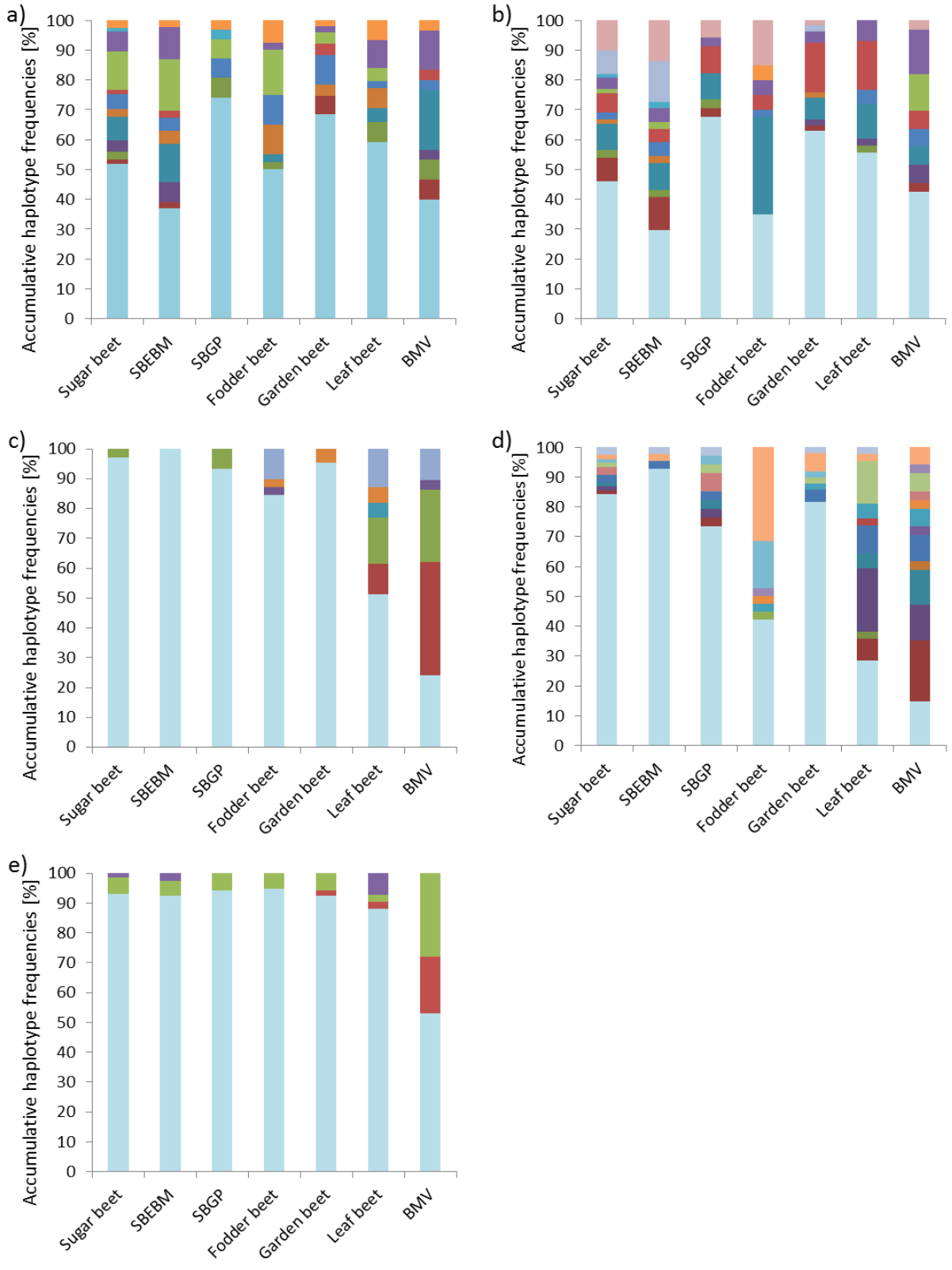

Figure 13: Haplotype diversity diagram. Shown is the haplotype distribution for the amplicons a) FT1a, b) FT1b, c) FL1a, d) FL1b and e) BTC1. The percent of haplotypes for each amplicon is given for each B. vulgaris form. Haplotypes are colour coded. The reference haplotype H0 is always shown in light blue, while non-reference haplotypes are shown in various colours. The B. vulgaris form is given on the x-axis. SBEBM = Sugar beet elite breeding material, SBGP = Sugar beet germplasm, BVM = *B. vulgaris* ssp. *maritima*.

5.4.4 *BvFL1* sequence variations are associated with bolting and survival rate

Based on the Q matrix for $k = 3$, associations with BR, SR, and SR with BR as cofactor were each significant ($P \leq 0.05$) for the amplicon FL1a and for the amplicon FL1b (Table 16). Dunnet comparisons revealed that BVM accessions with haplotype FL1a_H6 and FL1b_H5 had a significantly higher BR of 55% ($P < 0.0001$) and 75% ($P < 0.0001$), respectively, compared to 1% for FL1a_H0 and 2% for FL1b_H0 (Supplementary table 14). Furthermore, garden beet accessions with haplotype FL1b_H6 bolted before winter with a BR of 6% ($P < 0.0001$) compared to 0% non-bolting accessions with the reference haplotype FL1b_H0 (Figure 14). Dunnet comparisons for SR revealed that BVM accessions with haplotype FL1a_H6 had a significantly lower SR of 13% ($P = 0.015$) compared to 39% observed for accessions with the reference haplotype FL1a_H0 (Supplementary table 18). By contrast, leaf beet accessions with the haplotype FL1b_H3 had a significantly higher SR of 37% ($P = 0.012$) compared to 19% of accessions with the reference haplotype FL1b_H0 (Figure 15). DNA sequences of significant haplotypes of *BvFL1* are shown in Supplementary figure 2.

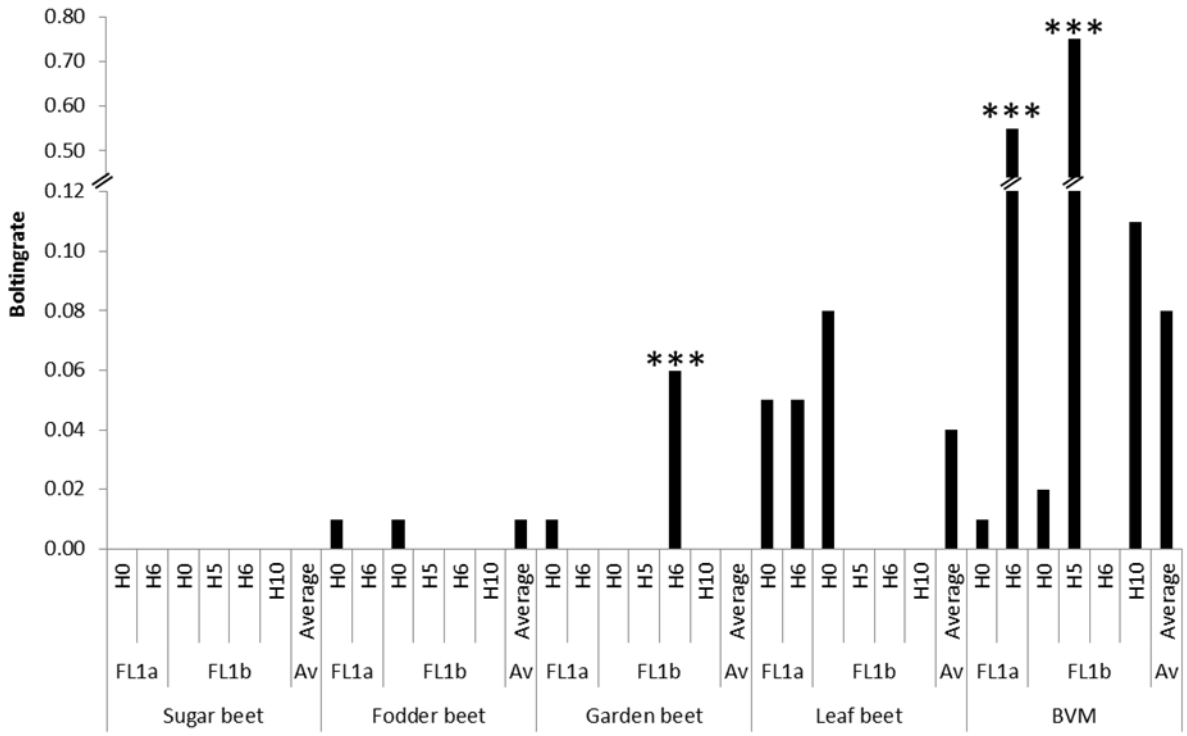

Figure 14: Effect of the *BvFL1* haplotypes FL1a_H6, FL1b_H5, FL1b_H6 and FL1b_H10 on bolting rate (BR) before winter. Bolting rate is shown for FL1a_H6 and the reference haplotype in the five beet forms. *** marks the haplotype with a significantly different bolting rate FL1a_H0 and FL1b_H0 (p= <0.0001); BVM = *Beta vulgaris* ssp. *maritima*.

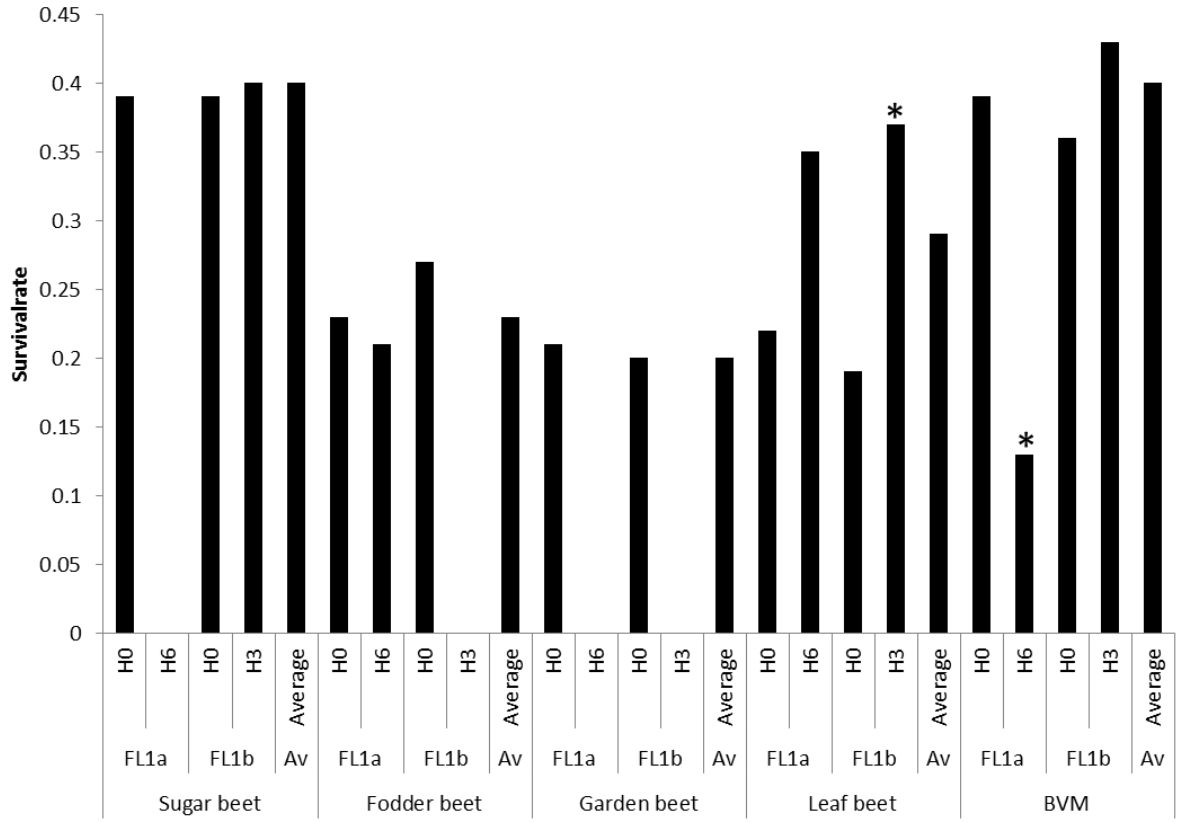

Figure 15: Effect of the *BvFL1* haplotypes FL1a_H6 and FL1b_H3 on survival rate (SR) after winter. * marks the haplotype with a significantly different survival rate from FL1a_H0 and FL1b_H0 (p= <0.05); BVM = *Beta vulgaris* ssp. *maritima*.

5.5 Discussion

This is the first report of EcoTILLING applied to *B. vulgaris*. We established EcoTILLING based on a panel of 268 accessions representing the wild and domesticated gene pool of *B. vulgaris*. In this panel we successfully screened the allelic variation in three genes that are candidates for regulators of vernalization requirement and/or winter hardiness. As a result we were able to provide a snapshot of the species-wide diversity within these genes. Further, we identified haplotypes that are associated with bolting rate before winter and with survival rate, which in turn might be useful for improvement of winter hardiness in sugar beets. Our results show that EcoTILLING is a suitable and cost effective method for allele mining in *B. vulgaris*.

In most EcoTILLING protocols heteroduplexed DNA is digested by purified *CEL*I endonuclease. Instead of the purified enzyme, Till et al. (2006c) and Galeano et al. (2009) used celery juice obtained from salted out or dialyzed crude celery extract for EcoTILLING screens of *Arabidopsis thaliana* and *Phaseolus vulgaris*, respectively. We went a step further and used the crude celery extract (CCE) without further processing, and observed the same activity as compared to the commercial *CEL*I enzyme Surveyor® (data not shown). Also, CCE was very stable and kept its activity for weeks even when stored at 4°C. As using CCE eliminates the need for special enzyme purification steps like chromatography and specialized laboratory equipment, it further increases the cost efficiency of EcoTILLING.

We were able to show that once suitable primers are designed, EcoTILLING provides a high throughput method for the analysis of natural nucleotide diversity in *B. vulgaris*. Also, EcoTILLING is a rather cost effective method. When evaluating LI-COR gels, signals can be grouped according to size and pattern, and only a limited number of samples per group need

to be sequenced to break down the detected variation to the nucleotide level. This drastically reduces the sequencing costs, in our case by 1/3. If only SNPs/haplotypes with effect on the phenotype of interest are sequenced, costs can be further reduced.

Table 16: Significant associations of bolting and survival rate with amplicons of the *FLC* homolog *BvFL1* in beet.

Trait	Amplicon	F[d)]	P[e)]	R2 [f)]
BR[a)]	FL1_a	9.04**	6.47E-11	0.22
BR	FL1_b	11.28**	2.65E-23	0.44
SR[b)]	FL1_a	7.17**	1.44E-08	0.13
SR	FL1_b	2.91**	7.72E-05	0.13
SR/BR[c)]	FL1_a	6.54**	9.52E-08	0.11
SR/BR	FL1_b	2.90**	1.14E-04	0.12

a) BR = bolting rate, b) SR = survival rate c) SR with BR as cofactor, d) F value from the F test on marker, e) P value from the F Test on marker, f) R^2 is the fraction of the total variation explained by the marker.**(P<0.001) indicates the amplicon is highly significantly associated with traits. **(P < 0.001) indicates the amplicon is highly significantly associated with trait.

A caveat is that EcoTILLING is prone to false negative detection because some fragment sizes are masked by background "noise", due to miss-priming, or because of weaker fluorescence toward the top of each lane and increasing fluorescence "noise" toward the bottom (Greene et al., 2003; Till et al., 2006a; Till et al., 2010). The false negative rate in our case was 5.0 % which is similar to rates reported in human (Till et al., 2006b) and banana (Till et al., 2010) EcoTILLING.

The population structure analysis from this study indicates that the *Beta* accessions can be grouped into three groups (k = 3), a sugar beet group, a fodder beet and garden beet group, and a group comprising leaf beets and wild sea beets. This corresponds to the evolutionary history of Beta and the selection intensity during the past 200 years of beet breeding. A similar structure has also been described by Jung et al. (1993) and by McGrath et al. (1999) after genotyping with completely different marker systems. Both groups report that sugar beets can be clearly distinguished from *B. vulgaris* ssp. *maritima*. In our study, a few accessions were classified differently by genotype (according to AFLP analysis) than by phenotype (Figure 11). Regarding *B. vulgaris* ssp. *maritima* this could hint at gene flow from cultivated forms into wild material, either in their natural habitat or during propagation by gene banks. At the same time, classification by phenotype was sometimes ambiguous. For instance 'Patak' accessions from India (PI 116809 and PI 121838), although cultivated, showed a plant habitus whose classification into *B. vulgaris* ssp. *maritima* seems more reasonable than classification into any of the cultivated forms. Interestingly, both approaches to account for population structure resulted in significant associations of the same amplicons, hinting at the robustness of the results. Nevertheless, to account for genotyping errors as a source for putative misclassification by STRUCTURE, genotypic outliers were removed from the dataset for a further analysis by TASSEL. These outliers were sugar beet, fodder beet and table beet accessions with an estimated portion of non-cultivated beet genome >50% and *B. vulgaris* ssp. *maritima* accessions with an estimated portion of cultivated beet genome >50% (Figure 11). In this analysis previous associations were still highly significant (data not shown).

Comparing all five *B. vulgaris* forms we observed the highest genetic diversity for the investigated genes in *B. vulgaris* ssp. *maritima*. This is indicated by a mean NNF of 0.36 compared to 0.19 in leaf beets followed by fodder beets (0.12), sugar beets (0.08) and garden beets (0.07). The same trend is observed when looking at the average NHF and Ht. Our findings are in accordance with Jung et al. (1993) and Fénart et al. (2008) who reported a higher genetic diversity in *B. vulgaris* ssp. *maritima* compared to sugar beets. As selection results in a loss of genetic diversity, it is not surprising that the genetic diversity in *B. vulgaris* ssp. *maritima* appears to be higher not only compared to sugar beet but also in comparison to all four cultivar groups taken together. Crop evolution is best understood for sugar beet which has been affected by founder effects as it was derived from a single fodder beet population and also by genetic bottlenecks through introgression of a series of traits from a limited number of genetic resources (Lewellen, 1992;Savitsky, 1952; Biancardi et al., 2002). This explains why sugar beet together with red table beet showed the lowest diversity.

In our study the genetic diversity in sugar beet based on Ht ranged from 0.03 to 0.28 for the single amplicons with an average of 0.17. These estimates are likely to be upward biased as we could not distinguish between the occurrence of non-reference nucleotides in the heterozygous or homozygous state. Nevertheless, gene diversity in our study is lower compared to Li et al. (2011) and McGrath et al. (1999). However, in contrast to Li et al. and McGrath et al. we estimated the genetic diversity for nucleotide polymorphisms in three genes that may have been under selective pressure. It is especially the case for *BvFL1* where we estimated Ht values of 0.03 and 0.12 for FL1a and FL1b, respectively, and also for *BTC1* (Ht = 0.06). This could be the effect of selection for bolting resistance to prevent bolting caused by late frosts after sowing in spring. Comparing sugar beet elite breeding material with sugar beet germplasm, the genetic diversity turned out to have been further decreased by selection for *BTC1* and *BvFL1* (Supplementary Table 16). At the same time *BvFT1* showed even more diversity in SBEBM indicating that this gene is obviously not under selective pressure. This is somehow surprising, as *BvFT1* was shown to play a key role in bolting suppression under non-inductive conditions (Pin et al., 2010). Still, these data have to be interpreted with care, as sample sizes are moderate and the SBEBM material represents only one breeding company.

For one of the three genes investigated, *BvFL1*, we were able to detect an effect on bolting. Four haplotypes of this gene (FL1a_H6, FL1b_H5, FL1b_H6 and FL1b_H10) had a significant impact on BR before winter in BVM and/or garden beets. Although variation in *FLC* is known to affect flowering time in *A. thaliana* (Michaels et al., 2003), the role of *FLC*-like genes outside the *Brassicaceae* is not well understood (Jung and Müller, 2009), and a functional analysis of *BvFL1* in *B. vulgaris*, e.g. through mutational or transgenic approaches, is still lacking. Effects on bolting rate were not observed for all beet forms, which in part may be due to the absence of the divergent haplotypes which affect BR in BVM or garden beet. The complete absence of these haplotypes in sugar beet may reflect the breeding history of sugar beet, during which breeders strongly selected against bolting before vernalization (Biancardi et al., 2010; McGrath et al., 2007).

As *BTC1* is known to be a major factor controlling bolting without prior vernalization in beets (Pin et al., 2012), we expected an effect of *BvBTC1* sequence variations on bolting before winter. However, this was not observed here. This may be due in part to an underrepresentation of annual *BTC1* alleles in our panel or the fact that the current analysis was limited to a relatively small portion of the coding sequence of *BTC1* (15%; Table 15) and did not include the promoter. Although *BvFT1* is known to respond to vernalization and is down-regulated by cold temperatures, which in turn enables induction of flowering (Pin et al., 2010), we also did not detect an effect of haplotype variation in this gene on bolting rate. While other reasons for this cannot be excluded, as discussed for *BvFL1* and *BTC1*, it is also

conceivable that possible phenotypic effects of haplotype variation at *BvFT1* are difficult to detect under the environmental conditions present in the current study.

Besides significant effects on bolting rate, plants with haplotypes FL1a_H6 and FL1b_H3 showed a significant impact on survival rate after winter in *B. vulgaris* ssp. *maritima* and leaf beets, respectively. A similar effect has been shown before for *A. thaliana* ecotypes, where a SNP in intron 1 of *FLC* led to a 1.6-fold increase in winter survival rates in genotypes carrying a functional *FRI* allele (Korves et al., 2007). The authors suggested that survival after winter is associated with time to bolting. Similarly, we found that the survival rate of truly biennial (vernalization requiring) leaf beet accessions with the FL1b_H3 haplotype was higher (by 20%) when compared to the reference haplotype FL1_H0 ($p = 0.006$, data not shown). Hence, winter hardiness in sugar beet might be improved by introgressing FL1b_H3 from leaf beet. Interestingly, *B. vulgaris* ssp. *maritima* accessions with FL1a_H6, which had a lower survival rate than accessions with the reference haplotype, also showed an increased bolting rate. Furthermore, after removal of *B. vulgaris* ssp. *maritima* accessions which bolted before winter, the accessions with haplotype FL1a_H6 did not have a significant effect anymore. Therefore, the lower survival rate observed for this haplotype might be a direct physiological effect of bolting before winter as plants in the generative phase are more frost sensitive (Fowler et al., 1993). However, by using bolting rate as a cofactor in a further TASSEL analysis, we can exclude that increased frost sensitivity in the generative phase is the mere cause for association of *BvFL1* with survival rate since this association stayed significant. Interestingly, Seo et al. (2009) reported that transient cold temperatures and overexpression of CBFs lead to elevated *FLC* expression and delayed flowering, suggesting a possible role of *FLC* in cold stress response in *A. thaliana* and, by analogy, a possible explanation for the detected effect of *BvFL1* haplotypes on survival rate in *B. vulgaris*. Effects on survival rate could not be observed consistently for both haplotypes throughout all *B. vulgaris* forms. This may in part be due to the absence of the two haplotypes in some of the other *B. vulgaris* forms (Supplementary table 15). Similar to BR, the absence of haplotype effects on SR in some cultivar forms might also be due to the polygenic inheritance of SR.

Among the SNPs underlying haplotypes FL1a_H6, FL1b_H3, FL1b_H5, FL1b_H6, and FL1b_H10, two are located in an exon. The SNP in exon 3 is synonymous, whereas the SNP in exon 4 is non-synonymous, leading to an amino acid substitution from valine to isoleucine. The other SNPs identified in *BvFL1* are silent as they are located in introns, including intron 1. These SNPs might influence gene function by affecting the transcriptional regulation of *BvFL1*, as was reported for intronic polymorphisms in *FLC* in Arabidopsis (Sheldon et al, 2002; Choi et al, 2011). Also, Heo and Sung (2011) reported that the regulatory non-coding RNA *COLDAIR* is expressed from intron 1 of *FLC*. Finally, the increase in winter survival rates observed for an allelic variant of *FLC* (Korves et al., 2007) was also associated with polymorphisms in intron 1. As with association studies in general, it cannot be excluded that the functional polymorphisms for the traits investigated are located outside the amplified gene regions and that the SNPs detected here are merely linked to these polymorphisms.

5.6 Conclusions

In conclusion, our study demonstrates that EcoTILLING can be successfully employed in *B. vulgaris* to survey a large panel of plant accessions for allelic variants in different candidate genes. Our data also provide the first genetic indication that an *FLC* homolog indeed may also affect flowering time (and winter survival) in a species which is only distantly related to *A. thaliana*. The above described panel of diverse *B. vulgaris* forms is an excellent resource to identify allelic variation in additional flowering time control genes such as *BvFT2* or candidate genes for agronomic traits such as stress response and plant architecture. Allelic

variants identified by EcoTILLING can be used to introduce new genetic variation into elite beet breeding material.

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6 Closing Discussion

6.1 Research perspectives for the analysis of winter-hardiness and frost tolerance in Beta species

A long-term aim in sugar beet breeding is to develop a winter sugar beet that tolerates cold temperate conditions. The requirements of such winter beets would be sufficient winter-hardiness as well as an effective bolting control system. Bolting and flowering in sugar beet have been intensively investigated in recent years and a system for bolting control might be developed in the near future (Jung and Müller, 2009; Pin et al., 2010). A profound knowledge of beet genetics is also required for successful breeding of winter beets, but prior to this study little was known about the genetics of winter-hardiness and frost tolerance in *B. vulgaris*.

A prerequisite for improving winter-hardiness is genetic variation of the trait. Therefore, in this study I analyzed the genetic variation of winter-hardiness in a diversity panel of *B. vulgaris*. Sugar beets were identified as the most winter-hard cultivar group, whereas the wild beet *B. vulgaris ssp. maritima* displayed the largest genetic variation, including the most winter hard accessions (chapter 2). Since studies in *A. thaliana* revealed that cold tolerance and flowering time regulation are interlinked (Eriksson and Webb, 2011; Nakamichi et al., 2009; Seo et al., 2009), we analyzed three major flowering regulators *BvFT1*, *BvBTC1* and *BvFL1* in *B. vulgaris* for their effect on annual bolting and winter-hardiness. In this study for the first time we could show that the *FLC* homologue *BvFL1* had an effect on flowering time outside the Brassicaceae (chapter 5). Moreover, the results indicate that *BvFL1* plays a role in cold response like its *Arabidopsis* homologue *FLC* (Korves et al., 2007*).* Furthermore I identified QTL for frost tolerance in sugar beets (chapter 3) and present first data on a segregating population tested for winter-hardiness under field conditions (chapter 4).

The ultimate scientific goal of the genetic dissection of any trait is the identification, verification and characterization of the underlying genes. However, even though thousands of QTL studies were published in the last 20 years, until recently very few QTL have been cloned (Salvi and Tuberosa, 2005). The classical road map from a QTL to the identification of causative genes can be understood as an iterative approach. In a first step, a limited number of large chromosome intervals with major effects are identified. This primary QTL identification is described for winter-hardiness in Chapter 4 and for frost tolerance in Chapter 3. The identification of primary QTL is classically followed by high resolution mapping of regions containing major QTL with an increased number of individuals and markers. However, the completion of sequencing the genomes of several crop species has improved the power to map and clone candidate genes drastically by integrating genetic and physical maps (Buckler et al., 2009; Huang et al., 2010; Jackson et al., 2011).With the sugar beet genome sequence being available candidate genes within QTL intervals can be identified via homology to previously characterized genes of model organisms. This will enhance the speed of identifying the causative genes underlying QTLs tremendously.

As only a low proportion of the analyzed sequences with homology to flowering time and cold responsive genes were polymorphic in the mapping population it remains unclear to which extend the identified candidate sequences could contribute to frost tolerance and winter-hardiness. Therefore the candidate genes have to be further verified in a wider genetic background. This verification is necessary due to a major limitation of linkage mapping. Bi-parental populations represent only two alleles at a given locus while in natural and breeding populations many more alleles with impact on the given trait are present (Mackay, 2001) Therefore, the results of a linkage study might deviate from the natural and breeding populations as follows: i. epistasis may cause different effect sizes of a given QTL in different genetic backgrounds; ii. number of QTL controlling the trait of interest might vary and iii. expected

allele frequencies in bi-parental F_2 mapping populations are 0.5 for any segregating locus, while in broader populations the allele frequencies can vary, and therefore the penetrance of a QTL is unknown (Flint and Mott, 2001). One strategy to validate the effect of QTL or candidate genes in a wide genetic background is to apply association mapping, as already done to verify yield related QTL in sugar beets (Stich et al., 2008). The application of an association approach to dissect winter-hardiness and frost tolerance in a diversity panel described in Chapter 2 could verify the QTL identified in chapter 3 and 4 and exploit the full range of diversity for winter-hardiness and frost tolerance present in the panel.

An addition to association approaches, the analysis of gene expression profiles could help to identify causal functions of candidate genes. For a limited number of candidate genes expression patterns can be analyzed via quantitative real time PCR. A successful example of such a quantitative expression analysis was given by Reeves et al. (2007) who characterized the expression of the flowering time gene *FL1* in *B. vulgaris* in response to cold. Moreover, expression analysis on a transcriptome-wide scale would be useful to identify genes that are differentially expressed after cold exposure and therefore give evidence for a functional role in cold response. In sugar beet, a macroarray analysis has been used to identify several genes which are predominantly expressed in sugar beet roots and affect root physiology and morphology (Bellin et al., 2002). More recently microarrays have shown to contribute to the knowledge about expression of candidate genes in sugar beet. Trebbi and McGrath (2009) identified several genes that contribute to the root development of sugar beet. However, even though microarrays are well suited for the identification of differentially expressed genes, follow up experiments are needed to elucidate the functional role of the differentially expressed genes.

Another experimental strategy, first shown in *A. thaliana*, was to combine linkage mapping with gene expression data resulting in the identification of expression QTL (eQTL) (DeCook et al., 2006). This combination of high resolution genetics and high throughput genomics helps to understand how genetic variation affects gene expression (Holloway & Lin, 2010; Jackson et al. 2011). Using microarray gene chips Jordan et al. (2007) identified 542 eQTL contributing to seedling development in wheat. Another approach to differentiate gene expression on a genome wide scale is based on sequencing of small expressed tags, called SuperSage. SuperSage has been applied successfully for the analysis of transcriptional changes caused by drought in chickpea (*Cicer arietinum*) (Molina et al., 2008). SuperSage has the major advantage, that no prior knowledge of expressed genes is required. Therefore SuperSage would not only be suited for the verification of known candidate genes, but any gene with a differential expression pattern.

Like expression analyses on a candidate gene- and transcriptome-wide scale the abundance of proteins can be analyzed for functional evidence. Hajheidari *et al.* (2005) investigated the role of 500 sugar beet proteins and identified 79 proteins which showed significant changes between control and drought stress treated plants. A similar approach would be feasible to identify cold induced proteins in sugar beet but can be overcome by combining linkage data with protein analyses. This approach has been used by Dumont and coworkers (2009) who identified protein quantitative loci which co-localize with QTL for winter-hardiness and frost tolerance in peas.

The sugar beet genome sequence opens the door to apply next generation sequencing (NGS) for genetic and functional analysis in *B. vulgaris*. By applying whole genome re-sequencing in rice to map a causative gene for plant height it was demonstrated that the NGS approach was 20 times faster and 35 times more accurate compared to a linkage analysis based on 287 PCR-markers (Huang et al., 2009). Moreover, it has been shown in recombinant inbred lines of rice that a whole mapping population of similar size (N=238) to our mapping population (Chapter 3 and 4) can be sequenced with a genome coverage of 0.5 per line in one sequencing run (Xie et al., 2010).

Another NGS strategy that was applied in *A. thaliana* allowed the identification of the causative gene for phenotypic variation in a mapping population is called SHOREmap. Once the mapping population is established, SHOREmap is suited to identify a causative polymorphism within eight working days including DNA isolation, sample preparation, sequencing and data analysis (Schneeberger and Weigel, 2011). Like SHOREmap, MutMap applies next generation sequencing of divergent phenotypic pools for the identification of candidate genes (Abe et al., 2012). MutMap is based on the identification of ethylmethansulfonat (EMS) induced mutations in a segregating population that are derived from a cross of a sequenced reference line with an EMS mutagenized individual of the reference line (Abe et al., 2012). The major advantage of MutMap compared to SHOREmap is that all polymorphisms have to be caused by the mutagen treatment and therefore the background noise by none causative polymorphisms is reduced. However, even though MutMap was proposed as a method for identifying genes underlying polygenic traits, the application for the identification of winter-hardiness candidate genes will highly depend on the availability of a suited phenotyping platform to screen thousands of mutant lines for winter-hardiness or frost tolerance. However, even though both SHOREmap and MutMap apply next generation sequencing for candidate gene identification, both approaches suffer from the major limitation of bulked segregant analyses: They are most promising to traits with clearly distinguishable phenotypes and its efficiency for quantitative traits has to be demonstrated yet.

Beside a tremendous impact on gene identification NGS technologies will have major impact on whole-transcriptome analysis of plants in the near future (Jain, 2012). The principle of RNA deep sequencing is based on the assumption that sequencing all transcripts at sufficient coverage will identify all expressed genes and their expression levels (Wang et al., 2009). Recently RNA deep sequencing was applied to characterize the expression of meiosis-specific genes in Arabidopsis (Chen et al., 2010; Yang et al., 2011). Moreover, RNA sequencing was applied in sugar beets to establish a reference transcriptome and perform a transcriptome wide analysis of vernalization response (Mutasa-Göttgens et al, 2012).

Applying RNA deep sequencing for the investigation of frost tolerance of sugar beets requires a frost tolerant and a susceptible genotypic group. Both groups have to undergo frost- and non-frost control treatments over a timeline to obtain various RNA pools. By sequencing the RNA pools, those transcripts that are differentially expressed in response to frost can be identified by this procedure. To identify those transcripts that contribute to differences in frost tolerance of sugar beets a subtractive analysis of transcripts expressed in the tolerant and the susceptible pool is needed. These differentially expressed genes are highly promising candidates to increase frost tolerance in sugar beets and will help to gain knowledge on the regulatory networks underlying frost tolerance. In *A. thaliana* the combined application of new sequencing and transcriptome analysis techniques combined with mapping approaches allowed the construction of a regulatory network controlling flowering time via a mapping of genome wide gene expression QTLs (Keurentjes et al., 2007).

The above mentioned examples of fast and accurate identification and characterization of causative genes in model organisms and crop species indicate that most likely in the near future marker based genotyping will be at least partially replaced by sequencing for QTL analysis.

Even though the identification and characterization of causative genes underlying QTL is of great interest, classical QTL approaches are limited in their power to dissect highly quantitative traits like winter-hardiness and frost tolerance (Mackay et al., 2009). Beside classical approaches focusing on genes underlying major QTL, genome wide approaches have gained importance in recent years. Studies in model organisms and humans have demonstrated, that with increasing mapping resolution the number of QTL increased and the effect of these QTL decreased (Flint and Mackay, 2009). In particular this has been observed for highly quantitative traits. Therefore, it cannot be ruled out that the detected QTL for winter-hardiness and frost tolerance will

segregate into smaller QTL after increasing population size and marker density. Moreover, even high resolution mapping rarely results in direct positional cloning of the causative genes underlying QTL, but rather the identification of a number of candidate genes (Mackay et al., 2009). An alternative to QTL mapping followed by fine mapping offers genome wide association mapping. The publication of the sugar beet genome sequence and recent progress in sequencing technologies opens the door towards marker saturation that allow the application of GWAS to analyze even highly quantitative traits. Even though GWAS in sugar beet was limited by the number of markers, several marker trait associations have been identified for physiological traits (Würschum et al., 2011a; Würschum et al., 2011b). Moreover, GWAS of frost tolerance in barley (von Zitzewitz, 2010) and flowering time in maize have demonstrated the power to dissect highly quantitative traits (Buckler et al., 2009; von Zitzewitz, 2010). Applying GWAS to a diverse panel of sugar beet (for example the diversity panel described in chapter 2) would answer the question whether the limited number of QTL detected in chapter 3 and 4 is an effect of limited genetic variation in the mapping population or the consequence of many QTL with minor effects. However, to benefit from the ultra-high mapping resolution demonstrated by GWAS in model organisms the number of polymorphic markers has to be increased by magnitudes (Kump et al., 2011). A major limitation of GWAS is that even with ultra-high marker densities and dozens of marker trait associations detected, the population size and cumulative effects of these associations are by far too small to explain the full observed genetic variation (Houle et al., 2010). The most likely explanations for the small proportion of explainable variability is the fact that population wide marker effects are diluted if the marker allele occurs at low frequencies or if genetic effects are small (Maher, 2008). However, with the marker saturations suited for GWAS genomic selection (GS) comes into perspective, which allows the prediction of performance based on the analysis of all markers simultaneously instead of single marker-trait associations (see also chapter 6.3).

With the genomic resources available for fast and accurate genotyping, accurate and fast phenotyping of winter-hardiness and frost-tolerance becomes the limiting factor. The results of chapters 2 and 4 have shown that winter-hardiness is highly affected by environmental effects. It is known from previous studies that winter field trial conditions can be highly variable for example due to different snow coverage. Fowler (1979) reported that within one wheat field trial the variation due to environmental factors, was almost as high as the genetic variation observed and that winter stress levels can change dramatically within a few meters. These large environmental differences within microenvironments were to a large extent caused by differences in the height of snow cover. Moreover, a major limitation of phenotyping winter-hardiness is the dependence on contrasting conditions, which are rarely predictable. As these factors cannot be controlled field studies on winter survival suffer from high experimental errors and low repeatability which in turn requires multi-location experiments (Fowler, 1979; Saulescu and Braun, 2001).

To be independent of irregular occurrence of natural contrasting conditions artificial frost tests have been developed (Saulescu and Braun, 2001; Sutka, 1981). A clear down side of growth chamber experiments is the constrained space, which limits the number of plants that can be tested in one experiment. In addition, previous studies show conflicting results of SRs in controlled and field environments (Dexter 1956, Dumont 2009). A comparative study in wheat revealed that even though the number of variable factors is limited under controlled conditions field trials provide lower experimental errors and higher reproducibility (Fowler et al., 1981). However, with high throughput genotyping techniques available, phenotypic characterization is lagging behind and the demand for accurate season independent characterization and selection of frost tolerance increases. The artificial frost test described in Chapter 3 revealed large variation in pre-frost development of sugar beets which affects frost tolerance. Moreover, a large proportion of the phenotypic variation could be assigned to the differences in seed quality. Therefore, future artificial frost experiments of sugar beets have to control environmental effects more rigorously. This implies that plants have to be grown in growth chambers before frost to

exclude the seasonal effect of day length. Furthermore, emphasis has to be put on the frost chamber itself by developing a system with minimal positional effects caused by air circulation and light. However, as recently concluded by Gusta and Wisniewski (2012) frost tolerance is a trait of high complexity integrating many environmental cues. Therefore the reduced complexity of artificial frost tests is both, its major advantage and its pitfall, as the results may reflect the environmental conditions of the artificial test but not necessarily the mechanisms for winter-hardiness under field conditions.

Considering the status quo and the future research perspectives, it can be concluded that only the first step towards understanding winter-hardiness and frost tolerance in *B. vulgaris* has been taken but many more have to follow. Recently developed genomic resources offer a wide range of opportunities to investigate the genetics of winter-hardiness and frost tolerance in sugar beets. However, to take full advantage of the benefits of the "genomics-age" precise phenotyping becomes most important in the future.

6.2 Comparative frost tolerance analyses between Beta species and other plant species

This is the first study on frost tolerance and winter-hardiness in Beta species described so far. Nevertheless, frost tolerance has been extensively studied in *A. thaliana* and cereals (for reviews see Thomashow (2010) and Dhillon et al. (2010)). Even though none of the identified QTL co-localizes with any of the identified candidate genes in *B. vulgaris*, the identification of 37 candidate genes in *B. vulgaris* with homology to known cold regulated genes in *A. thaliana* give first evidence for a possible conservation of cold responsive genes (Chapter 3).

Upon cold stress, plants react with a major reprogramming of the transcriptome by changing the expression of hundreds of genes including 170 transcription factors (Kilian et al., 2007; Maruyama et al., 2009). Some of the most prominent and best investigated cold responsive transcription factors are the *CBF* genes, which gave name to the corresponding pathway (Thomashow, 2010). The *CBF* pathway has been shown to be conserved across a wide range of species, including both, frost tolerant and susceptible species (Choi et al., 2002; Jaglo et al., 2001; Qin et al., 2004). In this study, I present first evidence that *CBF* pathway genes are conserved in *B. vulgaris* by the identification of homologues to 22 *CBF* pathway genes (Chapter 3). Interestingly, one identified *B. vulgaris* candidate gene shows homology to CAP160, a cold acclimation protein identified in spinach (*Spinacia oleracea*) (Kaye et al., 1998). Spinach like sugar beet is a member of the *Chenopodiaceae* family and therefore the closest relative investigated for cold response so far.

In the study presented here, ten homologous sequences of flowering time genes in *A. thaliana* were analyzed as candidate genes for their effect on frost tolerance and winter-hardiness in *B. vulgaris* (Chapter 3 and Chapter 4). These sugar beet candidates represent homologs of vernalization responsive and thermo-sensory pathway genes, of which four (*FLC*, *FRI*, *SOC1*, *FVE*) have been shown to affect either *CBF* expression or frost tolerance in *A. thaliana*. Further evidence for the interactions between flowering time control and cold response is given by the fact that several genes that are primarily known for their effect on frost tolerance affect flowering time. With sugar beet candidates for *HOS1*, *CBF1*, *CBF2*, *LOV1* four homologs of these cold responsive genes with dual role were integrated into the QTL analyses of frost tolerance and winter-hardiness. Interestingly, all flowering time genes with effect on cold tolerance and all cold responsive genes with effect on flowering time have in common that they either combine a late flowering phenotype with increased frost tolerance or early flowering with frost sensitivity (Deng et al., 2011; Gilmour et al., 2004; Kim et al., 2004; Korves et al., 2007; Lee et al., 2001; Seo et al., 2009; Yoo et al., 2007). This coincidence gives rise to the speculation that these double functions contribute to the plant's fitness by avoiding the transition into the more frost sensitive reproductive phase until temperatures have increased. Consistent observations have

been made in cereals where one of the major QTL for frost tolerance has been proposed to have a pleiotropic effect of the vernalization response locus *VRN1* (Dhillon et al., 2010). Moreover, a QTL for frost tolerance in peas has been mapped to the same region like the vernalization response locus *HR* (Lejeune-Henaut et al., 2008). Additional evidence for the interaction of flowering time and cold response is given by microarray analyses in *A. thaliana* that have indicated a feedback loop between the flowering time genes *FLC* and *SOC1* and the major regulator of cold response *CBF* (Seo et al., 2009). Even though no information about expression levels of *CBF* pathway genes are available for *B. vulgaris*, the identification of sequence variants in the *FLC* homolog *FL1* that affect winter-hardiness and bolting time give rise to the speculation that the interaction between flowering time and cold responsive genes might be conserved in sugar beets, too. This conserved interaction is not unlikely as many of the environmental cues and stresses that affect cold tolerance have been shown to affect also developmental processes including flowering time regulation (Fowler et al., 2005; Gusta and Wisniewski, 2012). Considering the interactions of winter-hardiness and flowering time in diverse species it is of high interest for the development of autumn sown winter beets to elucidate whether late bolting sugar beets display superior winter-hardiness. Therefore, a covariate analysis of winter-hardiness and flowering time in a biennial sugar beet panel covering a large variation for flowering time would give first evidence whether late flowering time contributes to winter-hardiness in sugar beets.

Comparisons of winter-hardiness across species have to be handled with care due to varying conditions across field trials. However, the results of field trials can contribute to a comparative estimation of the cold tolerance across species in cases were experimental conditions did not deviate substantially. Therefore, the assessment of winter-hardiness in the *Beta vulgaris* diversity panel described in Chapter 2 and in a rye association mapping panel (Li et al., 2011b) under similar field conditions in the same winter in Minsk 2009, (Belarus) allow a comparison of the levels of winter-hardiness. With a median SR of 70% the rye panel clearly demonstrated the superior winter-hardiness compared to the *B. vulgaris* panel with a median of 0% (Li et al., 2011b; Kirchhoff et al., 2012, Chapter 2). However, it is known that winter conditions can change on small scale levels and these experiments were not conducted on the same field. However, these results are not surprising as rye is known to be the most winter-hard cereal (Hömmö, 1994). In congruence Geisler (1988) reported that physiologically inactive plants of rye are capable to survive -25°C followed by winter wheat with -20°C. More susceptible to harsh winter conditions is barley with a reported minimal temperature for surviving of -12°C (Geisler, 1988). For spring sown *B. vulgaris* Geisler (1988) reported the ability to survive temperatures of down to -7°C which is identical to the temperature applied in the artificial frost test (Chapter 3). Even though no minimal temperature for survival of *Brassica napus* under field conditions could be found in the literature, it worth mentioning that farmers consider the developmental stage BBCH 19 as ideal for overwintering of oil seed rape. This corresponds to the observations made in chapter 2 and 4 for *B. vulgaris*.

Like the environmental cues affecting winter-hardiness, experimental designs for artificial frost tests vary largely between experiments (e.g. supplementary table 19). However, the minimal temperatures applied in the different artificial frost test can give a rough estimation for the level of frost tolerance of the species under investigation. In congruence with the observations under field conditions most adverse frost conditions were applied to rye in artificial frost tests (Li et al., 2011b). Protocols developed for assessing the frost tolerance of winter wheat and barley have treated the plants with temperatures of -15°C and -12°C, respectively (Pan et al., 1994; Sutka, 1981). Frost tolerance studies under controlled and semi-controlled conditions have reported lethal frost effects on *Brassica napus* over a wide spectrum of temperatures ranging from -4°C to -19°C (Kole et al., 2002; Rife and Zeinali, 2003; Markowski and Rapacz, 1994). Comparing these reports with the results of chapters 3, 2 and 4 *B. vulgaris* displays relatively little frost tolerance and winter-hardiness compared to traditional winter crops. Therefore, the cultivation of

autumn sown sugar beets under continental conditions requires major breeding efforts to improve winter-hardiness.

6.3 Perspectives for breeding winter beets

Competitive winter beets have to combine high yield and quality parameters with an efficient system for bolting control and high levels of winter-hardiness. Our experiments have shown that despite substantial genotypic variation SRs highly depend on environmental conditions and genotype by environment interaction. Taken together winter-hardiness of sugar beet might be sufficient for cultivation under maritime conditions. However, with a mean SR of sugar beets across eight environments of 39.7 % (Chapter 2) winter-hardiness *per se* in sugar beets has to be substantially increased for cultivation under continental conditions. Strategies to increase winter-hardiness by recurrent selection within sugar beets and introgression of exotic germplasm are discussed in Chapter 2.

In addition to these classical approaches the improvement of winter-hardiness in sugar beet could benefit from the application of MAS. As discussed by Xu and Crouch (2008) MAS is most preferentially suited for traits which are difficult to target by classical selection. This applies mostly to traits with complex inheritance where improvements require the pyramiding of the several QTL. Moreover, MAS has been proposed as a promising strategy for traits which can be assessed only under specific environmental conditions (Xu and Crouch, 2008). However, even though MAS has demonstrated its power for major genes, it has been shown to be inefficient to cope with minor QTL (Kearsey and Farquhar, 1998).

An approach that overcomes these limitations of MAS is genomic selection (GS). In contrast to MAS GS does not rely on significant marker trait associations, but GS utilizes all available markers to predict the performance of an individual (Heffner et al., 2010; Meuwissen et al., 2001). Therefore, high density marker information allows the prediction of breeding values even for complex quantitative traits where marker trait associations and MAS have been shown to be ineffective (Jannink et al., 2010). GS uses phenotypic and genotypic data of a relatively small training population for model fitting to predict genomic estimated breeding values (GEBV) of individuals from which only genomic data are available and the phenotype is unknown (Meuwissen et al., 2001). Therefore, GS is suited to select individuals based on genomic data without phenotypic data. The concept of GS relies on the hypothesis that any individual under selection is composed of alleles that are represented in the training population. Each of these alleles is phenotyped in a wider range of environments and in more replications than it would be feasible for the individual itself. Therefore, the prediction of GEBV is possible even under the presence of genotype by environment interactions (Heffner et al., 2009). Interestingly, simulations of Meuwissen (2001) and Muir (2007) indicate that even for polygenic traits with low h^2 correlation between GEBVs and true breeding values exceeded 0.70, indicating a high accuracy of GS. To compare the efficiency of GS with phenotypic selection not only the accuracy of the prediction is important but the expected selection response (Bernardo, 2008) which was defined by Becker (2011) as follows:

$$R = (1/Y)a\ i\ h\ r\ \sigma_g$$

Where R is the expected selection response; Y is the number of years required per selection cycle; a is a factor determining whether selection takes place in one or both sexes; i is the selection intensity; h is the square root of the heritability; r is the correlation between the GEBV of the selected individuals and their true breeding values and σ_g is the square root of the genomic variance. Assuming one phenotypic selection cycle per year (Y=1) and three cycles of genomic selection (Y=0.3) with i≥0.5 for the GS, Lorenzana and Bernardo (2009) concluded that gains of GS would approach 1.5 compared to phenotypic selection. Interestingly, Heffner *et al.* (2009) have chosen winter-hardiness as an example to discuss the power of GS, as winter-

hardiness is highly affected by genotype x environment interactions and selective conditions are rarely predictable (Saulescu and Braun, 2001). Moreover, GS is a promising approach to improve winter-hardiness and frost tolerance because harsh winters in field tests might delay propagation of even superior genotypes. This can be overcome if phenotyping for winter-hardiness is done in the training population and selection is based on the predicted performance based on marker data.

Until now, no non-bolting autumn sown sugar beet was ever investigated under field condition for quality and yield parameters including sugar yield and sugar content. Moreover, nothing is known about the processing quality of non-bolting autumn sown beets, which is determined by non-sugar ingredients like potassium, sodium and amino-nitrogen. However, analysis of autumn sown beets before winter revealed that these beets displayed high dry matter, sodium and potassium contents and low sugar content (Hoffmann and Kluge-Severin, 2011). This is in congruence with observations of winter beets around the Mediterranean (Ruiz-Holst et al., 2003) and Hoffmann and Kluge-Severin (2011) speculated that these changes in quality parameters are effects of cold adaptation. If these speculations could be confirmed by the analyses of non-bolting beets, these beets would have to over-compensate lower quality with increased yield. However, Hoffmann and Kluge-Severin (2011) speculated that the number of cambium rings is determined and therefore, the sink capacity of the sugar beet root is limited (Hoffmann and Kluge-Severin, 2011). If these speculations are true it has to be seriously questioned whether the yield increase of winter beets can meet the expectations driven by simulations, which were solely based on the increased light absorption and did not account for limiting factors (Hoffmann and Kluge-Severin, 2011; Jaggard and Werker, 1999). However, even though it is uncertain, whether autumn sown sugar beets will contribute to significant yield increases, a major advantage of cultivating autumn sown sugar beet would be the extension of the processing campaign due to early harvest in summer.

In contrast to the improvement of winter-hardiness the development of a system for bolting control most likely depends on genetic modification (Jung and Müller, 2009). One strategy to modify bolting has been proposed by Jung and Müller and is based on the genetic transformation of both hybrid components. This has severe consequences for the breeding perspectives of winter sugar beets in Europe. Taken the missing public acceptance of genetic modified plants in wide parts of Europe into account, breeders have to decide, whether the required economic, social and political expenses for the market introduction are justified and the point of breakeven can be reached.

In this study I have started to elucidate the genetics of winter-hardiness in lights of developing winter sugar beets. However, further research is needed to dissect the genetics of flowering time and winter-hardiness as well as their interactions. Moreover, a technological impact assessment has to elucidate the effects of altering the vegetation period of sugar beets under social, economic and ecological aspects. Finally the perspectives of autumn sown winter beets are highly dependent on the relative competitive ability compared to conventional sugar beets and other crops. Only if breeders, farmers and processing industry expect to benefit from autumn sown winter beets, winter beets will contribute to future sugar and biofuel production.

7 Summary

Under cool-temperate conditions, sugar beets (*Beta vulgaris* L.) are cultivated as a spring crop. Like in many other spring sown crops yield is limited by slow plant development at the beginning of the vegetation period. An increased leaf area index in early spring results in higher light absorption and photosynthesis rates. One strategy to extend the vegetation period of beets is to grow winter beets which are sown in autumn and harvested in the following year. This elongated vegetation period is expected to contribute to a substantially higher yield potential compared to spring sown sugar beets. However, cultivating winter beets requires winter hardiness and bolting control because conventional sugar beets are bolting after the exposure to a prolonged cold period. This research focussed on the phenotypic and genotypic dissection of winter-hardiness and frost tolerance in *B. vulgaris*.

A beet diversity panel of 396 *B. vulgaris* accessions was grown in the field in 2008/09 and 2009/2010 in eight environments at five locations. The evaluation of winter-hardiness in a diversity panel revealed wide genetic variation and a high heritability of winter-hardiness in the *B. vulgaris* gene pool. The diversity panel comprised 100 sugar beet, 61 fodder beet, 90 garden beet and 62 leaf beet accessions as well as 56 wild beet (*B. vulgaris* ssp. *maritima* L., BVM) and 27 uncharacterized *B. vulgaris* accessions. Survival rates (SR) were determined as the rate of living plants after winter. SR was highly affected by genotypes, environments and genotype × environment interaction. Sugar beet accessions had an average survival rate of 39.7%. Both, highest survival rates and the largest variation were found among the BVM (8.8% - 65.6%). SRs between locations ranged from 0.7% to 86.3%. The data demonstrate that substantial genetic variation for winter-hardiness exists among cultivated beets. The level of winter-hardiness might be sufficient for the cultivation of winter beets under maritime but not under continental conditions.

I selected 42 accessions due to their contrasting winter-hardiness and frost tolerance resulting in 107 crossing combinations, of which eleven were selected for the production of F_2 populations. Based on the genetic variation observed within the sugar beet accession a bi-parental mapping population of 238 $F_{2:3}$ families was established.This mapping population was phenotyped for artificial frost tolerance and winter-hardiness. For QTL mapping a linkage map (674cM) based on 127 AFLP, SSR, SNP and CAPs markers was established. Thirtyseven sequences with high homology to cold regulated genes in *Arabidopsis thaliana* were identified from the beet genome sequence and eight candidate gene markers for putative cold regulated genes could be integrated into the linkage map.

For the artificial frost test 226 of the $F_{2:3}$ families were cultivated under 20°C for six weeks, followed by a two week acclimation period at 4°C and 48h at -7°C. Frost effects were measured as SRs and an index for frost damages (frost severity index, FSI) under semi-controlled conditions. Three QTL for FSI were mapped to chromosomes 1, 3 and 8 explaining 17.3% of the total phenotypic variance. The QTL on chromosome 3 and 8 co-segregated with two QTL for SRs in the frost chamber that explained 13.1 % of the phenotypic variation.

Surviving plants three weeks after the last frost (rate of living plants, RLP) and SR in mid-April were recorded for the 238 $F_{2:3}$ families under field conditions. The field trails suffered from wet sowing and harsh winter conditions. Therefore, the field trials could only partially be evaluated. Consequently, only two preliminary QTL for RLP were identified on chromosomes 4 and 6 explaining 11.7% of the total phenotypic variation.

In a 3rd experiment, a subset of 268 accessions of the previously described *B. vulgaris* diversity panel was grown in the field to determine winter-hardiness and annual bolting in eight environments at five different locations in Germany and Belarus in the winters of 2008/09 and 2009/10. The panel was genotyped at three major flowering time loci via EcoTILLING. EcoTILLING revealed polymorphisms within the *BvFL1* gene that were associated with

annuality and winter-hardiness. These associations provide the first genetic indication for the function of *BvFL1* in beet.

8 Zusammenfassung

In gemäßigten Klimaten werden Zuckerrüben (*Beta vulgaris* L.) im Frühjahr ausgesät und im darauf folgenden Herbst geerntet. Wie viele andere Kulturen, deren Aussaat im Frühjahr erfolgt, ist der Ertrag durch die langsame Jugendentwicklung am Beginn der Vegetationsperiode begrenzt. Durch einen höheren Blattflächenindex im Frühjahr ließe sich die Lichtabsorption und die Photosyntheserate steigern. Einen Ansatz, um die Vegetationsperiode von Zuckerrüben zu verlängern, bietet der Anbau von Zuckerrüben, die im Herbst gesät und erst im darauf folgenden Jahr geerntet werden. Durch die Verlängerung des Vegetationszeitraumes wird eine deutliche Steigerung des Ertragspotentials von Zuckerrüben erwartet. Notwendige Voraussetzungen für den Anbau von Winterrüben sind allerdings eine ausreichende Winterhärte und ein System zur Schosskontrolle. Die Schosskontrolle wird benötigt, da bei Zuckerrüben der winterliche Kältereiz die Streckung der Sprossachse induziert. Ziel dieser Arbeit war die phänotypische und genotypische Untersuchung von Winterhärte und Frosttoleranz in *B. vulgaris*.

In den Wintern 2008/09 und 2009/2010 wurde ein Sortiment von 396 *B. vulgaris* Akzessionen in acht Umwelten an fünf Standorten auf Winterhärte im Feld untersucht. Die Akzessionen unterschieden sich deutlich in ihrer Winterhärte und die Erblichkeit für dieses Merkmal war sehr hoch. Das untersuchte *B. vulgaris* Sortiment setzte sich aus 100 Zuckerrüben, 61 Futterrüben, 90 rote Beten und 56 Wildrüben (*B. vulgaris* ssp. *maritima* L., BVM), sowie 27 nicht näher charakterisieren *B. vulgaris* Akzessionen zusammen. Überlebensraten wurden definiert als der Anteil an Pflanzen, die den Winter überlebten. Die Akzessionen, Umwelten, sowie die Interaktion von Akzessionen und Umwelten hatten einen hohen Einfluss auf die Überlebensraten. Die durchschnittliche Überlebensrate über alle Zuckerrüben betrug 39,7%. Die Wildrübenakzessionen zeigten neben der höchsten Überlebensrate auch die größte Variation (8,8% - 65,6%). Die durchschnittlichen Überlebensraten in den einzelnen Umwelten variierten von 0,7% bis 86,3%. Diese Ergebnisse zeigen deutliche genetische Variation für das Merkmal Winterhärte in Kulturrüben. Die beobachte Winterhärte ist vermutlich ausreichend für den Winterrübenanbau in maritimen Regionen, jedoch nicht für den Anbau unter kontinentalen Bedingungen.

Auf Grund ihrer Differenzierung für Winterhärte und Frosttoleranz wurden 42 Akzessionen in 107 Kombinationen gekreuzt. Von elf dieser Kombinationen wurden F_2 Populationen produziert. Auf Grund der beobachteten Variation wurde eine bi-parentale Zuckerrübenpopulation ausgewählt und es wurden 238 $F_{2:3}$ Familien erstellt. Die Kartierungspopulation wurde in einer Frostkammer auf Frosttoleranz und im Feld auf Winterhärte untersucht. Für die QTL Kartierung wurde eine genetische Karte (674cM) basierend auf 127 AFLP-, SSR-, SNP- und CAPs-Markern entwickelt. Darüber hinaus wurden 37 Sequenzen mit hoher Homologie zu kälteregulierten Genen in *Arabidopsis thaliana* identifiziert, von denen acht als Marker für die Kandidatengene in die genetische Karte integriert werden konnten.

Für die Untersuchung der Frosttoleranz in der Frostkammer wurden 226 $F_{2:3}$ Familien für sechs Wochen bei 20°C angezogen und anschließend für zwei Wochen bei 4°C kälteakklimatisiert. Die Frostbehandlung erfolgte für 48 Stunden bei -7°C. Anschließend wurden die Überlebensrate (SR) und ein Index für die Frostschädigung (FSI) erfasst. Für das Merkmal FSI wurden drei QTL auf den Chromosomen 1, 3 und 8 identifiziert, die gemeinsam 17,3% der phänotypischen Variation erklärten. Die QTL auf Chromosom 3 und 8 kosegregieren mit zwei QTL für SR, die wiederum 13,1% der beobachteten Variation erklärten.

Unter Feldbedingungen wurden die Überlebensraten (SR) und der Anteil an Pflanzen, die drei Wochen nach dem letzten Frostereignis noch lebten (RLP), in 238 $F_{2:3}$ Familien erfasst. Leider wurde die Auswertbarkeit der Feldversuche durch die nassen Aussaatbedingungen und den

strengen Winter erheblich eingeschränkt. Daher sind die beiden auf Chromosom 4 und 6 für das Merkmal RLP identifizierten QTL auch als vorläufig zu betrachten. Gemeinsam erklärten die beiden QTL 11,7% der phänotypischen Variation.

In einem dritten Experiment wurde in den Wintern 2008/09 und 2009/2010 eine aus 268 Akzessionen bestehende Auswahl des obigen Sortiments in acht Umwelten an fünf Standorten auf Winterhärte und einjähriges Schossen untersucht. Anschließend wurde das Sortiment an fünf Loci von drei wichtigen Blühregulationsgenen mit Hilfe von EcoTILLING genotypisiert. Es konnten Sequenzvariationen in dem Gen *BvFL1*identifiziert werden, die sowohl einen Einfluss auf das Schossverhalten, als auch auf Winterhärte hatten. Diese Assoziationen liefern einen ersten Hinweis auf eine Funktionalität von *BvFL1* in *B. vulgaris*.

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Supplementary table 1: Literature survey on the genetic analyses of stress tolerances in sugar beet

Stress	Population structure	Number of plants investigated	Mapped gene	mapped QTL or loci	Chromosome of Gene or QTL	Marker technology	Number of markers	Mapsize [cM]	Reference
Cyst nematode *Heterodera schachtii*	progenies of monosomic addition lines of *B. procumbens*, *B.webbiana* and *B. patellaris*	11	isoenzyme marker on *B. procumbens* chromosome carrying the resistance gene			Isozyme	3		Jung et al.; 1986
Rhizomania resistance	four-way cross	49	*Rz1*		IV	RFLP	111	540	Barzen et al.; 1992
			monogermity locus m		IX				
			red hypocotyl *R*		VII				
Cyst nematode *Heterodera schachtii*	progenies of translocation plant	83	Nema		II	RAPD, isozyme, morphological marker, isozyme,	13		Uphoff et al.; 1992
Rhizomania	four-way cross	49	*Rz1*		IV	RFLP, RAPD	301	815	Barzen et al;. 1992
Cyst nematode *Heterodera schachtii*	3 segregating populations	84; 47; 93	*Nema*		I	RAPD, Isozyme, RFLP, morphological markers	142	738	Uphoff et al.; 1995
			red hypocotyl *R*		I				
			bolting locus *B*		I				

Stress	Population structure	Number of plants investigated	Mapped gene	mapped QTL or loci	Chromosome of Gene or QTL	Marker technology	Number of markers	Mapsize [cM]	Reference
			Restorer gene *X* for Owen CMS		III				
Cyst nematode *Heterodera schachtii*	4 segregating populations	48; 61; 96; 101	*Hs1*$^{pro-1}$		IV	RFLP	10		Heller et al.; 1996
			Hs1^{web-1}		IV				
			Hs2^{web-7}		IV				
Cyst nematode *Heterodera schachtii*	F2 population	208	*Hs1*$^{pro-1}$		IV	RAPD	165		Hallden et al.; 1997
Rhizomania	F1 and BC1 populations	88; 95; 98; 105; 112		4 rhizomania resistance loci	NA	RAPD,STS	26		Scholten et al.; 1997
Cercospora	F2-3 families and test cross progenies	193		Cercospora resistance	I	RFLP, AFLP	261	688.4	Nilsson et al.; 1999
				Cercospora resistance	II				
				Cercospora resistance	III				
				Cercospora resistance	III				
				Cercospora resistance	IX				
Cercospora	F2-3 families and test cross progenies	185 F3 families and 181 Test cross progenies		Cercospora resistance	II	RFLP, AFLP, SCAR, SSR	224	567	Schäfer-Pregl et al.; 1999

Stress	Population structure	Number of plants investigated	Mapped gene	mapped QTL or loci	Chromosome of Gene or QTL	Marker technology	Number of markers	Mapsize [cM]	Reference
				Cercospora resistance	VI				
				Cercospora resistance	IX				
				Cercospora resistance based on F2 data only	IV				
				Cercospora resistance based on F2 data only	V				
Cercospora	F2 population	86 F3 famiiles under field conditions; 196 F2 plants in leaf test		Cercospora resistance	III	AFLP, RFLP	261	458	Setiawan et al.; 2000
				Cercospora resistance	IV				
				Cercospora resistance	VII				
				Cercospora resistance	IX				
Cercospora Rhizomania	2 F2 populations	156; 211		Rhizomania resistance	III	RFLP, AFLP, STS	260	744	Weber et al.; 2000
				Potassium	II				
				amino-N	VII				
				amino-N	VIII				

Stress	Population structure	Number of plants investigated	Mapped gene	mapped QTL or loci	Chromosome of Gene or QTL	Marker technology	Number of markers	Mapsize [cM]	Reference
Rhizomania	BC1S1 population	120	Rhiomania resistance gene *Rz3*			AFLP, SSR	13	40	Gidner et al.; 20005
Rhizomania	2 F2 populations	117	Rhiomania resistance gene *Rz1*		III	SNP, RFLP	7	50	Lein et al.; 2007
Rhizomania	full sib mapping population	158	Rhiomania resistance gene *Rz4*			SNP, AFLP, RAPD	233	497.2	Grimmer et al.; 2007
Rizoctonia	F2-3 families	95 families		Rhizoctonia solani resistance	I	SSR, RFLP, CAPS, STS	101		Lein et al.; 2008

Supplementary table 2: Literature survey on genetic mapping of traits with relevance for agronomy and sciences in sugar beets

Reference	Mapping strategy	Marker technology	traits, focus of study	Number of markers	Mapsize [cM]
Jung et al.; 1986	detection of foreign chromatin	isozyme	nematode resistance	3	
Abe et al.; 1992	segregation analysis in F_2 and B_1F_1 population	isozyme, morphological markers	fertility restoration, self-compatibility, bolting locus B, monogermy	13	
Barzen et al.; 1992	mapping in four-way cross	RFLP	Rhizomania resistance, monogermy	111	540
Pillen et al.; 1992	segregation analysis in F_2 Poulation	RFLP, morphological markers, isozyme	development of an integrated map	115	789
Uphoff et al.; 1992	segregation analysis of progenies of translocation plant	RAPD, isozyme, morphological marker	nematode resistance	13	
Wagner et al.; 192	mapping in segregating population	isozyme, morphological marker	pollen fertility, monogermy	22	
Pillen et al.; 1995	segregation analysis in F_2 and B_1F_1 population	RFLP, morphological markers, isozyme	CMS restoration	177	1057
Boudry et al.; 1995	segregation analysis in back cross populations	RFLP	Bolting Locus B	9	
Barzen et al.; 1995	mapping in four-way cross	RFLP, RAPD	extend existing maps	301	815
Uphoff et al.; 1995	mapping in 3 segregating populations I01	RAPD, Isozyme, RFLP, morphological markers	nematode resistance, construction of linkage map	142	738
Hallden et al.; 1996	segregation analysis in 2 F_2 population	RFLP	integration of RFLP markers in linkage map	413	621
Heller et al., 1996	mapping in 4 segregating populations, Bulked segregant analysis	RFLP	Nematode resistance genes $Hs1^{pro-1}$, $Hs1^{web-1}$, $Hs2^{web-7}$	10	

First Author	Mapping strategy	Marker technology	traits, focus of study	Number of markers	Mapsize [cM]
Schondelmaier et al.; 1996	segregation analysis in F_2 population	AFLP, RFLP, Isozyme, morphological markers	integration of AFLP markers in linkage map	336	557
Hallden et al.; 1997	Bulked segregant analysis	RAPD	Nematode resistance genes $Hs1^{pro-1}$	165	
Nilsson et al.; 1997	segregation analysis in F_2 population	RFLP, RAPD	integration of RAPD markers in linkage map	408	508
Scholten et al.; 1997	Bulked segregant analysis in F1 and BC_1 populations	RAPD,STS	BNYVV	26	
Schumacher et al.; 1997	segregation analysis in 3 Populations	AFLP, RFLP, Isozyme, morphological markers	integration of existing maps	600	557
Nilsson et al.; 1999	segregation analysis in F_{2-3} families and test cross progenies	RFLP, AFLP	*Cercospora* resistance	261	688.4
Schäfer-Pregl et al.; 1999	segregation analysis in F_{2-3} families and test cross progenies	RFLP, AFLP, SCAR, SSR	*Cercospora* tolerance	224	567
Schneider et al.; 1999	segregation anaylsis of EST derived candidate sequences in 4 F2 populations	CAPS, DFLP, SSCP, HD, RFLP, SSR	identification and mapping of yield-related genes	45	
Kraft et al.; 2000	LD mapping in 9 sugar beet breeding lines	AFLP	determination of Linkage disequilibrium	451	
Rae et al.; 2000	segregation analysis in 2 F_2 populations	RFLP, SSR	integration of SSR markers in linkage map	62	
Setiawan et al.; 2000	segregation analysis in F_2 population	AFLP, RFLP	*Cerospora* resistance	261	458

First Author	Mapping strategy	Marker technology	traits, focus of study	Number of markers	Mapsize [cM]
Hansen et al.; 2001	Association mapping in 106 *B. vulgaris ssp. maritima*	AFLP	Bolting Locus *B*	440	
El-Mezawy et al.; 2002	segregation analysis in F_{2-3} families and bulked segregant analysis	RFLP, AFLP	Bolting Locus *B*	249	
Hjerdin-Panagopoulos et al.; 2002	segregation analysis in F_{2-3} families based on three crosses	RFLP	CMS restoration	51	822.3
Schneider et al.; 2002	segregation analysis in F_{2-3} families	CAPS, SNP, RFLP, SSR	sugar content, yield and quality related genes	174	490.3
Hohmann et al.; 2003	physical mapping of BACs	AFLP, YAC ends	Fine mapping of bolting gene B	12	446
Hunger et al.; 2003	segregation analysis in 2 F_2 populations	AFLP, RFLP, STS	Resistance Gene Analogons	47	
Touzet et al.; 2004	Analysis of segregating families, bulked segregant analysis	AFLP, SSR	CMS restoration locus G	13	
Möhring et al.; 2005	segregation analysis in 3 F_2 populations	SNP	development of linkage group specific SNP markers	52	
Gidner et al.; 2005	segregation analysis in BC_1S_1 population	AFLP, SSR	BNYVV	13	40
Hagihara et al.; 2005	Bulked segregant analysis	AFLP, RAPD	male sterility	10	
Lein et al.; 2007	segregation analysis in 2 F2 populations	SNP, RFLP	Resistance Gene Analogons co-segregating with the Rhizomania resistance locus Rz1	7	50
Schneider et al.; 2007	segregation analysis in 3 F2 populations	SNP	development of a SNP-based map	524	555

Reference	Mapping strategy	Marker technology	traits, focus of study	Number of markers	Mapsize [cM]
Grimmer et al.; 2007	segregation analysis in full sib mapping population	SNP, AFLP, RAPD	BNYVV resistance	233	664.3
Lein et al.; 2008	segregation analysis in F2-3 families	SSR, RFLP, CAPS, STS	Rizoctonia resistance	101	497.2
Stich et al.; 2008	Association mapping in 19 segregating population	SSR	yield and quality related genes	26	
Stich et al.; 2008	Association mapping				
Stevanato et al.; 2009	bulked segregant analyis of segregating F_1 population	AFLP	root yield and related traits	27	
Lange et al.; 2010	segregation analysis in F2 population	SNP, representational oligonucleotide microarray analysis (ROMA)	microarray based marker development	595	
Reif et al.; 2010	Joint linkage association mapping				
Würschum et al.; 2011	Association mapping in 924 breeding lines	SNP	White sugar yield, sugar yield, sugar content, beet yield, Ka, Na	677	

Supplementary table 3: Mean survival rates of 396 B. vulgaris accessions tested in overwintering field trials in Germany and Belarus in 2008/09 and 2009/10 across eight environments.

Accession	Kiel code	SR^+ [%]	Descriptor	Donor
2122	080377	41.85	fodder beet	Vavilov Institute of Plant Industry
PI142814	080405	36.72	fodder beet	US Department of Agriculture, Sugar beet Research Unit
PI142817	080407	36.27	fodder beet	US Department of Agriculture, Sugar beet Research Unit
PI140354	080395	35.82	fodder beet	US Department of Agriculture, Sugar beet Research Unit
733	080351	35.27	fodder beet	Vavilov Institute of Plant Industry
BETA 1700	080295	35.25	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
143	080346	34.98	fodder beet	Vavilov Institute of Plant Industry
BETA 70	080201	34.59	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI140361	080399	31.26	fodder beet	US Department of Agriculture, Sugar beet Research Unit
PI140362	080400	31.11	fodder beet	US Department of Agriculture, Sugar beet Research Unit
PI142823	080411	30.82	fodder beet	US Department of Agriculture, Sugar beet Research Unit
751	080353	30.22	fodder beet	Vavilov Institute of Plant Industry
1828	080369	30.11	fodder beet	Vavilov Institute of Plant Industry
PI140355	080396	27.84	fodder beet	US Department of Agriculture, Sugar beet Research Unit
1866	080374	27.74	fodder beet	Vavilov Institute of Plant Industry
840	080354	27.70	fodder beet	Vavilov Institute of Plant Industry
PI113306	080386	27.22	fodder beet	US Department of Agriculture, Sugar beet Research Unit
54V	080324	26.05	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
BETA 149	080208	25.85	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
58V	080326	24.74	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
38V	080321	24.67	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
BETA 172	080215	24.22	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
750	080352	24.05	fodder beet	Vavilov Institute of Plant Industry
PI142812	080403	23.90	fodder beet	US Department of Agriculture, Sugar beet Research Unit
128V	080318	23.61	fodder beet	Institute for Agricultural and Fisheries Research, PLANT

Accession	Kiel code	SR^{+} [%]	Descriptor	Donor
36V	080320	23.47	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
3609	080331	23.40	fodder beet	University of Warwick-Genetic Resources Unit
PI142818	080408	22.54	fodder beet	US Department of Agriculture, Sugar beet Research Unit
PI140359	080397	22.40	fodder beet	US Department of Agriculture, Sugar beet Research Unit
BETA 1922	080306	22.31	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI142816	080406	22.16	fodder beet	US Department of Agriculture, Sugar beet Research Unit
732	080350	21.60	fodder beet	Vavilov Institute of Plant Industry
BETA 1407	080281	21.55	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 171	080214	21.49	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
64V	080327	21.40	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
44V	080322	20.98	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
PI142813	080404	20.34	fodder beet	US Department of Agriculture, Sugar beet Research Unit
56V	080325	20.11	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
PI142821	080410	20.03	fodder beet	US Department of Agriculture, Sugar beet Research Unit
50V	080323	19.39	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
BETA 277	080244	19.16	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI109040	080385	18.97	fodder beet	US Department of Agriculture, Sugar beet Research Unit
BETA 175	080216	18.88	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI140360	080398	18.46	fodder beet	US Department of Agriculture, Sugar beet Research Unit
125V	080317	18.15	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
BETA 61	080200	18.02	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 176	080217	17.86	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 2220	080313	17.74	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 959	080250	16.82	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
930234	081768	16.81	fodder beet	Christian Albrechts University Kiel
720	080349	16.53	fodder beet	Vavilov Institute of Plant Industry
883	080355	14.85	fodder beet	Vavilov Institute of Plant Industry

Accession	Kiel code	SR+ [%]	Descriptor	Donor
175V	080319	14.79	fodder beet	Institute for Agricultural and Fisheries Research, PLANT
BETA 275	080243	14.57	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1596	080291	13.50	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1335	080272	13.13	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1326	080270	13.06	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI229683	080440	11.46	fodder beet	US Department of Agriculture, Sugar beet Research Unit
1836	080370	42.39	fodder beet	Vavilov Institute of Plant Industry
1749	080365	42.35	fodder beet	Vavilov Institute of Plant Industry
BETA 1566	080289	27.56	fodder beet	Leibniz Institute of Plant Genetics and Crop Plant Research
806,080	080545	56.67	leaf beet	Agroscope Changins-Wädenswil ACW
NGB 570	070520	46.75	leaf beet	Nordic Genetic Resource Center
806,042	070524	45.73	leaf beet	Agroscope Changins-Wädenswil ACW
805,948	093335	42.66	leaf beet	Agroscope Changins-Wädenswil ACW
BETA 225	070326	42.65	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
806,081	080546	42.58	leaf beet	Agroscope Changins-Wädenswil ACW
805,583	080547	41.99	leaf beet	Agroscope Changins-Wädenswil ACW
BETA 1617	080293	41.35	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
806,141	080548	40.51	leaf beet	Agroscope Changins-Wädenswil ACW
BETA 40	080193	40.43	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 2164	960009	39.91	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 259	080239	39.64	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
806,142	080549	38.59	leaf beet	Agroscope Changins-Wädenswil ACW
BETA 215	080226	38.55	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 225	080231	38.41	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
806,021	080544	38.36	leaf beet	Agroscope Changins-Wädenswil ACW
BETA 1716	080297	38.25	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1228	080267	37.81	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research

Accession	Kiel code	SR^{+} [%]	Descriptor	Donor
806,013	080543	37.56	leaf beet	Agroscope Changins-Wädenswil ACW
BETA 261	080240	35.36	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
825,523	080550	34.50	leaf beet	Agroscope Changins-Wädenswil ACW
PI590606	080481	34.33	leaf beet	US Department of Agriculture, Sugar beet Research Unit
BETA 216	080227	33.87	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 235	080235	33.33	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
33	080343	32.27	leaf beet	Vavilov Institute of Plant Industry
BETA 3816	080314	31.99	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 157	080211	31.52	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 230	080233	31.05	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 247	080237	29.70	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
970024	960074	28.84	leaf beet	Christian Albrechts University Kiel
806,009	080541	27.00	leaf beet	Agroscope Changins-Wädenswil ACW
BETA 229	080232	26.99	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
805,906	080539	26.39	leaf beet	Agroscope Changins-Wädenswil ACW
BETA 1090	080258	25.90	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
806,011	080542	25.71	leaf beet	Agroscope Changins-Wädenswil ACW
835,949	080551	25.46	leaf beet	Agroscope Changins-Wädenswil ACW
PI181859	080432	23.96	leaf beet	US Department of Agriculture, Sugar beet Research Unit
BETA 1334	080271	23.45	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI116810	080389	23.05	leaf beet	US Department of Agriculture, Sugar beet Research Unit
BETA 290	080245	22.86	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI504171	080443	21.54	leaf beet	US Department of Agriculture, Sugar beet Research Unit
BETA 1014	080253	21.23	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
1785	080368	19.91	leaf beet	Vavilov Institute of Plant Industry
BETA 1377	080277	18.99	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 293	080246	18.70	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research

Accession	Kiel code	SR^{+} [%]	Descriptor	Donor
BETA 1057	080256	18.64	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1995	080310	18.27	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1714	080296	17.59	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1447	080285	17.44	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1128	080262	16.94	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 231	080234	15.47	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1091	080259	15.44	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1384	080278	12.64	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI504174	080444	11.11	leaf beet	US Department of Agriculture, Sugar beet Research Unit
BETA 1348	080275	10.30	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1610	080292	9.99	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI504175	080445	8.81	leaf beet	US Department of Agriculture, Sugar beet Research Unit
BETA 249	080238	8.41	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 202	080223	8.17	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1558	080288	7.64	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI179179	080428	7.05	leaf beet	US Department of Agriculture, Sugar beet Research Unit
BETA 1432	080282	6.87	leaf beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1901	070323	33.75	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
3070	080329	32.99	garden beet	University of Warwick-Genetic Resources Unit
BETA 179	080218	32.33	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI105335	080383	32.29	garden beet	US Department of Agriculture, Sugar beet Research Unit
BETA 42	080195	31.17	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 125	080205	30.14	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 49	080197	30.11	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
3067	080328	29.54	garden beet	University of Warwick-Genetic Resources Unit
202	080347	29.18	garden beet	Vavilov Institute of Plant Industry
BETA 41	080194	27.41	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research

Accession	Kiel code	SR^{+} [%]	Descriptor	Donor
BETA 144	080207	27.03	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
6862	080334	26.54	garden beet	University of Warwick-Genetic Resources Unit
BETA 154	080209	26.28	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI140353	080394	25.67	garden beet	US Department of Agriculture, Sugar beet Research Unit
PI164292	080421	25.24	garden beet	US Department of Agriculture, Sugar beet Research Unit
BETA 98	080202	23.96	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
1679	080364	23.73	garden beet	Vavilov Institute of Plant Industry
2049	080376	23.64	garden beet	Vavilov Institute of Plant Industry
BETA 188	080220	23.36	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
2900	080381	23.32	garden beet	Vavilov Institute of Plant Industry
2946	080382	23.07	garden beet	Vavilov Institute of Plant Industry
7	080340	23.03	garden beet	Vavilov Institute of Plant Industry
BETA 48	080196	22.94	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
2226	080378	22.93	garden beet	Vavilov Institute of Plant Industry
BETA 212	080224	22.83	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI590607	080482	22.22	garden beet	US Department of Agriculture, Sugar beet Research Unit
BETA 124	080204	22.07	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
1586	080361	21.93	garden beet	Vavilov Institute of Plant Industry
BETA 264	080241	21.21	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 136	080206	21.20	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1343	080273	21.13	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 2158	080312	21.08	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
1672	080362	20.92	garden beet	Vavilov Institute of Plant Industry
BETA 1618	080294	20.87	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI164805	080427	20.76	garden beet	US Department of Agriculture, Sugar beet Research Unit
PI590596	080478	20.66	garden beet	US Department of Agriculture, Sugar beet Research Unit
1370	080357	20.18	garden beet	Vavilov Institute of Plant Industry

Accession	Kiel code	SR^{+} [%]	Descriptor	Donor
BETA 1172	080264	19.62	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 190	080221	19.55	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 354	080247	19.51	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI163178	080415	19.47	garden beet	US Department of Agriculture, Sugar beet Research Unit
PI590598	080479	19.40	garden beet	US Department of Agriculture, Sugar beet Research Unit
BETA 244	080236	19.18	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 51	080198	19.15	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 213	080225	19.02	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
2228	080379	18.99	garden beet	Vavilov Institute of Plant Industry
PI163182	080419	18.88	garden beet	US Department of Agriculture, Sugar beet Research Unit
BETA 111	080203	18.79	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI181718	080431	18.61	garden beet	US Department of Agriculture, Sugar beet Research Unit
358	000358	18.53	garden beet	Christian Albrechts University Kiel
BETA 1224	080344	18.15	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
39	960093	18.15	garden beet	Vavilov Institute of Plant Industry
PI144675	080412	17.93	garden beet	US Department of Agriculture, Sugar beet Research Unit
PI163179	080416	17.70	garden beet	US Department of Agriculture, Sugar beet Research Unit
BETA 28	070327	17.52	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
1323	080356	17.32	garden beet	Vavilov Institute of Plant Industry
1561	080359	17.18	garden beet	Vavilov Institute of Plant Industry
111	080345	16.87	garden beet	Vavilov Institute of Plant Industry
BETA 221	080228	16.76	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
1860	080373	16.58	garden beet	Vavilov Institute of Plant Industry
1	080339	16.51	garden beet	Vavilov Institute of Plant Industry
BETA 222	080229	16.48	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
6859	080333	16.32	garden beet	University of Warwick-Genetic Resources Unit
BETA 1159	080263	15.96	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research

Accession	Kiel code	SR^+ [%]	Descriptor	Donor
7209	080336	15.94	garden beet	University of Warwick-Genetic Resources Unit
BETA 185	080219	15.89	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
1678	080363	15.79	garden beet	Vavilov Institute of Plant Industry
2882	080380	15.07	garden beet	Vavilov Institute of Plant Industry
BETA 1905	080303	15.06	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
10	080341	14.87	garden beet	Vavilov Institute of Plant Industry
3084	080330	14.70	garden beet	University of Warwick-Genetic Resources Unit
11	080342	14.70	garden beet	Vavilov Institute of Plant Industry
6867	080335	14.44	garden beet	University of Warwick-Genetic Resources Unit
BETA 32	080192	14.40	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI164659	080425	14.07	garden beet	US Department of Agriculture, Sugar beet Research Unit
BETA 30	080191	13.94	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
597	080348	13.62	garden beet	Vavilov Institute of Plant Industry
BETA 3818	080315	13.36	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
930185	080305	13.14	garden beet	Christian Albrechts University Kiel
BETA 1910	930185	13.14	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
1581	080480	12.86	garden beet	Vavilov Institute of Plant Industry
PI590600	080360	12.86	garden beet	US Department of Agriculture, Sugar beet Research Unit
PI181930	080433	11.25	garden beet	US Department of Agriculture, Sugar beet Research Unit
BETA 52	080199	10.96	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1203	080265	10.80	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1899	080302	10.63	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 155	080210	10.45	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
6774	080332	9.13	garden beet	University of Warwick-Genetic Resources Unit
BETA 1478	080286	9.08	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 2105	080311	8.13	garden beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI467869	080442	50.67	sugar beet	US Department of Agriculture, Sugar beet Research Unit

Accession	Kiel code	SR+ [%]	Descriptor	Donor
BETA 1049	070318	49.76	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
950542	950542	46.98	sugar beet	Christian Albrechts University Kiel
BETA 1567	080290	45.92	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1261	080268	45.65	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
1523	080358	45.45	sugar beet	Vavilov Institute of Plant Industry
BETA 1375	080276	45.14	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1906	080304	44.95	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
940027	940027	44.78	sugar beet	Christian Albrechts University Kiel
PI220506	093170	44.72	sugar beet	US Department of Agriculture, Sugar beet Research Unit
BETA 1049	080255	44.63	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 14-2008	080501	44.62	sugar beet	Strube Research GmbH & Co. KG
WR CAU 20-2008	080507	44.41	sugar beet	Strube Research GmbH & Co. KG
WR CAU 05-2008	080492	44.36	sugar beet	Strube Research GmbH & Co. KG
2034	080375	44.32	sugar beet	Vavilov Institute of Plant Industry
1837	080371	43.61	sugar beet	Vavilov Institute of Plant Industry
WR CAU 47-2008	080534	43.54	sugar beet	Strube Research GmbH & Co. KG
DH-2-Minsk	020581	43.46	sugar beet	National Academy of Sciences of Belarus, Institute of Genetics and Cytology
BETA 1046	080254	43.24	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 07-2008	080494	43.20	sugar beet	Strube Research GmbH & Co. KG
BETA 1375	070321	42.81	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 31-2008	080518	42.78	sugar beet	Strube Research GmbH & Co. KG
BETA 165	080212	42.53	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI590614	080483	42.37	sugar beet	US Department of Agriculture, Sugar beet Research Unit
PI251042	080441	42.30	sugar beet	US Department of Agriculture, Sugar beet Research Unit
WR CAU 29-2008	080516	42.18	sugar beet	Strube Research GmbH & Co. KG
BETA 224	092479	42.11	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 10-2008	080497	41.91	sugar beet	Strube Research GmbH & Co. KG

Accession	Kiel code	SR^{+} [%]	Descriptor	Donor
WR CAU 46-2008	080533	41.88	sugar beet	Strube Research GmbH & Co. KG
WR CAU 16-2008	080503	41.34	sugar beet	Strube Research GmbH & Co. KG
BETA 1773	091645	41.29	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 32-2008	080519	41.28	sugar beet	Strube Research GmbH & Co. KG
WR CAU 22-2008	080509	41.13	sugar beet	Strube Research GmbH & Co. KG
WR CAU 51-2008	010243	41.08	sugar beet	Strube Research GmbH & Co. KG
WR CAU 36-2008	080523	40.72	sugar beet	Strube Research GmbH & Co. KG
BETA 268	080242	40.63	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 02-2008	080490	40.44	sugar beet	Strube Research GmbH & Co. KG
WR CAU 37-2008	080524	40.40	sugar beet	Strube Research GmbH & Co. KG
WR CAU 27-2008	080514	40.29	sugar beet	Strube Research GmbH & Co. KG
PI193457	080434	40.20	sugar beet	US Department of Agriculture, Sugar beet Research Unit
WR CAU 25-2008	080512	40.20	sugar beet	Strube Research GmbH & Co. KG
PI222970	080439	40.18	sugar beet	US Department of Agriculture, Sugar beet Research Unit
WR CAU 18-2008	080505	40.02	sugar beet	Strube Research GmbH & Co. KG
1770	080506	39.93	sugar beet	Vavilov Institute of Plant Industry
WR CAU 19-2008	080367	39.93	sugar beet	Strube Research GmbH & Co. KG
WR CAU 44-2008	080531	39.93	sugar beet	Strube Research GmbH & Co. KG
PI109038	080384	39.86	sugar beet	US Department of Agriculture, Sugar beet Research Unit
BETA 2145	070324	39.85	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 24-2008	080511	39.83	sugar beet	Strube Research GmbH & Co. KG
Theresa	090023	39.70	sugar beet	KWS Saat AG
1838	080372	39.62	sugar beet	Vavilov Institute of Plant Industry
940269	940269	39.45	sugar beet	Christian Albrechts University Kiel
WR CAU 35-2008	080522	39.34	sugar beet	Strube Research GmbH & Co. KG
BETA 166	080213	39.21	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 23-2008	080510	39.07	sugar beet	Strube Research GmbH & Co. KG

Accession	Kiel code	SR+ [%]	Descriptor	Donor
WR CAU 34-2008	080521	39.05	sugar beet	Strube Research GmbH & Co. KG
PI590281	930183	38.94	sugar beet	US Department of Agriculture, Sugar beet Research Unit
PI590838	950528	38.54	sugar beet	US Department of Agriculture, Sugar beet Research Unit
BETA 1214	080266	38.52	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 13-2008	080500	38.44	sugar beet	Strube Research GmbH & Co. KG
WR CAU 28-2008	080515	38.43	sugar beet	Strube Research GmbH & Co. KG
BETA 1803	080300	38.38	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 06-2008	080493	38.27	sugar beet	Strube Research GmbH & Co. KG
WR CAU 09-2008	080496	37.93	sugar beet	Strube Research GmbH & Co. KG
WR CAU 50-2008	080537	37.83	sugar beet	Strube Research GmbH & Co. KG
1769	080366	37.81	sugar beet	Vavilov Institute of Plant Industry
WR CAU 38-2008	080525	37.51	sugar beet	Strube Research GmbH & Co. KG
WR CAU 15-2008	080502	37.39	sugar beet	Strube Research GmbH & Co. KG
PI633949	080487	37.34	sugar beet	US Department of Agriculture, Sugar beet Research Unit
WR CAU 40-2008	080527	37.22	sugar beet	Strube Research GmbH & Co. KG
WR CAU 45-2008	080532	36.87	sugar beet	Strube Research GmbH & Co. KG
WR CAU 48-2008	080535	36.57	sugar beet	Strube Research GmbH & Co. KG
WR CAU 11-2008	080499	36.56	sugar beet	Strube Research GmbH & Co. KG
WR CAU 12-2008	080498	36.56	sugar beet	Strube Research GmbH & Co. KG
BETA 1446	080284	36.49	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
WR CAU 30-2008	080517	36.39	sugar beet	Strube Research GmbH & Co. KG
WR CAU 26-2008	080513	36.10	sugar beet	Strube Research GmbH & Co. KG
WR CAU 17-2008	080504	35.96	sugar beet	Strube Research GmbH & Co. KG
WR CAU 04-2008	080553	35.54	sugar beet	Strube Research GmbH & Co. KG
WR CAU 52-2008	930176	35.54	sugar beet	Strube Research GmbH & Co. KG
WR CAU 41-2008	080528	35.24	sugar beet	Strube Research GmbH & Co. KG
WR CAU 21-2008	080508	35.21	sugar beet	Strube Research GmbH & Co. KG

Accession	Kiel code	SR^{+} [%]	Descriptor	Donor
WR CAU 01-2008	080489	35.15	sugar beet	Strube Research GmbH & Co. KG
Avignon	010240	34.84	sugar beet	KWS Saat AG
WR CAU 33-2008	080520	34.82	sugar beet	Strube Research GmbH & Co. KG
WR CAU 08-2008	080495	34.81	sugar beet	Strube Research GmbH & Co. KG
WR CAU 03-2008	080491	34.65	sugar beet	Strube Research GmbH & Co. KG
WR CAU 49-2008	080536	34.29	sugar beet	Strube Research GmbH & Co. KG
WR CAU 39-2008	080526	34.08	sugar beet	Strube Research GmbH & Co. KG
940268	940268	34.05	sugar beet	Christian Albrechts University Kiel
PI633945	080485	34.00	sugar beet	US Department of Agriculture, Sugar beet Research Unit
WR CAU 42-2008	080529	33.97	sugar beet	Strube Research GmbH & Co. KG
PI633950	080488	33.86	sugar beet	US Department of Agriculture, Sugar beet Research Unit
WR CAU 43-2008	080530	33.56	sugar beet	Strube Research GmbH & Co. KG
PI142820	080409	33.36	sugar beet	US Department of Agriculture, Sugar beet Research Unit
BETA 1228	970024	32.47	sugar beet	Leibniz Institute of Plant Genetics and Crop Plant Research
PI633947	080486	31.88	sugar beet	US Department of Agriculture, Sugar beet Research Unit
7213	080338	28.90	sugar beet	University of Warwick-Genetic Resources Unit
PI558506	080467	28.84	sugar beet	US Department of Agriculture, Sugar beet Research Unit
PI142808	080401	26.79	sugar beet	US Department of Agriculture, Sugar beet Research Unit
BETA 1083	080257	43.16	uncharacterized *B. vulgaris*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI116906	080390	40.11	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
BETA 3867	080316	37.37	uncharacterized *B. vulgaris*	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1839	080301	35.97	uncharacterized *B. vulgaris*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI148625	080413	33.23	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI164553	080424	30.45	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
BETA 1927	080307	29.60	uncharacterized *B. vulgaris*	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1927	960036	29.60	uncharacterized *B. vulgaris*	Leibniz Institute of Plant Genetics and Crop Plant Research
7211	080337	28.64	uncharacterized *B. vulgaris*	University of Warwick-Genetic Resources Unit

Accession	Kiel code	SR^{+} [%]	Descriptor	Donor
PI169023	980319	28.39	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI164172	080420	28.22	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI164747	080426	28.22	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI140350	080393	26.70	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI142810	080402	26.18	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI116808	080387	25.99	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI124404	080392	25.99	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI164363	080422	25.99	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI180409	080429	25.99	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI215577	080435	25.99	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
BETA 1444	080283	18.15	uncharacterized *B. vulgaris*	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 371	080248	13.65	uncharacterized *B. vulgaris*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI163176	080414	13.65	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI164495	080423	13.65	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI181011	080430	13.65	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
BETA 200	080222	11.30	uncharacterized *B. vulgaris*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI217964	080436	8.81	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI163180	080417	8.13	uncharacterized *B. vulgaris*	US Department of Agriculture, Sugar beet Research Unit
PI540678	080461	65.60	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI546429	080466	65.36	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI540671	093237	62.86	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI540668	080457	60.96	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 1344	080274	57.79	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI540675	080459	53.28	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 1429	960037	53.15	*B. vulgaris* ssp. *maritima*	Christian Albrechts University Kiel
BETA 1101	080260	52.06	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI518421	080455	51.37	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit

Accession	Kiel code	SR^{+} [%]	Descriptor	Donor
BETA 315	080143	49.63	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI540682	080462	48.84	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 992	080251	48.78	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI540676	080460	48.77	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 1960	960069	48.61	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI518306	080449	48.06	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 1127	080261	46.50	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI540604	080456	45.24	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI546413	080465	44.60	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 316	080144	43.94	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI518412	080454	42.98	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 1942	080308	41.94	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI540684	080463	41.79	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI518355	080452	41.48	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 1002	080252	38.72	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1762	080298	38.43	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1550	080287	37.67	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 314	080142	37.52	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI518324	080450	36.23	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI562581	080469	34.92	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI546394	080464	33.47	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 319	080145	33.04	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI504245	080447	33.02	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 1320	080269	33.00	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 957	080249	29.43	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
BETA 1960	080309	28.84	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI613273	080484	28.84	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit

Accession	Kiel code	SR[+] [%]	Descriptor	Donor
PI562590	080471	28.22	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI518336	080451	28.19	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 321	080538	27.39	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI163181	080418	25.99	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI218063	080437	25.99	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI562586	080470	25.99	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI518411	080453	24.60	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI504282	080448	24.59	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI562595	080473	19.85	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI562579	080468	17.29	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 1400	080280	15.67	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI562600	080476	15.47	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI121838	080391	15.01	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI504204	080446	14.54	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI562597	080474	13.65	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI562602	080477	13.65	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
BETA 1397	080279	10.74	*B. vulgaris* ssp. *maritima*	Leibniz Institute of Plant Genetics and Crop Plant Research
PI562591	080472	10.44	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI116809	080388	9.10	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit
PI562599	080475	8.81	*B. vulgaris* ssp. *maritima*	US Department of Agriculture, Sugar beet Research Unit

[+]SR= Survivalrate

Supplementary table 4: Parameters of variation for the survival rates [%] of different *B. vulgaris* cultivar groups and *B. vulgaris* ssp. *maritima* (BVM) observed in overwintering experiments across eight environments.

***B. vulgaris* form**	**Min[+]**	**1st Qu[*]**	**Median**	**Mean**	**3rd Qu[#]**	**Max[§]**	**Range**
Fodder Beets	11.46	18.88	22.54	24.26	27.84	42.39	30.93
Leaf Beets	6.87	17.93	26.99	27.30	38.31	56.67	49.80
Red Table Beets	8.13	15.25	19.01	19.48	22.94	33.75	25.62
Sugar Beets	26.79	36.54	39.70	39.28	42.21	50.67	23.88
BVM	8.81	25.99	34.92	35.29	48.06	65.60	56.79
Uncharacterized *B. vulgaris*	8.13	14.77	26.09	24.90	29.41	43.16	35.03

[+]Min= Minimum. [*]1st Qu= 1st Quantile. [#]3rd Qu=3rd Quantile. [§]Max=Maximum.

Supplementary table 5: Timeline of artificial frost tests performed from Sept. 2011 to March 2012 in Kiel.

Abbreviation of trial	Sowing date	Transfer to acclimation	Frost treatment	1. phenotypic evaluation	2. phenotypic evaluation	3. phenotypic evaluation	4. phenotypic evaluation
FC N	2011-09-07	2011-10-24	2011-11-07	2011-11-14	2011-11-21	2011-11-28	2011-12-05
FC O	2011-09-05	2011-10-26	2011-11-09	2011-11-16	2011-11-23	2011-11-30	2011-12-07
FC P	2011-10-17	2011-11-28	2011-12-12	2011-12-19	2011-12-26	2012-01-02	2012-01-09
FC Q	2011-11-09	2011-12-19	2012-01-02	2012-01-09	2012-01-16	2012-01-23	2012-01-30
FC R	2011-11-28	2012-01-09	2012-01-23	2012-01-30	2012-02-06	2012-02-13	2012-02-20
FC S	2011-12-19	2012-01-30	2012-02-13	2012-02-20	2012-02-27	2012-03-05	2012-03-12

Supplementary table 5: Timeline of artificial frost tests performed from Sept. 2011 to March 2012 in Kiel.

Abbreviation of trial	Sowing date	Transfer to acclimation	Frost treatment	1. phenotypic evaluation	2. phenotypic evaluation	3. phenotypic evaluation	4. phenotypic evaluation
FC N	2011-09-07	2011-10-24	2011-11-07	2011-11-14	2011-11-21	2011-11-28	2011-12-05
FC O	2011-09-05	2011-10-26	2011-11-09	2011-11-16	2011-11-23	2011-11-30	2011-12-07
FC P	2011-10-17	2011-11-28	2011-12-12	2011-12-19	2011-12-26	2012-01-02	2012-01-09
FC Q	2011-11-09	2011-12-19	2012-01-02	2012-01-09	2012-01-16	2012-01-23	2012-01-30
FC R	2011-11-28	2012-01-09	2012-01-23	2012-01-30	2012-02-06	2012-02-13	2012-02-20
FC S	2011-12-19	2012-01-30	2012-02-13	2012-02-20	2012-02-27	2012-03-05	2012-03-12

Supplementary table 6: Anchor marker used for chromosome determination. SNP marker data was provided by Strube research GmbH.

Chr.+	Marker name	Marker type*	PCR Primers	PCR Condition	PCR fragment size in SCK [bp]	Detection	Marker allele in SCK [bp]	Marker allele in SC2 [bp]
1	BB00953a_466	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	EBS0312a_139	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	K653_C1	CAPS	K653/K654	Standard PCR	536	*Ase*I + gel electrophoresis	464+70	536
1	LHP1	CAPS	K105/K106	Standard PCR	552	*EcoR*I + gel electrophoresis	552	163+389
2	B161_C2	CAPS	B161/B162	Standard PCR	500	*Pst*1 + gel electrophoresis	500	300+200
2	BB02074a_613	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	EBS0126a_151	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	K73_C2	CAPS	K73_K74	Standard PCR	524	*Mnl*I + gel electrophoresis	330+214	310+214
3	A989_C3	SSR	A989/A990	Touchdown PCR	138	Gel electrophoresis	138	150
3	BB04258a_204	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	BB09196a_305	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
3	N69_C3	CAPS	N69/N70	Standard PCR	367	BcnI + gel electrophoresis	367	180+137+50
3	N97_C3	CAPS	N97/N98	Standard PCR	316	DdeI + gel electrophoresis	175	141+175
4	A991_C4	SSR	A991/A992	Touchdown PCR	240	Gel electrophoresis	240	220

Chr.+	Marker name	Marker type*	PCR Primers	PCR Condition	PCR fragment size in SCK [bp]	Detection	Marker allele in SCK [bp]	Marker allele in SC2 [bp]
5	EBS0104a_457	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	EBS0226a_537	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	GolS3_C5	CAPS	K51/K52	Standard PCR	558	*Tai*I + gel electrophoresis	558	290+268
5	K545_C5	CAPS	K545/K546	Standard PCR	538	*Ear*I + gel electrophoresis	538	400+138
5	A978_C5	SSR	A978/A979	Touchdown PCR	158	Gel electrophoresis	158	184
5	K941_C5	CAPS	K941/K942	Standard PCR	546	*Bpi*I + gel electrophoresis	257+213+76	546
6	B011_C6	SSR	B011/B012	Touchdown PCR	139	Gel electrophoresis	139	134
6	BB01936b_577	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
6	EBS0192a_172	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
6	K359_C6	CAPS	K359/K360	Standard PCR	570	*Eco91*I + gel electrophoresis	350+240	570
6	K501_C6	CAPS	K501/502	Standard PCR	671	*Alu*I + gel electrophoresis	331+110+230	360+311
7	BB06357a_113	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7	BB07454a_238	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7	K217_C7	CAPS	K217/K218	Standard PCR	647	*Cai*I + gel electrophoresis	304+339	647
7	N115_C7	SSR	N115/N116	Touchdown PCR	180	Gel electrophoresis	180	245
8	B015_C8	SSR	B015/016	Standard PCR	279	Gel electrophoresis	279	295

Chr.+	Marker name	Marker type*	PCR Primers	PCR Condition	PCR fragment size in SCK [bp]	Detection	Marker allele in SCK [bp]	Marker allele in SC2 [bp]
8	BB00963a_544	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	BB06903a_252	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	BB08644a_547	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	BB09203a_210	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	BB09285a_640	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	EB00867a_246	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	EBS0033a_247	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	EBS0252a_404	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	EBS0264a_300	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
9	B017_C	SSR	B017/B018	Standard PCR	331	Gel electrophoresis	331	355
9	EBS0110a_177	SNP	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
9	FT1_C9	SSR	N33/N34	Touchdown PCR	618	Gel electrophoresis	618	697

+= Chromosome. * SSR= Simple Sequence repeats; CAPS= Cleaved Amplified Polymorphic DNA. n.a. = not available

Supplementary table 7: AFLP primer combinations used for map construction.

M Primer	P Primer	IRD Channel	Number of polymorphisms	markers integrated into linkage map
31	34	700	7	6
31	35	700	4	4
32	33	700	6	4
33	40	700	6	2
37	34	700	4	2
40	40	700	5	5
42	42	700	5	2
44	31	700	9	8
45	34	700	3	2
46	34	700	6	0
47	35	700	6	0
48	35	700	3	2
31	40	800	5	2
31	44	800	4	1
32	35	800	4	3
32	36	800	3	3
38	36	800	2	0
39	40	800	3	3
42	41	800	3	3
42	46	800	3	0
44	39	800	6	6
44	43	800	4	2
45	33	800	6	5
46	33	800	6	2
47	36	800	7	1
48	36	800	4	4
		SUM	124	72

Supplementary table 8: Primers used to amplify sugar beet candidate sequences with homology to known cold regulated and flowering time genes in *Arabidopsis thaliana*.

***A. thaliana* Homolog**	**Primer**	**Sugar Beet EST**	**Genome Scaffold**	**Sequence (5'->3')**
CBF1	K029	GBQ88_GBQ42_CauKiel_c1913	scaffold00066	CTATAGGCGTAAACCTCGTGTC
CBF1	K030	GBQ88_GBQ42_CauKiel_c1913	scaffold00066	CCGCAGAAGTACCCTTAATAC
CBF1	K031	GBQ88_GBQ42_CauKiel_c1913	scaffold00066	CTACTCCAGAGATGGCTG
CBF1	K032	GBQ88_GBQ42_CauKiel_c1913	scaffold00066	ATTAACCACGTCTTGTCCAC
CBF2	K023	GBQ88_GBQ42_CauKiel_lrc15447	scaffold00432	CGTTAAGCTGATGGTCGG
CBF2	K024	GBQ88_GBQ42_CauKiel_lrc15447	scaffold00432	GCCTACACAAGCATCCTC
CBF2	K025	GBQ88_GBQ42_CauKiel_lrc15447	scaffold00432	CCCCACTATAGAGCCTGC
CBF2	K026	GBQ88_GBQ42_CauKiel_lrc15447	scaffold00432	GGCGAAGCGTGAAGAACG
CCT2	K011	GBQ88_GBQ42_CauKiel_c16204	scaffold00121	CTACCCAAGGTGCATCAG
CCT2	K012	GBQ88_GBQ42_CauKiel_c16204	scaffold00121	CGGTGGTTTGTGATGTGTTC
CCT2	K013	GBQ88_GBQ42_CauKiel_c16204	scaffold00121	GGACCTGCATCAAGACCAC
CCT2	K014	GBQ88_GBQ42_CauKiel_c16204	scaffold00121	GTGTGCAGATGGGTTGATG
Cor15a	K019	GBQ88_GBQ42_CauKiel_c13676	scaffold00187	GGCAAGGATGAGTCTCATG
Cor15a	K020	GBQ88_GBQ42_CauKiel_c13676	scaffold00187	CAGAAAGAATCAGGCAGGC
Cor15a	K021	GBQ88_GBQ42_CauKiel_c13676	scaffold00187	GAGTTTGTTTTACTTCTGCG
Cor15a	K022	GBQ88_GBQ42_CauKiel_c13676	scaffold00187	CGACCATATGAGTTCTAGG
COR15b	K033	GBQ88_GBQ42_CauKiel_c14648	scaffold00505	GCTAGTAATGCCCATTTACC
COR15b	K034	GBQ88_GBQ42_CauKiel_c14648	scaffold00505	CTGCTAAGAAGAAGGCTGG
COR15b	K035	GBQ88_GBQ42_CauKiel_c14648	scaffold00505	CTTGAGCCTTATCTTTAGC
COR15b	K036	GBQ88_GBQ42_CauKiel_c14648	scaffold00505	CAAGCGAAATGAGTGGAC
COR47	K125	GBQ88_GBQ42_CauKiel_c410	scaffold00500	CTTCCTCATCGGACTGCAG
COR47	K126	GBQ88_GBQ42_CauKiel_c410	scaffold00500	CAACCTAAACGGAATGAAAAGGG
COR47	K127	GBQ88_GBQ42_CauKiel_c410	scaffold00500	CCATTCCACTAGGATACC
COR47	K128	GBQ88_GBQ42_CauKiel_c410	scaffold00500	CGGTATCGAAAAGGTGCAC
COR8.6	K095	GBQ88_GBQ42_CauKiel_c13982	scaffold00171	GAGCCAAACTTCCACTAC
COR8.6	K096	GBQ88_GBQ42_CauKiel_c13982	scaffold00171	GTCTTCTAATCTACTCCTTC

A. thaliana Homolog	Primer	Sugar Beet EST	Genome Scaffold	Sequence (5'->3')
COR8.6	K097	GBQ88_GBQ42_CauKiel_c13982	scaffold00171	GTTGCCTCGTTTTGTGTGC
COR8.6	K098	GBQ88_GBQ42_CauKiel_c13982	scaffold00171	GGTAGTTCTTAGTTATGAGGC
CORI3	K117	GBQ88_GBQ42_CauKiel_c213	scaffold00559	CCTTGTGGAAGTGTATATAC
CORI3	K118	GBQ88_GBQ42_CauKiel_c213	scaffold00559	GGGCTTGCTACCAAAAGTG
CORI3	K119	GBQ88_GBQ42_CauKiel_c213	scaffold00559	GAATGAGCCGGAAGGTTC
CORI3	K120	GBQ88_GBQ42_CauKiel_c213	scaffold00559	CCGACAGTAGAACCTATTC
CORI3	S003	GBQ88_GBQ42_CauKiel_c213	scaffold00559	GAATGAGCCGGAAGGTTC
CORI3	S004	GBQ88_GBQ42_CauKiel_c213	scaffold00559	CCGACAGTAGAACCTATTC
ERD7	K133	GBQ88_GBQ42_CauKiel_c5557	scaffold00213	AGACGCCATTGTTGTGAAG
ERD7	K134	GBQ88_GBQ42_CauKiel_c5557	scaffold00213	GAACTCAGGGGTACCTCA
ERD7	K135	GBQ88_GBQ42_CauKiel_c5557	scaffold00213	GTGCAAGAGCATAAGTGC
ERD7	K136	GBQ88_GBQ42_CauKiel_c5557	scaffold00213	CTTGACCCTTGTGAGAAG
ESK1	K121	GBQ88_GBQ42_CauKiel_c3664	scaffold00219	GCTATGTTTGGTGGATGTAC
ESK1	K122	GBQ88_GBQ42_CauKiel_c3664	scaffold00219	GAATCATGAATGGATGAGC
ESK1	K123	GBQ88_GBQ42_CauKiel_c3664	scaffold00219	CCGGCTTTTACCATGTTC
ESK1	K124	GBQ88_GBQ42_CauKiel_c3664	scaffold00219	CTCCTGGCAAACACCAATG
FCA	K041	GBQ88_GBQ42_CauKiel_c3362	scaffold00120	CTGATGCAGCTATTGAGG
FCA	K042	GBQ88_GBQ42_CauKiel_c3362	scaffold00120	CTTGCAGCTAGAACTCTC
FCA	K043	GBQ88_GBQ42_CauKiel_c3362	scaffold00120	GTTCAAGCAGGCCCTGTTG
FCA	K044	GBQ88_GBQ42_CauKiel_c3362	scaffold00120	GAATGGAGACTCACAGGATC
FCA	K045	GBQ88_GBQ42_CauKiel_c3362	scaffold00120	GGAGCGAATCTATTTGTTGG
FCA	K046	GBQ88_GBQ42_CauKiel_c3362	scaffold00120	CTGGCATCAAAGCACTTGTG
FLC	K059	GBQ88_GBQ42_CauKiel_c1903	scaffold00521	GTGTATGACCAAGCCCTTC
FLC	K060	GBQ88_GBQ42_CauKiel_c1903	scaffold00521	CAGTCAAAAGGACCTGGCC
FLC	K061	GBQ88_GBQ42_CauKiel_c1903	scaffold00521	GATTACAAGGGAAGAAGAGAACC
FLC	K062	GBQ88_GBQ42_CauKiel_c1903	scaffold00521	CAATACCTACAGCCATGATAAAG
FLC	K063	GBQ88_GBQ42_CauKiel_c1903	scaffold00521	CTCTCTGATTGACAAAAGGGAC

A. thaliana Homolog	Primer	Sugar Beet EST	Genome Scaffold	Sequence (5'->3')
FLC	K064	GBQ88_GBQ42_CauKiel_c1903	scaffold00521	ATCCTCTTACTGGCTTACCAAG
FLC	K065	GBQ88_GBQ42_CauKiel_c1903	scaffold00521	GACTTACACATCAGTATGAG
FLC	K066	GBQ88_GBQ42_CauKiel_c1903	scaffold00521	CTTGACTAGGTATGCCAGC
FRI	K113	GBQ88_GBQ42_CauKiel_c2059	scaffold00012	CATTAACCTCATTGCTCTG
FRI	K114	GBQ88_GBQ42_CauKiel_c2059	scaffold00012	CTAAACCTGGAAATGTATCC
FRI	K115	GBQ88_GBQ42_CauKiel_c2059	scaffold00012	GTGCATTGGGAGATGAAGG
FRI	K116	GBQ88_GBQ42_CauKiel_c2059	scaffold00012	CTTACAAGATGCCAGGTCAG
FVE	K155	GBQ88_GBQ42_CauKiel_c7625	scaffold00023	GGCTCGCTAACCACAATC
FVE	K156	GBQ88_GBQ42_CauKiel_c7625	scaffold00023	CGTCTGCATATCAAAGCATCA
FVE	K157	GBQ88_GBQ42_CauKiel_c7625	scaffold00023	CGTGACTCGTGTGGTGGTTG
FVE	K158	GBQ88_GBQ42_CauKiel_c7625	scaffold00023	CGAGCTTCTTCATTAAACTGC
GolS3	K051	GBQ88_GBQ42_CauKiel_c7651	scaffold00308	GAGCAGGTACTCATCATCTC
GolS3	K052	GBQ88_GBQ42_CauKiel_c7651	scaffold00308	GCTTCTGTAGGAGGATCAG
GolS3	K053	GBQ88_GBQ42_CauKiel_c7651	scaffold00308	GAAGTGGTGGGATATCTACAATG
GolS3	K054	GBQ88_GBQ42_CauKiel_c7651	scaffold00308	GCCAAGATTGGTATGGTCATG
hos 9	K001	GBQ88_GBQ42_CauKiel_c10075	scaffold00139	GAGATTTAACCACGCACATCTG
hos 9	K002	GBQ88_GBQ42_CauKiel_c10075	scaffold00139	CCCAAGTTGAAAGTCACG
HOS1	K145	GBQ88_GBQ42_CauKiel_c6314	scaffold00432	GTTGTGTATTATCTCCAGG
HOS1	K146	GBQ88_GBQ42_CauKiel_c6314	scaffold00432	TGCAACCACCAGATTGTC
HOS1	K147	GBQ88_GBQ42_CauKiel_c6314	scaffold00432	GAGCAGGATTGGTGGTAAG
HOS1	K148	GBQ88_GBQ42_CauKiel_c6314	scaffold00432	CTGCTCAAAAGAGCACCAC
HOS15	K081	GBQ88_GBQ42_CauKiel_c10981	scaffold00022	CATGAGATGTGTGCGTTTGG
HOS15	K082	GBQ88_GBQ42_CauKiel_c10981	scaffold00022	CTTGCAATACTAACACTTCCCC
HOS15	K083	GBQ88_GBQ42_CauKiel_c10981	scaffold00022	CAGGCCATCAGGTCTGTTTAC
HOS15	K084	GBQ88_GBQ42_CauKiel_c10981	scaffold00022	CCTTTGCAGTGCTATCATC
HSP70	K085	GBQ88_GBQ42_CauKiel_c1245	scaffold00052	GAACCGACGCATCACTGAAC
HSP70	K086	GBQ88_GBQ42_CauKiel_c1245	scaffold00052	GCAACATCGACCATTTTGAG

A. thaliana Homolog	Primer	Sugar Beet EST	Genome Scaffold	Sequence (5'->3')
HSP70	K087	GBQ88_GBQ42_CauKiel_c1245	scaffold00052	CGAAACAAGGGGATGAACG
HSP70	K088	GBQ88_GBQ42_CauKiel_c1245	scaffold00052	CCAACACAGGAGTAAGTGG
ICE1	K129	GBQ88_GBQ42_CauKiel_c5225	scaffold00843	GTTGTTTTGGGGCCGGGTTC
ICE1	K130	GBQ88_GBQ42_CauKiel_c5225	scaffold00843	CTGAACCACCCGAGGATTCTG
ICE1	K131	GBQ88_GBQ42_CauKiel_c5225	scaffold00843	CCACTACTAAACGAATGGGTC
ICE1	K132	GBQ88_GBQ42_CauKiel_c5225	scaffold00843	CACCTACTGTCAAGTGTCAAC
ICE1	S001	GBQ88_GBQ42_CauKiel_c5225	scaffold00843	CATGCCTCATCATCTTCCTC
ICE1	S002	GBQ88_GBQ42_CauKiel_c5225	scaffold00843	CGATGAGAGATTGAAGACGG
LEA14	K077	GBQ88_GBQ42_CauKiel_c3439	scaffold00001	GTGATATTCTGGATTACCCTG
LEA14	K078	GBQ88_GBQ42_CauKiel_c3439	scaffold00001	GGTAAGAAGAAATCTTGTGGAC
LEA14	K079	GBQ88_GBQ42_CauKiel_c3439	scaffold00001	GATGTAAGTGCATGTTTTGAGTG
LEA14	K080	GBQ88_GBQ42_CauKiel_c3439	scaffold00001	CCGGACAATATCACCCTGC
LEA4-5	K055	GBQ88_GBQ42_CauKiel_c1888	scaffold00780	CCTCTCGACTCATAATGG
LEA4-5	K056	GBQ88_GBQ42_CauKiel_c1888	scaffold00780	GTGTTGTACCACTAACTG
LEA4-5	K057	GBQ88_GBQ42_CauKiel_c1888	scaffold00780	CTTGTCAGGTGTGTGTCAC
LEA4-5	K058	GBQ88_GBQ42_CauKiel_c1888	scaffold00780	CGGTTGTACTAATTCATAGC
LHP1	K103	GBQ88_GBQ42_CauKiel_c16987	scaffold00062	GTGGTGAACCTAGCTGTTAC
LHP1	K104	GBQ88_GBQ42_CauKiel_c16987	scaffold00062	CTCTAGGTCTCTTCATCG
LHP1	K105	GBQ88_GBQ42_CauKiel_c16987	scaffold00062	GCCATTGTTGTAGAGCATG
LHP1	K106	GBQ88_GBQ42_CauKiel_c16987	scaffold00062	GGCAGAAGGATTAGCATAC
LHP1	K107	GBQ88_GBQ42_CauKiel_c16987	scaffold00062	GCACACCCTTATAAGGTGG
LHP1	K108	GBQ88_GBQ42_CauKiel_c16987	scaffold00062	GATGTGGAAGAATGAGTCAC
LOV1	K037	GBQ88_GBQ42_CauKiel_c16210	scaffold00183	GCCATCCATATCAGACTTAATG
LOV1	K038	GBQ88_GBQ42_CauKiel_c16210	scaffold00183	CACATATACTAGACTACCCG
LOV1	K039	GBQ88_GBQ42_CauKiel_c16210	scaffold00183	CAATTCCTCCAAGGCGGTG
LOV1	K040	GBQ88_GBQ42_CauKiel_c16210	scaffold00183	GCCATCAGGGTTTGAGAAG
LTI30	K167	GBQ88_GBQ42_CauKiel_lrc19699	scaffold00059	CTCCGACTGCAATTTTCG

A. thaliana Homolog	Primer	Sugar Beet EST	Genome Scaffold	Sequence (5'->3')
LTI30	K168	GBQ88_GBQ42_CauKiel_lrc19699	scaffold00059	CACTATGCCAACACTAATTG
LTI30	K169	GBQ88_GBQ42_CauKiel_lrc19699	scaffold00059	GAAACGGAGGAAGTATAAC
LTI30	K170	GBQ88_GBQ42_CauKiel_lrc19699	scaffold00059	TGGGAGCTCCAGTTCTAG
MYB15	K047	GBQ88_GBQ42_CauKiel_c14024	scaffold00030	GAAGTTTCCCCTCTTAATATCTGG
MYB15	K048	GBQ88_GBQ42_CauKiel_c14024	scaffold00030	CAATATCCACCCATTTCTCGTC
MYB15	K049	GBQ88_GBQ42_CauKiel_c14024	scaffold00030	CGTTGTAGAAGATTGTTGAG
MYB15	K050	GBQ88_GBQ42_CauKiel_c14024	scaffold00030	CTACAACTTTTCCCACATC
RAP2.1	K151	GBQ88_GBQ42_CauKiel_c6562	scaffold00619	TACTCCGCCGCCACCACC
RAP2.1	K152	GBQ88_GBQ42_CauKiel_c6562	scaffold00619	GGTTAGGTTCATATTCAACACCAG
RAP2.1	K153	GBQ88_GBQ42_CauKiel_c6562	scaffold00619	GCTGTATCATAAGCTCTTGC
RAP2.1	K154	GBQ88_GBQ42_CauKiel_c6562	scaffold00619	CTGCTATCGACAGTAAATACC
RD22	K149	GBQ88_GBQ42_CauKiel_c6499	scaffold00234	CCGAATTCCGAAGAAGCTAAGG
RD22	K150	GBQ88_GBQ42_CauKiel_c6499	scaffold00234	CACCATATATGCGGACGTAGCC
RD29a	K069	GBQ88_GBQ42_CauKiel_c1863	scaffold00357	GTCAGCACCCTTATCCACAG
RD29a	K070	GBQ88_GBQ42_CauKiel_c1863	scaffold00357	CAGATTTCTGCTGAAAGGGAG
RD29a	K071	GBQ88_GBQ42_CauKiel_c1863	scaffold00357	CACAACTGCCGACTTCGCATC
RD29a	K072	GBQ88_GBQ42_CauKiel_c1863	scaffold00357	CGACTCTCTCACTCACTCACAG
RD29b	K099	GBQ88_GBQ42_CauKiel_c14152	scaffold00081	CCAGATAACACCTCACATCAC
RD29b	K100	GBQ88_GBQ42_CauKiel_c14152	scaffold00081	CAAAGATGCTGCTGGTGCCTG
RD29b	K101	GBQ88_GBQ42_CauKiel_c14152	scaffold00081	CCTCCGTAGCCTAGTTTTG
RD29b	K102	GBQ88_GBQ42_CauKiel_c14152	scaffold00081	CAGGTGGTGAAGAAATAGGG
RLP33	K015	GBQ88_GBQ42_CauKiel_c14744	scaffold00357	GCACGTGCCAATCCAACTC
RLP33	K016	GBQ88_GBQ42_CauKiel_c14744	scaffold00357	GCTATAGGGCGATTACACTC
RLP33	K017	GBQ88_GBQ42_CauKiel_c14744	scaffold00357	GCGAGAGATTAAGATGAC
RLP33	K018	GBQ88_GBQ42_CauKiel_c14744	scaffold00357	CTTGTGGTATTGAAACTCC
SFR6	K137	GBQ88_GBQ42_CauKiel_c5737	scaffold00057	CTGCACCTCTAGGCCTAG
SFR6	K138	GBQ88_GBQ42_CauKiel_c5737	scaffold00057	CATTAGCGGAAATGTGCC

A. thaliana Homolog	Primer	Sugar Beet EST	Genome Scaffold	Sequence (5'->3')
SFR6	K139	GBQ88_GBQ42_CauKiel_c5737	scaffold00057	CAAACGCCCCTTATCCAC
SFR6	K140	GBQ88_GBQ42_CauKiel_c5737	scaffold00057	GTCCACATATGCAGGTATC
SOC1	K067	GBQ88_GBQ42_CauKiel_c1912	scaffold00184	GGAGAAGATGATGAGAGC
SOC1	K068	GBQ88_GBQ42_CauKiel_c1912	scaffold00184	CCACATCACATTCTTGCAC
SOC1	K109	GBQ88_GBQ42_CauKiel_c1912	scaffold00184	CTGTTAAATCTCATAAATGCAGC
SOC1	K110	GBQ88_GBQ42_CauKiel_c1912	scaffold00184	GAGTTCTGCAGCTCTTCTAG
SOC1	K111	GBQ88_GBQ42_CauKiel_c1912	scaffold00184	GATCCAGCATATGTTGACTCTG
SOC1	K112	GBQ88_GBQ42_CauKiel_c1912	scaffold00184	GGCGATAAAGGAGCTAGAAC
SOC1	K027	GBQ88_GBQ42_CauKiel_c1912	scaffold00184	CTTCACCCTCTACCTCATC
SOC1	K028	GBQ88_GBQ42_CauKiel_c1912	scaffold00184	GTAGTCTATCTGGTGGCTAG
SVP	K159	GBQ88_GBQ42_CauKiel_c7919	scaffold00041	GCTCTCTATTTCTCTGATCTG
SVP	K160	GBQ88_GBQ42_CauKiel_c7919	scaffold00041	CTTCCTCTTCCTGACATTTTTAC
SVP	K161	GBQ88_GBQ42_CauKiel_c7919	scaffold00041	CAGGAGCTAAGGTGATTATTG
SVP	K162	GBQ88_GBQ42_CauKiel_c7919	scaffold00041	CCTTTCAACTTTCTGCATGAG
VIN3	K163	GBQ88_GBQ42_CauKiel_c9002	scaffold00001	CATTGTGTACAGATGCTTTCAGG
VIN3	K164	GBQ88_GBQ42_CauKiel_c9002	scaffold00001	CTTGCATTCTGCTTGCTATTGTC
VIN3	K165	GBQ88_GBQ42_CauKiel_c9002	scaffold00001	GTTGCTAGTTGTGTCTTGTG
VIN3	K166	GBQ88_GBQ42_CauKiel_c9002	scaffold00001	CTCTGGCCTCAGAACAATG
VRN1	K141	GBQ88_GBQ42_CauKiel_c6065	scaffold00266	GCCACACTTCTTTGTTGAC
VRN1	K142	GBQ88_GBQ42_CauKiel_c6065	scaffold00266	GGGAAAAAGAAGATACACCC
VRN1	K143	GBQ88_GBQ42_CauKiel_c6065	scaffold00266	GTCTGGGGTTCTCTTTTC
VRN1	K144	GBQ88_GBQ42_CauKiel_c6065	scaffold00266	GATGGTACTTTCTGCCAGC
VRN2	K089	GBQ88_GBQ42_CauKiel_c12452	scaffold00240	AGCAGAGTATTTTGGGCG
VRN2	K090	GBQ88_GBQ42_CauKiel_c12452	scaffold00240	CTCCAACACCTGCAGAAG
VRN2	K091	GBQ88_GBQ42_CauKiel_c12452	scaffold00240	CCTGCTCTAATTTGGTAAG
VRN2	K092	GBQ88_GBQ42_CauKiel_c12452	scaffold00240	CTATTGACCACTAATAGAAGG
VRN2	K093	GBQ88_GBQ42_CauKiel_c12452	scaffold00240	GAACTCTTTCGTAAGGAAGC

***A. thaliana* Homolog**	**Primer**	**Sugar Beet EST**	**Genome Scaffold**	**Sequence (5'->3')**
VRN2	K094	GBQ88_GBQ42_CauKiel_c12452	scaffold00240	CAAGATAGAGATCCAGGAC
ZAT12	K073	GBQ88_GBQ42_CauKiel_c15677	scaffold00049	GACTGTGGAACCATGTTCC
ZAT12	K074	GBQ88_GBQ42_CauKiel_c15677	scaffold00049	GGTCTTGGATAAGAGGATTAG
ZAT12	K075	GBQ88_GBQ42_CauKiel_c15677	scaffold00049	CCAAGACCTCTAACTCGACC
ZAT12	K076	GBQ88_GBQ42_CauKiel_c15677	scaffold00049	GAGTACTCATATGGTTGGAG

Supplementary table 9: Characteristics of 17 cold responsive *A. thaliana* genes, their homologous *B. vulgaris* ESTs and the most homologous cold related sequences identified by *in silico* analysis of EST derived genomic sequence of *B. vulgaris* amplified in the parents of the mapping population SCK and SC2.

***A. thaliana* gene**	***B. vulgaris* EST**	**Putative *B. vulgaris* gene**	**Accession ID**	**Description**	**Function of identified homolog**	**Reference of tblastx Hit**	**e-value**	**Score**
FLC	GBQ88_GBQ42_CauKiel_c1903	*B.v.FL1*	EF036526	*Beta vulgaris FLC*-like 1 (*FL1*) gene, alternatively spliced	flowering time gene , effect on cold in *B. vulgaris*	Reeves et al., 2007	0	591
ice1	GBQ88_GBQ42_CauKiel_c5225	*B.v.ice1*	FJ379754	*Lotus japonicus* putative basic helix-loop-helix protein BHLH22	Activator of *CBF*	Karsas et al., 2009 (http://www.ncbi.nlm.nih.gov /nuccore/FJ379754)	$4.77E^{-32}$	243
rd22	GBQ88_GBQ42_CauKiel_c6499	*B.v.RD22*	XM_002516535	*Ricinus communis* Dehydration-responsive protein *RD22*	dehydration & heat response	Chan et al., 2009 (http://www.ncbi.nlm.nih.gov /nuccore/XM_002516535)	$2.63E^{-27}$	174
esk1	GBQ88_GBQ42_CauKiel_c3664	*B.v.ESK1*	NM_115457	*Arabidopsis thaliana* uncharacterized protein (*ESK1*)	CBF independent freezing tolerance	Xin et al., 2007	$4.06E^{-21}$	233
GolS3	GBQ88_GBQ42_CauKiel_c7651	*B.v.GolS3*	FJ407183	*Brassica napus* galactinol synthase (*GolS1*)	Raffinose synthetase	Li et al., 2011	$7.97E^{-20}$	54
VIN3	GBQ88_GBQ42_CauKiel_c9002	*B.v.VIN3*	XM_002283740	*Vitis vinifera* protein VERNALIZATION INSENSITIVE	flowering time gene (Vernalization pathway)	(http://www.ncbi.nlm.nih.gov /nuccore/XM_002283740)	$1.46E^{-19}$	221
FVE	GBQ88_GBQ42_CauKiel_c7625	*B.v.FVE*	AF498101	Arabidopsis thaliana *MSI4* (*FVE*) gene	regulator of *CBF* and flowering time gene (autonomous pathway)	Kim et al., 2004	$4.93E^{-18}$	131
FRI	GBQ88_GBQ42_CauKiel_c2059	*B.v.FRIGIDA*	XM_003634798	*Vitis vinifera* protein *FRIGIDA*-like	flowering time gene (Vernalization pathway)	http://www.ncbi.nlm.nih.gov/ nuccore/XM_003634798	$1.42E^{-15}$	193
rd29b	GBQ88_GBQ42_CauKiel_c14152	*B.v.rd29b*	AF035535	*Spinacia oleracea* cold acclimation protein (CAP160)	*COR* gene downstream of *CBF*	Kaye et al., 1998	$1.88E^{-15}$	116
MYB15	GBQ88_GBQ42_CauKiel_c14024	*B.v.MYB15*	FJ475127	*Panax ginseng* myb-related transcription factor (MYB) mRNA	regulator of *CBF*	Agarwal et al., 2006; (http://www.ncbi.nlm.nih.gov /nuccore/FJ475127)	$1.11E^{-12}$	42

***A. thaliana* gene**	***B. vulgaris* EST**	**Putative *B. vulgaris* gene**	**Accession ID**	**Description**	**Function of identified homolog**	**Reference of tblastx Hit**	**e-value**	**Score**
CCT2	GBQ88_GBQ42_CauKiel_c16204	*B.v.CCT2*	AB056715	*Arabidopsis thaliana AtCCT2* gene	Membrane remodeling	Inatsugi et al., 2009	$1.66E^{-11}$	40
hos1	GBQ88_GBQ42_CauKiel_c6314	*B.v.HOS1*	XM_002264715	*Vitis vinifera* E3 ubiquitin-protein ligase HOS1-like	regulator of CBF, Suppressor of flowering time gene *FLC*	Ishitani et al., 1998; (http://www.ncbi.nlm.nih.gov/nuccore/XM_002264715)	$2.61E^{-11}$	161
LHP1	GBQ88_GBQ42_CauKiel_c16987	*B.v.LHP1*	AB292419	*Malus* x *domestica MdLHP1*	flowering time gene (regulator of *FLC)*	Mimida et al., 2009	$1.39E^{-08}$	141
CBF1	GBQ88_GBQ42_CauKiel_c1913	*B.v.CBF1*	XM_002303265	*Populus trichocarpa* AP2/ERF domain-transcription factor (*DREB76*)	*CBF* gene	Tuskan et al., 2006	$1.44E^{-08}$	35
CBF2	GBQ88_GBQ42_CauKiel_lrc15447	*NPR1*	EF101866	*Beta vulgaris* RNA-directed DNA polymerase gene, partial cds; and heat stress transcription factor HSF, *NPR1* (*NPR1*)	dehydration & heat response	Kuykendall et al., 2009	$2.64E^{-07}$	33
RAP2.1	GBQ88_GBQ42_CauKiel_c6562	*B.v.DREB3*	JN711506	*Solanum tuberosum* dehydration-responsive element-binding protein *DREB3* (*DREB3*) mRNA	CBF gene	Bouaziz, D. Gargouri Bouzid, 2012 (http://www.ncbi.nlm.nih.gov/nuccore/358008881)	$2.97E^{-05}$	110
sfr6	GBQ88_GBQ42_CauKiel_c5737	*B.v.sfr6*	CU233237	*Populus* EST from severe drought-stressed opposite wood	dehydration & heat response	Leple et al., 2007 (http://www.ncbi.nlm.nih.gov/nuccore/CU233237)	$8.53E^{-03}$	72
hos 9	GBQ88_GBQ42_CauKiel_c10074	B.v.WUSCHEL-related homeobox 4-like	XM_002284891	*Vitis vinifera* WUSCHEL-related homeobox 4-like	CBF independent freezing tolerance	(http://www.ncbi.nlm.nih.gov/nuccore/XM_002284891)	0.07	24

Supplementary table 10: Marker information on eight candidate genes integrated into the linkage map.

putative *B. vulgaris* gene	Marker type	PCR Primers	EST	PCR fragment size in SCK [bp]	Detection	Marker allele in SCK [bp]	Marker allele in SC2 [bp]
CBF1	CAPS	K29/K30	GBQ88_GBQ42_CauKiel_c1913	493	Gel electrophoresis + Bful	493	297+196
LOV1	CAPS	K37/K38	GBQ88_GBQ42_CauKiel_c16210	700	Gel electrophoresis + PsyI	700	520+180
FRI	CAPS	K113/K114	GBQ88_GBQ42_CauKiel_c2059	600	Gel electrophoresis + AluI	400+200	600
LHP1	CAPS	K105/K106	GBQ88_GBQ42_CauKiel_c16987	600	Gel electrophoresis + EcoRI	600	400+200
GolS3	CAPS	K51/K52	GBQ88_GBQ42_CauKiel_c7651	520	Gel electrophoresis + TaiI	260+260	260+130+130
Hos1	CAPS	K145/K146	GBQ88_GBQ42_CauKiel_c6314	495	Gel electrophoresis + MluI	495	275+126
SVP	CAPS	K161/162	GBQ88_GBQ42_CauKiel_c7919	791	Gel electrophoresis + BsrI	791	245+504
FT1	SSR	N33/N34	-	620	Gel electrophoresis	620	700

Supplementary table 11: Details of the two Field test locations for winter-hardiness with 238 $F_{2:3}$ Families.

Environment	Year	Location	Coordinates	height above sea level [m]	Rep	Sowing Date
Gö 11/12	2011/12	Göttingen	51.47, 9.91	159	3	2011-08-29
Sö 11/12	2011/12	Söllingen	52.27,10.94	142	3	2011-08-30

Rep: Replication

Supplementary table 12: Daily precipitation, average and minimal daily temperatures recorded at the field trial location in Göttingen from August 2011 to April 2012.

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
August 1, 2011	0.0	18.1	14.0
August 2, 2011	0.0	19.2	10.2
August 3, 2011	0.8	20.3	12.9
August 4, 2011	1.7	21.4	16.7
August 5, 2011	2.3	20.3	16.6
August 6, 2011	3.4	20.3	14.5
August 7, 2011	0.8	18.0	14.3
August 8, 2011	3.2	15.0	13.0
August 9, 2011	8.8	14.6	12.8
August 10, 2011	0.1	15.4	11.7
August 11, 2011	0.0	20.0	16.0
August 12, 2011	3.4	17.9	16.0
August 13, 2011	0.2	17.7	15.1
August 14, 2011	5.5	18.7	15.6
August 15, 2011	0.0	17.7	13.1
August 16, 2011	0.1	15.5	10.4
August 17, 2011	0.0	19.9	12.8
August 18, 2011	5.0	21.5	14.1
August 19, 2011	18.9	17.4	11.6
August 20, 2011	0.0	16.6	8.4
August 21, 2011	0.4	18.7	9.7
August 22, 2011	0.2	19.7	15.3
August 23, 2011	0.4	21.5	15.0
August 24, 2011	1.8	21.6	16.5
August 25, 2011	1.3	20.0	15.0

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
August 26, 2011	0.7	23.5	17.1
August 27, 2011	0.5	15.1	13.1
August 28, 2011	0.5	14.9	11.9
August 29, 2011	0.2	13.9	10.1
August 30, 2011	0.2	13.1	9.9
August 31, 2011	0.2	13.8	8.1
September 1, 2011	0.2	12.9	6.9
September 2, 2011	0.0	15.9	5.9
September 3, 2011	0.0	19.6	11.0
September 4, 2011	0.4	20.0	14.3
September 5, 2011	0.2	17.5	13.0
September 6, 2011	0.1	16.8	12.2
September 7, 2011	0.1	15.1	13.0
September 8, 2011	0.1	12.9	11.8
September 9, 2011	0.2	16.5	12.7
September 10, 2011	0.3	20.6	16.7
September 11, 2011	0.1	19.9	15.7
September 12, 2011	0.2	17.7	13.6
September 13, 2011	0.1	18.0	14.3
September 14, 2011	0.1	14.4	11.3
September 15, 2011	0.1	13.0	6.8
September 16, 2011	0.2	12.5	4.3
September 17, 2011	0.2	16.8	12.0
September 18, 2011	0.1	12.9	8.9
September 19, 2011	0.1	11.5	7.0
September 20, 2011	0.1	12.5	5.6
September 21, 2011	0.0	13.6	6.6

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
September 22, 2011	0.2	15.0	9.0
September 23, 2011	0.0	11.9	6.7
September 24, 2011	0.0	11.7	4.3
September 25, 2011	0.0	13.6	5.2
September 26, 2011	0.0	16.5	8.0
September 27, 2011	0.6	16.3	11.5
September 28, 2011	0.0	15.1	9.6
September 29, 2011	0.0	14.0	7.2
September 30, 2011	0.0	15.1	6.0
October 1, 2011	0.0	15.2	6.4
October 2, 2011	0.0	15.5	7.0
October 3, 2011	0.0	16.5	7.7
October 4, 2011	0.0	17.8	12.5
October 5, 2011	0.0	16.8	14.7
October 6, 2011	5.0	14.4	8.7
October 7, 2011	6.1	8.9	7.4
October 8, 2011	3.9	7.3	3.5
October 9, 2011	3.8	6.9	1.4
October 10, 2011	0.4	14.2	8.7
October 11, 2011	10.5	14.5	10.3
October 12, 2011	9.0	9.8	8.1
October 13, 2011	0.1	7.2	2.1
October 14, 2011	0.1	4.2	1.0
October 15, 2011	0.0	4.5	-1.3
October 16, 2011	0.0	5.8	-1.1
October 17, 2011	0.0	6.6	1.4
October 18, 2011	1.4	8.9	5.9

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
October 19, 2011	0.7	7.9	5.7
October 20, 2011	0.0	5.7	1.1
October 21, 2011	0.0	3.7	0.3
October 22, 2011	0.0	3.3	-0.8
October 23, 2011	0.0	4.4	-2.0
October 24, 2011	0.0	7.4	-1.3
October 25, 2011	0.0	8.8	6.1
October 26, 2011	0.0	7.7	1.6
October 27, 2011	0.0	6.8	-1.2
October 28, 2011	0.0	10.3	5.6
October 29, 2011	0.0	9.4	4.3
October 30, 2011	0.1	11.3	8.9
October 31, 2011	0.0	10.7	5.8
November 1, 2011	0.0	8.8	4.7
November 2, 2011	0.0	8.9	7.6
November 3, 2011	0.0	10.2	6.6
November 4, 2011	0.0	9.1	4.9
November 5, 2011	0.0	7.3	2.3
November 6, 2011	0.0	6.9	1.2
November 7, 2011	0.0	6.1	1.7
November 8, 2011	0.1	3.6	2.3
November 9, 2011	0.0	4.7	2.6
November 10, 2011	0.0	4.9	2.3
November 11, 2011	0.0	3.0	1.0
November 12, 2011	0.0	0.5	-4.0
November 13, 2011	0.1	-0.9	-4.3
November 14, 2011	0.0	-1.4	-5.0

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
November 15, 2011	0.1	-1.6	-4.9
November 16, 2011	0.0	0.4	-1.0
November 17, 2011	0.0	1.8	0.1
November 18, 2011	0.0	5.1	1.9
November 19, 2011	0.0	5.2	0.2
November 20, 2011	0.0	3.7	0.2
November 21, 2011	0.0	4.0	-1.1
November 22, 2011	0.0	1.6	-3.1
November 23, 2011	0.1	3.5	0.5
November 24, 2011	0.0	4.6	1.6
November 25, 2011	0.0	2.9	0.2
November 26, 2011	0.0	6.4	4.3
November 27, 2011	0.0	7.3	6.1
November 28, 2011	0.1	3.7	-1.8
November 29, 2011	0.1	2.4	-2.2
November 30, 2011	0.0	6.2	1.3
December 1, 2011	1.5	7.3	1.3
December 2, 2011	2.2	8.0	2.8
December 3, 2011	5.8	5.3	2.0
December 4, 2011	0.6	7.3	6.1
December 5, 2011	4.2	3.4	2.3
December 6, 2011	1.7	2.9	2.0
December 7, 2011	4.4	4.3	1.6
December 8, 2011	1.2	5.9	4.5
December 9, 2011	5.5	5.5	2.7
December 10, 2011	0.9	2.8	1.2
December 11, 2011	0.0	1.0	0.1

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
December 12, 2011	3.8	4.6	1.7
December 13, 2011	4.3	6.0	4.7
December 14, 2011	2.3	6.2	4.5
December 15, 2011	0.3	5.2	4.2
December 16, 2011	9.5	3.9	1.7
December 17, 2011	1.2	3.2	2.4
December 18, 2011	0.7	2.0	1.5
December 19, 2011	0.2	1.2	0.1
December 20, 2011	2.1	1.4	0.2
December 21, 2011	1.4	4.0	3.4
December 22, 2011	6.3	4.5	3.3
December 23, 2011	0.3	8.0	7.4
December 24, 2011	3.1	4.7	3.0
December 25, 2011	0.4	5.0	3.3
December 26, 2011	0.0	8.0	7.1
December 27, 2011	0.3	7.9	6.1
December 28, 2011	0.1	5.8	4.9
December 29, 2011	9.7	4.8	3.5
December 30, 2011	9.7	3.0	2.2
December 31, 2011	1.1	3.1	1.6
January 1, 2012	0.8	10.3	6.0
January 2, 2012	10.2	8.2	3.6
January 3, 2012	5.3	6.6	3.8
January 4, 2012	2.7	5.2	3.8
January 5, 2012	19.9	4.7	2.6
January 6, 2012	4.2	4.2	3.3
January 7, 2012	11.4	4.4	2.9

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
January 8, 2012	5.8	4.5	3.6
January 9, 2012	2.7	5.2	3.9
January 10, 2012	0.7	5.6	3.5
January 11, 2012	0.0	7.6	6.4
January 12, 2012	2.8	7.2	3.9
January 13, 2012	5.3	3.3	1.1
January 14, 2012	0.4	2.2	-0.9
January 15, 2012	0.2	-1.2	-3.8
January 16, 2012	0.0	-0.9	-2.7
January 17, 2012	0.0	0.1	-2.0
January 18, 2012	2.2	0.5	-4.6
January 19, 2012	8.7	4.2	2.5
January 20, 2012	3.4	2.8	1.7
January 21, 2012	12.2	3.5	0.6
January 22, 2012	9.5	4.8	3.9
January 23, 2012	2.8	3.5	2.1
January 24, 2012	0.1	2.6	1.3
January 25, 2012	0.0	1.1	-1.8
January 26, 2012	0.0	0.6	0.1
January 27, 2012	0.5	0.8	0.1
January 28, 2012	0.0	0.5	-1.4
January 29, 2012	0.0	-2.4	-4.5
January 30, 2012	0.0	-4.5	-6.8
January 31, 2012	0.0	-6.6	-8.7
February 1, 2012	0.0	-8.1	-10.7
February 2, 2012	0.0	-10.9	-14.3
February 3, 2012	0.0	-10.8	-14.7

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
February 4, 2012	0.0	-10.2	-14.5
February 5, 2012	0.0	-11.6	-15.9
February 6, 2012	0.0	-13.3	-17.6
February 7, 2012	0.0	-12.7	-19.0
February 8, 2012	0.0	-7.2	-12.2
February 9, 2012	0.5	-7.0	-13.4
February 10, 2012	0.1	-8.5	-14.7
February 11, 2012	0.0	-10.8	-15.0
February 12, 2012	0.0	-8.2	-15.6
February 13, 2012	2.7	-2.2	-4.2
February 14, 2012	1.9	0.1	-1.8
February 15, 2012	4.7	1.9	0.3
February 16, 2012	0.5	1.0	-2.7
February 17, 2012	0.4	3.9	2.5
February 18, 2012	0.0	4.3	1.6
February 19, 2012	2.3	2.0	0.2
February 20, 2012	0.0	0.7	-1.5
February 21, 2012	0.0	1.8	0.2
February 22, 2012	0.0	4.4	1.1
February 23, 2012	0.3	5.0	2.4
February 24, 2012	2.1	7.1	5.6
February 25, 2012	0.0	4.4	2.7
February 26, 2012	0.1	2.9	-2.3
February 27, 2012	0.0	1.9	-3.4
February 28, 2012	0.0	6.2	3.9
February 29, 2012	0.0	8.5	7.4
March 1, 2012	0.0	8.2	6.4

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
March 2, 2012	0.2	5.8	1.2
March 3, 2012	0.0	5.5	0.2
March 4, 2012	0.0	7.8	5.4
March 5, 2012	0.2	4.8	2.2
March 6, 2012	0.1	3.2	-1.8
March 7, 2012	2.9	3.6	-2.0
March 8, 2012	1.5	4.4	0.4
March 9, 2012	0.0	4.9	0.0
March 10, 2012	0.0	8.3	6.4
March 11, 2012	0.0	8.0	6.8
March 12, 2012	0.0	7.6	6.5
March 13, 2012	0.0	6.7	5.7
March 14, 2012	0.0	5.8	4.9
March 15, 2012	0.0	7.2	2.7
March 16, 2012	0.0	10.0	0.8
March 17, 2012	0.0	12.9	6.2
March 18, 2012	3.9	8.8	5.3
March 19, 2012	0.0	6.1	1.1
March 20, 2012	0.0	6.5	-0.1
March 21, 2012	0.0	9.2	1.9
March 22, 2012	0.0	8.6	2.8
March 23, 2012	0.0	8.8	1.2
March 24, 2012	0.0	10.0	1.7
March 25, 2012	0.0	9.3	0.8
March 26, 2012	0.0	8.8	0.9
March 27, 2012	0.0	9.1	0.9
March 28, 2012	0.0	11.4	2.9

Date	Precipitation [mm]	Average daily temperature in 2 m height [°C]	Minimum daily temperature in 2 m height [°C]
March 29, 2012	0.0	8.4	6.6
March 30, 2012	0.3	7.8	6.8
March 31, 2012	0.9	5.6	0.1
April 1, 2012	0.0	3.8	-2.8
April 2, 2012	0.0	6.3	1.5
April 3, 2012	0.0	7.7	-0.8

Supplementary table 13: Calculation of Delta K for subpopulation. Table output of the Evanno method results. Shown are the number of subpopulations k, the mean Log probability and the respective standard deviation (SD), as well as the Delta K (ΔK).

# k	Mean L(K)	SD L(K)	ΔK
1	-8098.50	0.81	NA
2	-7837.60	4.61	21.76
3	-7676.98	2.76	60.04
4	-7681.92	21.98	0.85
5	-7668.15	28.68	14.82
6	-8079.55	105.92	0.35
7	-8454.38	51.58	8.30
8	-8401.23	82.29	0.25

Supplementary table 14: Results of EcoTILLING screens in the three genes *BvBTC1*, *BvFL1*, and *BvFL1*. Listed for each amplicon are the number of successfully screened accessions, the number of detected SNPs, the corresponding SNP density, the number of detected haplotypes, the mean frequency of the reference haplotype H0 from accession 93161P and the range of non-reference haplotype frequency (NHF). SNP densities were calculated as the number of polymorphic SNP loci divided by the total length of screened sequence in kb. NHF were calculated for each haplotype as the number of accessions with haplotype deviating from reference haplotype H0.

Amplicon	**screened accessions**	**Detected SNPs**	**SNP density**	**Haplotypes detected per amplicon**	**Mean frequency of H0**	**Range of NHF**
FL1a	219	3	3.28	7	0.77	0.03 – 0.38
FL1b	239	7	9.82	18	0.57	0.01 - 0.32
FT1a	242	4	4.09	12	0.55	0.01 – 0.20
FT1b	248	5	7.91	14	0.49	0.01 – 0.33
BTC1	237	2	2.01	4	0.87	0.01 – 0.28
Over all amplicons		21	5.3*	55	0.65	

* mean SNP density over all amplicons

Supplementary table 15: Non-reference nucleotide frequencies (NNFs) in three flowering time genes BvFL1, BvFT1, and BvBTC1 in divergent B. vulgaris forms. Listed is the NNF for each gene and each B. vulgaris form as well as the mean NNF for the entire panel. NNFs were calculated as the number of accessions with an allele carrying a SNP (compared to the reference allele in 93161P) divided by the total number of screened accessions.

Gene	NNF Sugar beet	NNF Fodder beet	NNF Garden beet	NNF Leaf beet	NNF BVM[a)]	Mean NNF
BvFL1	0.05	0.11	0.04	0.36	0.55	0.18
BvFT1	0.14	0.21	0.12	0.15	0.23	0.17
BvBTC1	0.05	0.03	0.04	0.06	0.3	0.07

a) BVM = *Beta vulgaris* ssp. *maritima*

Supplementary table 16: Statistics of haplotypes for the two amplicons of BvFL1 with significant differences in bolting rate (BR) compared with the respective reference haplotypes (FL1a_H0 or FL1b_H0). Shown are the observed haplotypes, their occurrence (n) in each B. vulgaris form, the average BR, and the corresponding p-value for comparison with the respective reference haplotype. The p-value is Bonferroni corrected to account for the experiment-wise error rate.

haplotype		**Sugar beet**	**Fodder beet**	**Garden beet**	**Leaf beet**	**BVM[b)]**
All	average BR before winter	0%	1%	0%	4%	8%
FL1a_H0	n[a)]	66	32	42	20	7
	average BR before winter	0%	1%	1%	5%	1%
FL1a_H6	n	-	4	-	5	3
	average BR before winter	-	0%	-	5%	55%
	p-Value	-	0.93	-	1	<1e-06
FL1b_H0	n	64	16	40	12	5
	average BR before winter	0%	1%	0%	8%	2%
FL1b_H5	n	-	-	-	-	1
	average BR before winter	-	-	-	-	0.75
	p-Value	-	-	-	-	<1e-04
FL1b_H6	n	-	-	2	4	2
	average BR before winter	-	-	6%	0%	0%
	p-Value	-	-	<1e-09	0.461	1
FL1b_H10	n	-	1	1	2	2
	average BR before winter	-	0%	0%	0%	11%
	p-Value	-	1	1	0.434	0.0356

a) n: Number of accessions carrying the given haplotype

b) BVM = *Beta vulgaris* ssp. *maritima*

Supplementary table 17: Comparison of average gene diversity (Ht) and standard errors (SE) within amplicons of divergent *B. vulgaris* forms. Shown are the gene diversity (Ht) and the standard error (SE) value for each amplicon and cultivated form, all amplicons and cultivated form, as well as for the whole panel. Gene diversity was calculated with the genetic distance and phylogenetic analysis package DISPAN (Ota, 1993).

	Cultivated Forms															
	Sugar beet		**SBEBM[a)]**		**SBGP[b)]**		**Fodder beet**		**Garden beet**		**Leaf beet**		**BVM[c)]**		**Whole panel**	
Amplicons	**Ht**	SE	**Ht**	SE	**Ht**	SE	**Ht**	SE	**Ht**	SE	**Ht**	SE	**Ht**	SE	**Ht**	SE
FL1a	**0.03**	0.01	**0**	0	**0.07**	0.03	**0.1**	0.04	**0.02**	0.02	**0.38**	0.03	**0.51**	0.01	**0.21**	0.02
FL1b	**0.12**	0.02	**0.08**	0.04	**0.32**	0.05	**0.2**	0.07	**0.12**	0.02	**0.46**	0.04	**0.47**	0.02	**0.34**	0.04
FT1a	**0.28**	0.04	**0.38**	0.08	**0.17**	0.01	**0.34**	0.04	**0.26**	0.02	**0.27**	0.02	**0.34**	0.07	**0.3**	0.04
FT1b	**0.26**	0.06	**0.33**	0.07	**0.16**	0.06	**0.29**	0.1	**0.16**	0.07	**0.22**	0.08	**0.3**	0.07	**0.25**	0.07
BTC1	**0.06**	0.04	**0.04**	0.04	**0.19**	0.1	**0.05**	0.05	**0.09**	0.05	**0.12**	0.04	**0.4**	0.11	**0.15**	0.06
All Amplicons	**0.17**	0.03	**0.19**	0.04	**0.2**	0.03	**0.22**	0.04	**0.14**	0.03	**0.32**	0.03	**0.4**	0.03	**0.27**	0.03

a) SBEBM = Sugar beet elite breeding material
b) SBGP = Sugar beet germplasm
c) BVM = *Beta vulgaris* ssp. *maritima*

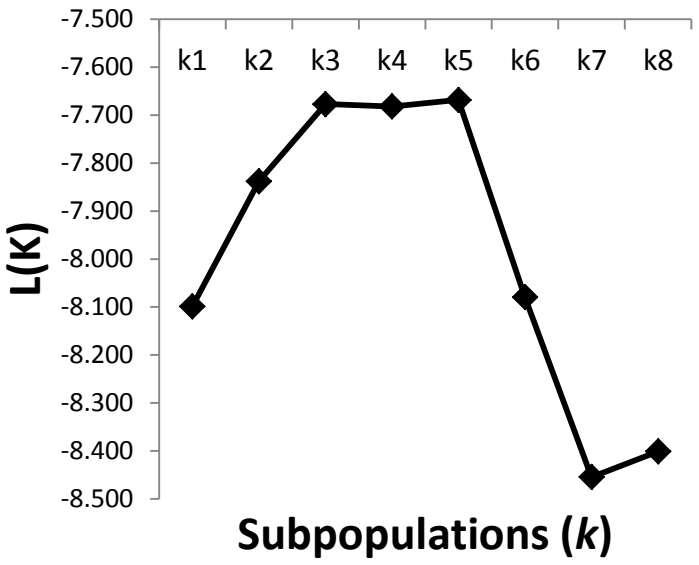
Supplementary table 18: Statistics of haplotypes for the two amplicons of *BvFL1* with significant differences in survival rate (SR) compared with the respective reference haplotypes (FL1a_H0 or FL1b_H0). Shown are the observed haplotypes, their occurrence (n) in each *B. vulgaris* form, the average SR, and the corresponding p-value for comparison with the respective reference haplotype. The p-value is Bonferroni corrected to account for the experiment-wise error rate.

		Sugar beet	**Fodder beet**	**Garden beet**	**Leaf beet**	**BVM[b)]**
All	average SR	40%	23%	20%	29%	40%
FL1a_H0	n[a)]	66	32	42	20	7
	average SR	39%	23%	21%	22%	39%
FL1a_H6	n	-	4	-	5	3
	average SR	-	21%	-	35%	13%
	p-Value	-	1	-	0.339	0.0143
FL1b_H0	n	64	16	40	12	5
	average SR	39%	27%	20%	19%	36%
FL1b_H3	n	1	-	-	9	4
	average SR	40%	-	-	37%	43%
	p-Value	1	-	-	0.0116	1

Supplementary table 19: Literature survey of previously published QTL studies on frost tolerance.

Species	Reference	number of genotypes	number of markers	number of detected QTL	experimental conditions	Nursery	Cold acclimation	Frost	Recovery cool	Recovery warm
faba bean (*Vicia faba*)	Arbaoui et al.; 2007	101	108	2						
					Number of days	14	7	5.5	0.42	-
					Daylength day/ night [h]	n.a.	12/12	12/12	12/12	-
					Temperature day/night [°C]	10/3	2.5/0	2.5/ 6 steps from -7°C to -21°C	2.5/-	-
					Light intensity [µmol/m^{2}s]	natural light	200	200	200	-
pea (*Pisum sativum*)	Lejeune-Henaut et al.; 2009	164	363	6						
					Number of days	11	11	4	-	8
					Daylength day/ night [h]	10/14	10/14	8/16	-	10/14
					Temperature day/night [°C]	19/12	10/2	6/-8	-	16/5
					Light intensity [µmol/m^{2}s]	300	250	250	-	300
Barley (*Hordeum vulgare*)	Francia *et al.*; 2004	136	128	2						
					Number of days	42	7+2	2	0.3 + 0.7	18
					Daylength day/ night [h]	20-8/4-16	20/4 + 0/24	0/24	0/24	14/10
					Temperature day/night [°C]	15-2/10--2	2/-2 + -4/-4	-10 and -12	thawing by increasing temperature by 2°C/h to 1°C	16/15

Species	Reference	number of genotypes	number of markers	number of detected QTL	experimental conditions	Nursery	Cold acclimation	Frost	Recovery cool	Recovery warm
Arabidopsis thaliana	Gery et al.; 2011	2x164	na	2-6	Light intensity [µmol/m^{2}s]	260	260	-	-	na
					Number of days	14	7	2	-	
					Daylength day/ night [h]	na	12/12		-	na
					Temperature day/night [°C]	na	5/5	-8	-	na
					Light intensity [µmol/m^{2}s]	natural light	70	na	-	natural light



Supplementary figure 1: Mean Log probability *L*(*K*) of results from six parallel calculations for each hypothetic number of subpopulations (*k*) in the range of *k*=1 to *k*=8. The *x-axis* shows subpopulations (*k*). The *y*-axis shows the Log probability *L*(*K*).

A)

```
FL1a_H0         TCGGACTTTCCCTATAAGCTTAAGAAAATAAATGAATGATACTATAAATAGATAAAAATA
FL1a_H6         TCGGACTTTCCCTATAAGCTTAAGAAAATAAATGAATGATACTATAAATAGATAAAAATA

FL1a_H0         GCCTAAATAGGGCATCCACAGCTAGTAAGGAGATAAACTGATAAACTAAAAGTCTAAAAC
FL1a_H6         GCCTAAATAGGGCATCCACAGCTAGTAAGGAGATAAACTGATAAACTAAAAGTCTAAAAC

FL1a_H0         TAAAACGAAAACGATAAGTTGCACTAAAATCTAATACTTATCTAACATTTCCACGTGTAA
FL1a_H6         TAAAACGAAAACGATAAGTTGCACTAAAATCTAATACTTATCTAACATTTCCACGTGTAA

FL1a_H0         TAAATAAAACATGGGGCCCAATGATTAATCTTCAACTGACCTCTCCCTCCAATCGCGTAT
FL1a_H6         TAAATAAAACATGGGGCCCAATGATTAATCTTCAACTGACCTCTCCCTCCAATCGCGTAT

FL1a_H0         TCTAAAAATCATTTCACTATCTCTTTCATCTTTTTTTTTCCGGCATTTTTTTTTCATTTC
FL1a_H6         TCTAAAAATCATTTCACTATCTCTTTCATCTTTTTTTTTCCGGCATTTTTTTTTCATTTC

FL1a_H0         CTCTCTCTCTTACTCTATACTACTCTAGGGTCTATATGATACTAGTATTCAGTAGTAGCA
FL1a_H6         CTCTCTCTCTTACTCTATACTACTCTAGGGTCTATATGATACTAGTATTCAGTAGTAGCA

FL1a_H0         GTAATAGAAGTACTATCAGTTTTCTCTTCCTTTTGAAATAAAATTAGTATTCTATTCAAT
FL1a_H6         GTAATAGAAGTACTATCAGTTTTCTCTTCCTTTTGAAATAAAATTAGTATTCTATTCAAT

FL1a_H0         TAAAATCGATCCACTAGTCTAAATACTAGTACTATTATCAGAGGAGAAGAAAGGACAGAG
FL1a_H6         TAAAATCGATCCACTAGTCTAAATACTAGTACTATTATCAGAGGAGAAGAAAGGACAGAG

FL1a_H0         AGAGTGAGAGAAATTGCAGCGACGAAGACAGAGAAAGGTATTTGGATAAGGATGGGAAGA
FL1a_H6         AGAGTGAGAGAAATTGCAGCGACGAAGACAGAGAAAGGTATTTGGATAAGGATGGGAAGA

FL1a_H0         AGGAAGATAGAGATGAAAAGAATTGAAGATAAAAGTAGTCGTCARGTTACATTTTCAAAG
FL1a_H6         AGGAAGATAGAGATGAAAAGAATTGAAGATAAAAGTAGTCGTCARGTTACATTTTCAAAG

FL1a_H0         CGGCGTTCTGGTCTTATCAAAAAAGCTCGCGAACTCTCTATCCTTTGTGATGTCGATGTT
FL1a_H6         CGGCGTTCTGGTCTTATCAAAAAAGCTCGCGAACTCTCTATCCTTTGTGATGTCGATGTT

FL1a_H0         GCTGTTCTTGTTTTCTCTAATCGTGGCCGTCTTTACGAATTCGTCAATAGTTCTTCTTCT
FL1a_H6         GCTGTTCTTGTTTTCTCTAATCGTGGCCGTCTTTACGAATTCGTCAATAGTTCTTCTTCT

FL1a_H0         TCCAGGTTTTTCTTCATCTCTTTCCTTGTTATTAGTTTTTTCTTTTTCAATTTTCAAGTT
FL1a_H6         TCCAGGTTTTTCTTCATCTCTTTCCTTGTTATTAGTTTTTTCTTTTTCAATTTTCAAGTT
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FL1a_H0
TAATTTTAATGGTATGCTTTGTTGAATAGTTTCGGTTGTTAATGGCGGAATTTTGTTGTT
FL1a_H6
TAATTTTAATGGTATGCTTTGTTGAATAGTTTCGGTGGTTAATGGCGGAATTTTGTTGTT
                                                          *

FL1a_H0
TAGTTTTTTCGGTGTGTTTTGTTGTTTTGTGATTCTAGGTTTTAGATGATTTTGCTTGAT
FL1a_H6
TAGTTTTTTCGGTGTGTTTTGTTGTTTTGTGATTCTAGGTTTTAGATGATTTTGCTTGAT


FL1a_H0
TTCATGAATTTTGAATTTTGGAGGTTTTTGGTTTTACCGTATAAAATTGAATGATTTTGT
FL1a_H6
TTCATGAATTTTGAATTTTGGAGGTTTTTGGTTTTACCGTATAAAATTGAATGATTTTGT


FL1a_H0          CTGTAACGATTCACGTG
FL1a_H6          CTGTAACGATTCACGTG
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B)

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FL1b_H0
GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTATT
FL1b_H03
GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTATT
FL1b_H05
GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTATT
FL1b_H06
GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTATT
FL1b_H10
GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTATT


FL1b_H0
CAAGGCCATGAATAAATTTTCCTACTTTGAATATTCATAATTTCATATCCTTCATGCTTC
FL1b_H03
CAAGGCCATGAATAAATTTTCCTACTTTGAATATTCATAATTTCATATCCTTCATGCTTC
FL1b_H05
CAAGGCCATGAATAAATTTTCCTACTTTGAATATTCATAATTTCATATCCTTCATGCTTC
FL1b_H06
CAAGGCCATGAATAAATTTTCCTACTTTGAATATTCATAATTTCATATCCTTCATGCTTC
FL1b_H10
CAAGGCCATGAATAAATTTTCCTACTTTGAATATTCATAATTTCATATCCTTCATGCTTC


FL1b_H0
TGTCCGTACAACTTTTCGTTCTATTCTCTGACAATTCTGGAGTTCTTTTTGCTTTTGATA
FL1b_H03
TGTCCGTACAACTTTTCGTTCTATTCTCTGACAATTCTGGAGTTCTTTTTGCTTTTGATA
FL1b_H05
TGTCCGTACAACTTTTCGTTCTATTCTCTGACAATTCTGGAGTTCTTTTTGCTTTTGATA
FL1b_H06
TGTCCGTACAACTTTTCGTTCTATTCTCTGACAATTCTGGAGTTCTTTTTGCTTTTGATA
FL1b_H10
TGTCCGTACAACTTTTCGTTCTATTCTCTGACAATTCTGGAGTTCTTTTTGCTTTTGATA


FL1b_H0
GCAGAGTTCACCTTCTAGTTGTGCAGAAGTTCAAACATGTGGTGAGCTAGTAAAATCAGT
FL1b_H03
GCAGAGTTCACCTTCTAGTTGTGCAGAAGTCCAAACATGTGGTGAGCTAGTAAAATCAGT
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FL1b_H05
GCAGAGTTCACCTTCTAGTTGTGCAGAAGTCCAAACATGTGGTGAGCTAGTAAAATCAGT
FL1b_H06
GCAGAGTTCACCTTCTAGTTGTGCAGAAGTCCAAACATGTGGTGAGCTAGTAAAATCAGT
FL1b_H10
GCAGAGTTCACCTTCTAGTTGTGCAGAAGTTCAAACATGTGGTGAGCTAGTAAAATCAGT
                                                 *

FL1b_H0
TGAAGGGCAAGTACTCAATATTCTATTTACTTCTGACGATGACTTCTCCATGTTCCATAA
FL1b_H03
TGAAGGGCAAGTACTCAATTTTCTATTTACTTCTGACGATGACTTCTCCATGTTCCATAA
FL1b_H05
TGAAGGGCAAGTACTCAATTTTCTATTTACTTCTGACGATGACTTCTCCATGTTCCATAA
FL1b_H06
TGAAGGGCAAGTACTCAATTTTCTATTTACTTCTGACGATGACTTCTCCATGTTCCATAA
FL1b_H10
TGAAGGGCAAGTACTCAATTTTCTATTTACTTCTGACGATGACTTCTCCATGTTCCATAA
                                      *

FL1b_H0
TTATGGTCAACTTACAGGTACCTAGAAGGACCAGAGCTTGAAAATCTTAGGCTTGAGGAC
FL1b_H03
TTATGGTCAACTTACAGGTACCTAGAAGGACCAGAGCTTGAAAATCTTAGGCTTGAGGAC
FL1b_H05
TTATGGTCAACTTACAGGTACCTAGAAGGACCAGAGCTTGAAAATCTTAGGCTTGAGGAC
FL1b_H06
TTATGGTCAACTTACAGGTACCTAGAAGGACCAGAGCTTGAAAATCTTAGGCTTGAGGAC
FL1b_H10
TTATGGTCAACTTACAGGTACCTAGAAGGACCAGAGCTTGAAAATCTTAGGCTTGAGGAC
FL1b_H0
TTCATGAGGCTGGAGAGGCAACTAGCTGATGCCCTTATACAGACCAGAACCCGAAAGGTT
FL1b_H03
TTCATGAGGCTGGAGAGGCAACTAGCTGATGCCCTTGTACAGACCAGAACCCGAAAGGTT
FL1b_H05
TTCATGAGGCTGGAGAGGCAACTAGCTGATGCCCTTATACAGACCAGAACCCGAAAGGTT
FL1b_H06
TTCATGAGGCTGGAGAGGCAACTAGCTGATGCCCTTATACAGACCAGAACCCGAAAGGTT
FL1b_H10
TTCATGAGGCTGGAGAGGCAACTAGCTGATGCCCTTGTACAGACCAGAACCCGAAAGGTT
                                                        *

FL1b_H0
CTCTTCTTCCCTTGTAAACCATTTAGTTTGATGATCTACCACGTGGTTGGGTTGGAAGCT
FL1b_H03
CTCTTCTTCCCTTGTAAACCATTTAGTTTGATGATCTACCACGTGGTTGGGTTGGAAGCT
FL1b_H05
CTCTTCTTCCCTTGTAATCCATTTAGTTTGATGATCTACCACGTGGTTGGGTTGGAAGCT
FL1b_H06
CTCTTCTTCCCTTGTAATCCATTTAGTTTGATGATCTACCACGTGGTTGGGTTGGAAGCT
FL1b_H10
CTCTTCTTCCCTTGTAATCCATTTAGTTTGATGATCTACCACGTGGTTGGGTTGGAAGCT
                                     *

FL1b_H0
GGATAGTTTATATGTCTTTACCCTTTCTCTATGCTTACACTTTGCATATTTGATGGATGG
FL1b_H03
GGATAGTTTATATTTCTTTACCCTTTTTCTACGCTTACACTTTGCATATTTGATGGATGG
FL1b_H05
GGATAGTTTATATTTCTTTACCCTTTCTCTACGCTTACACTTTGCATATTTGATGGATGG
FL1b_H06
GGATAGTTTATATGTCTTTACCCTTTCTCTACGCTTACACTTTGCATATTTGATGGATGG
FL1b_H10
GGATAGTTTATATTTCTTTACCCTTTTTCTACGCTTACACTTTGCATATTTGATGGATGG
                                  *            *    *
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FL1b_H0
AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG
FL1b_H03
AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG
FL1b_H05
AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG
FL1b_H06
AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG
FL1b_H10
AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG


FL1b_H0          ACTGTATTTTATGCATCGTGGTGTTGGAGTCA
FL1b_H03         ACTGTATTTTATGCATCGTGGTGTTGGAGTCA
FL1b_H05         ACTGTATTTTATGCATCGTGGTGTTGGAGTCA
FL1b_H06         ACTGTATTTTATGCATCGTGGTGTTGGAGTCA
FL1b_H10         ACTGTATTTTATGCATCGTGGTGTTGGAGTCA
```

Supplementary figure 2: Sequences of the BvFL1 haplotypes with impact on survival and bolting rate. A) Sequence alignment of reference haplotype FL1a_H0 and FL1b_H6. B) Multiple sequence alignment of reference haplotype FL1b_H0, FL1b_H3, FL1b_H5, FL1b_H6, and FL1b_H10. Asterisks indicate a single nucleotide polymorphism. Reference nucleotides are marked in yellow and changed nucleotides are marked in red.

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DON`T PANIC

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13 Declaration of co-authorship

The chapters of this thesis are published (Chapter 2), in review (Chapter 5) or in preparation (Chapters 3 and 4).

The PhD candidate Martin Kirchhoff contributed to the publication as follows:

Chapter 2: High degree of genetic variation of winter hardiness in a panel of *Beta vulgaris* L.

Kirchhoff, M., A. Svirshchevskaya, C. Hoffmann, A. Schechert, C. Jung, and F.J. Kopisch Obuch. 2012. High Degree of Genetic Variation of Winter Hardiness in a Panel of Beta vulgaris L. Crop Sci. 52:179-188.

- Design, planning, preparation and conduction of field experiments in Germany 80%
- Data Collection in Germany 100%
- Design, planning and preparation of experiments trails in Belarus 50%
- Statistical analysis and interpretation of Data 85%
- Preparation of Manuscript, Revision of the manuscript including all figures and tables under supervision of Dr. Friedrich J. Kopisch-Obuch 100%

Chapter 3: Genetic dissection of frost tolerance in sugar beets (*B. vulgaris* L.): climate chamber trials

- Design, planning, preparation and conduction of artificial frost test 100 %
- Statistical analysis and data interpretation 100%
- Bioinformatic identification of sugar beet candidate genes with homologies to cold regulated genes in *A. thaliana* 100%
- Amplification and anchoring of candidate sequences in the parental inbred lines used for the development of the mapping population 85%
- Construction of the Linkage Maps including AFLP analysis, development of anchor markers and markers for candidate genes 100%
- QTL Analysis 100%
- Preparation of Manuscript including all figures and tables under supervision of Dr. Friedrich J. Kopisch-Obuch 100%

Chapter 4: Mapping of quantitative trait loci for winter-hardiness in sugar beets (*B. vulgaris* L.): field trials

- Design, planning, preparation and conduction of field experiments 100%
- Collection, statistical analysis and interpretation of field data 100%
- QTL Analysis 100%
- Writing of Chapter 4 for incorporation into a manuscript on a multi-environment QTL study for winter-hardiness in sugar beet. The Manuscript including all figures and tables was prepared under supervision of Dr. Friedrich J. Kopisch-Obuch 100%

Chapter 5: EcoTILLING in *Beta vulgaris* reveals polymorphisms in the *FLC*-like gene *BvFL1* that are associated with annuality and winter hardiness. (In review, Biomed Central, 2012)

Frerichmann, S. L. M., **M. Kirchhoff**, A. E. Müller, Scheidig, A. E.; C. Jung and F. J. Kopisch-Obuch

- Design, planning, preparation and conduction of field experiments 100%
- Collection of field data 100%
- Statistical analysis and data interpretation of phenotypic data 100%
- Statistical analysis of genotypic data 50%
- Writing of the parts of the manuscript concerning phenotypic and statistical data analysis 70%
- Minor contributions to introduction and discussion 10%