

Aus dem Institut für Pflanzenbau und Pflanzenzüchtung
der Christian-Albrechts-Universität zu Kiel

**Detection of new sequence variants for flowering and bolting
time genes in species of the genus *Beta***

Dissertation

Zur Erlangung des Doktorgrades

Der Mathematisch-Naturwissenschaftlichen Fakultät

der Christian-Albrechts-Universität zu Kiel

vorgelegt von

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Kiel, 2013

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Tag der mündlichen Prüfung: 06. Mai 2013
Zum Druck genehmigt: Kiel,

gez.

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List of abbreviations

°C	Degree Celsius
µl	Micro-liter
X ²	chi-squared
1D	one dimensional
2D	two dimensional
A	Adenine
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
<i>API</i>	<i>APETALA1</i>
<i>At</i>	<i>Arabidopsis thaliana</i>
Az	sodium azide
Az-MNU	sodium azide plus methylnitrosourea
B	Bolting locus of <i>Beta vulgaris</i>
BAC	Bacterial artificial chromosome
bp	Base pairs
BLAST	Basic local alignment search tool
<i>BTC1</i>	BOLTING TIME CONTROL 1
<i>Bv</i>	<i>Beta vulgaris</i>
<i>BvGI</i>	<i>Bv</i> gene index
C	Cytosine
CBF	CRT/DRE-binding factor
<i>CCA1</i>	<i>CIRCADIAN CLOCK ASSOCIATED 1</i>
CCM	cleavage mismatch method
CCT	<i>CO</i> , <i>CO</i> -like, <i>TOC1</i>
<i>CDF1</i>	<i>CYCLING DOF FACTOR</i>
CE	capillary electrophoresis
CEL1	CELERY ENDONUKLEASE 1
cDNA	Complementary DNA
cM	centiMorgan
<i>cf.</i>	compare
CMS	cytoplasmatic male sterility
<i>CO</i>	<i>CONSTANS</i>
<i>COPI</i>	<i>CONSTITUTIVE PHOTOMORPHOGENIC 1</i>
COR	Cold responsive
CRT	C-repeat
CTAB	Cetrylmethylammoniumbromid
cv.	Cultivar
<i>cyp</i>	cryptochrome
DH	Doubled haploids
DMS	dimethyl sulfate

DNA	Deoxyribonucleic acid
dNTP	Di nucleotide tetra phosphate
DRE	Dehydration responsive element
DREB	DRE-binding protein
e.V.	eingetragener Verein
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic translation initiation factor 4E
EMS	Ethyl methanesulfonate
ENU	N-ethyl-N-nitrosourea
EST	Expressed sequence tag
et al.	et alii
EU	European union
F ₁	First generation after cross
F ₂	Second generation after cross
FAO	<i>Food and Agriculture Organization</i> of the United Nations
<i>FCA</i>	<i>FLOWERING LOCUS CA</i>
Fig.	Figure
FISH	fluorescence <i>in situ</i> hybridisation
<i>FLC</i>	<i>FLOWERING LOCUS C</i>
<i>FLD</i>	<i>FLOWERING LOCUS D</i>
<i>FLK</i>	<i>FLOWERING LOCUS KH DOMAIN</i>
<i>FPA</i>	<i>FLOWERING LOCUS PA</i>
<i>FRI</i>	<i>FRIGIDA</i>
<i>FT</i>	<i>FLOWERING LOCUS T</i>
<i>FVE</i>	<i>FLOWERING LOCUS VE</i>
G	Guanine
GABI	German Agri-Biotech Initiative
GABI-PD	GABI-Primärdatenbank
GC	gas chromatographie
GMO	genetically modified organism
ha	Hectare
HII	heavy ion irradiation
HPLC	high-performance liquid chromatography
HPLC-MS	HPLC-mass spectrometry
HRM	High Resolution DNA Melting Analysis
HRXCT	high resolution X-ray computed tomography
HTP	High-throughput phenotyping
IAEA	International Atomic Energy Agency
IR	infrared
kb	kilo base
L.	Linné

LD	long day
<i>LD</i>	<i>LUMINIDEPENDES</i>
<i>LDL1</i>	<i>LYSINE-SPECIFIC HISTONE DEMETHYLASE1 LIKE1</i>
<i>LHP1</i>	<i>LIKE HETEROCHROMATIN PROTEIN 1</i>
<i>LHY</i>	<i>LATE ELONGATED HYPOCOTYL</i>
M	Mol
M ₁	First generation after mutagenesis treatment
M ₂	Second generation after mutagenesis treatment
M ₃	Third generation after mutagenesis treatment
MALDI	Matrix-assisted laser desorption/ionization
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
MAS	marker assisted selection
Mbp	Million base pairs
MDF	Mutation detection frequency
MF	Mutation frequency
Min	Minutes
MNU	N-methyl-N-nitrosourea
mRNA	Messenger ribonucleic acid
MS	mass spectrometry
n.a.	Not applicable
NaN ₃	sodium azide
nb	Non-bolting
NCBI	National center for biotechnology information
NGS	next generation sequencing
NIR	near infrared
ng	Nano gram
NMR	nuclear magnetic resonance
ORF	Open reading frame
P	parental generation
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
<i>PEP1</i>	<i>PERPETUAL FLOWERING 1</i>
pH	<i>potential hydrogenii</i>
<i>phyA</i>	phytochrome A
<i>PRR</i>	<i>PSEUDO-RESPONSE REGULATOR</i>
QTL	quantitative trait loci
RACE	rapid amplification of cDNA ends
RAPD	rapid amplification polymorphic DNA
RFLP	restricted fragment length polymorphism
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease

ROMA	representational oligonucleotide microarray analysis
rpm	Rounds per minute
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative real time PCR
SA	sodium azide
SD	short day
SNP	Single nucleotide polymorphism
<i>SOCI</i>	SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1
ssp.	subspecies
SSR	simple sequence repeat
STS	sodium thiosulfate
suppl.	supplementary
<i>SVP</i>	<i>SHORT VEGETATIVE PHASE</i>
<i>SWP</i>	<i>STRUWELPETER</i>
T	Thymine
t	ton
Tab.	Table
TAIR	The Arabidopsis information resource
TE	Tris EDTA buffer
TILLING	Targeted induced local lesions in genomes
Tris	Tris(hydroxymethyl)amino methane
UTR	un-translated region
UV	Ultra violet
v/v	Volume per volume
<i>VIN3</i>	<i>VERNALIZATION INSENSITIVE 3</i>
Vol	Volume
w/v	Weight per volume
YAC	yeast artificial chromosome

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1 General Introduction

1.1 Sugar beet: biology and agronomical importance

Sugar beets (*Beta vulgaris* ssp. *vulgaris* L. var. *altissima*) are herbaceous, dicotyledonous plants that belong to the Amaranthaceae family (formerly Chenopodiaceae). The genus *Beta* is divided into the two sections *Corollinae* and *Beta*, which are further divided into wild sea beets (*B. vulgaris* ssp. *maritima* L.), wild beets (*B. vulgaris* ssp. *adanensis*) and cultivated beets (*B. vulgaris* ssp. *vulgaris*) comprising the cultivar forms fodder beet (var. *crassa*), leaf beet (var. *flavescens*), garden beet (var. *conditiva*) and sugar beet (var. *altissima*) (Kadereit et al., 2006). The *Beta vulgaris* subspecies *vulgaris*, *maritima*, and *adanensis* are cross compatible, and ssp. *maritima* is a major source for resistance and tolerance genes against biotic and abiotic stresses (Frese et al., 2001; Panella and Lewellen, 2007). Species from the genus *Beta* are annual, biennial or perennial plants. Jung et al. (1993) and Hjerdin et al. (1994) have confirmed by molecular data that the wild sea beet is the progenitor of the cultivated beets in the subspecies *vulgaris*. Sugar beets are biennial plants that grow vegetatively and produce a storage root with high sucrose concentrations in the first year. In the second year, the beet undergoes transition to the reproductive state after receiving vernalization over winter by exposure to cold temperature for at least ten to 14 weeks. In this state, nutrients stored in the root are remobilized to produce shoots, flowers and seeds (McGrath et al., 2007).

Sugar beets are the only crop plants used for sugar production in Europe. Seeds are sown in spring and beets are harvested in autumn. In Germany, sugar beets were cultivated on 367,000 ha yielding on average 65 t/ha in the 2009/2010 growing season (FAO, 2012a). For seed production, the beets are mostly grown in southern France or northern Italy, where relatively mild winters ensure a maximum survival rate.

The history of sugar beet domestication began in the end of the 18th century, when Franz C. Archard built the first beet sugar factory and developed the “White Silesian” beet, which is the predecessor of all modern sugar beet cultivars today (Fischer, 1989). After a few years of expansion, the crop acreage decreased in the following years in favor of sugar from cane, because of easier cultivation and harvesting. By the mid-19th century beet cultivation and factory construction resumed in Germany, mainly due to improved sugar yield by crop rotation (Biancardi et al., 2010). In the beginning of the 20th century, cultivation was further improved by chemical fertilizers and steam tractors, which tremendously improved soil management. Due to the introduction of monogermity (Savitsky, 1952) and cytoplasmic male sterility (CMS) (Owen, 1945) the production and commercialization of sugar from beets was further pushed forward. While monogermic seed facilitated mechanization of sugar beet cultivation, CMS allowed the development of hybrid cultivars and therefore the exploitation of heterosis for yield improvement. Almost all sugar beet varieties grown today are hybrids and only a minor part are open pollinated cultivars. While most hybrid cultivars are composed of inbred lines, a slight minority is composed of doubled haploids (DH's). DHs in sugar beets were first described by Bossoutrot and Hosemanas (1985), but due to the higher production costs and the more problematic production, by parthenogenesis and colchicine treatment of ovules, DHs were never as successful in sugar beet breeding as DHs in the breeding programs of barley, rape seed, wheat, and maize (Mackay et al., 1999).

B. vulgaris is a diploid species and has a basic haploid chromosome number of $x=9$. Schondelmaier and Jung (1997) have standardized chromosome nomenclature, defined in genetic linkage maps. Genome sizes of higher plants vary tremendously. The variation can be accounted in parts to polyploidy, but even in diploid organisms, the total amount of nuclear DNA can range from 63 million base pairs (Mbp) in the carnivorous plant *Genlisea margaretae* (corkscrew plant) (Greilhuber et al., 2006) to 157 Mbp in the model plant *Arabidopsis thaliana* (Bennet et al., 2003), while the size of the

crop species wheat is estimated to be 16,500 Mbp (Arumuganathan and Earle, 1991). The genome sizes of other crop plants such as rice and rapeseed are 430 Mbp (Goff et al., 2002; Yu et al., 2002) and 1,200 Mbp (Johnston et al., 2005) respectively. The genome size of *B. vulgaris* is reported to be between 714 and 758 Mbp per haploid genome ($n=x=9$), which can be regarded as midsize compared to the afore-mentioned crop species. The number of genes for *B. vulgaris* was estimated to be around 28,000 (Dohm et al., 2011), whereas the gene number in *Oryza sativa* was estimated to be slightly over 41,000 (Sterck et al., 2007) and for *A. thaliana* a gene number of 33,600 is documented in the TAIR10 (The Arabidopsis Information Resource 10) release (Swarbreck et al., 2008; <http://www.Arabidopsis.org>).

The exploitation of the sugar beet genome has experienced considerable advances by molecular marker analysis resulting in several genetic maps for *B. vulgaris* (Barzen et al., 1992; Barzen et al., 1995; El-Mezawy et al., 2002; Gidner et al., 2005; Grimmer et al., 2007; Hjerdin et al., 1994; Lein et al., 2007; Pillen et al., 1992; Rae et al., 2000; Schafer-Pregl et al., 1999; Schneider et al., 2007; Uphoff and Wricke, 1995) with various marker types such as restriction fragment length polymorphic (RFLP), randomly amplified DNA polymorphic (RAPD), amplified fragment length polymorphic (AFLP) markers, simple sequence repeats (SSR), single nucleotide polymorphisms (SNPs), and representational oligonucleotide microarray analysis (ROMA). The map sizes range from 457 cM (Rae et al., 2000) to 822 cM (El-Mezawy et al., 2002) with an average of 622 cM. The molecular markers on these maps can be used in a map-based and/or positional cloning strategy to tag single genes and/or quantitative trait loci (QTL) associated with important agronomical traits being important for e.g. transgenic approaches. Marker-assisted breeding can be successfully implemented for four main topics in sugar beet breeding: (a) marker assisted backcrosses (selection for target or genome proportion), (b) fingerprinting (revision of hand crosses, assignment of gene pools), (c) quality control (purity check seed development), and (d) marker assisted selection (MAS) for important diseases such as rhizomania, but also for targeting major genes or poly-genes. MAS is a well suited strategy for traits that are difficult to target by classical selection (Xu and Crouch, 2008). Furthermore, MAS is a valuable method for complex traits where normally a pyramiding of several QTLs is needed to improve the trait (Bernardo, 2008). In addition, MAS has been proposed to be a well-suited strategy for traits that require special environmental conditions.

For gene discovery by positional cloning it is mandatory to have a physical map of the target region. Physical maps can be constructed from large insert clone libraries such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), fosmid, and cDNA libraries. YAC libraries for sugar beet were constructed first (Del-Favero et al., 1994; Evers et al., 1992; Klein-Lankhorst et al., 1994; Kleine et al., 1995), but were subsequently substituted by BAC libraries (Fang et al., 2004; Gindullis et al., 2001; Hagihara et al., 2005; Hohmann et al., 2003; Jacobs et al., 2009; McGrath et al., 2004; Schulte et al., 2006) due to vector instabilities. Lange et al. (2008) recently have constructed a fosmid library for sequencing of the sugar beet genome in the GABI project BEETSEQ (<http://gabi.de/projekte-alle-projekte-neue-seite-144.php>), which is supported by high resolution FISH (fluorescent *in situ* hybridization) (Jacobs et al., 2009). In comparison to BAC libraries, fosmid libraries have the advantage of easier manageability because of their smaller insert sizes (40kb fosmid and up to 300 kb BAC). Besides genetic and physical maps, expressed sequence tag (EST) databases are an important resource for the identification and analysis of candidate genes. To date, there are three publicly accessible EST databases in which sugar beet-derived expressed sequences are stored: the Gene Index Project (<http://compbio.dfci.harvard.edu/tgi/>; formerly TIGR Gene Index) with the BvGI gene index consisting of 17,186 unique entries (Release 4.0 March 2012); NCBI dbEST (<http://www.ncbi.nlm.nih.gov>) with about 30,235 ESTs (March 2012), including data from the just recently sequenced *Beta vulgaris* genome project (Dohm et al., 2011); and the GABI primary database (<http://www.gabipd.org>) holding 20,389 sequence entries (August 2011).

1.2 The impact of mutations for plant breeding

Artificially induced mutations are an important source for increasing the genetic variation in a species and it is especially valuable for the production of new cultivars in plant breeding. First, mass selection was used in plant breeding to select traits of economic importance (Evans, 1993). This selection also altered the plants in new ways, since human selection and selection in nature may have conflicting aims (Hillman and Davies, 1990). Charles Darwin (1859) created the term “artificial selection” in 1859 to emphasize the difference between natural and human selection, as he and others realized that domesticated plants were not to be considered natural, in terms of genetic background. Later, de Vries introduced the term “mutation” to the scientific community, by which he meant a sudden change of existing traits (de Vries, 1901), helping to explain the natural diversity found in plants and animals. Furthermore, de Vries (1901) predicted: “that it may become possible in the future to artificially induce mutations in (cultivated) plants and animals”. Due to systematic selection wild crop relatives are today often classified into completely different taxa than their cultivated counterpart (Sikora et al., 2011). Greater yields from the domesticated crops helped to secure the access to food and therefore enabled an increased human population density, formation of communities, and work specialization in areas other than food production within those communities. The move from foraging to agriculture also brought many negative consequences for humankind, including new infectious diseases and epidemics caused by the increased population density and trade, coupled with a decrease in food diversity (Gepts, 2002). Today, we rely on only about 14-21 crop plant species for world food production (FAO, 2012b).

During crop domestication and further breeding, there has been a continuous process leading to a reduction of a species’ genetic diversity, especially during the past decades as breeders have focused on so-called “elite” cultivars. This substantial reduction in genetic diversity for the sake of yield increase has eventually become a bottleneck in plant breeding and various techniques to induce variation have emerged in the middle of the last century. The classical method applied in plant breeding is the cross of the elite breeding material with genotypes of the primary or secondary gene pool to induce or enhance genetic diversity by re-introducing genes, which have not been in the focus of human selection during domestication. Another, more modern method is the introduction of traits by transformation with the underlying genes or the silencing of undesired traits by transformation with antisense RNA or RNA interference (RNAi) targeting the respective gene or by transformation. These processes result in a genetically modified organism (GMO). Due to the huge potential to change and/or enhance single or multiple traits, the application of GMOs is among the most favored techniques, but due to legal constraints and an almost non-existent public acceptance in European countries, GMOs are currently of minor relevance.

Until now, the most widely used method to create new genetic variation beyond crossing are artificially induced mutations. Artificially induced mutations can lead to DNA base changes, nucleotide changes, intragenic deletions, large deletions (>100 kb), translocations, inversions or chromosomal aberrations. Mutations can be induced rapidly and easily, and once identified they can be maintained and integrated into breeding programs. Furthermore, mutation breeding can be applied to any crop species.

First reports of an induced mutation in plants were made by Koernicke (1904a; 1904b), who described gamma irradiation of seeds from *Vicia faba* (field bean) to have an impact on germination and growth speed when compared to non-treated controls. The first applications of chemical mutagens were reported by Wolf (1909) and Schiemann (1912), who showed that a range of chemicals could induce mutations in bacteria and fungi. The proof that mutations were definitively inducible was presented by the zoologist Muller (1927) at the fifth international congress of genetics in Berlin, where he reported on successful irradiation applied to *Drosophila melanogaster* (fruit fly) to introduce mutational changes. Stadler was the first plant geneticist who reported successful mutation induction by X-rays

and radium rays in *Hordeum vulgare* (barley) and X-rays in *Zea mays* (maize, 1928a; 1928b). The pioneers in mutation induction for plant breeding purposes were H. Stubbe (*Solanum lycopersicum* (tomato), *Glycine max* (soybean)) and Å. Gustafson (*H. vulgare*). Stubbe showed that mutagenic treatments resulted in a significant increase of gene mutations and that a clear correlation existed between the dose applied and the mutation frequency of *Antirrhinum majus* (snapdragon, Stubbe, 1930a; Stubbe, 1930b; Stubbe, 1930c; Stubbe, 1935). Gustafson might be called “father of mutation breeding” (van Harten, 1998). He made barley the model crop for mutation research and identified and documented one of the most famous barley mutant cultivars, which is “Pallas” a stiff straw mutant (Gustafson, 1941; Gustafson, 1954; Gustafson, 1963). The first commercial mutant cultivar “Chlorina” was released in 1934. Tollenaar (1934) reported a chlorophyll-defective *Nicotiana tabacum* (tobacco) plant, which in 1936 already comprised 10% of all tobacco grown in the Dutch East Indies (Indonesia) (Tollenaar, 1938). Leaves from this cultivar appeared to be light green and were preferred as wrapper for cigars. The first disease resistant mutant barley cultivar “M66” showing resistance against powdery mildew was released by Freisleben and Lein (1942). After 1942, mutation breeding was pushed forward and further insights into the action of induced mutations were gained, as for instance by Oehlkers (1946; 1956) who introduced various types of chromosome aberrations by ethyl urethane treatment of developing buds of cut inflorescences of *Oenothera*. Auerbach and Robson (1944; 1946) reported a mutagenic effect of mustard gas on *D. melanogaster* and concluded, that chemical substances could be as effective as X-ray in inducing mutations and chromosome rearrangements.

This understanding of mutagenesis was further expanded when Watson and Crick (1953) presented their model of the double helix to explain the structure of the DNA. From then on, the action of mutagens could be related to changes on the DNA level. Mutation induction in *Z. mays* led to the identification of the class II transposons *Activator (Ac)* and *Dissociator (Dc)* by Barbara McClintock. She reported that maize kernels showed unexpected change in pigmentation after mutagenesis treatment (McClintock, 1950; 1953), but it was not until the identification of transposable elements in *D. melanogaster* by Jacob and Monod (1961), that McClintock’s work on transposable elements was acknowledged by the scientific community. Today we know that transposons do not move unless the cell is subjected to stress (Knippers, 2006), such as mutagenesis treatment.

Since the 1960s, many symposia and meetings have been held for practical applications of radiation and the use of chemical mutagens in plant breeding. Here, data regarding choice of the most appropriate starting material, quantity of seeds or plants to be treated, optimal dose for various mutagens, and optimal treatment conditions were reported to the research community. The combined efforts in mutant research resulted in the establishment of an advisory group on mutation breeding of the joint Food and Agriculture Organization (FAO) / International Atomic Energy Agency (IAEA) division in 1964 (see *Mutation Breeding Newsletter* 34, 1989). In 1969, a FAO/IAEA symposium on induced mutations in plant breeding marked the turning point from fundamental research on mutations to mutation breeding. In this perspective, Sigurbjörnsson and Micke (1969) presented a list of 77 mutant cultivars obtained by mutagenic treatment. Today the Joint FAO/IAEA division mutant variety and genetic stocks database holds 2,889 varieties generated by chemical or physical mutagens or a combination of both (377 chemical, 2,476 physical, combined 36) (IAEA, 2011). Several protocols on mutation treatment of the various mutagens for several plant species are available to the public and workshops are held to train especially people from developmental countries in mutation techniques and mutation breeding at the FAO/IAEA in Vienna, Austria.

The goal in mutagenesis breeding is to cause maximal genomic variation in traits like yield potential, yield stability, stress resistance against biotic (pests and diseases) and abiotic factors (drought, heat, cold, water), herbicide tolerance, advanced quality (starch, oil, protein etc.), and physiological characteristics (plant height, harvested organ, shoot length, flowering time, bolting resistance, etc.) with a minimum decrease in viability. Currently the most commonly applied methods are physical agents (different types of irradiation), chemical mutagens, and tDNA-based mutations. For irradiation,

mostly γ -ray, X-ray and electrons are used, but recently ion beam mutagenesis has come into use for plants (Magori et al., 2010). The most common chemical mutagens are ethyl methanesulfonate (EMS), N-ethyl-N-nitrosourea (ENU), dimethyl sulfate (DMS), N-methyl-N-nitrosourea (MNU), and sodium azide (NaN_3) (Ceccarelli et al., 2009). Throughout the years many other methods have been used, such as heat shock or aging of seed and pollen grains, but those methods have been proved to be far less efficient than irradiation or mutagenic chemicals (Lindgren, 1972; van Harten, 1998). Mutagenesis has been applied to major crop species such as rice (*O. sativa*), bread wheat (*T. aestivum*), maize (*Z. mays*) and soybean (*G. max*), fruits, vegetables and ornamentals. It has generated a vast amount of genetic variability and has played a significant role in plant breeding programs throughout the world. Mutation breeding has been most successfully applied in *O. sativa* resulting in more cultivars than in any other crop species (Rutger and McKill, 2001). The most important of all rice mutant cultivars is the semi-dwarf cultivar “Reimei” (Futsuhara et al., 1967) which was developed by gamma irradiation of cultivar “Fujiminori” and released in 1966. Dwarfing in “Reimei” is caused by the *sd1* gene and leads to stiff-straw, increased fertilizer responsiveness, lodging resistance, day-length–insensitivity and no seed dormancy (Powell et al., 2004). Today, the majority of all semi-dwarfs in major rice-growing areas carry the mutated *sd1* allele (Kawai and Amano, 1991). However, mutation breeding may result in negative effects as well: (1) many of the mutations induced are recessive and cannot be detected in the first generation after mutagenesis treatment (M_2) and therefore selection for desired phenotypes can be difficult, and (2) the vast majority of the mutations are undesirable, such as mutations limiting yield, plant height, growth, or root morphology.

1.3 Mutation research in plant genetics

1.3.1 Mutation induction

Mutation induction can be achieved by physical or chemical mutagens. Table 1 lists the most commonly used mutagens and their effects after treatment.

Table 1: Types of mutations, mutagens and their causative effects.

Mutagen type	Mutagen	Effect
Physical	γ -ray	large deletions (>100 kb), chromosomal aberrations (translocations, inversions)
	X-ray	
	electrons	
	ion beam	
Chemical	EMS (ethyl methanesulfonate)	Single base pair changes, single nucleotide polymorphisms (transitions and transversions)
	ENU (N-ethyl-N-nitrosourea)	
	MNU (N-methyl-N-nitrosourea)	
	NaN_3 (sodium azide)	

The choice of mutagen is dependent on the desired effect. Radiation is mostly used if small intragenic deletions, large deletions (greater than 100 kb) and chromosomal aberrations such as translocations and inversions are desired. Chemical mutagens mostly introduce single base-pair (bp) changes resulting in single-nucleotide polymorphisms (SNPs), and in most cases will lead to a higher mutation frequencies in comparison to radiation (van Harten, 1998). One other critical aspect of choosing the

right mutagen lies in the nature of the mutagen itself. Chemical mutagens are characterized by a specific threshold value, after which the survival rate of the treated organism rapidly declines, whereas for radiation, a more gradual decline of survival rate is observed (Dertinger and Jung, 1970).

Once the mutagen of choice has been applied to the desired organism, there are two methods of investigating mutations in the mutagenized population. One method is based on phenotypic observation and the other on genotypic observations.

1.3.2 Mutation detection by phenotype

The classical approach to identify mutants affecting a certain trait is to phenotype large mutant populations. Once the needle in the haystack is identified, the mutant can be used for breeding or for further research on the genetics underlying the trait of interest. In research, this approach has been termed forward genetics (Konopka and Benzer, 1971), where based on a mutant phenotype the genetics of a certain trait is analyzed. Many traits can be phenotyped by visual scoring that can be done by a person or by machine. These phenotyping platforms have improved greatly over the last years, but most phenotyping platforms are not yet capable of screening the hundreds of accessions necessary to identify genotypes with high impact phenotypes. There are two main high throughput phenotyping methods, depending on the analytical objectives. One is the quantitative analysis by X-ray through computed tomography; the second is the visualization using different cameras and acquisition modes (visual light, near infrared NIR/SWR, infrared IR, ultra-violet UV, and fluorescent imaging). Recently, Hartmann et al. (2011) demonstrated that it is possible to monitor different barley cultivars using image capture and analysis in a high-throughput phenotyping (HTP) drought stress experiment. X-ray has been used for a long time in plant science. Heeraman et al. (1997) used X-ray to quantify rooting spatial distribution and root length of bush bean, whereas Dhondt et al. (2010) present a modified X-ray method (high resolution X-ray computed tomography HRXCT), demonstrating three-dimensional images of morphological traits of *A. thaliana* seedlings. For important traits, that are not detectable by eye, such as seed oil composition, different techniques need to be applied: (1) techniques that are destructive and require chemical manipulation (gas chromatography (GS), GC-mass spectrometry (GC-MS), and high-performance liquid chromatography-MS (HPLC-MS)) and (2) non-destructive techniques (nuclear magnetic resonance (NMR) or near-infra-red (NIR) spectroscopy).

Non-destructive techniques are attractive for studies where loss of viability is inconvenient. With NIR the information content is high; however NIR analysis is a secondary method and requires careful and comprehensive calibrations. Furthermore, seed variability can complicate NIR data interpretation. NMR has been applied for many years as a reliable method for the measurement of seed oil content. Hutton et al. (1999) uses NMR to measure the seed oil composition of canola seeds in intact transformants and showed that by comparing GS and NMR, NMR is the faster and more reliable method. Velasco et al. (1999) came to the same conclusion by comparing NIR and GS for the measurement of seed weight, oil content and fatty acid composition.

The steps required to identify a gene responsible for the desired phenotype in a forward genetic approach are: (1) selection of a biological process or trait, (2) production of a highly mutagenized population, (3) screening for mutants with desired phenotype, (4) mapping and cloning the gene responsible for the phenotype. Step four requires an especially enormous effort (Peters et al., 2003). Map based cloning has become less labor intensive than 20 years ago, but still can often take more than a year for a skilled scientist to move from a mutant to the affected gene (Alonso and Ecker, 2006). Therefore, when the gene responsible for the desired effect or phenotype is already known, mutation detection by genotype is by far the preferred method.

1.3.3 Mutation detection methods by genotype

In addition to mutation detection by phenotype, mutations can also be detected by genotype on the DNA level. This approach is termed reverse genetics (Shortle et al., 1981). Reverse genetic approaches have been primarily developed in order to understand gene function. A typical reverse genetic approach involves the following steps: (1) selection of gene or genes of interest, (2) production of a highly mutagenized population, (3) development and conduction of sequence based mutations screens, (4) analysis of the phenotype of the mutants. Knowledge of the sequence is a mandatory aspect in reverse genetic screens, which is normally gained from homology searches, expressed sequence tag (EST) databases, sequencing or transcript profiling.

High-Resolution DNA melting analyses

One method for mutation scanning in plants on the DNA level is high-resolution DNA melting analysis (HRM), where a PCR is performed in the presence of saturating fluorescent DNA dye and unlabeled oligonucleotides. Afterwards the fluorescent melting curves of PCR amplicons and amplicon-probe duplexed are analyzed (Gundry et al., 2003). The technique allows identification of single nucleotide polymorphisms (SNPs) and small insertions as well as deletions in desired accessions. Gady et al. (2009) have used HRM to exploit the mutation frequency of a *S. lycopersicum* EMS population. They identified two mutations (one stop- and one missense mutation) in the *phytoene synthase 1* gene leading to altered fruit color and carotenoid content (Gady et al., 2012). However, HRM also suffers from some drawbacks. According to Gady et al. (2009), the ratio of detecting false positives was rather high, which meant a lot of proof-reading and re-screening.

Chemical cleavage mismatch method / TILLING

Another reverse genetic technique, which gained major attention in recent years, is the chemical cleavage mismatch method (CCM), where point mutations in heteroduplexed DNA are identified by cleaving the modified base at the mismatch site. Subsequently, the cleaved products can be visualized by gel electrophoresis. One of the most popular CCM methods is Targeted Induced Local Lesions IN Genomes (TILLING) depicted in Figure 1. This method was first described by McCallum et al. (2000a; 2000b) for *A. thaliana*. In this technique gamma irradiation or chemical mutagens such as EMS, Az-MNU or MNU are used to introduce point mutations in the genome of interest. After mutagenesis of seeds, pollen (Till et al., 2004) or ovaries, the plants are grown and DNA is sampled, extracted and pooled, most commonly with an 8-fold pooling depth. To make the identification of putative mutants easier with fewer sequencing steps necessary to confirm mutations, a two dimensional (2D) pooling strategy was introduced by Till et al. (2006a). The pooling depth strongly depends on the aim of the project, since the higher the number of plants per pool, the fewer mutations will be identified after PCR amplification. After PCR for a gene of interest, heteroduplex formation is carried out and the samples are digested by S1 endonucleases such as CEL1 (Colbert et al., 2001) or *ENDO1* (Triques et al., 2008; Triques et al., 2007). Digested PCR products are then analyzed by polyacrylamide gel electrophoresis (PAGE) or on a capillary sequencer.

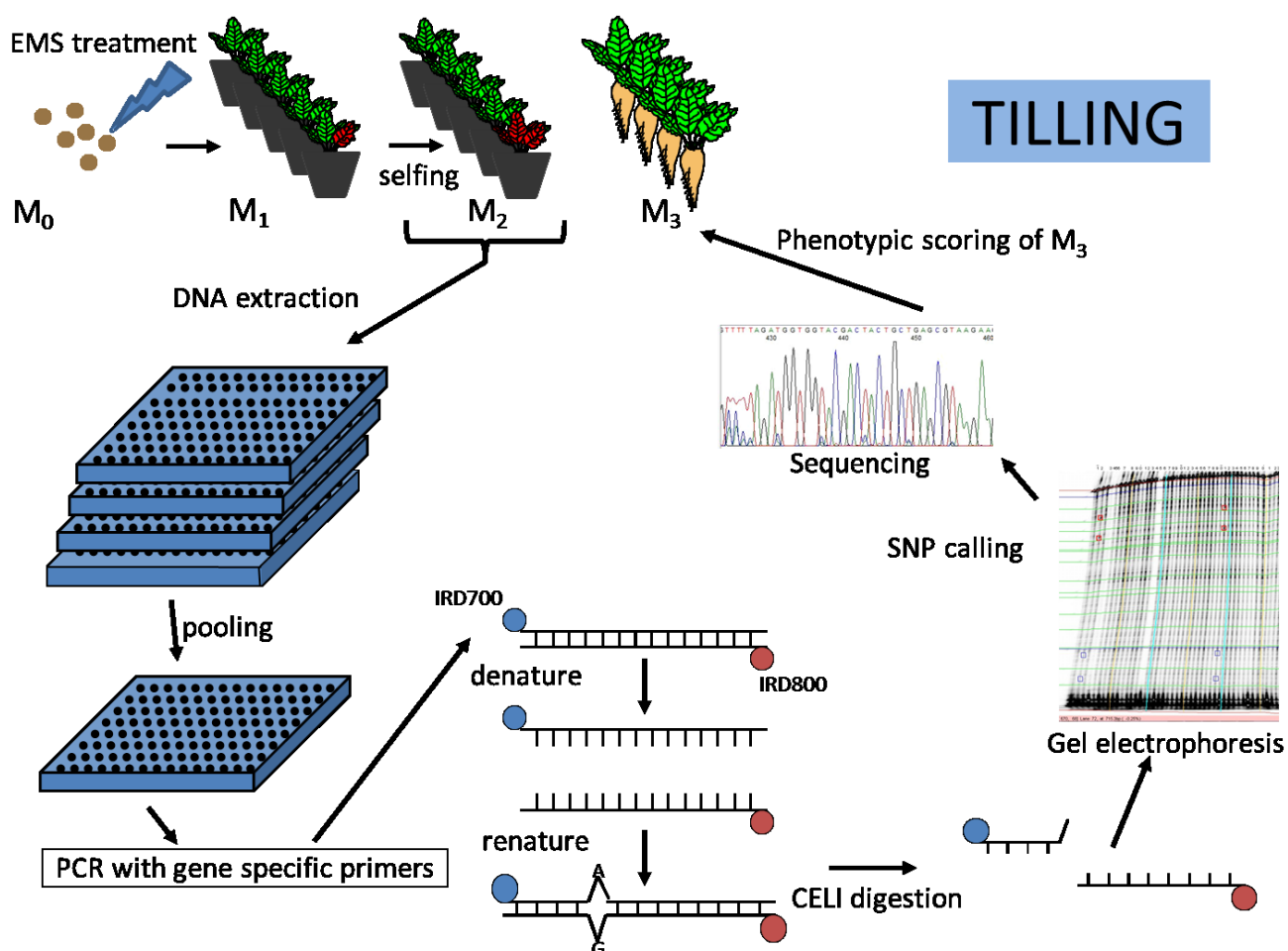


Figure 1: High-throughput TILLING procedure. Procedure is started with mutagenesis of seeds, pollen or ovaries. M₁ plants are grown and selfed. From selfed M₁ plants M₂ plants are grown, selfed, M₃ seeds are harvested, and samples are taken for DNA extraction. DNA is pooled and PCR is carried out with gene specific primers. After PCR, a heteroduplex formation is carried out to allow non-homologous strands to pair. After heteroduplex formation endonuclease digestion (CEL1) is conducted. Endonuclease digested fragments are analyzed via gel electrophoresis, SNP calling is performed and identified SNPs are sequenced to check for type of mutation. Interesting mutants are grown for phenotypic analyses.

Table 2 provides an overview of all TILLING projects known to date together with the analyzed species, the number of plants identified and the mutation frequency. As shown in the table, the mutation frequency varies substantially in different plant species. For most of the studied species, the analysis of several genes revealed a frequency of 1 mutation per 200-500 kb screened. However, there are also mutation frequencies of 1/16 kb e.g. for *Brassica napus* (rapeseed, Harloff et al., 2011) or 1/967 kb for *Arachis hypogaea* (peanut, Knoll et al., 2011). This variation in mutation frequency depends mostly on four factors: (1) the choice of mutagen, (2) the dose of mutagen, and (3) the number and G/C content of investigated genes, and (4) the tolerance of each genotype/species to mutations, which includes the capability of each organism to deal with mutations and the repair of important DNA domains with specialized DNA repair mechanisms. In addition, the mutation density can vary from cultivar to cultivar in a given species as reported for *G. max*, where EMS treatment of two different cultivars with the same dose resulted in a 4-fold difference in mutation frequency (Cooper et al., 2008).

Table 2: Published mutant populations in various plant species investigated via TILLING technique.

Species (ploidy level) ¹⁾	Mutagen ²⁾	Concentration ³⁾	Screening method ⁴⁾	Size of M ₂ population	No. of genes analyzed	Total No. of mutations detected	Mutation frequency ⁵⁾	Reference
<i>Arabidopsis thaliana</i> (2x)	EMS	20 - 40 mM	LI-COR [®]	3,712	14	450	1/89 kb	Martin et al., 2009
				6,912	1,090	1,774	1/170 kb	Till et al., 2003
				3,072	192	1,890	1/300 kb	Greene et al., 2003
		20 mM	HRM	10,000	2	12	1/415 kb	Bush and Krysan 2010
<i>Arachis hypogea</i> (2x)	EMS	32/80 mM	LI-COR [®]	3,420	3	27	1/967 kb	Knoll et al. 2011
<i>Avena sativa</i> (6x)	EMS	73 mM	MALDI-TOF	2,600	2	16	1/33 kb	Chawade et al., 2010
<i>Brassica napus</i> (4x)	EMS	48/80 mM	LI-COR [®]	5,355	4	498	1/16 kb	Harloff et al., 2011
		80 mM		3,488	2	94	1/61 kb	Harloff et al., 2011
		16 - 96 mM		1,344	1	19	1/86 kb	Wang et al., 2008
<i>Brassica oleracea</i> (2x)	EMS	32 mM	LI-COR [®]	2,263	15	25	1/447 kb	Himelblau et al., 2009
<i>Brassica rapa</i> (2x)	EMS	16 - 40 mM	CE	9,216	6	617	1/60 kb	Stephenson et al., 2010
<i>Cucumis melo</i> (2x)	EMS	80 - 240 mM	LI-COR [®]	4,023	11	134	1/573 kb	Dahmani-Mardas et al., 2010
<i>Glycine max</i> (2x)	EMS	40 mM	LI-COR [®]	529	7	32	1/140 kb	Cooper et al., 2008a
	MNU	2.5 mM		768	7	47	1/140 kb	Cooper et al., 2008a
	EMS	40 mM		768	7	12	1/550 kb	Cooper et al., 2008a
<i>Helianthus annuus</i> (2x)	EMS	68 mM	LI-COR [®]	1,152	4	9	1/475 kb	Sabetta et al. 2011
<i>Hordeum vulgare</i> (2x)	Az-MNU	N/A	ND	10,000	12	174	1/235 kb	Kurowska et al., 2011
	Az	10 mM	LI-COR [®]	4,906	4	22	1/374 kb	Talame et al., 2008
	MNU	N/A	ND	1,372	2	9	1/486 kb	Kurowska et al., 2011
	EMS	20 - 60 mM	LI-COR [®]	10,279	6	81	1/500 kb	Gottwald et al., 2009
	EMS	20/30 mM	dHPLC	9,216	2	10	1/1,000 kb	Caldwell et al., 2004
	NaN ₃	1.5 mM	CE	9,573	2	5	1/2,500 kb	Lababidi et al., 2009
	Gamma rays	N/A	ND	1,753	1	1	1/3,297 kb	Kurowska et al., 2011
<i>Lotus japonicus</i> (2x)	EMS	N/A	LI-COR [®]	4,904	61	576	1/502 kb	Perry et al., 2009

Species (ploidy level) ¹⁾	Mutagen ²⁾	Concentration ³⁾	Screening method ⁴⁾	Size of M ₂ population	No. of genes analyzed	Total No. of mutations detected	Mutation frequency ⁵⁾	Reference
<i>Medicago truncatula</i> (2x)	EMS	12/16 mM	CE	4,350	56	456	1/424 kb	Le Signor et al. 2009
		12/16 mM	LI-COR [®]	4,500	56	456	1/485 kb	Le Signor et al. 2009
<i>Oryza sativa</i> (2x)	MNU	1 mM	CE	767	3	24	1/135 kb	Suzuki et al., 2008
	Az-MNU	1 mM Az 15mM MNU	LI-COR [®]	768	10	30	1/265 kb	Till et al., 2007
	EMS	120 mM	LI-COR [®]	768	10	27	1/294 kb	Till et al., 2007
	Az-MNU	1 mM Az 15mM MNU	NGS	768	13	81	N/A	Tsai et al.2011 *
	EMS	64/80 mM	LI-COR [®]	2,000	10	na	1/1,000 kb	Wu et al. 2005
<i>Pisum sativum</i> (2x)	EMS	16/20/24 mM	LI-COR [®]	4,717	1	50	1/193 kb	Triques et al., 2008
<i>Solanum lycopersicum</i> (2x)	EMS	80 mM	LI-COR [®] , CE	4,741	7	25	1/322 kb	Minoia et al., 2010
		68 mM		1,926	7	41	1/574 kb	Minoia et al., 2010
		48 mM	NGS	15,000	1	2	N/A	Rigola et al., 2009 *
		48 mM	LI-COR [®]	4,759	19	256	1/574 kb	Piron et al., 2010
		80 mM	HRM	8,225	5	44	1/737 kb	Gady et al., 2009
<i>Solanum tuberosum</i>	EMS	80 mM	SS	2,748	1	19	1/91 kb	Muth et al., 2008
<i>Sorghum bicolor</i> (2x)	EMS	8 - 48 mM	LI-COR [®]	1,600	4	5	1/526 kb	Xin et al., 2008
<i>Triticum aestivum</i> (6x)	EMS	60/80 mM	LI-COR [®]	10,000	2	196	1/24 kb	Slade et al., 2005
		73/80 mM		1,536	3	186	1/38 kb	Uauy et al., 2009
	HII	50 Gy	TaqMan	20,000	1	294	1/84 kb	Fitzgerald et al., 2010
<i>Triticum durum</i> (4x)	EMS	60/80/96 mM	LI-COR [®]	8,000	2	50	1/40 kb	Slade et al., 2005
		57/60 mM	PAGE	1,386	2	93	1/51 kb	Uauy et al., 2009
			NGS	1,386	5	112	N/A	Tsai et al.2011 *
<i>Zea mays</i> (2x)	EMS	80 mM	LI-COR [®]	750	11	17	1/485 kb	Till et al., 2004

1) Plant species used for developing the mutant population; 2) Mutagen used for inducing mutations: EMS (ethyl methanesulfonate), Az-MNU (sodium azide plus methylnitrosourea), Az (sodium azide) MNU (methylnitrosourea), and HII (heavy ion irradiation); 3) Concentration of mutagen used for developing the mutant population; 4) Mutation screening methods: CE (capillary electrophoresis), HRM (high-resolution melting), PAGE (polyacrylamide gel electrophoresis), MALDI-TOF (Matrix-assisted laser desorption/ionization – time of flight), NGS (next generation sequencing, GS-Flex), and SS (Sanger sequencing); 5) The mutation frequency was calculated as follows: [(amplicon size – 100 bp) x total number of screened samples] / total number of identified SV/mutations; 100 bp were subtracted because of the diminished ability to detect mutations in the upper and lower 50 bp;

TILLING has led to the successful identification of mutants for such important traits as for sex determination in *Cucumis melo* (muskmelon). Here a missense mutation in the *andromonoecious* gene leads to an andromonoecious phenotype (Boualem et al., 2008). Further TILLING in 4,032 melon M₂ plants revealed a missense mutant in the *CmACO1-ACC* oxidase 1, responsible for delayed ripening and yellowing with improved shelf life (Dahmani-Mardas et al., 2010). Thus results from TILLING made this mutant economically important as fruit ripening and softening are key traits that have an effect on food supply, fruit nutritional value and consequently, human health. Another investigated trait was spike morphology in *H. vulgare* (Gottwald et al., 2009). By TILLING of 7,348 M₂ plants of the cultivar “Barke”, it was possible to identify three mutants in the *HvHOX1* gene, which exhibit either six-rowed or an intermedium-spike phenotype. Here a direct link between the mutated gene and the phenotype could be established. TILLING in *T. aestivum* has greatly improved the quality trait starch. By screening 1,920 allohexaploid and allotetraploid wheat individuals, 246 alleles of the *waxy* gene could be identified through TILLING (Slade et al., 2005). Among those 246 alleles was one missense mutation with drastically reduced amylose content. Waxy starches with high levels of amylopectin and low levels of amylose have economically valuable functional qualities for the food industry for making breads and pastas, the paper industry for enhancing the strength and printing properties of paper products, and the adhesives industry as a component of glues and adhesives (Burrell, 2003). In *Avena sativa* (oat) mutation in genes responsible for grain quality and human consumption (phenylalanine ammonia-lyase and cellulose synthase-like β -glucan biosynthesis) as well as genes for lignin content (Chawade et al., 2010) helped to substantially push the nutritional benefits in human. TILLING in *Solanum tuberosum* (potato) has led to a small revolution, as through a mutation in the granule-bound *starch synthase I* gene a loss of function mutation was introduced and this led to the establishment of a high amylopectin elite breeding line (Muth et al., 2008). This elite breeding potato line produces pure amylopectin, a starch used in the paper, textile and food industry. This demonstrates how economically successful TILLING can be as a reverse genetic tool and that this technology provides breeders with a new and sophisticated tool for crop improvement.

TILLING was modified by Comai et al. (2004) to allow discovery of polymorphisms in target genes using natural, i.e. non-mutagenized, populations. In the pilot screen, the authors investigated the genetic variability in *A. thaliana* and detected 55 haplotypes in 150 accessions. Since the screening was done in ecotypes rather than in mutants, they termed the “new” method EcoTILLING. Since its introduction, EcoTILLING has been used for the characterization of the genetic variability in 14 genes of *Musa* ssp. (banana, Till et al., 2010) for nine genes of *Populus trichocarpa* (black cottonwood, Gilchrist et al., 2006), for 37 genes of *Phaseolus vulgaris* (common bean, Galeano et al., 2009), and for ten genes of *Vigna radiata* (mung bean, Barkley et al., 2008). Furthermore, EcoTILLING has been successfully applied in *A. hypogaea* and *Arachis duranensis* (wild peanut, Ramos et al., 2009) to identify a hypoallergenic ortholog of the Ara h 2.01 gene, to identify allelic variants of eIF4E in *Cucumis* ssp. (Nieto et al., 2007) and *S. lycopersicum* (Rigola et al., 2009), and to *T. aestivum* (Wang et al., 2008a) to identify new allelic variants for kernel hardness. Additionally, it has been used as candidate gene based detection of new resistance alleles against powdery mildew in *H. vulgare* (Mejlhede et al., 2006) and allelic variation in genes underlying drought stress and salinity tolerance in *O. sativa* (Kadaru et al., 2006; Negrao et al., 2011) and *S. tuberosum* (Elias et al., 2009), while Wang et al. (2010a) used EcoTILLING to find natural sequence variants for seed erucic acid content in *Brassica oleracea* (wild cabbage). Moreover, EcoTILLING has been used to detect new alleles in the acetolactate synthase genes of *Monochoria vaginalis* (heartleaf, Wang et al., 2007).

Next generation sequencing

TILLING and EcoTILLING are both high throughput techniques for the detection of single nucleotide polymorphisms via gel electrophoresis (Sikora et al., 2011), but a sequencing step is always necessary to identify the true nature of the SNP underlying a mutation or natural allele. Therefore, direct sequencing seems to be the appropriate technique to evaluate the genetic diversity or identify new alleles as in the case for EcoTILLING or to identify mutants as in the case for TILLING. Since its introduction, Sanger sequencing (Sanger and Coulson, 1975) has led to many breakthrough results, such as the first sequencing of the human genome (Lander et al., 2001; Venter et al., 2001). Recent advances in sequencing technology have resulted in the introduction of next generation sequencing (NGS) techniques. NGS technology has surpassed the classical Sanger sequence method in throughput by 1000-fold and up to 100 fold in coverage.

Today there are second and third generation sequencing platforms available with the Genome Sequencer FLX Titanium (Roche Deutschland Holding GmbH, Grenzach-Wyhlen, GER), the Genome Analyzer IIX (Illumina Inc, San Diego, CA, USA), and the SOLiD™ 4 system (Life Technologies GmbH, Darmstadt, GER) as second generation sequencing platform. Second-generation platforms use light or fluorescent emission detection systems to identify incorporated nucleotides. All systems use the sequencing-by-synthesis chemistry by which it is possible to generate more than 1 Gb of high quality sequence data per day for more than one million templates. Second generation platforms are highly efficient for the discovery of micro- and macro-lesions in complex genomes. It is possible to identify single or multiple nucleotide variations, small and large insertions, copy number variation, translocations, and complex genomic re-arrangements at single nucleotide resolution. For third generation sequencing there are two platforms commercial available: the HeliScope (Helicos BioSciences Corporation, Cambridge, MA, USA) and the PacBio RS (Pacific Bioscience, Menlo Park, CA, USA). These platforms use real-time detection of fluorescent dyes in the polymerase active site during incorporation to detect the base. Although NGS has revolutionized sequencing, it is not the ultimate ratio. It has been proposed that the \$1,000 genome should be available soon (Pareek et al., 2011), but until then, it is an expensive method compared to TILLING. Still NGS has been applied in TILLING of bread-wheat, durum-wheat, rice (Tsai et al., 2011), tomato (Rigola et al., 2009), and tobacco (Reddy et al., 2012), see Figure 2 for NGS TILLING workflow. In the tomato TILLING project KeyPoint™ technology (Keygene NV, Wageningen, Netherlands) was used to detect two mutations for the eukaryotic translation initiation factor 4E (*eiFE4*) in a population of 15,040 M₂ plants. KeyPoint™ makes use of GS-FLX machines to directly amplify genes of interest. Each gene specific primer carries tags to assign sequence reads to individual mutant plants for each multidimensional pool or to assign sequence haplotypes to pooled or individual samples. For bread-wheat 1,536 M₂ plants, for durum-wheat 1,386 M₂ plants, and for rice 768 M₂ plants were sequenced on a GAI in three different pooling schemes (1D, 2D, 3D) (Tsai et al., 2011). The method created by Tsai and co-workers is called Coverage Aware Mutation-Calling using Bayesian analysis (CAMBa). CAMBa allowed for the identification of several mutations that were missed by classical CCM based TILLING and confirmed already known mutations with fewer false positives. For tobacco also a GAI was used and 3,072 M₂ plants were screened after 3D-pooling (Reddy et al., 2012).

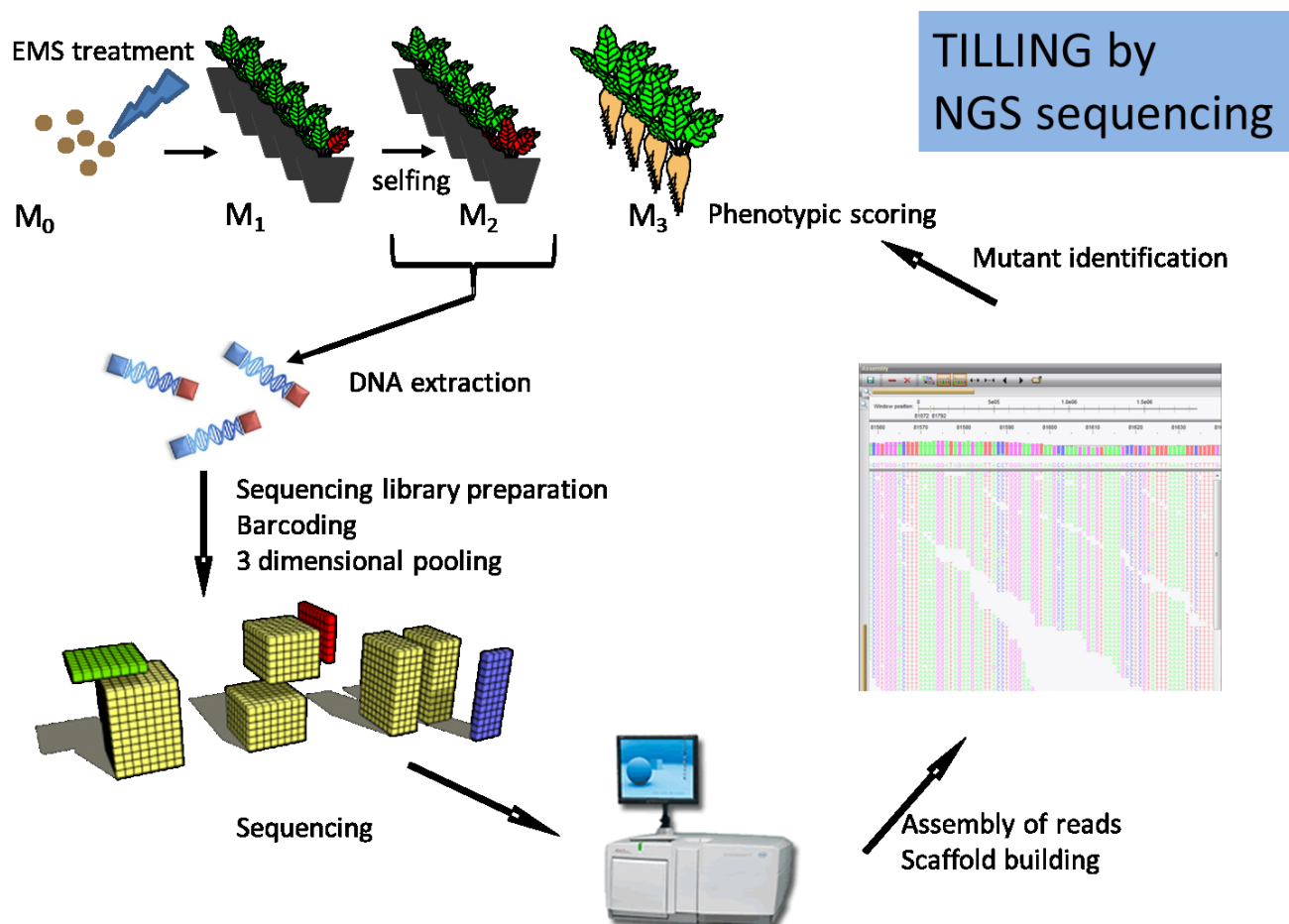


Figure 2: TILLING by NGS sequencing workflow. The procedure starts with mutagenesis of seeds, pollen or ovaries. M_1 plants are grown and selfed. From selfed M_1 plants M_2 plants are grown, selfed, M_3 seeds are harvested, and samples are taken for DNA extraction. DNA is barcoded, prepared for NGS sequencing and pooled 3 dimensional to ensure mutant verification. NGS sequencing is performed and reads are mapped to non-mutated genotype to enable SNP calling and scaffold building. Once a mutant SNP is identified and the responsible genotype mapped, the mutant genotype is grown for phenotypic analyses.

1.3.4 DNA repair mechanisms

Mutations are crucial for evolution but they can also be harmful to an organism as they can damage the DNA substantially and DNA integrity is important for the survival of an organism. Therefore DNA damage can lead to the death of a cell and ultimately to the death of the organism itself. To protect itself from DNA damage every cell has developed several DNA repair mechanisms to alleviate the toxic effects of the accumulation on DNA damage. Each mechanism is specialized for a certain type of DNA damage. In plants, the two main mechanisms are termed photoreactivation and dark-repair (Britt, 1999; Tuteja et al., 2001a). Photoreactivation, which is mediated by photolyase, is thought to be the major DNA repair pathway for cyclobutane pyrimidine dimers and photoproducts in higher plants (Dany et al., 2001; Tuteja et al., 2001a). Photolyases bind specifically to these DNA lesions and remove them directly by absorbing light in the 300-600 nm range (Kimura et al., 2004). The dark repair includes nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and other DNA repair pathways such as repair of DNA-double-strand breaks (DSB). Dark repair pathways have been identified in many plant species and the involved genes have been analyzed (Córdoba-Cañero et al., 2009; Tuteja et al., 2009) and it is highly correlated to proliferating tissue (Kimura et al., 2004).

Ionizing radiation and oxidative agents mainly cause DSB. Repair of DSB is critical for the survival of any organism and they have to be eliminated before the genome can be replicated. There are two main pathways for the elimination of DSB in plants, either via homologous recombination (HR) or by non-homologous end-joining (NHEJ). Many studies in plants focused on HR, as HR can be used as an approach for targeted mutagenesis (Tzfira and White, 2005). Homologous information for repair can be copied from four different locations: (1) from elsewhere in the genome, (2) from the homologue as in meiosis, (3) from the same chromosome, or after replication (4) from the sister chromatid (Puchta, 2005). For HR there are two models: (1) the synthesis-dependent strand annealing-mechanism (SDSA) (Formosa and Alberts, 1986) and (2) the single-strand annealing mechanism (SSA) (Lin et al., 1984). A DSB is repaired in the following way for the SDSA model:

- A 3'-single-strand overhang is produced in the broken double strand (acceptor) via an exonuclease-catalyzed digestion
- A D-loop is formed in the double stranded donor, the free 3' end of the acceptor invades the loop and repair synthesis is carried out by DNA-polymerases

The main difference between SDSA and SSA is that there is no donor molecule. After an exonuclease-catalyzed digestion of the blunt ends, a 3'-single-strand overhang is produced. If the overhangs carry complementary sequences they can anneal and a chimeric DNA molecule is formed. In some cases the chimeric molecule has overhanging ends; those are resected in the process. If there are no complementary sequences, the putative single-stranded regions are filled in by DNA synthesis. Therefore all information at the region of the break is lost in the reaction (Puchta, 2005). The newest results for DSB repair in plants are reviewed by Puchta (2005) and Gorbunova and Levy (1999).

DNA damages caused by UV light and alkylating agents such as EMS are mainly repaired by NER. In NER up to 25 nucleotides are removed by a DNA helicase, which is bound to an enzyme complex that recognizes a wide range of damages. After removal of the oligonucleotides the gap is filled by a DNA-polymerase and an DNA-ligase (de Laat et al., 1999). In NER there are two subpathways, (1) the global genomic repair (GGR) which repairs the DNA damage over the entire genome, and (2) the transcription-coupled repair (TCR) which is selective for the transcribed DNA strand in expressed genes (Kimura et al., 2004). The first evidence for NER pathways in plants came from studies of EMS treated *A. thaliana* plants (Harlow et al., 1994). Here M₂ plants with a mutation in the *uvh1* gene were hypersensitive to ionizing radiation, and were therefore defective in DNA repair. Many putative (evolutionary conserved) NER compound homologs can be found in plant genome databases. In *Arabidopsis* the DNA unwinding helicase *AtXPB* and *AtXPD* are crucial for DNA repair, further null mutations in *Atxpd* are lethal (Boubriak et al., 2008).

The mechanism of BER is similar to the one known from bacteria and mammalian cells. In bacteria and mammals, alkylated bases, and in the case of EMS mutagenesis O⁶-methylguanine, are repaired by the O⁶-Methylguanidin-DNA-methyltransferase (MGMT). The transferase recognizes methylated bases and transfers the methyl-group to a serine residue of its own active site (Engelbergs et al., 2000). So far no MGMT has been found in plants (Britt, 1996), but DNA glycosylases in plants fulfill the same function. As studies in *A. thaliana* showed (Santerre and Britt, 1994). Here the damaged base is removed by lesion specific DNA glycosylases. The DNA glycosylases function in the first step of BER to cleave the 'incorrect' or damaged base from the sugar-phosphate backbone, leaving an abasic (AP) site that is repaired by other enzymes. A more specialized enzyme is known from rice. Here the delta-polymerase has also a deoxyribose activity allowing the enzyme to remove the sugar left by a monofunctional DNA glycosylase (Uchiyama et al., 2004), the gap in the DNA strand is then filled by the polymerase. In BER there also exist two sub pathways, (1) the short-patch BER, which is beta-polymerase dependent and (2) the long-patch BER, which is delta/epsilon-polymerase dependent.

One last important repair mechanism is the mismatch repair (MMR). MMR re-reads newly replicated DNA to improve proof reading function from 10^6 - 10^7 of DNA polymerase to $10^9 - 10^{10}$ (Leonard et al., 2003). The MSH (MutS homologs) protein subunits recognize the rare sporadic mismatches in replicating DNA to prevent the establishment of mutations in the genome by discriminating between the existing correct nucleotide in the template strand and the incorrect nucleotide inserted into the replicating DNA strand (de Wind and Hays, 2001).

1.4 Bolting and flowering time regulation in *Arabidopsis thaliana* and *Beta vulgaris*

As in other crop species, flowering behavior in sugar beet has direct impact on the yield and quality traits of agronomic importance. It is widely accepted that in sugar beet, there is a physiological and temporal distinction between bolting and flowering, such that plants can bolt without flowering, but rarely flower without bolting (Mutasa-Gottgens et al., 2008). In sugar beet production, late frost periods after seedling emergence in spring can serve as the vernalization signal, thus causing sugar beets to bolt already in the first year. As a result in these early bolters, root yield is tremendously decreased (Hoffmann and Kluge-Severin, 2011). The gene underlying annuality in beet was first described by Munerati (1931), whereas the term “bolting gene” (*B*) was later introduced by Abegg (1936). In the presence of the dominant allele *B* in homozygous or heterozygous state, bolting is induced in long days, whereas the homozygous recessive genotype *bb* is present in biennial beets that require vernalization for bolting and transition to flowering (Abe et al., 1997). Bolting behavior can be influenced by environmental factors such as light quality, day length and interacting genes responsible for photoperiod (Abe et al., 1997; Sadeghian and Johansson, 1992). Therefore, early on beet breeders already selected for “resistance” to early bolting. Recently, bolting control in sugar beet has again gained attention, this time in the context of a so-called winter sugar beet.

Winter beets are supposed to have up to 28 % higher yield potential compared to spring sown beets (Jaggard and Werker, 1999). The gain in yield is achieved through the earlier canopy closure in early spring and a therefore photosynthetic activity of leaves, which will lead to a faster root development (Jaggard et al., 1983). The main obstacle in breeding winter beets is the combination of two contrasting breeding goals: (1) bolting repression after cold treatment, and (2) for seed propagation of winter beets, requiring bolting and flowering. Jung and Müller (2009) proposed a model where bolting is regulated through a transgene, which only becomes active when bolting is desired. Recent results in the cloning and identification of several genes of the flowering and bolting time pathway (Abou-Elwafa et al., 2012; Büttner et al., 2010; Chia et al., 2008; Mutasa-Gottgens et al., 2008; Pin et al., 2010; Pin et al., 2012; Reeves et al., 2007), have a high impact on the functional understanding of these pathways and will ultimately lead to the construction of a transgenic winter sugar beet. Since not all important candidate genes have been identified yet, candidate-gene based functional analysis and identification has come into focus of research. Recently, the sugar beet genome has been sequenced by NGS technology and is available to the public (Dohm et al., 2011; Weisshaar et al., 2011). The genome sequence provides extensive molecular resources for sugar beet and enables future high-resolution trait mapping, gene identification, and cross-referencing to regions sequenced in other plant species. This is a milestone in sugar beet science, but cross-referring to candidate genes in other species will still have a major impact. Therefore, further exploitation of the flowering and bolting pathway of the model species *A. thaliana* is still an important issue.

1.4.1 Flowering and bolting time regulation in *Arabidopsis thaliana*

The reproductive success of flowering plants growing in temperate regions is mainly dependent on the ability to adjust vegetative and reproductive growth to local environmental cues such as day length and temperature. Flowering is in general regulated by four main pathways in *A. thaliana* (Andres and Coupland, 2012), the autonomous, the vernalization, the photoperiodic, and the gibberellic acid (GA) pathway. All four pathways are combined by the floral integrators *SUPPRESSOR OF*

OVEREXPRESSION OF CONSTANS 1 (SOC1), FLOWERING LOCUS T (FT) and FD, which in a second step activate the floral meristem identity genes *LEAFY (LFY)*, *APETALA 1 (AP1)*, *CAULIFLOWER (CAL)*, and *FRUITFUL (FUL)* (Figure 3).

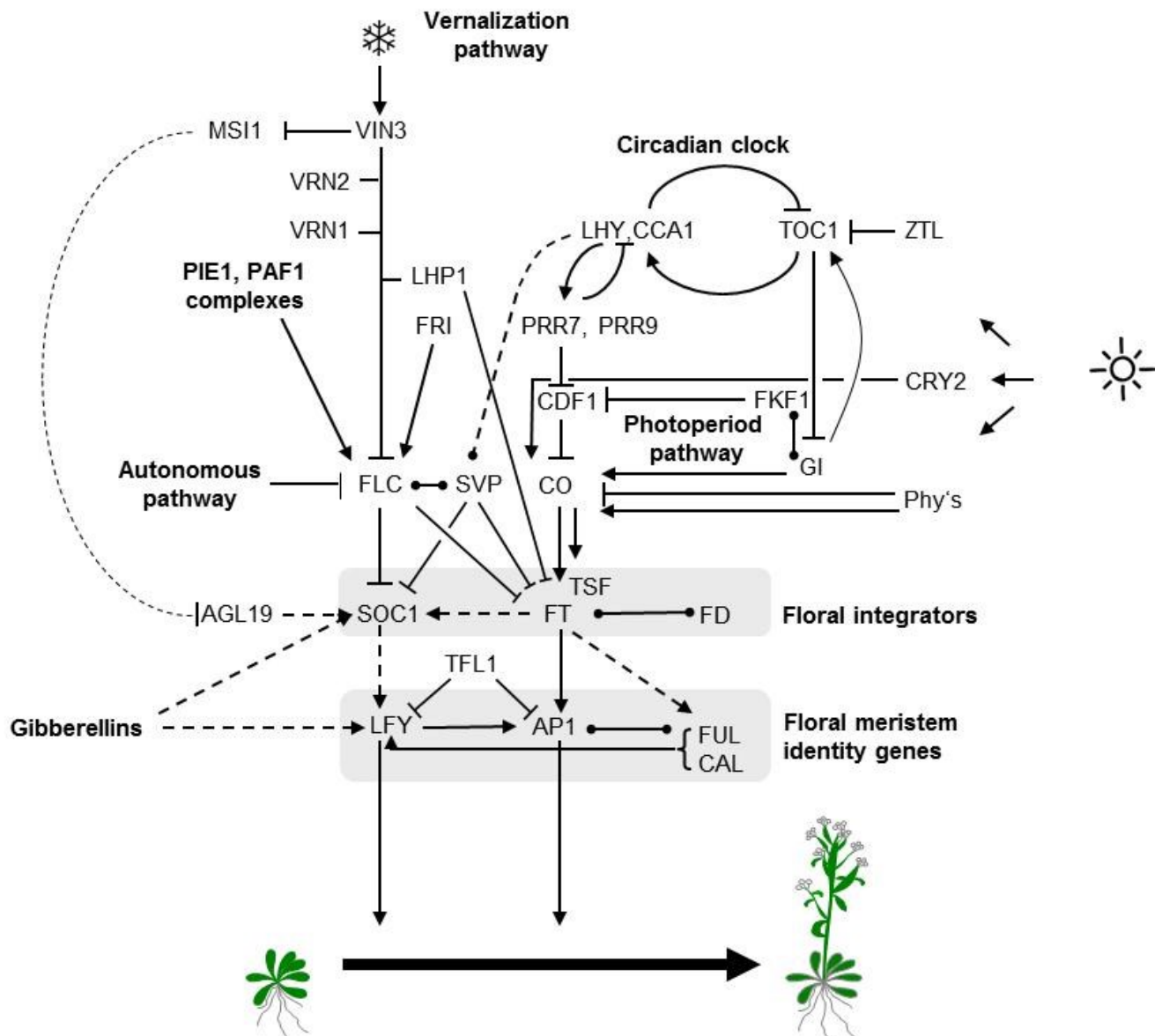


Figure 3: Flowering time control in *Arabidopsis thaliana*. Exogenous signals (cold and light) are indicated by symbols. Arrows and lines with bars, respectively indicate positive and negative regulatory actions. Dashed lines designate interactions that are more speculative. Lines with filled circles at either end indicate protein–protein interactions. (from Jung and Müller, 2009)

Another important group of regulatory molecules are small RNAs including microRNAs (miRNAs). These regulatory molecules play an important role throughout the plant life cycle. MiRNAs are non-coding RNAs (20–24 nucleotides in length) that negatively regulate expression of their target genes via either sequence-specific degradation or translational repression (Jones-Rhoades et al., 2006). A number of miRNAs have been identified that play a critical role in root, flower, and leaf development, as well as in developmental transition (Jones-Rhoades et al., 2006). Furthermore it has been reported that ambient temperature-responsive miRNAs regulate flowering time under non-stress temperature conditions (Lee et al., 2010).

The ability to adjust to temperature is controlled by genes belonging to the vernalization pathway and the autonomous pathway. The key gene in the vernalization pathway is the floral inhibitor *FLOWERING LOCUS C (FLC)*, which maintains the vegetative state without vernalization (Michaels and Amasino, 1999). Prolonged cold activates *VERNALIZATION (VRN1-2)* and *VERNALIZATION INSENSITIVE 3 (VIN3)*, which down-regulate and maintain the repressed state of *FLC* by histone deacetylation and methylation (Sung and Amasino, 2004b). For the stable repression of *FLC* after the cold period, the heterochromatin binding protein *LIKE HETEROCHROMATIN PROTEIN 1 (LHP1)* is needed (Mylne et al., 2006; Sung et al., 2006). By down-regulation of *FLC* the floral integrator, *SOCI* is activated. *SOCI* prevents the premature differentiation of the floral meristem and mainly regulates *LEAFY (LFY)*, one of the floral meristem identity genes (Lee and Lee, 2010). Furthermore, Seo et al., (2009) reported the involvement of *SOCI* in the negative regulation of cold responsive genes termed CBF (C-repeat/dehydration responsive element (DRE)-binding factor).

FLC is also the only target known today for the autonomous pathway (Simpson, 2004). *FLC* is either regulated at the RNA level by eliminating the accumulation of *FLC* mRNA, or epigenetically through histone modifications (Turck and Coupland, 2011) by several genes of the autonomous pathway.

The ability to sense the day length and the photoperiod are regulated by the photoperiodic pathway and by the circadian clock and its key genes *CONSTANS (CO)* *PSEUDO-RESPONSE REGULATOR (PRR7, 9)*, *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, and *LATE ELONGATED HYPCOTYL (LHY)* (Nakamichi et al., 2007). Here, *CO* plays a specific role because it connects the circadian clock and the photoperiodic pathway. *CO* is regulated at the transcriptional level by several genes that are part of the circadian clock or are under circadian control. Activation of *CO* occurs when high light intensities (far red to low red/ratio) coincide with long days (LD) by cryptochromes (*cyp2, 1*) and phytochrome A (*phyA*) (Valverde et al., 2004) and through repression of *CYCLING DOF FACTOR 1 (CDF1)* by *PRR7* and *9* (Nakamichi et al., 2007). During short days (SD), the *CO* mRNA is not stabilized by light and will be methylated by the E3 ligase *CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)*, which causes degradation by the proteasome (Liu et al., 2008). *PRR7/9*, *CCA1* and *LHY* regulate each other by a feedback loop. *CCA1* and *LHY* are constitutively expressed through the day and are transcriptionally repressed by *PRR7/9* protein from morning until midnight. To close the loop, *CCA1* and *LHY* proteins activate *PRR7/9* transcription by directly binding to their promoters (Nakamichi et al., 2010).

The ability to sense day length and photoperiod is essential, but the transition from vegetative to generative growth requires a genetic reprogramming at the shoot apical meristems to develop shoots and flowers instead of leaves. This is achieved by the phloem-mobile protein of *FLOWERING LOCUS T (FT)* which represents the long sought florigen (Amasino and Michaels, 2010). *FT* is activated during LD by interaction of the *CO* protein with the *FT* promoter in phloem companion cells. The active protein is transported to the shoot apex through the phloem, where it forms a heterodimer with *FLOWERING LOCUS D (FD)* and activates *SOCI* and *APETALA1 (API)* (Adrian et al., 2010; An et al., 2004). By maintaining the activation of *API* and *LFY* the flower meristem identity is established and by that action flower development can take place at the precursor of shoot apical meristem (Causier et al., 2010).

1.4.2 Flowering and bolting time regulation in *Beta vulgaris*

Research on the molecular identification of flowering and bolting time genes in sugar beets has led to the identification of several genes. Reeves et al. (2007) could identify the *AtFLC* homolog in sugar beets (*Beta vulgaris FLC-LIKE 1*). Overexpression of *BvFL1* in *flc* mutants in Arabidopsis could restore flowering repression. Therefore, they assumed a similar function of *BvFL1* in sugar beets. Chia et al. (2008) were able to identify several *CONSTANS-LIKE (COL)* genes in sugar beet, and overexpression of *BvCOL1* could rescue the late flowering phenotype of *A. thaliana co-2* mutants.

These findings suggested an important role of *BvCOL1* in the photoperiod response of sugar beet. Another breakthrough was the identification of two paralogous copies of the *A. thaliana* gene *AtFT*, termed *BvFT1* and *BvFT2* by Pin et al. (2010). In *BvFT2*, the function of *AtFT* is conserved and promotes flowering. In contrast, *BvFT1* acts antagonistically and represses flowering. As in *A. thaliana*, both *BvFT* paralogs play key roles in the integration of vernalization and photoperiodic pathways. Recently Pin et al. (2012) have reported cloning of the *B* gene which they termed *BOLTING TIME CANDIDATE 1 (BTC1)*. *BTC1* is a homologue of the PRR 3/7 clade and differs by 11 non-synonymous SNPs and one large insertion in the promotor region of the biennial allele from the annual allele. Regarding the function, Pin et al. (2012) report that annuals lost their ability to bolt when *BTC1* was down regulated by RNAi. Plants carrying the dominant *BTC1* allele bolt and flower under long day conditions without vernalization, whereas plants carrying the recessive *BTC1* allele require vernalization in order to bolt. In biennial beets, down-regulation of *BvFL1* by vernalization and LD-dependent induction of *BTC1* removes the *BvFT1* dependent repression of *BvFT2* and thereby induces the transition into the generative state (Pin et al., 2010). Further research at the Plant Breeding Institute in Kiel has led to the identification of the *FLOWERING LOCUS K (FLK)* homolog *BvFLK* (Abou-Elwafa et al., 2012). When overexpressed in *A. thaliana*, *BvFLK* accelerates bolting in the Col-0 background and fully complements the late-bolting phenotype of a *flk* mutant through repression of *FLC*. Additionally, four homologous genes *BvFVE1*, *BvLD*, and *BvLDL1* to the Arabidopsis autonomous pathway genes *FVE*, *LUMINIDEPENDENS (LD)*, and *LYSINE-SPECIFIC HISTONE DEMETHYLASE1 LIKE1 (LDL1)*, respectively, were identified in sugar beets (Abou-Elwafa et al., 2012).

1.5 Objectives

Beta vulgaris ssp. *vulgaris* is an agronomical important crop for sugar production in Europe with a diploid genome of moderate size that has a relative short history of breeding, compared to other important crops species such as maize or wheat. In addition, genetic variation is rather limited in sugar beet as it is derived from a single population of fodder beet. Given this, generation of new genetic variability by mutagenesis is an important method to broaden the genetic base of sugar beet germplasm. In classical mutation breeding, selection for favorable mutations by phenotyping large mutant populations has suffered from low throughput. More efficient is mutagenesis coupled with high throughput screening of offspring genotypes and phenotypes that can be accomplished using the chemical cleavage mismatch (CCM) method Targeted Induced Local Lesions IN Genomes (TILLING), this has not yet been applied to *B. vulgaris*.

One important goal in sugar beet research is the understanding of the mechanism underlying the regulation of flowering and bolting to optimize beet production in order to address the underlying genes in selection.

The four main pathways regulating vernalization response, bolting and flowering have been thoroughly characterized and many genes have been already identified in the model plant organism *A. thaliana*, and recent advances in sugar beet research have resulted in the characterization of several of the *B. vulgaris* homologues. This knowledge allows for the identification of induced or natural mutants on the DNA level via TILLING and EcoTILLING, respectively.

The objectives of the work were:

- To generate a EMS mutant population from the biennial sugar beet doubled haploid (DH) line SynDH1
- To establish a TILLING protocol for sugar beet
- To isolate and test different endonucleases for their suitability in TILLING
- To identify point mutations in candidate genes of the bolting and flowering pathway, as well as virus resistance genes and genes responsible for storage root formation
- To identify phenotypic effects caused by these mutations
- To produce double mutants from desired phenotypes
- To establish an EcoTILLING protocol and screen for natural nucleotide polymorphisms in *Beta* accessions
- To identify natural sequence variants in the bolting and flowering pathway

2 TILLING in *Beta vulgaris* ssp. *vulgaris* L. - Detection of new sequence variants for flowering and bolting time genes

2.1 Introduction

Induced mutations have been a driving force in breeding programs since the early 1960s. They provided breeders with additional genetic variation for the development of new cultivars. Beyond that, mutations have been an important tool in genetics. The traditional way of identifying new traits was to analyze phenotypic changes caused by mutations and link these phenotypic changes to a gene (forward genetic approach; Konopka and Benzer, 1971). During the last 5 decades, large mutant collections have been developed for various model and crop plant species. After linking phenotypic traits to genetic information and identification of the respective gene, the corresponding sequence information can be retrieved by e.g. map-based cloning. Although this method is time-consuming and labor-intensive, map-based cloning has been successfully applied in forward genetic approaches in crop species with large genomes such as wheat (Keller et al., 2005).

The forward genetic approach in mutation analysis studies has been complemented by a reverse genetic strategy (Shortle et al., 1981), in which a gene of interest and its alteration in structure or activity are analyzed for an impact on phenotype. Applied techniques for reverse genetics are for example transposon/retrotransposon tagging or gene silencing using RNA interference (RNAi). Although these techniques are generally applicable, the majority of these techniques are currently only applied to model organisms with small genomes. A decade ago, McCallum et al., (2000a) presented TILLING (Targeting Induced Local Lesions in Genomes) as an alternative reverse genetic technique to identify point mutations in a mutant population of *Arabidopsis thaliana*. In the TILLING procedure, chemical mutagens such as EMS (ethyl-methanesulfonate) or MNU (N-nitroso-N-methylurea) are applied to seeds, pollen, or ovaries to induce genome-wide point mutations. Point mutations are discovered by pooling DNA from several mutant plants, amplifying candidate genes by PCR and detecting sequence variation after heteroduplex digest of the PCR products by endonucleases such as CEL1 or *EndoI* and subsequent fragment length analysis, e.g. on capillary sequencers (Stephenson et al., 2010), by HPLC (Caldwell et al., 2004), by MALDI-TOF (Chawade et al., 2010) or by high-resolution DNA melting analysis (HRM) (Bush and Krysan, 2010). TILLING is a high throughput technique that combines advantages of classical induced mutagenesis, sequence availability and high-throughput screening for nucleotide polymorphisms. One major advantage of TILLING is based on the fact that it can be applied to almost any organism, regardless of ploidy level, genome size or method of propagation. Furthermore, the use of chemical mutagens is favored over insertional mutagenesis, since the former is able to create a broad spectrum of mutations, including missense changes, truncations and mutations in splice junctions, whereas the latter mostly creates knockouts. Therefore, chemical mutagens do not only generate loss of function alleles but also gain of function and hypomorphic alleles that can provide a range of phenotypes. Hypomorphic alleles have a reduced level of gene expression, but not a complete loss (van Harten, 1998). Additionally, the induced mutations are stable, which is not always the case for alternative methods of reverse genetics such as RNAi silencing or transposon tagging (Almeida and Allshire, 2005).

Although TILLING was developed as a discovery tool for functional genomics, it has been successfully applied for crop species as a valuable tool for manipulation of genes underlying traits of agronomic importance. Today, insertional mutagenesis can be regarded as a golden standard for gene manipulation for most plant researchers because genomic resources are readily available and many plant genomes are small. However, it requires an efficient transformation system, which is not available for each plant species. Furthermore, insertional mutagenesis is often not saturable and therefore requires large population sizes (Wang et al., 2012). In addition, artificially induced mutations through chemical mutagens do not fall under the regulation of genetically modified organisms (GMO).

This is especially valuable for Europe, which has the broadest and most stringent regulations for GMO field trials in the world. Since in the EU the assumption, that all GMOs carry an inherent risk (Gómez-Galera et al., 2012), TILLING can be regarded as a potential alternative to the transgenic approach and has already been successfully established for a variety of crop species as described in section 1.3.1 above. Mutagenized populations are a useful resource for crop improvement and TILLING is a valuable technique to explore mutations, which can in further steps, be introgressed into elite breeding material. Furthermore TILLING is a good alternative to direct DNA modification techniques such as insertional mutagenesis. The advantages of TILLING together with the valuable resource of a mutagenized sugar beet population will provide a basis for the evaluation of useful mutations for crop improvement in sugar beet.

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) is a diploid species with $2n=2x=18$ chromosomes and a relatively small genome size of 758 Mbp. It has a biennial growth habit as described in chapter 1.1 Sugar beet is an allogamous species with an efficient gametophytic self-incompatibility system based on a series of sterility alleles (*S1-Sn*, *Z1-Zn*) (Biancardi et al., 2005). Thus, sugar beet populations are highly heterogeneous and heterozygous. Seed set after selfing can be increased by introducing the S^F -self compatibility allele, which suppresses the *S*-alleles. Sugar beet is the only crop for sucrose (i.e. sugar) production in the temperate climate zone, which accounts for about one quarter to one third of the worldwide sugar production (FAO, 2012). Furthermore sugar beet is becoming an important biofuel alternative to fossil energy. A number of genomic tools as described in chapter 1.1 supports genetic research in sugar beet.

A promising strategy for increasing its yield potential is to grow sugar beet as a winter crop (Jaggard and Werker, 1999). Besides sufficient winter hardiness, the cultivation of winter beets requires the suppression of bolting after winter. For seed production, however, reactivation of bolting is required (Jung and Müller, 2009). To understand the mechanisms of bolting and to achieve bolting control, mutants could be helpful.

To gain a better picture of bolting and flowering, substantial information on the underlying mechanisms is needed. As revealed in the model plant *A. thaliana*, the two major mechanisms in the regulation of flowering time of plants are the vernalization and the photoperiodic pathway as reviewed by Amasino and Michaels (2010) and Srikanth and Schmid (2011). While the vernalization pathway responds to long periods of cold temperature, the photoperiod pathway controls flowering by the ability to distinguish long days (LD) from short days (SD). Underlying genes of these pathways have been reported in Arabidopsis and a number of homologues to these genes have been identified in the sugar beet genome. As the gene *FLC* (*FLOWERING LOCUS C*) in the vernalization pathway of *A. thaliana*, the sugar beet homologue *BvFL1* (*Beta vulgaris FLC-LIKE 1*) seems to play a major role in floral repression as well (Reeves et al., 2007). In *A. thaliana*, *FLC* functions as a floral repressor maintaining the vegetative state of the growing apex (Dennis and Peacock, 2007; Michaels and Amasino, 1999). *FLC* is stable repressed by histone deacetylation and methylation through the polycomb group proteins *VRN1*, and *VRN2* (*VERNALIZATION 1* and *2*, respectively) (Choi et al., 2005). For the initiation of the repression of *FLC* the gene *VIN3* (*VERNALIZATION INSENSITIVE 3*), whose expressions increases proportionally with the length of cold, is necessary (Sung and Amasino, 2004a). Eventually, *LHP1* (*LIKE HETERCHROMATIN PROTEIN 1*) is required to maintain the repressed state of *FLC* after the end of the cold period (Mylne et al., 2006; Zhang et al., 2007). The up-regulation of *FLC* transcript is controlled by *FRI* (*FRIGIDA*) (Michaels and Amasino, 2001). *FRI* builds a transcription activator complex with other genes such as *FRI1* (*FRIGIDA LIKE 1*), *FES1* (*FRIGIDA ESSENTIAL 1*), and *FLX* (*FLC EXPRESSOR*) and recruits as such the general transcription machinery and chromatin modification factors (Choi et al., 2011). *FLC* is also regulated by RNA-based control mechanisms and/or chromatin modifications through different genes assigned to the autonomous flowering time pathway (Bäurle and Dean, 2008; Lim et al., 2004; Simpson, 2004). *FLOWERING LOCUS CA* (*FCA*), *FLOWERING LOCUS PA* (*FPA*), and *FLOWERING LOCUS KH*

DOMAIN (FLK) regulate through RNA regulatory processing while *FLOWERING LOCUS VE (FVE)* and *FLOWERING LOCUS D (FLD)* control flowering as part of an histone deacetylase complex (Ausin et al., 2004).

In the photoperiod pathway, the floral promoter gene *CO (CONSTANS)* mostly controls flowering. Several *CO*-like genes (*BvCOL1*) have been described for sugar beet. One of these genes was shown to be functionally related to *CO* (Chia et al., 2008), but none of the *CO*-like genes in beet appear to be true orthologs. *CO* is regulated at the transcriptional level by several genes that are part of the circadian clock, like *CCA1 (CIRCADIAN CLOCK ASSOCIATED 1)* and *LHY (LATE ELONGATED HYPCOTYL)* or are under circadian control as the *PRR (PSEUDO-RESPONSE REGULATOR)* genes. Activation occurs when high light intensities (far red to low red/ratio) coincide with LDs by *cyp2, 1* (cryptochromes) and *phyA* (phytochrome A) (Valverde et al., 2004) and through repression of *CDF1 (CYCLING DOF FACTOR 1)* by *PRR7* and *9* (Nakamichi et al., 2007). During SDs, the *CO* mRNA is not stabilized by light and will be methylated by the E3 ligase *COPI (CONSTITUTIVE PHOTOMORPHOGENIC 1)*, which causes the degradation by the proteasome (Liu et al., 2008). *PRR7/9*, *CCA1* and *LHY* regulate each other by a feedback loop. *CCA1* and *LHY* are constitutively expressed through the day and are transcriptionally repressed by *PRR7/9* protein from morning until midnight. To close the loop, *CCA1* and *LHY* proteins activate *PRR7/9* transcription by directly binding to their promoters (Nakamichi et al., 2010). A *PRR* gene, *BOLTING TIME CONTROL 1 (BTC1)*, has been also identified for beet (Pin et al., 2012). The dominant *BTC1* allele promotes bolting in annual plants in response to LD by differential regulation of the floral integrators, while in biennial beets vernalization is needed to mediate the promotive effect. This is due to a partial-loss-of-function allele which is not able to differentially regulate the floral integrators (Pin et al., 2012) without prior cold treatment.

These floral integrator genes which include *FT (FLOWERING LOCUS T)* and *SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1)*, act as strong floral promoters and are antagonistically regulated by *CO* and *FLC*. *FT* is the so-called “florigen” (Turck et al., 2008; Zeevaart, 2008) and transports the signal to flower from the phloem companion cells of the leaf to the shoot apical meristem. *CO* achieves this process firstly through the transcriptional activation of *FT*. After transcription *FT* is transferred to the apical meristem (Corbesier et al., 2007; Jaeger and Wigge, 2007) and induces flowering by building a complex with the bZIP transcription factor *FD* to transcriptionally activate *SOC1* and *API (APETELAI)* (Abe et al., 2005; Lee and Lee, 2010; Wang et al., 2009). In *B. vulgaris* there are two *FT* paralogs, *BvFT1* and *BvFT2*, which integrate the vernalization and the photoperiodic pathway (Pin et al., 2010). *BvFT1* represses flowering, while *BvFT2* promotes flowering. Flower induction is achieved by the repression of *BvFT1* through the down-regulation of *BvFL1* by vernalization and LD-dependent induction of *BTC1* (Pin et al., 2012).

Thus, a number of sugar beet genes have been cloned and characterized due to their similarity in function to *A. thaliana* homologs from the autonomous, vernalization, photoperiodic, and flowering pathways. These *Beta vulgaris* genes are important candidates for TILLING in order to obtain mutants with altered flowering behavior that could be utilized in sugar beet breeding. In this chapter, I will present a protocol for routine identification of mutants in the offspring of EMS-treated sugar beets. Furthermore, results of the TILLING screens in the genes *BTC1*, *BvCOL1*, *BvFL1*, *BvFLK*, *BvFT1*, *BVFT2*, *BvFVE*, *BvLHP1*, *BvSWP* and *BvTON1*, will be presented.

2.2 Material and Methods

2.2.1 Plant material

The annual sugar beet population 930190 and the biennial sugar beet populations SynDH1 (seed code 062457) (Syngenta Seeds AB Landskrona, Sweden) as well as 940043 were used in this study.

The mutant population 930190 (93167P) has been described by Hohmann *et al.* (2005). In short, this population was derived from the annual sugar beet genotype 930190 (Saatzucht Dieckmann, Nienstädt, Germany). 930190 is homozygous for the dominant bolting allele (*BB*) and bolting is initiated under LD conditions 9 – 12 weeks after germination. Seeds from this line were treated for 12 hours with EMS concentrations ranging from 0.5 to 1.0 %.

The doubled haploid sugar beet line ‘SynDH1’ provided by Syngenta Seeds AB (Landskrona, Sweden) was used for the creation of the second mutant population. SynDH1 exhibits a vernalization requirement of about 12 weeks for bolting.

The biennial sugar beet line 940043 (93168P) provided by Saatzeit Dieckmann (Nienstädt, Germany) was used for the creation of a third mutant population. 940043 exhibits a vernalization requirement of about 12 weeks for bolting.

2.2.2 EMS mutagenesis and population propagation

Two different EMS mutagenesis treatments were carried out. Mutagenesis of population 930190 is described by Hohmann *et al.* (2005). Here batches of 100 seeds were transferred to 50 ml Sarstedt tubes (SARSTEDT AG & Co., Nümbrecht, Germany) which were covered by a nylon mesh instead of a lid. Several tubes were dipped for 8 hours into 200 ml distilled water at room temperature, to allow soaking of the seeds. Pre-soaked seeds were then transferred to 200 ml sodium phosphate buffer (0.1 M, pH 7.4) with two different EMS concentrations (0.5 % and 1 %) for 4, 6, 8 and 12 hours, while gently shaken (100 rpm). Following mutagenesis, the seeds were washed twice with sodium thiosulphate buffer (0.1 M, pH 7.4) for 20 min at room temperature while gentle shaking (100 rpm). Finally, the seeds were washed in 200 ml distilled water and then rinsed under running tap water for 30–40 min. The seeds were dried and kept on wet filter paper (Whatman) overnight.

The mutagenesis treatment for the population SynDH1 and 940043 was as follows: Prior to mutagenesis, M_0 seeds were germinated on wet paper towels at 20°C for 24 hours. Batches of 200 seeds were transferred to 200ml Erlenmeyer flasks filled with 150 ml sodium phosphate buffer (0.1 M, pH 7.4) and an EMS concentration of 1 % (v/v). The treatment was carried out for 12 hours while gently shaken at 100 rpm. Following mutagenesis, the seeds were washed twice with sodium thiosulphate buffer (0.1 M, pH 7.4) for 30 min at room temperature while gentle shaking (100 rpm). Finally, the seeds were rinsed under running tap water for 40–50 min. Seeds were dried on filter paper (Whatman) over night at room temperature.

For germination M_1 seeds were planted in standard potting soil (Einheitserde Typ ED73, Einheitserdewerk Uetersen, Uetersen, Germany) covered with sand and treated with 0.25 % Previcur[®] N (Syngenta, Maintal, Germany) to control seedling diseases. M_1 plants were grown in a greenhouse at Kiel and in Landskrona (Sweden) in small pots or in flats with 250 to 400 seeds per flat. After 2 to 3 weeks, plantlets were transplanted to 11 x 11 cm plastic pots and grown for 6 weeks in the greenhouse. M_1 plants from the biennial populations (SynDH1 and 940043) received an additional vernalization of 12 weeks at 4°C. After vernalization the plants were transferred to the field (Kiel 2005, 2006, 2008, and 2009) or to 13 x 13 cm plastic pots and grown in the greenhouse (Kiel and Landskrona 2008 - 2010), depending on number of plants and weather conditions. All bolting plants were bagged to ensure selfing. Shoots with flowering branches were isolated using plastic bags and after seed

production, selfed M_2 seeds were harvested by hand. Depending on the seed availability, between one and eight M_2 plants per $M_{1:2}$ family were grown for M_3 seed production by using identical conditions as described above.

2.2.3 DNA extraction and pooling strategy

In the TILLING project, two entire plant populations comprising 2,688 and 3,148 plants were to be screened and this required a large amount of DNA. To evaluate the best DNA extraction method, three different approaches were compared for cost effectiveness and DNA yield. DNA yield is a mandatory goal in TILLING since the DNA should last for a large number of screens for different genes and sequencing reactions of positively tested mutants.

NucleoSpin® 96 Plant II DNA extraction:

DNA was isolated from one small leaf taken from each M_2 plant. After sampling, leaves were put into 2 ml Eppendorf tubes (SARSTEDT AG & Co.) or into 8-well Eppendorf stripes (0.5 μ l) (SARSTEDT AG & Co.) and freeze-dried. Subsequently, 50 mg freeze-dried leaves were ground using a ball mill and DNA was extracted with the NucleoSpin® 96 Plant I or NucleoSpin® 96 Plant II Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions on a Tecan Freedom EVO® Robot (Tecan Group Ltd., Crailsheim, Germany). For evaluation purposes, DNA concentration was measured by a NanoDrop 2000 (Fisher Scientific GmbH, Schwerte, Germany) as well as on 3% agarose gels, on which a DNA standard marker (FastRuler™ Middle Range DNA Ladder, Fermentas, St. Leon-Rot, Germany) allowing for DNA concentration determination was used. For pool construction, DNA concentration was also measured by the Tecan Freedom EVO® Robot using a photometer and SYBR® Green dye (Invitrogen GmbH, Darmstadt, Germany) and subsequently normalized with DNase free water to a concentration of 10 ng/ μ l in a total volume of 160 μ l. Normalized DNA samples were pooled to 8x 2D pools in 96-well plates.

CTAB DNA extraction:

Genomic DNA was extracted according to the CTAB method described by Rogers and Bendich (1985). 50 mg fresh leaf material was ground into a fine powder in liquid nitrogen, dispersed in 300 μ l of pre-warmed extraction solution, and incubated at 65°C for 30 to 60 min with occasional mixing by gentle swirling. After cooling the suspension on ice for 4-5 min, 1 volume of chloroform/isoamylalcohol (24:1, v/v) was added; the solution was then mixed by inversion and centrifuged at 4,500 x g for 10 min at 4°C. The supernatant was transferred to a new tube. The DNA was precipitated with 1 volume of cold isopropanol and centrifuged at 3,200 x g for 5 min at 4°C. The supernatant was discarded, and the DNA was transferred into a new micro centrifuge tube containing washing solution I. After 10 min incubation at room temperature, the tube was centrifuged at 3,200 x g for 5 min; the supernatant was again discarded before addition of washing solution II. After 10 min incubation at room temperature the tube was centrifuged at 3,200 x g for 5 min and the supernatant was discarded. After air-drying the DNA-pellet for about 10 to 20 min, DNA was eluted into 100 μ l TE buffer. In order to remove RNA, RNase (10 μ l, 10 μ g/ml) treatment was applied for 30 min at 37°C. The final step was the measurement of DNA concentration by the NanoDrop 2000 (Fisher Scientific GmbH) and by agarose gel electrophoresis with a DNA standard marker (FastRuler™ Middle Range DNA Ladder, Fermentas) allowing for DNA concentration determination.

Extraction solution		Washing solution I	
Tris-buffer, pH 7.5	200 mM	Sodium acetate	200 mM
NaCl	1.4 M	Ethanol	76 % (v/v)
EDTA pH 8.0	20 mM		
CTAB	2 % (w/v)	Washing solution II	
β -mercaptoethanol*	0.2 % (v/v)	Ammonium acetate	10 mM
* added immediately before use		Ethanol	76 % (v/v)

MagAttract® 96 DNA Plant Core DNA extraction:

DNA was isolated from one small leaf taken from each M₂ plant. After sampling, leaves were put into 2 ml Eppendorf tubes (SARSTEDT AG & Co.) and freeze dried. 50 mg freeze-dried leaves were ground using a ball mill and DNA was extracted with the MagAttract® 96 DNA Plant Core (QUIAGEN, Hilden, Germany) following the protocol specified by the manufacturer. After elution, DNA concentration was measured using the NanoDrop 2000 (Fisher Scientific GmbH) spectrophotometer and 3% agarose gels, on which a DNA standard marker (FastRuler™ Middle Range DNA Ladder, Fermentas) allowing for DNA concentration determination was used.

DNA pooling:

Pooling size and depth is another important issue when performing genomic screens on large populations, especially when performing TILLING screens. Here it is of major importance to identify a mutated individual in a mixture of different DNAs. To evaluate the optimal pooling depth for the construction of DNA pool plates for TILLING screens, nine different pooling strategies were tested. A test DNA pool from two individual sugar beets (930190 and 940043) was generated according to Table 3.

Table 3: Pooling size and depth.

Pooling depth	1x	8x	6x	4x	2x	4x	6x	8x	1x
Pooling ratio	8:0	1:7	1:5	1:3	1:1	3:1	5:1	7:1	0:8
930190	0	0.1	0.2	0.3	0.5	0.7	0.8	0.9	1
940043	1	0.9	0.8	0.7	0.5	0.3	0.2	0.1	0

The two accessions differed by two SNPs and one indel in the *BvTON1* gene. Therefore, three fragments after CEL1 digestion were expected.

Pooled DNA samples were amplified by PCR. PCR amplification was performed in a total volume of 20 μ l containing 2 ng pooled DNA, 1x Taq buffer, 1.5 mM MgCl₂, 2 mM dNTPs (Invitrogen), 0.2 units recombinant Taq DNA polymerase (Invitrogen) and 0.8 pmol of each primer (Eurofins MWG Operon, Ebersberg, Germany). 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 3 min followed by 30 cycles of a 20 sec denaturation step at 95°C, 30 sec annealing at 65°C, and 60 sec elongation at 72°C. The final PCR step was a 5 min elongation at 72°C before cooling the samples to 8°C.

For SNP identification, a subsequent heteroduplex analysis was performed. The PCR product was denatured at 95°C for 10 min and slowly re-annealed by cooling down to 85°C at a ramping rate of 2°C per sec and further cooling down to 25°C using ramping rates of 0.5°C per sec. The re-annealed PCR product was digested at 42°C for 15 min with CCE containing 0.6 μ l CCE and 5.4 μ l CCE buffer for each 20 μ l reaction. The reaction was stopped by addition of 3 μ l 200 mM EDTA. Digested PCR samples were separated on a 1% ethidium bromide stained agarose gel for 20 min at 100 V.

Visualization was performed with a Gel Doc™ XR molecular imager (Bio-Rad Laboratories GmbH, München, Germany).

2D pool plates were constructed in the following manner: eight M₂ samples in each of the columns 1 A-H to 8 A-H of the DNA plate 1 were pipetted into column 1 A-H of the 2D pool plate. Eight M₂ samples in each of the rows A 1-8 to H 1-8 were then pipetted in the 2nd column (2 A-H) of the 2D pool plate (row pool), and the following samples were transferred accordingly for DNA plates 2 to 5 (Figure 4). The remaining two columns (11 and 12) included positive and negative controls for endonuclease digestion. DNA pools were stored at -20°C while the normalized DNA samples were stored at -70°C in 96 well plates sealed with adhesive foil.

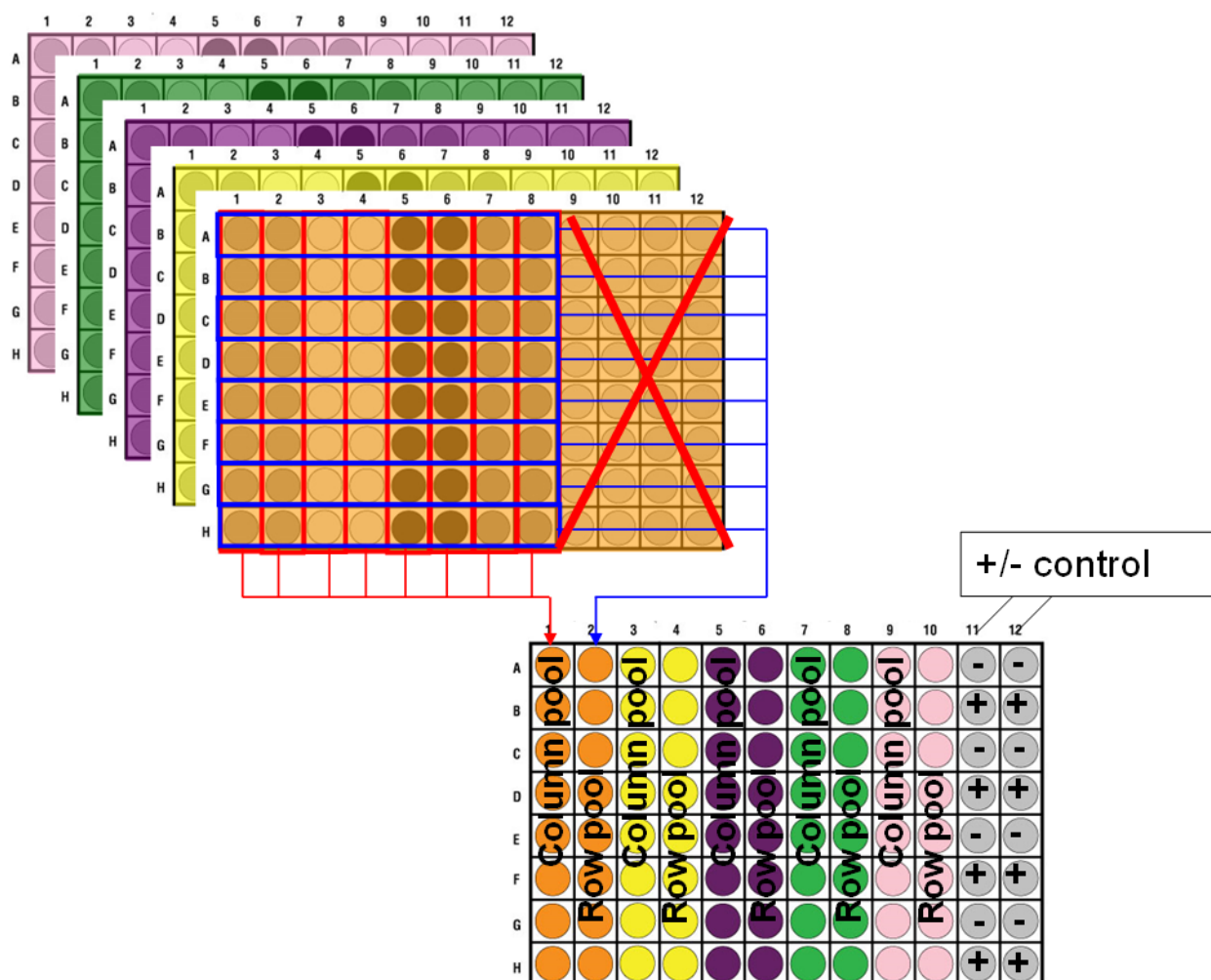


Figure 4: Construction of an 8x 2D pool. Samples of columns 1 A-H to 8 A-H of DNA plate 1 (orange) was pipetted into column 1 A-H of the 2D pool plate. Samples of rows A 1-8 to H 1-8 were pipetted in the 2nd column (2 A-H) of the 2D pool plate (row pool). Transfer of samples from DNA plates 2 to 5 (yellow, purple, green, and pink) was performed accordingly. The remaining two columns (11 and 12) were used for positive and negative controls of the endonuclease digestion.

2.2.4 RNA extraction and reverse transcription quantitative real time PCR

To test whether the nucleotide change had an effect on *BvFTI* expression, leaf samples were collected from all plant families shown in Table 19 at BBCH-13 at different time points (Zeitgeber ZT 0, 2, 4, 6, 8, 10, 14, and 16) as mentioned in Pin et al. (2010). Total RNA was isolated from fresh leaves with the “RNeasy® Plant Mini Kit” (QUIAGEN GmbH) as described by the manufacturer and DNase treated (DNase I, Fermentas). The amount of RNA was validated through optical measurement with a NanoDrop ND-2000 (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and by visual control on a

1% agarose gel. RNA was reverse transcribed with RevertAid™ (Fermentas) to cDNA and the amount and quality was checked via optical measurement as above and visual on a 1% agarose gel. The cDNA was normalized to 5ng/μl and reverse transcription quantitative real time PCR (RT-qPCR) was performed on a Biorad FX Cyclor (Bio-Rad Laboratories GmbH). For expression normalization, the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (*BvGAPDH*) was used. The PCR programs used for expression analyses of *BvFT1* and *BvGAPDH* are given in Table 4. The RT qPCR results were evaluated with the program Biorad FX Manager (Bio-Rad Laboratories GmbH).

Table 4: Reverse transcription quantitative real time PCR conditions for the expression analyses of *BvFT1* and *BvGAPDH*. The table shows the required temperature, the time, the number of cycles, and the status.

Temperature [°C]	Time [min]	Cycles	Status
95.0	3.0	1	Denature
95.0	0.10	40	Denature
61.0 (<i>BvGAPDH</i>)	0.30		Annealing
64.5 (<i>BvFT1</i>)	0.30		Elongation
72.0			Elongation
95.0	0.10	1	Melt curve
65.0 to 95 increment 0.5	0.05	1	

2.2.5 Primer design and primer tests

In both sugar beet populations, bolting and flowering time regulatory genes were targeted by TILLING (Table 5). Sequence information for the candidate genes *BTC1*, *BvColl*, *BvFLK*, *BvFT1*, *BvFVE*, *BvLHP1*, *BvSWP*, and *BvTON1* was provided by Dr. Andreas Müller (Plant Breeding Institute, Kiel, Germany) in collaboration with the GABI Genoflor project. Sequence information for *BvFL1* was obtained from Reeves et al. (2007) and sequence information for *BvFT2* was obtained from Pin et al. (2010). The above-mentioned candidate genes *BvColl*, *BvFLK*, *BvFVE*, *BvLHP1*, *BvSWP*, and *BvTON1* are homologues of the respective *A. thaliana* flowering genes. The genes *BTC1*, *BvColl*, *BvFT1*, *BvFT2*, and *BvFL1* share an amino acid identity of up to 85 % to the *A. thaliana* genes *PRR3/7*, *CO*, *FT1*, and *FLC*, respectively.

Table 5: Sugar beet flowering time genes investigated by TILLING using populations 930190 and SynDH1. Indicated in the columns are the gene names, their assumed effect on bolting / flowering and the length of their genomic sequence. Furthermore, the number of already screened M₂ families per candidate gene is shown (population sizes: 930190= 2,688; SynDH1 = 3,418). The column “part of genomic sequence screened via TILLING” gives estimation on the percentage proportion of the whole genomic sequence investigated via TILLING during this study. *BvSWP* and *BvTONI* were used as control genes.

<i>B. vulgaris</i> gene name	Effect on bolting / flowering	Length of genomic sequence	Population	# of tested / successfully tested primers	# of screened M ₂ plants (CEL1)	genomic sequence screened via TILLING	reading frame investigated via TILLING
Bolting time control 1 (<i>BTC1</i>)	promoting	12,480 bp	SynDH1	10/03	3,418	1,437 bp (11.5%)	49.4%
			930190	32/18	2,688	1,437 bp (11.5%)	49.4%
Constans Like gene 1 (<i>BvCol1</i>)	promoting	4,018 bp	930190	15/01	1,536	1,253 bp (31.2%)	67.4%
Flowering Locus K (<i>BvFLK</i>)	promoting	11,901 bp	SynDH1	16/12	3,418	2,284 bp (19.2%)	68.2%
FLC-Like 1 (<i>BvFL1</i>)	repressing	36,165 bp	930190	18/06	2,688	966 bp (2.7%)	34.5%
			SynDH1	06/02	3,418	966 bp (2.7%)	34.5%
Flowering Locus T1 (<i>BvFT1</i>)	repressing	11,466 bp	SynDH1	30/04	3,418	1,687 bp (14.7%)	100%
			930190	30/04	2,688	1,687 bp (14.7%)	100%
Flowering Locus T2 (<i>BvFT2</i>)	promoting	4,893 bp	SynDH1	08/02	3,418	1,237 bp (25.3%)	71%
			930190	10/02	2,688	1,237 bp (25.3%)	71%
Flowering Locus E (<i>BvFVE</i>)	promoting	13,880 bp	SynDH1	04/02	3,418	1,131 bp (8.2%)	23.4%
			930190	12/03	1,152	1,857 bp (13.4%)	40.1%
Like Heterochromatin Protein 1 (<i>BvLHPI</i>)	promoting	8,466 bp	SynDH1	08/02	3,418	899 bp (10.6%)	36.8%
			930190	26/03	2,688	1,262 bp (14.9%)	69.8%
Stuwelpeter (<i>BvSWP</i>)	indirect promoting of flowering	3,721 bp	930190	11/02	1,536	1,502 bp (40.4%)	62.4%
TONNEAU 1 (<i>BvTONI</i>)	unknown	11,300 bp	SynDH1	10/02	3,418	907 bp (8.0%)	40%
			930190	12/03	2,688	907 bp (8.0%)	40%

Locus-specific primer for PCR amplification of target regions in candidate genes were designed with the programs FastPCR[®] (Kalendar et al., 2009), Primer3Plus (Untergasser et al., 2007), and PearlPrimer (Marshall, 2004) and the best suited program was evaluated. The program parameters were the same in all three tested programs:

- Length range (bp): 18 – 26
- Tm°C range (°C): 56 – 64
- Tm°C at 3'-end (°C): 29 - 48
- GC% range (%): 45 – 70

For the “primer test”, four primers for *BvLHP1* were designed with each of the above-named programs. To identify a target region in which a mutation has the highest predicted impact on gene function, candidate gene sequences were investigated with the Program CODDLe (Choosing codons to Optimize Discovery of Deleterious Lesions; <http://www.proweb.org/coddle/>).

After *in silico* primer design testing, for each candidate gene, up to 42 primer pairs were selected and pre-screened for single amplification products in a so called “crash-test” following the PCR conditions described by Weil and Monde (2007) on a DNA Engine DYAD Thermal cycler (MJ Research Inc.). PCR amplification was performed in a total volume of 10 µl containing 0.9 ng/µl template DNA, 1x Taq buffer, 0.8 mM MgCl₂, 2 mM dNTPs (Invitrogen), 0.2 units recombinant Taq DNA polymerase (Invitrogen) and 1.0 pmol primer (Eurofins MWG Operon). Primer combinations that produced single amplicons of the expected size and yielded high quality sequencing chromatograms were re-tested with IRDye labeling (IRDye 700 for forward primers and IRDye 800 for reverse primers, IRDye primers obtained from Biomers (biomers.net GmbH, Ulm, Germany)). Only primer combinations passing the labeling test were further used for TILLING.

2.2.6 AFLP analyses

Amplified fragment length polymorphism (AFLP) is a DNA amplification based method for genotyping. In this study, AFLP was used to evaluate the level of heterozygosity in the EMS-treated sugar beet population 930190. For this study, an AFLP protocol was conducted as described by Vos *et al.*, (1995). Except that for restriction, *PstI* instead of *EcoRI* was used. The protocol consisted of five stages: i) extraction of genomic DNA, ii) digestion of genomic DNA with restriction enzymes, iii) adapter ligation, iv) pre-amplification with primers P01 and M01, and v) selective amplification with primers M31-M38 in combination with primers P31-P46. Oligomers were obtained from biomers.net GmbH. Fragment analyses was performed on a LI-COR[®] 4300 DNA analyzer using a 6.5 % KB^{Plus} gel matrix (LI-COR[®]). AFLP fragment sizes were determined by comparison with the IRDye[®] sizing standard 50-700 bp (LI-COR[®]). Acquired data were analyzed using the software Gelbuddy (Zerr and Henikoff, 2005).

2.2.7 Mutation screening

For mutation detection, the above-mentioned 8x 2D pooled DNA was used as template for PCR. PCR amplification was performed as described in 2.2.3, with the difference that IRDye labeled primer (IRDye primers obtained from biomers.net GmbH) were used. The primers were used in a labeled versus unlabeled ratio of 3:2 for IRDye 700 and 4:1 for IRDye 800 (Till et al., 2006a). PCR was performed on a DNA Engine DYAD thermal cycler (MJ Research Inc.). PCR samples were endonuclease digested (see page 25 SNP identification). PCR products were purified after endonuclease digestion by Sephadex purification. For this, Milipore MultiScreen 96 Filter plates[®] (BioLabProducts GmbH) were loaded with Sephadex G50 fine (GE Healthcare Europe GmbH, Munique, Germany) and the PCR products were loaded on the columns followed by centrifugation at 500 x g for 3 min.

One bunch of fresh celery was washed in fresh water and processed at 4°C in a cool room as follows: the celery was passed through a juicer and the juice was centrifuged at 2,600 x g for 20 min. The supernatant was transferred to a new tube. 1 M Tris-HCL and phenylmethanesulfonylfluoride (PMSF) was added to a final concentration of 0.1 M Tris-HCL, pH 7.7, 100 µM PMSF. The solution was aliquoted to 1.6 ml in 2 ml Eppendorf tubes and stored at – 20°C.

10x CCE buffer

BSA	20 mg ml ⁻¹
Triton X-100	10% (vol/vol)
KCL	2 M
HEPES	1 M pH 7.5
MgSO ₄	1 M

Fragment analysis was performed on a LI-COR[®] 4300 DNA analyzer using a 6.5 % KB^{Plus} gel matrix (LI-COR[®]). Before gel loading, 2 µl of the digestion product was mixed with 2 µl formamide loading dye and denatured for 3 min at 95°C. Of each sample, 0.3 µl was manually loaded with an 8-channel pipette on a 100 lanes membrane comb. The gel run was performed at 1,500 V, 40 mA and 40 W for 4 hours and 15 minutes. Acquired data were analyzed using the software Gelbuddy (Zerr and Henikoff, 2005).

DNA Pools with signals detected by the Gelbuddy software were further analyzed by sequencing. Sequence analysis was performed using dye-terminator chemistry (Applied Biosystems, Foster City, CA, USA) on a 3730 xL DNA Analyzer (Applied Biosystems). For detected 4x pools, each of the four M₂ plants represented by the 4x pool were sequenced. All four sequences were aligned and checked for single nucleotide polymorphisms using the software module SeqMan from the Lasergene software package (DNA Star Version 7.1, Inc., Madison, WI, USA) in order to identify individual M₂ plants showing a sequence variation (SV). For detected 8x 2D pools, only one plant per pool had to be sequenced and aligned to the non-mutated parent in order to verify a sequence variation. SVs were further investigated with the software tools ParseSNP (Taylor and Greene, 2003) and SIFT (Ng and Henikoff, 2003) to predict their impact on the gene function.

2.2.8 Sequence variant frequency calculation

The sequence variant (SVF) and mutation frequency (MF) was estimated as the total number of sequence variants/mutations divided by the total number of base pairs screened (Cooper et al., 2008a):

$$SVF/MF[1/kb] = 1/\left(\frac{(\text{amplicon size [bp]} - 100) \times (\text{number of } M_2 \text{ plants})}{(\text{number of mutations}) \times 1,000}\right) \quad (1)$$

For each target region, 100 bp were subtracted from the amplicon size to account for the 50 bp regions at the 5' and 3' ends of the amplicons, which are known to be difficult to analyze on a TILLING gel (Greene et al., 2003). Furthermore, we estimated the sequence variant (SVDF) and mutation detection frequency (MDF) per 1,000 bp:

$$SVDF/MDF = MF \times (\text{number of } M_2 \text{ plants} \times 1,000) \quad (2)$$

Further, the average frequency of mutations was calculated as follows:

$$AVF = \frac{1}{(\text{Population size } [M_2 \text{ plants}] \times \text{amplicon specific G/C content})} \times \text{number of mutations} \quad (3)$$

To estimate the number of base pairs susceptible to EMS the following equation was used:

$$Bp \text{ susceptible to EMS} = (\text{Genome size [bp]} \times \text{overall G/C content}) \quad (4)$$

Assuming that all G/C pairs are equally sensitive to EMS, the number of mutations expected for each M_2 plant can be calculated in the following way:

$$\text{Mutations per } M_2 \text{ plant} = (AVF \times \text{bp susceptible to EMS}) \quad (5)$$

With the binomial distribution:

$$P = 1 - (1 - F)^N \quad (6)$$

It is possible to calculate the probability of finding a mutation in a given G/C base pair in the two TILLING populations, where F is the average mutation frequency and N the number of M_2 plants.

$$P = 1 - (1 - AVF)^{\text{Number of } M_2 \text{ plants}} \quad (7)$$

2.2.9 Evaluation of bolting and flowering time

Bolting time was determined in the following way: M_3 seeds derived from M_2 plants detected in TILLING were grown in the greenhouse together with non-mutagenized seeds as control (930190 or SynDH1). Seeds were sown for germination in standard potting soil (Einheitserde Typ ED73, Einheitserdewerk Uetersen) covered with sand and treated with 0.25 % Previcur[®] N (Syngenta) to control seedling diseases. Up to forty seedlings per M_3 family were transplanted at growth stage BBCH 10 (cotyledon stage (Meier et al., 1993)) into 9 by 9 cm square pots filled with standard potting soil. Pots were arranged as a completely randomized design. The experiment was conducted in a greenhouse with 16 or 22 hours of artificial light (Osram Plantastar[®] 400 W / 725 $\mu\text{mol/s}$) and a temperature of approximately 18 – 21°C. Plants were fertilized with Folistar[®] (JOST GmbH, Iserlohn, Germany) once a week.

Experiments with plants derived from 930190 were kept in the greenhouse until bolting, while experiments with plants derived from SynDH1 were transferred after 8 weeks to a cold chamber for vernalization. After vernalization for 12 weeks at 4°C plants were transferred back to the greenhouse and kept until bolting at 22 hours of artificial light (Osram Plantastar[®] 600 W / 725 $\mu\text{mol/s}$).

Bolting and flowering was recorded as the days from cotyledon stage (BBCH 10; (Meier et al., 1993)) to shoot emergence (BBCH 51), shoot elongation of 10 cm (BBCH 52), and beginning of flowering (BBCH 60).

Leaf samples were taken at BBCH 19 (9 leaf stage (Meier et al., 1993)) and DNA was extracted and normalized as described above. A PCR was conducted with the TILLING primers for the corresponding candidate gene to determine the allelic state of the SV genotype in each plant in case of segregating M_3 families.

2.2.10 Codon evaluation

The codon (triplet) usage for each gene analyzed was evaluated by the web based program Countcodon (version 4; <http://www.kazusa.or.jp/codon/countcodon.html>) by the Kazusa DNA Research Institute (Chiba, Japan). The percentage of obtaining a truncation, missense, nonsense or silent mutation through EMS mutagenesis in the coding region was evaluated through the web based tool CODDLE (Choosing codons to Optimize Discovery of Deleterious Lesions; <http://www.proweb.org/coddle/>) and manually by hand.

2.3 Results

2.3.1 Plant Material

Three EMS-mutagenized sugar beet populations were developed for TILLING (Table 6). For a detailed description of the EMS population 930190 see Hohmann et al. (2005). In total, 26,620 M₁ plants were grown and M₁ plants from populations 930190 and SynDH1 were self-pollinated by bag isolation, and M₂ seeds were harvested. No M₁ plants from population 940043 were bag isolated, because all plants died in the field during the fall and winter. Of each M₂ family up to eight plants were grown to avoid loss of mutation due to the chimeric and heterozygous character of the M₁ plants. After bag isolation, M₃ seeds were harvested from 13,741 M₂ plants. Genomic DNA was isolated from leaves of 6,106 M₂ plants from population 930190 and SynDH1 representing 2,387 M₂ families and samples were normalized prior to pooling 2D eight-fold.

Table 6: Characteristic features of the EMS TILLING populations used during this study

Population	M ₁ seeds treated with EMS	M ₁ plants grown	M ₁ plants with >50 seeds	M ₂ plants grown	M ₂ families ¹⁾	M ₂ plants DNA isolated	M ₂ plants with >50 seeds
930190 ²⁾	15,000	10,066	3,238	12,400	1,550	2,688	10,033
SynDH1	20,000	11,571	720	6,024	837	3,418	2,251
940043	8,000	4,983	NA ³⁾	NA	NA	NA	NA

1) Up to 8 plants grown/family

2) Hohmann et al., 2005

3) Not available, all plants died in the field

2.3.2 DNA extraction methods

Ninety-six leaf samples were used to evaluate three different extraction methods (total samples: 288). For evaluation purposes, DNA extraction was performed with leaf samples from sugar beet and rapeseed. Elution volumes differed for the three methods. For the CTAB method and the NucleoSpin[®] 96 Plant Kit 200 µl were eluted, whereas 80 µl were eluted after extraction using the MagAttract[®] Kit. The DNA yield for each extraction method is given in Table 7.

Table 7: Results from DNA extraction test: DNA yield of three different DNA extraction methods for sugar beet and rapeseed. 50 mg of freeze dried leaves were used to evaluate DNA yield.

	CTAB		MagAttract [®]		NucleoSpin [®]	
	Yield mean	Range	Yield mean	Range	Yield mean	Range
Sugar beet	19.8 ng	1.2 – 63.5 ng	1.9	0.2 – 4.0 ng	8 ng	0.8 – 24.5 ng
Rapeseed	22.5 ng	1.9 – 68.3 ng	0.9	0.2 – 2.8 ng	6.3 ng	1.1 – 19.4 ng
Cost per extraction	0.32 Euro*		0.98 Euro*		1.13 Euro*	

*) Only consumables

Application of the CTAB method resulted in highest yields (sugar beet 19.8 ng, rapeseed 22.5 ng), followed by NucleoSpin[®] extraction (sugar beet 8 ng, rapeseed 6.3 ng), whereas extraction with MagAttract yielded the lowest DNA amount (sugar beet 1.9 ng, rapeseed 0.9 ng). Figure 5 illustrates the obtained DNA quality resulting from application of the different methods after control PCR and agarose gel analysis.

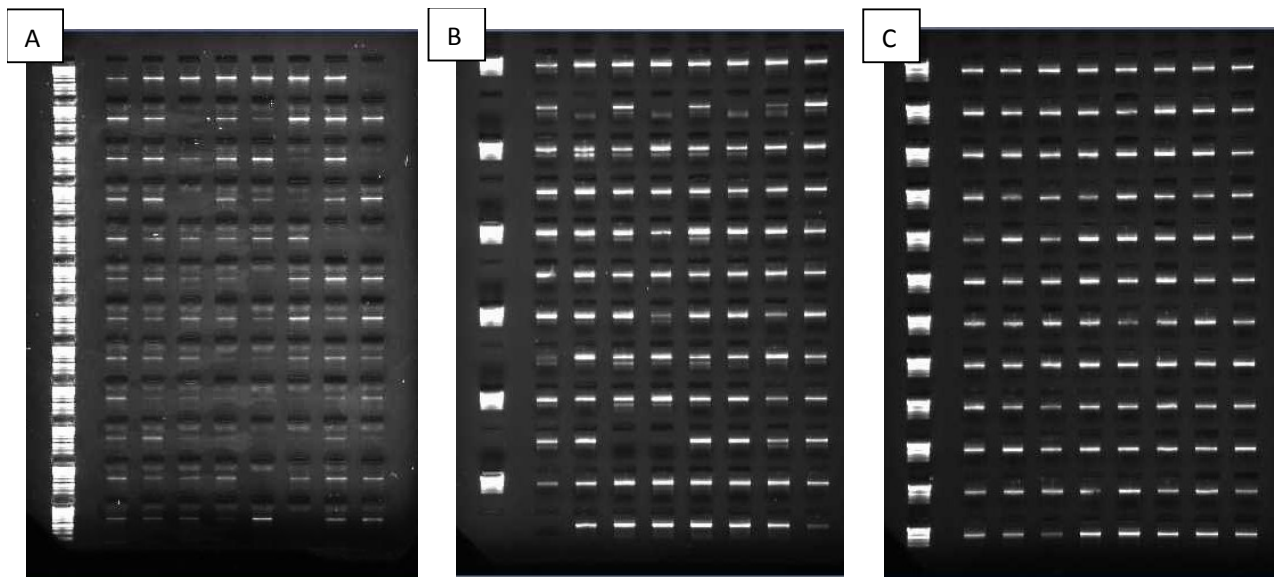


Figure 5: Control PCR from 96 sugar beet samples with the primer pair ZRT 115/117 after DNA extraction with A) CTAB, B) MagAttract[®], and C) NucleoSpin[®]. The expected sample size was 910 bp and the samples were analyzed in a 1% agarose gel, with a runtime of 20 min at 80 volt. The Fermentas FastRuler[™] Middle Range DNA Ladder was used as a marker.

2.3.3 Pooling size and pooling depth

During this study, an experimental approach was conducted in order to investigate maximum pooling depth for individual DNA samples with regard to the identification ability of a single SNP in the DNA mixture. Figure 6 shows the result of this pooling experiment and the successful amplification of all nine test amplicons on an acrylamide gel.

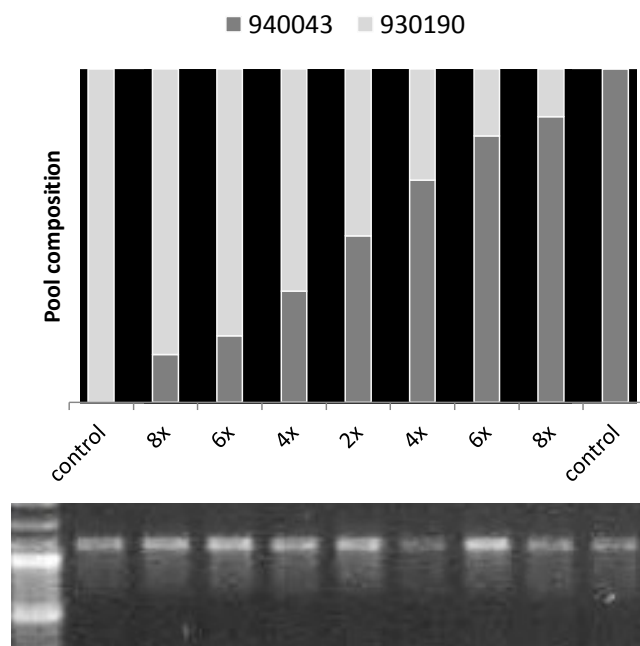


Figure 6: Results from the pooling test and corresponding 1% agarose gel with successful amplification of *BvTON1* PCR amplicons in the expected size of 1,055 bp for all seven test pools. Selected Pooling depths were: 1x (control), 2x, 4x, 6x, and 8x.

The results indicate that 8 x pooling for sugar beet TILLING is possible. Figure 7 demonstrates that the results obtained for the agarose gel detection can be transferred to the LI-COR[®]. According to the SNP position one digested fragment can be seen in the ird 700 channel.

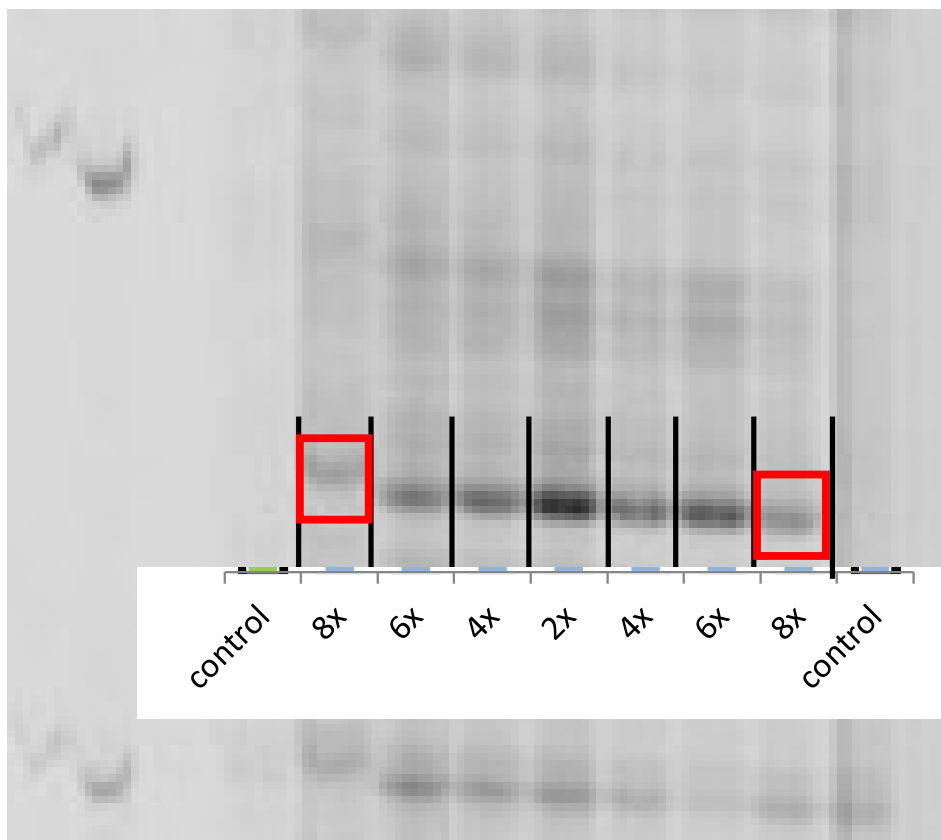


Figure 7: Pooling depth test. LI-COR[®] gel run of PCR products amplified with gene specific primers for *BvLHP1* after heteroduplex digestion with CEL1 in the IRDye 700 channel (forward primer). Due to clarity, only parts from 9 of 48 lanes are shown. Detection of digestion products is strongest in 2x pools but still sufficient even in 8x pools (red boxes).

2.3.4 Primer design and test

The practical application of primers designed with three different software programs showed differing results. Primers designed with FastPCR[®] gave the best results in the practical application experiment as shown in Figure 8. Primers designed with PerlPrimer and Primer3Plus showed amplification of single primer products as can be seen for the labels P1 and P2.

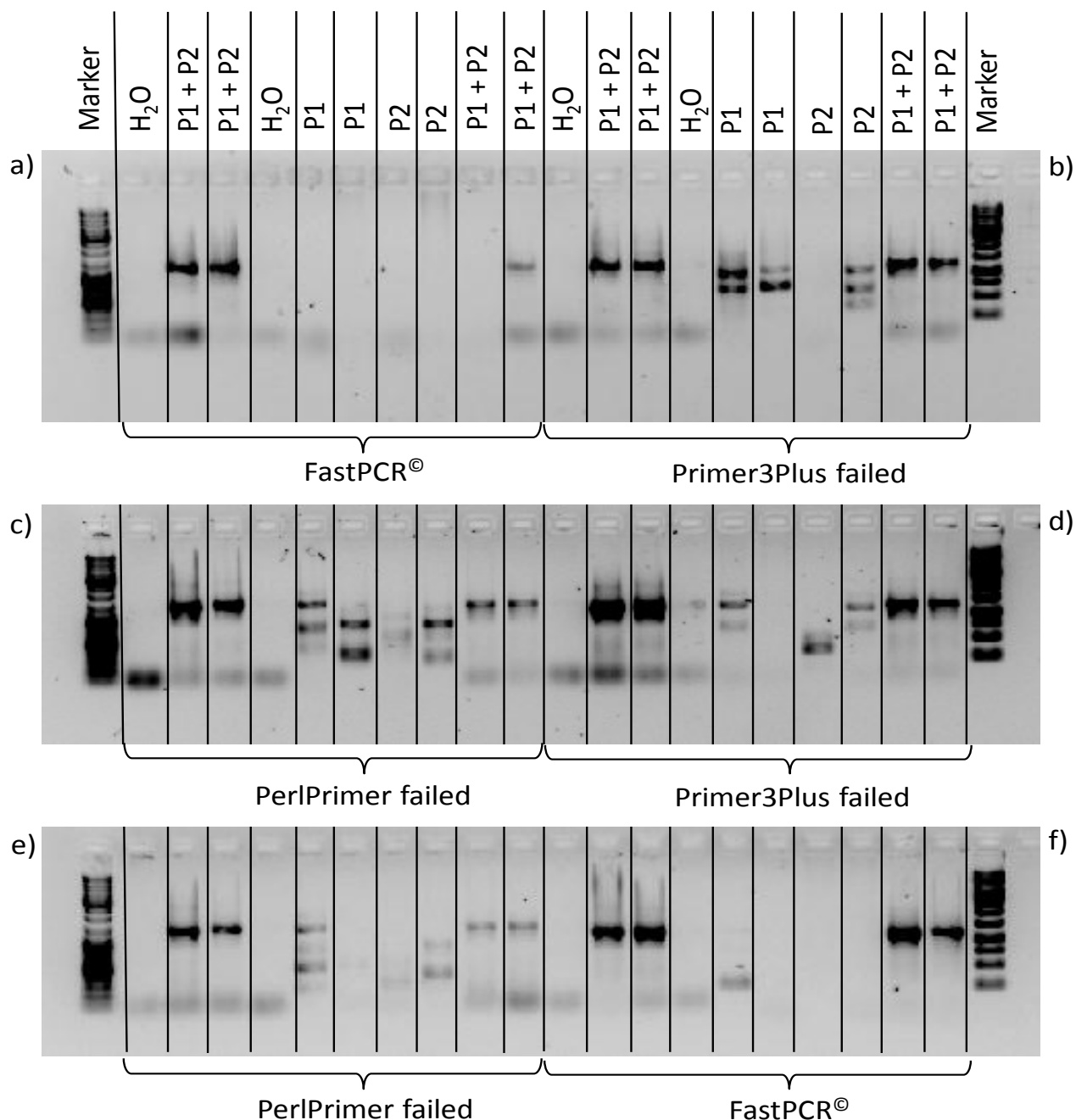


Figure 8: Primer design test. Shown are six primer "crash-tests" with identical primer pairs designed in three programs a) and f) FastPCR[®], b) and d) Primer3Plus, and c) and e) PerlPrimer. Lanes with amplicons in single primers (P1 or P2) are marked as failed. H₂O = no template included, P1 + P2 = both primers included, P1 = single forward primer, P2 = single reverse primer.

Table 8 displays the results of primer testing after the crash test. For each gene, up to 42 primers were designed in FastPCR[®] and tested for single product amplification. The success rate ranged from 14.7 for *BvLHP1* to 61.3 for *BvFL1*, and the overall success rate is 34.8.

Table 8: Primer success rates after “crash-test”. For each investigated gene the number of total primers designed as well as the resulting number of primer combinations is given. Furthermore, the number of working single primers as well as the number of not working single primers is given. The success rate is calculated as total number of primer working times 100 divided by total number of primer not working.

Gene	<i>BTC1</i>	<i>BvCOI1</i>	<i>BvFL1</i>	<i>BvFLK</i>	<i>BvFT1</i>	<i>BvFT2</i>	<i>BvFVE</i>	<i>BvLHP1</i>	<i>BvSWP</i>	<i>BvTON1</i>
# Primers	42	16	31	16	15	13	16	34	23	22
# Primer combinations	28	10	22	23	8	8	12	19	14	12
successful	21	4	19	7	4	6	5	5	6	5
not successful	21	12	12	9	11	7	11	29	17	17
Success rate	50.0%	25.0%	61.3%	43.8%	26.7%	46.2%	31.3%	14.7%	26.1%	22.7%

2.3.5 Detection and characterization of EMS mutations

In both populations, TILLING was performed targeting ten bolting and flowering time regulatory genes of which Table 5 gives an overview. The position of the SNP after CCE digestion in the polyacrylamide LI-COR[®] gel matched the position after Sanger sequencing with a precision of 3-24 bp. From the known function of the genes in *A. thaliana*, the candidate genes could promote (*BTCL1*, *BvColl1*, *BvFVE*, *BvFLK*, *BvLHP1*, and *BvFT1*) or repress (*BvFL1* and *BvFT2*) bolting and flowering in sugar beet. The complete screening of 6,106 M₂ plants, (3,418 for SynDH1 and 2,688 for 930190), revealed 75 sequence variations (SVs) (Table 9), including 52 (67%) located in exons. Twenty-four (32%) of the exon-located sequence variants induced a change of amino acid (AA) sequence, and one (1%) affected a splice site, the remaining 30 (40 %) were intron mutations. No stop mutation could be found. In the screening also 11 non G/C to A/T transitions were discovered in all regions screened (Table 9) and in some cases the identical mutations were found in different M₂ families. These “mutations” were subtracted for the calculation of mutation frequency (MF) as it is unlikely that these mutations are EMS derived. A complete list of all transition mutations/mutants is provided in Supplementary Table 1.

Table 9: Results of TILLING various candidate genes for bolting and flowering control in two sugar beet mutant populations derived from the annual sugar beet 930190 or the biennial sugar beet SynDH1. Listed are for each candidate gene the investigated population, the primer combination used, size of amplicons, and the number and distribution of mutation types. Furthermore, the sequence variation (SV) and mutation frequencies (MF) for each gene as well as the SV and mutation detection frequencies have been calculated.

Gene	Population	Amplicon size	Detected sequence variants (transitions)						Sequence variant frequency ⁵⁾	Mutation frequency ⁵⁾	SV detection frequency (per 1 kb) ⁶⁾	Mutation detection frequency (per 1 kb) ⁶⁾
			Total	Intron	syn ¹⁾	non-syn ²⁾	splice site ³⁾	stop ⁴⁾				
<i>BTC1</i>	930190	932 bp	4 (3)	3 (2)	-	(1)	-	-	1/559 kb	1/745 kb	5	4
		948 bp	(4)	(1)	(2)	(1)	-	-	1/570 kb	1/570 kb	5	5
	SynDH1	932 bp	(5)	(2)	2 (1)	(2)	-	-	1/569 kb	1/569 kb	7	6
		948 bp	(6)	(1)	(1)	(4)	-	-	1/483 kb	1/483 kb	7	7
<i>BvColl</i>	930190	1,253 bp	(2)	(1)	(1)	-	-	-	1/886 kb	1/886 kb	2	2
<i>BvFLK</i>	930190	801 bp	(3)	(1)	(2)	-	-	-	1/628 kb	1/628 kb	7	4
	SynDH1	801 bp	2 (1)	-	2 (1)	-	-	-	1/858 kb	1/1,716 kb	3	1
<i>BvFLI</i>	930190	966 bp	6 (5)	-	(4)	2 (1)	-	-	1/389 kb	1/466 kb	7	6
	SynDH1	966 bp	(4)	(1)	(2)	(1)	-	-	1/740 kb	1/740 kb	6	5
<i>BvFTI</i>	930190	939 bp	2 (1)	1	(1)	-	-	-	1/1,128 kb	1/2,255 kb	2	1
		713 bp	(2)	(1)	(1)	-	-	-	1/824 kb	1/824 kb	7	3
	SynDH1	939 bp	2 (1)	2 (1)	1	-	-	-	1/1,434 kb	1/2,868 kb	4	1
		713 bp	(4)	(1)	-	(2)	(1)	-	1/524 kb	1/524 kb	11	7

Gene	Population	Amplicon size	Detected sequence variants (transitions)						Sequence variant frequency ⁵⁾	Mutation frequency ⁵⁾	SV detection frequency (per 1 kb) ⁶⁾	Mutation detection frequency (per 1 kb) ⁶⁾
			Total	Intron	syn ¹⁾	non-syn ²⁾	splice site ³⁾	stop ⁴⁾				
<i>BvFT2</i>	930190	716 bp	(2)	2	(2)	-	-	-	1/828 kb	1/828 kb	7	3
		521 bp	3 (2)	1	-	(2)	-	-	1/377 kb	1/566 kb	7	5
	SynDH1	716 bp	3 (2)	1	(1)	(1)	-	-	1/702 kb	1/1,053 kb	5	3
		521 bp	(1)	1	-	(1)	-	-	1/1,439 kb	1/1,439 kb	5	2
<i>BvFVE</i>	930190	726 bp	(1)	-	(1)	-	-	-	1/1,683 kb	1/1,683 kb	2	2
<i>BvLHP1</i>	930190	1,262 bp	6 (5)	3 (2)	(1)	(2)	-	-	1/521 kb	1/624 kb	5	4
	SynDH1	899 bp	4 (3)	-	2 (1)	(2)	-	-	1/278 kb	1/371 kb	5	4
<i>BvSWP</i>	930190	1,502 bp	(1)	-	-	(1)	-	-	1/2,153 kb	1/2,153 kb	1	1
<i>BvTON1</i>	930190	907 bp	2 (1)	2 (1)	-	-	-	-	1/1,084 kb	1/2,169 kb	3	1
	SynDH1	907 bp	6 (5)	5 (4)	-	(1)	-	-	1/329 kb	1/395 kb	10	9

1) syn= synonymous mutations that do not alter the AA sequence of the protein; 2) non-syn = non-synonymous mutations that introduce a change of AA in the protein sequence; 3) splice site mutations = mutations that alter the splice junction, potentially resulting in a truncation of the protein; 4) stop mutations = mutations which generate a premature stop codon, potentially resulting in a truncation of the protein; 5) The SVF and MF for each amplicon is calculated as follows: [(size of amplicon – 100 bp) x total number of screened samples] / total number of identified SV; 100 bp were subtracted because of the diminished ability to detect mutations in the upper and lower 50 bp; 6) The SVDF and MDF is calculated as follows: SV frequency x total number of plants screened x 1,000 bp.

The SVF and MF varied greatly between the different amplicons. The lowest MF was found for *BvFT2* with one mutation every 2,868 kb. The highest frequency could be detected for *BvLHP1* with one mutation every 374 kb. Table 10 lists the SVF and MF for each population, and the SVDF / MDF. The differences in MF and MDF between the two populations are small, while the differences in SVF are greater (~ 160 kb).

Table 10: Total number of sequence variants discovered by TILLING for the two populations 930190 and SynDH1. Data from seven to 10 flowering time related genes have been evaluated. Given are the form of detected SV, the total No. of tested bp, the frequency of SV and MF detection, and the SV and MF frequency.

Population	Detected sequence variants (transitions)					No. of genes tested	Total No. of bp tested	SVDF / MDF (mutation per 1 kb) ⁴⁾	SVF / MF ⁵⁾
	total	Intro n	syn ¹⁾	non-syn ²⁾	trun ³⁾				
930190	38 (32)	16 (9)	(15)	9 (8)	-	10	13,273	4 / 3	1/895 kb / 1/1,108 kb
SynDH1	37 (32)	14 (10)	11 (7)	(14)	(1)	7	9,429	5 / 5	1/736 kb / 1/1,016 kb
Total	75 (64)	30 (19)	26 (22)	23 (22)	(1)	10	22,702	4 / 4	1/816 kb / 1/1,062 kb

1) syn= synonymous mutations that do not alter the AA sequence of the protein; 2) non-syn = non-synonymous mutations that introduce a change of AA in the protein sequence; 3) trun = truncation mutations that generate a premature stop codon or alter a splice junction, potentially resulting in a truncation of the protein ; 4) The SVDF and MDF is calculated as follows: MF x total number of plants screened x 1,000 bp; 5) The SVF and MF is calculated as follows: [(size of amplicon – 100 bp) x total number of screened samples] / total number of identified mutations; 100 bp were subtracted because of the diminished ability to detect mutations in the upper and lower 50 bp.

Table 11 gives information on possible percentage of EMS based mutations in the 10 genes analyzed. Coddle calculates based on codon usage the possibility of obtaining truncation, missense or silent mutation in coding regions. The calculation is based on the fact that EMS will lead to G/C → A/T transitions. Therefore the triplets TGG, CAA, CAG, CGA are of highest interest, as G/C→A/T transitions in this codons can lead to a premature STOP codon (TGA, TAG, and TAA), which will have a high impact on gene function. Supplemental tables 1-10 list the codon usage of *BTC1*, *BvCOL1*, *BvFLK*, *BvFL1*, *BvFT1*, *BvFT2*, *BvFVE*, *BvLHP1*, *BvSWP*, and *BvTON1*.

Table 11: Predicted possible detection of mutations by EMS mutagenesis. For each gene the percentage of obtaining a truncation (premature STOP or splice site mutation) or missense mutation is given. Furthermore, the effect of a missense change being deleterious for the protein is calculated.

Gene	part of gene	EMS mutation				
		Truncation		Missense	predicted Deleterious missense changes	Silent
Nonsense	Splice site					
<i>BTC1</i>	Genomic seq.	0.53	0.10	5.57	1.03	2.11
	CDS	2.79	0.55	29.36	5.41	11.11
<i>BvCOL1</i>	Genomic seq.	0.60	0.07	8.74	2.74	3.98
	CDS	2.17	0.27	31.79	9.96	14.49
<i>BvFLK</i>	Genomic seq.	0.45	0.11	4.73	0.67	1.48
	CDS	3.22	0.78	33.57	4.77	10.49
<i>BvFL1</i>	Genomic seq.	0.13	0.25	2.25	0.76	1.05
	CDS	1.55	2.95	26.05	8.84	12.09
<i>BvFT1</i>	Genomic seq.	0.10	0.08	1.89	1.13	0.46
	CDS	2.22	1.67	40.19	24.07	9.81
<i>BvFT2</i>	Genomic seq.	0.27	0.14	3.33	2.47	1.02
	CDS	2.46	1.33	30.87	22.92	9.47
<i>BvFVE</i>	Genomic seq.	0.32	0.23	3.07	1.22	1.15
	CDS	3.11	2.26	30.08	12.01	11.23
<i>BvLHP1</i>	Genomic seq.	0.30	0.12	4.13	0.31	1.82
	CDS	2.03	0.81	28.46	2.11	12.52
<i>BvSWP</i>	Genomic seq.	0.81	0.05	20.24	1.69	9.19
	CDS	1.20	0.08	30.24	2.53	13.73
<i>BvTON1</i>	Genomic seq.	0.12	0.11	1.90	0.18	0.83
	CDS	1.75	1.63	28.82	2.76	12.66
Total	Genomic seq.	0.36	0.13	5.58	1.22	2.31
	CDS	2.25	1.23	30.94	9.54	11.76

According to Table 11 the frequency of obtaining a nonsense mutation in the CDS over the ten genes is 2.25. The possibility for a nonsense mutation in the CDS of *BvFLK* is highest (3.22), while it is lowest in *BvSWP* (1.2). The same accounts for the frequency of obtaining a splice site mutation. The mean is 1.23 in the CDS over all genes, while it is highest in *BvFL1* (2.95) and lowest in *BvSWP* (0.08). *BvSWP* has only one exon, which explains the low value. The highest frequency of obtaining a missense mutation with a high impact on protein function can be obtained in *BvFT1* (24.07), while the lowest possibility can be seen for *BvLHP1* (2.11). The mean is 9.54.

Mutations were grouped into distinct classes to find out whether the mutations caused by EMS are distributed randomly over the different genes. Pastink et al. (1991) and Alcantara et al. (1996) report that only a limited number of G residues can be changed by EMS treatment. Table 12 lists the probability for the occurrence of mutations in each class based on G/C distribution and compares those with the frequency of mutation in each class. The probabilities and frequencies are expressed as percentage of total G/C and total mutations. In most cases, the observed values differ substantially from the expected ones. Only in three cases, for the SynDH1 screen, a similar too expected difference for *BvFT1*, *BvFT2* and *BvLHP1* from the observed and expected values can be shown.

Table 12: Predicted frequency properties of detected mutations. Probabilities for the occurrence of mutations in each class are calculated based on G/C distribution and compared with the frequencies of mutations in each class.

Gene/Screening		Silent [%]	Low codon usage [%]	AA change [%]	AA-STOP [%]
930190 screening					
<i>BTC1</i>	Expected	24.88	0.87	69.22	5.91
	Observed	71.43	0	28.57	0
<i>BvColl</i>	Expected	27.88	1.35	68.27	4.42
	Observed	100	0	0	0
<i>BvFLK</i>	Expected	22.15	1.77	71.65	6.20
	Observed	100	0	0	0
<i>BvFLI</i>	Expected	25.82	1.45	69.82	4.36
	Observed	80	0	20	0
<i>BvFT1</i>	Expected	23.18	3.00	72.10	4.72
	Observed	100	0	0	0
<i>BvFT2</i>	Expected	21.15	3.08	74.01	4.85
	Observed	50	0	50	0
<i>BvFVE</i>	Expected	24.01	1.58	69.67	6.32
	Observed	100	0	0	0
<i>BvLHP1</i>	Expected	28.04	2.06	68.41	3.55
	Observed	60	0	40	0
<i>BvSWP</i>	Expected	29.70	2.75	67.98	2.32
	Observed	0	0	100	0
<i>BvTONI</i>	Expected	26.03	2.47	70.41	3.56
	Observed	100	0	0	0
SynDH1 screening					
Gene/Screening		Silent [%]	Low codon usage [%]	AA change [%]	AA-STOP [%]
<i>BTC1</i>	Expected	24.88	0.87	69.22	5.91
	Observed	45.45	0	54.55	0
<i>BvFLK</i>	Expected	22.15	1.77	71.65	6.20
	Observed	100	0	0	0
<i>BvFLI</i>	Expected	25.82	1.45	69.82	4.36
	Observed	75	0	25	0
<i>BvFT1</i>	Expected	23.18	3.00	72.10	4.72
	Observed	40	0	60	0
<i>BvFT2</i>	Expected	21.15	3.08	74.01	4.85
	Observed	33.33	0	66.67	0
<i>BvLHP1</i>	Expected	28.04	2.06	68.41	3.55
	Observed	33.33	0	66.67	0
<i>BvTONI</i>	Expected	26.03	2.47	70.41	3.56
	Observed	80	0	20	0

From the 64 identified transitions mutations, 45 were exonic mutations. These 45 mutations were obtained by screening 10 genes that contain 5,726 guanines and cytosines. Based on those results, the estimated average frequency of mutations (AVF) (formula 3) is 1.65×10^{-6} . Given a genome size of 758 Mb and a 43.34% G/C content in the coding sequence of the sugar beet genome, there are 3.3×10^8 bp susceptible to EMS mutagenesis (formula 4). By estimating the number of mutations per M_2 plant (formula 5), approximately 375 mutations per M_2 plant can be expected. The estimated probability of finding one mutation in any given G/C base pair in the genome of both populations (formula 7) is therefore 1%. To increase the probability to 10%, a population of about 60,000 M_2 plants would be needed, which is an unmanageable number.

The amount of EMS and duration of treatment used to create the two populations differed by 0.5% EMS and 4 to 8 hours. For the creation of 930190 Hohmann et al. (2005) used 0.5% and 1.0% EMS and durations of 4, 6, 8, and 12 hours treatment, while 1.0% and a duration of 12 hours was used here to create the SynDH1 mutant population.

Regarding TILLING approaches, economic considerations are generally of major importance. Besides the MF, the cost of screening one amplicon of 1 kb in the SynDH1 population for 4x and 8x 2D pools were evaluated. Cost calculation only accounts for laboratory consumables, since other costs can substantially differ (e.g. work-related costs, different technical equipment etc.). Table 13 sums up the results of the evaluation with regard to different pooling depths. To screen the whole SynDH1 population and to sequence one mutation per pool plate, the cost is 105.50 Euros for the 4x pools and 83.30 Euros for the 8x 2D pools.

Table 13: Costs for TILLING one amplicon of 1 kb in the SynDH1 population of 3,418 M_2 plants showing the effect of different pool compositions on the required number of pool plates and LI-COR® runs. The number of required sequencing PCRs was estimated, taking into account the expectation of one positive signal per pool plate.

Pool complexity	No. of PCRs to screen whole population	No. of gel runs	Sequencing PCR's (if one signal occurs per pool plate)	Costs (in €)*
4x	5.5 x 96	3	24	105.48
8x 2D	6.5 x 96	4	7	83.29

*only consumables

2.3.6 Phenotypic screening of M_3 offspring

Mutations were detected in the following genes: *BTC1*, *BvFL1*, *BvFT1*, *BvFT2*, *BvFVE*, *BvLHP1*, and *BvTONI*. All of these mutations were selected for phenotypic characterization. A modified protein activity was expected if the point mutation led to a substitution of an amino acid depending on the conformation and the properties of the affected amino acid. We would expect a loss of protein function if the mutation leads to a premature stop codon or if a splice site is affected by the mutation, which was not the case for the identified mutations.

Screening of *BvLHP1* mutant families (930190)

Based on sequence variations in the *BvLHP1* candidate region, thirteen M_3 families (population 930190) showing nine different mutations were selected for phenotyping of bolting and flowering behavior.

Sequencing of M_2 plants indicated whether M_2 plants were homozygous or heterozygous for the mutation. The following families were expected to segregate at the mutated locus, because the M_2 plants were heterozygous for the mutation: 045037, 054654, 054757, 054877, 055470, and 056710.

The following families were expected not to segregate at the mutated locus, because the M_2 plants were homozygous for the mutation: 045137, 045573, 052922, 054658, 054876, 054692, and 055667.

An ANOVA revealed a significant variation between families for bolting time as well as flowering time ($p < 0.001$). The mean separation (Table 14) showed a delay in bolting start of eleven mutant families compared to the non-mutated parent 930190 ranging from 0.7 days to 8.0 days, of which nine lines were significant ($\alpha = 0.05$). Eleven families were also late in flowering compared to 930190 (up to 12.1 days), ten of them significantly and including all significant late bolters. Two mutant families started bolting slightly earlier, however not significantly. Early flowering was observed in two mutant families, of which one was significant with 3 days earlier than 930190. The following mutants are expected to have a *BvLHP1* protein with altered function because the introduced nucleotide changes lead to an amino acid exchange, which could have an impact on protein function (Table 14).

Table 14: Bolting and flowering behavior of 13 *BvLHP1* $M_{2,3}$ families compared to the non-mutated annual sugar beet line 930190. Shown are the means for delay in bolting start (BBCH 51) and beginning of flowering (BBCH 60) of each $M_{2,3}$ family compared to the annual parent 930190. Data were obtained in a greenhouse experiment (see methods). Genotypic segregation indicates whether the M_3 plants of a family are homozygous for the detected SV or segregating.

$M_{2,3}$ family	nucleotide exchange position (position on genomic DNA)	Bolting delay compared to annual parent [days]	Flowering delay compared to annual parent [days]	Genotypic segregation ¹
045037	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	8.02 ***	12.09 ***	Segregating
045137	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	0.65	-0.30	Non segregating
045573	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	3.05*	4.20 ***	Non segregating
052922	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	1.88	5.39 ***	Non segregating
054654	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	4.76 ***	4.71 ***	Segregating
054658	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	7.00 ***	8.09 ***	Non segregating

M_{2:3} family	nucleotide exchange position (position on genomic DNA)	Bolting delay compared to annual parent [days]	Flowering delay compared to annual parent [days]	Genotypic segregation¹
054757	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	-0.91	0.39	Segregating
054876	3643, 3644, 3685, 3840, 3950, 3972, 4034, 4072, 4123	4.96 ***	5.20 ***	Non segregating
054877	3643, 3644, 3685, 3840, 3950, 3972, 4034, 4072, 4123	4.89 ***	4.95 ***	Segregating
054962	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	1.37	3.49 **	Non segregating
055470	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	7.63 ***	8.30 ***	Segregating
055667	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	7.45 ***	9.04 ***	Non segregating
056710	3643, 3644, 3840, 3950, 3972, 4034, 4072, 4123	-0.34	-2.96*	Segregating

P-value: * < 0.003; ** < 0.0005; *** < 0.0001

¹: determined by sequencing

Figure 9 and Table 15 show the phenotypic variation within the 13 families. For each M₃ family, three early and the three late bolting plants were genotyped for the *BvLHPI* candidate region in order to identify families that were co-segregating for phenotype and genotype. This was done by sequencing of PCR amplicons using the primer combinations SF01/SF02.

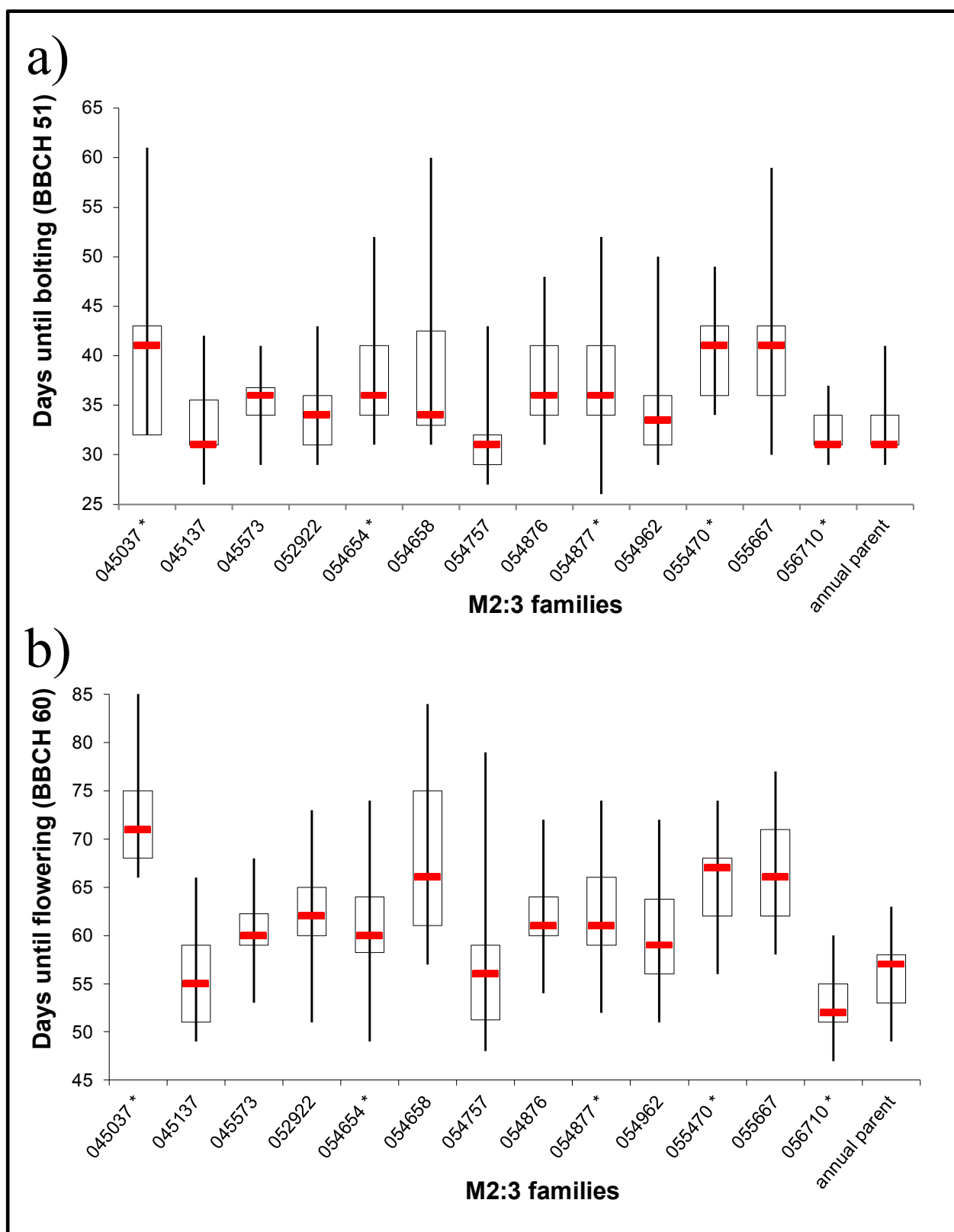


Figure 9: Box plot display of phenotypic variation for bolting time a) BBCH 51 and b) BBCH 60 within 13 *BvLHP1* M_{2:3} families and the wild type. Data were collected in a greenhouse experiment. Families segregating for detected SV are indicated by an asterisk.

Table 15: Standard deviations for bolting and flowering time of 13 M₃ families and the wild type 930190 with their respective confidence intervals. Plants were phenotyped in a greenhouse experiment and non-bolting plants were disregarded in the estimation of variances. M_{2,3} families segregating for sequence variations in *BvLHP1* detected in TILLING are marked with an asterisk.

M ₃ -family	Days to bolting from BBCH 10		Days to flowering from BBCH 10	
	Standard deviation	95% confidence interval	Standard deviation	95% confidence interval
045037*	7.66	6.08-10.36	4.45	3.49-6.14
045137	3.44	2.80-4.45	5.00	4.04-6.55
045573	2.83	2.30-3.66	3.46	2.81-4.51
052922	3.63	2.97-4.66	5.00	4.07-6.50
054654*	4.67	3.83-6.00	5.96	4.86-7.71
054658	10.56	8.17-14.95	8.51	6.34-12.96
054757	3.06	2.51-3.93	6.49	5.29-8.39
054876	3.98	3.26-5.11	3.61	2.94-4.67
054877*	5.40	4.27-7.35	4.95	3.88-6.84
054962	4.12	3.37-5.29	5.89	4.80-7.62
055470*	4.20	3.43-5.41	4.24	3.46-5.49
055667	5.49	4.45-7.16	5.45	4.38-7.21
056710*	2.03	1.66-2.60	2.82	2.31-3.62
control	2.35	1.93-3.02	3.99	3.26-5.14

*) Mutated M₃ families with non –bolting plants

It was possible to distinguish between plants heterozygous or homozygous for the mutations or plants carrying the wild type allele. This led to the identification of four M₃ families putatively co segregating for genotype and phenotype: 045037, 054877, 055470 and 056710. Each of these families was then completely genotyped in order to classify the plants into three genotypic classes Table 16.

A Chi-Square test to check for deviation from the expected 1:2:1 genotypic segregation was not significant ($\alpha = 0.05$) in any family. Non-bolting plants were observed in families 045037 (11 plants), 054877 (1 plant) and 055470 (1 plant) (Figure 10). To test the effect of sequence variants on bolting time in family 045037 with 11 non-bolting plants, similarity of genotypic frequencies (G0G0:G0G1:G1G1; GO = allele of the annual parent, G1 = sequence variant allele) in bolting and non-bolting plants was tested with a Chi-Square association test. This test was not significant ($p=0.61$) indicating no effect of sequence variants on bolting inhibition in the 11 non-bolting plants. For further statistical analysis, non-bolting plants were disregarded because bolting time of these plants could not be quantified. Two ANOVA tests were performed for each population to test the effect of genotype on bolting start and flowering start. Such an effect could only be detected in family 056710, where the homozygous sequence variant genotype showed a bolting delay of 3.5 days ($p=0.0039$) and a flowering delay of 4.4 days ($p=0.013$) compared with the two remaining genotypes. Interestingly, we could not detect significant effects ($\alpha = 0.05$) in 045037 and 055470, which both, other than 054877, segregate for the same mutation as 056710. To test a possible influence of the phenotypic background variation in the three families on the statistical detection, standard deviations were estimated for each family. Interestingly, the standard deviation was lowest in 056710 (2.04) compared to 045037 (7.47) and 055470 (4.23), not even considering the non-bolting plants of the latter two populations.

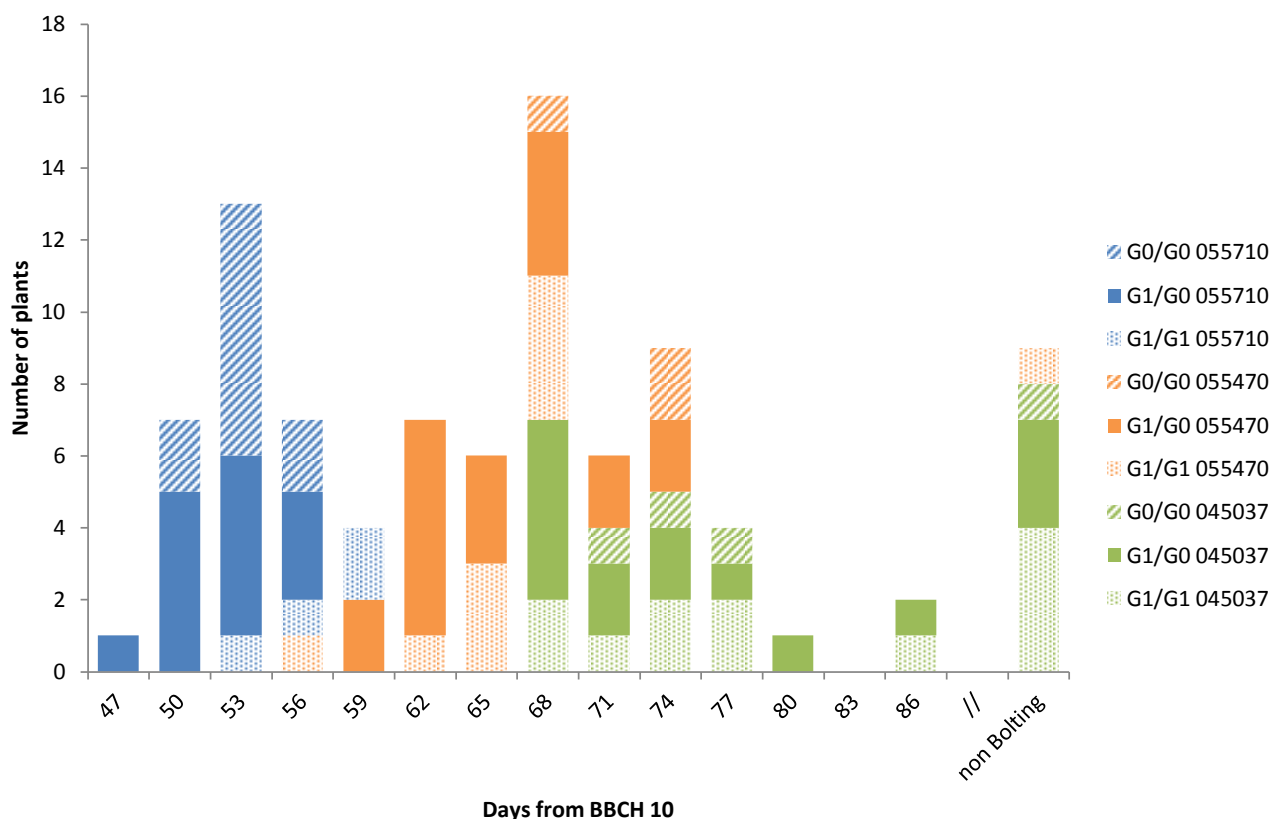


Figure 10: Histogram of M_3 families for *BvLHP1*. Segregation of M_3 families 045037, 055470, and 056710. Genotypes are in dependence of days from BBCH 10.

Table 17 summarizes the phenotypic observation for the 13 mutated M_3 families. The growth conditions are described as well as the growth period and the number of plants that were phenotyped in the experiment. The earliest and the latest bolting start of the individual plants per M_3 family are mentioned as well as the average number of days until bolting. Furthermore, any phenotypic deviation from the control is listed.

Table 17: Results of the phenotypic observation of M₃ families of the mutant population 930190, and one control family. The plants were grown in a greenhouse with 16hrs of artificial light until the onset of flowering. With the start of flowering, the plants were transferred to benches outside with natural light. The growing lasted from March until November for the investigated plants. The table gives information of the target gene, as well as the number of plants grown for each sequence variant detected via TILLING. Furthermore, the table shows the days to bolting for each mutation and any phenotypic observation.

M ₃ -Family	Target gene	No. of plants	Bolting start in days (earliest/latest)	Average no. days to bolting	Phenotypic observations
930190 (control)		40	44/56	47	
045037*	<i>BvLHP1</i>	40	47/75	56	
045137		40	42/56	47	Green to red leaves
045573		40	44/56	50	
052922		40	44/58	49	Long leaves
054654		40	46/67	52	
054658*		38	49/85	60	Small leaves
054757		40	42/58	46	Small growth habitus compared to other mutant families
054876		40	46/63	52	
054877		29	47/74	51	
054962		40	44/65	48	
055470*		40	49/64	55	
055667*		40	45/74	55	
056710		40	44/52	47	

*) Mutated M₃ families with non –bolting plants

Screening of BTC1, BvLHP1, BvTON1, BvFT1 and BvFLK mutant families (SynDHI)

Based on mutations in the genes *BTC1*, *BvLHP1*, *BvTON1* and *BvFLK* 14 M₃ families were selected for phenotyping of bolting and flowering time in a greenhouse experiment. Sequencing of M₂ plants indicated whether M₂ plants were homozygous or heterozygous for the sequence variant. The following M₃ families were expected to segregate at the mutated locus, because the M₂ plants were heterozygous for the mutation: 100408 and 100410. The following families were expected not to segregate at the mutated locus, because the M₂ plants were homozygous for the mutation: 100407, 100409, 100411, 100413, 100414, 100415, 100416, 100417, 100418, 100419 and 100421.

Table 18: Results of the phenotyping mutant population SynDH1, and one control family. The plants were grown for 10 weeks in a climate chamber with 22hrs. of artificial light, transferred to a 4°C vernalization chamber for 12 weeks with 22 hrs. of artificial light and finally grown until onset of flowering in a greenhouse with 22 hrs of artificial light, The growing period of the investigated plants lasted from May 2010 until June 2011. The table shows the target gene, as well as the number of plants grown for each sequence variant detected via TILLING. Furthermore, the table shows the days to bolting for each mutation and any phenotypic observation

M₃ family	Target gene	No. of plants	Bolting start in days after vernalization (earliest/latest)	Average no. days to bolting
control		14	17/26	21
100407	<i>BTC1</i>	33	26/43	33
100410		29	33/47	40
100413		15	24/42	32
100414		30	26/56	38
100415		19	30/50	41
100419		12	24/24	24
100421		9	Non-bolting	Non-bolting
100408		<i>BvTON1</i>	17	Non-bolting
100416	12		25/54	43
100417	17		21/47	29
100409	<i>BvLHP1</i>	22	24/53	37
100411		35	48/49	49
100418		26	16/47	29
100422	<i>BvFLK</i>	1	Non-bolting	Non-bolting

Table 18 summarizes the phenotyping experiment for the 15 mutated M₃ families. Since some of the plants did not start to bolt until January 2011, a second round of vernalization was started to ensure bolting of the remaining plants. The light conditions in the wintertime are not favorable to induce bolting even if artificial light is supplied. The second vernalization period of the non-bolting plants did not result in any further bolting plants. Overall, the experiment did not show any significant statistical results (data not shown). The mutations had no effect on bolting time.

Furthermore, one mutant family with a truncation mutation in *BvFT1* at base pair position 8,477 (Supplementary Table 1) was grown for phenotypic scoring and reverse transcription quantitative real time PCR quantification. Table 19 gives information on the results of the phenotypic scoring. The time to bolting was measured by visual scoring.

Table 19: Results of the phenotypic of *BvFT1* mutant M₃ family of the population SynDH1 and control plants. The plants were grown for 10 weeks in a greenhouse with 16 hrs of artificial light, transferred to a greenhouse with 22 hrs of artificial light for 4 weeks, transferred to a 4°C vernalization chamber for 12 weeks with 22 hrs of artificial light and finally grown until onset of flowering in a greenhouse with 22 hrs of artificial light. The growing period of the investigated plants lasted from February 2011 until August 2011. The table shows the target gene, as well as the number of plants grown for each plant family. Furthermore, the table shows the days to bolting and the average number of days to bolting for each family.

M₃-Family	Target gene	No. of plants	Bolting start in days after vernalization (earliest/latest)	Average no. days to bolting
Control SynDH1 110077		15	25/32	27
Control annual 930190		18	42/53*	48
Control biennial 020416		20	23/31	25
Control biennial 100043		20	26/37	29
110076	<i>BvFT1</i>	34	23/38	31

*) Annual growth type, no vernalization required, all plants bolted in the greenhouse.

No phenotypic difference for time to bolting was observed in the *BvFT1* mutant plants. No statistical differences were measured (data not shown).

2.3.7 Reverse transcription quantitative real time PCR experiment *BvFT1* splice site mutation

The identified mutation at base pair position 8,477 (Supplemental Table 1) should lead to a disruption of a splice site. Therefore, the M₃ plants carrying this mutation should have a reduced level of *BvFT1* transcript and should therefore have an early flowering phenotype as compared to control plants. I could not detect statistically significant transcript levels (data not shown) at any Zeitgeber time as described by Pin et al. (2010). The transcript levels were not significantly different from those of the control plants.

2.3.8 EMS population analysis

EMS population 930190:

Population 930190 had been produced by selfing individual plants of accession 93167P, which is an inbred line. After EMS mutagenesis G/C to A/T transversions were expected in the majority. Furthermore, those transversions were expected to be unique resulting in the ability to trace back a certain transversion to only one mutation event (i.e. one M₁ plant). However, these expectations could not be confirmed, which strongly indicated a contamination of the founder seeds, due to seed impurities. These seed impurities are difficult to trace back as the seeds were obtained by Saatzucht Dieckmann in 1993 and no propagation records are available. 93176P has been propagated by selfing individual plants, but it is not recorded if the plants were self-pollinated or open-pollinated.

In contrast, during TILLING screens with primer combinations targeting the genes *BvLHP1* and *BTC1*, up to 11 M₂ plants with identical nucleotide variations were observed. In addition, one M₂ plant (No. 054658) differed at three different nucleotide positions within 1 kb of the *BTC1* gene from the standard haplotype of the annual parent 9030190.

As these results cannot be explained by EMS mutagenesis alone and major contamination during bag isolation of M_1 and M_2 plants can be excluded, even so in 2005 18.3% (1110) of all M_2 plants were open pollinated due to major aphid infestation (Hohmann, 2007; Final Report GABI-TILL). I conclude a genetic heterogeneity of the 93167P seed lot used for EMS mutagenesis. To test this hypothesis, an AFLP assay was conducted for 36 plants grown from untreated 930190 founder seeds, 28 M_3 plants from the mutant family 054658 as well as 40 M_2 plants from EMS treated 930190 (930190-EMS). Eight AFLP primer combinations were chosen to investigate the population structure following the protocol of El-Mezawy *et al.* (2002). Table 20 gives an overview of the AFLP banding pattern resulting from these analyses and Figure 11 exemplarily shows an AFLP gel obtained during the heterogeneity analysis for population 930190. AFLP gels from all primer combinations are shown on the Supplementary Data on DVD.

Table 20: AFLP analysis of population structure of the untreated control family 930190 and the M₃ family 054658. Eight primer combinations were used. The test panel was composed of 36 randomly chosen 930190 plants. In addition, to 28 M₃ plants of the mutant family 054658. The number of total bands per primer combination is given as well as the number of monomorphic and polymorphic bands per primer combination.

930190				054658			930190-EMS			
Primer combination	No. of bands	monomorphic	polymorphic	No. of bands	monomorphic	polymorphic	Primer combination	No of bands	monomorph	polymorph
P46/M35 700	46	38	8	40	33	7	P45/M43 700	52	44	8
P39/M35 800	24	21	3	24	20	4	P39/M35 800	26	20	6
P46/M47 700	32	28	4	32	26	6	P45/M62 700	32	24	8
P39/M62 800	30	24	6	29	24	5	P39/M50 800	27	22	5
P33/M32 700	38	31	7	32	28	4	P41/M43 700	39	29	10
P33/M47 800	26	19	7	19	15	4	P46/M32 800	29	20	9
P33/M49 700	26	19	7	29	23	6	P41/M30 700	28	28	10
P33/M39 800	24	20	4	22	18	4	P46/M49 800	18	15	3
Total	246	200	46	227	187	40		251	202	59
Mean	31	25	6	28	23	5		31	25	7

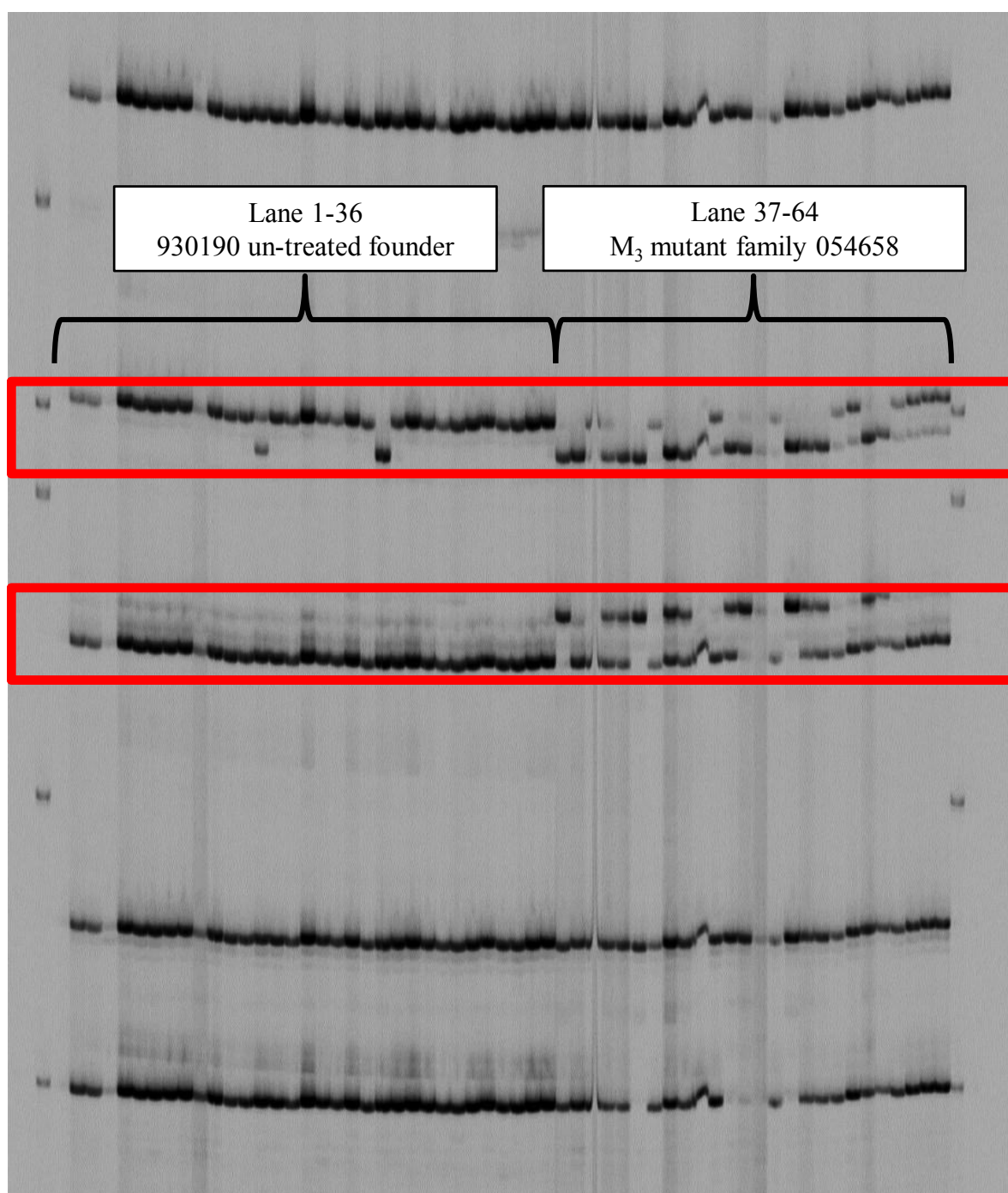


Figure 11: AFLP gel with primer combination P39/M35. Lines 1- 36: amplified DNA from sugar beet plants from the untreated 930190 annual parent, Line 37-64: DNA from M₃ plants of the mutant family 054685, which had 3 sequence variants in a 1 kb TILLING fragment.

The AFLP gels resulting from this experiment clearly show that the 930190 founder seed lot is heterogeneous. Off types could be detected in plants grown from non-treated 930190 founder seeds, in M₃ family 054658, as well as in 930190-EMS. The rate of polymorphic bands was estimated to be 18.7 % for 930190, 17.6% for 054658, and 22.6%, which means that one out of 12 plants in 930190 and 1 out of 20 plants in 930190-EMS are heterogenic.

To test for 3:1 segregation of the AFLP bands in 054658 I conducted a chi-square test.

	1	0
Expected ratio (Mendelian)	3	1
b (Sum observed)	23	5
e (Sum expected)	21	7
b - e	2	-2
(b-e) ²	4	4
(b-e) ² / e	0.19	0.57
χ^2	0.76	
p-value	0.38	

The chi-square test for a 3:1 segregation of AFLP bands for the mutant family 054658 was significant with a p-value $p < 0.5$.

EMS population SynDHI:

20,000 seed balls were treated with EMS as described (2.2.2 EMS mutagenesis and population propagation). Germination rates were determined for all 20,000 mutagenized seeds. The germination rate was 57.9%. 11,571 M₁ plants were vernalized for 12 weeks. 5,817 M₁ plants survived vernalization and were either transplanted to the field or grown in the greenhouse for M₂ seed production. 2,197 M₁ plants flowered and 837 of these set seed (Figure 12).

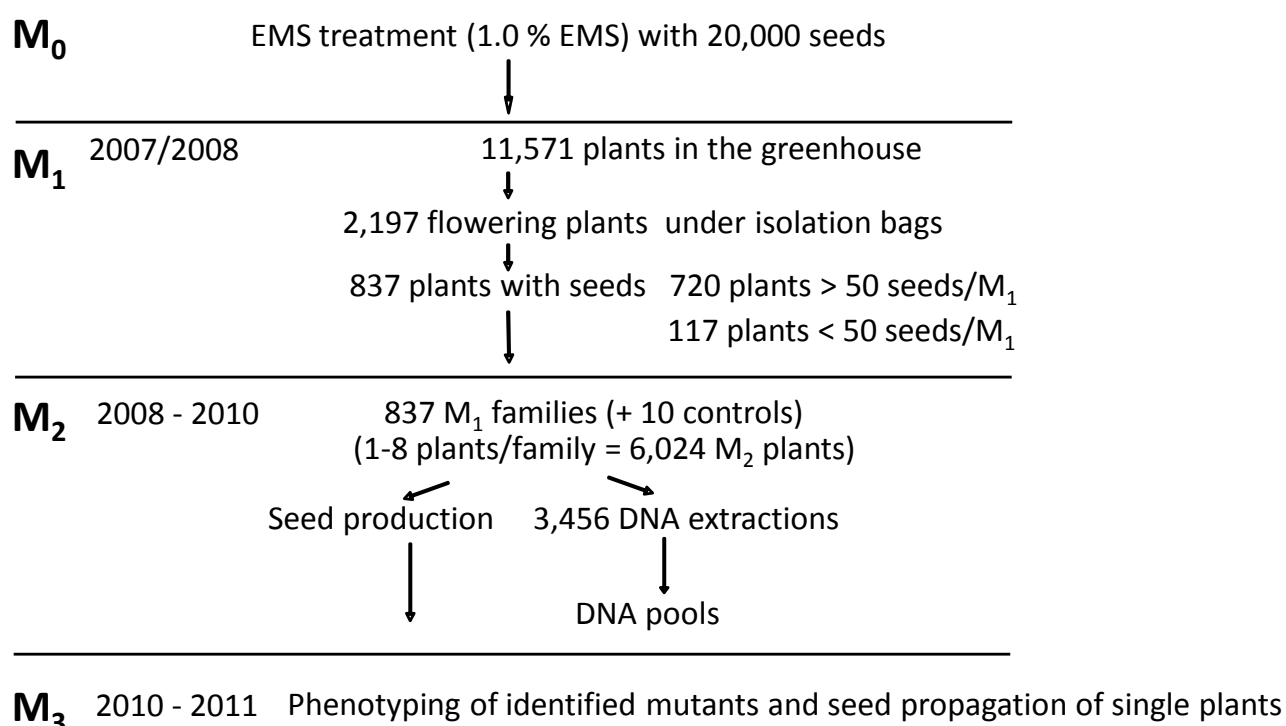


Figure 12: Propagation of mutant population after ethyl methanesulfonate (EMS) treatment of the doubled haploid sugar beet genotype SynDHI.

Between 1 and 3,705 seeds per plant were harvested from M₂ plants, 906 M₂ plants did not produce any seeds (Figure 13), 246 M₂ plants produced less than 50 seeds/plant, and 2,251 M₂ plants produced more than 50 seeds/plant (Table 6).

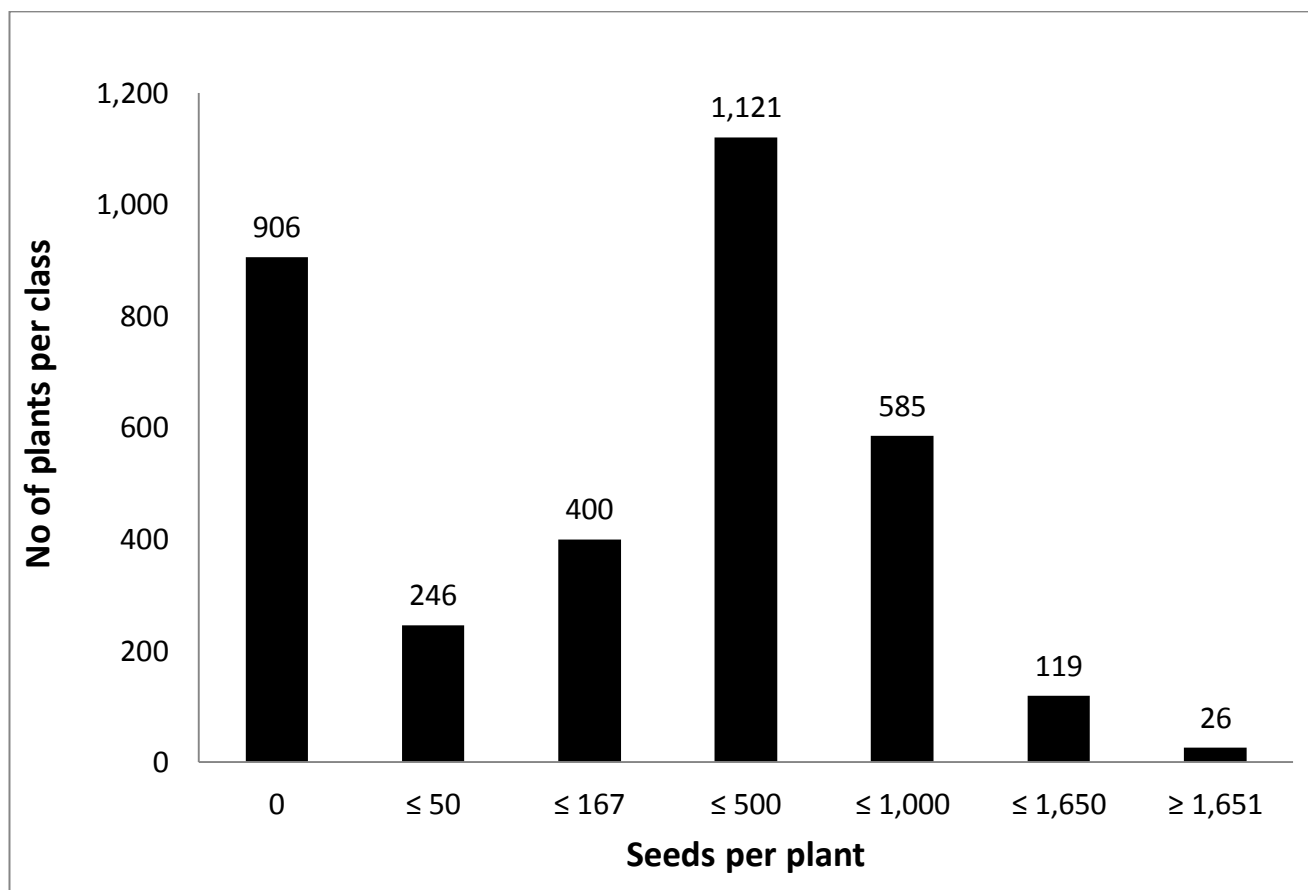


Figure 13: Histogram of seed amount harvested from 3,403 M2 plants of SynDH1. Numbers above each bar indicate number of plants per class.

In the years 2007 - 2008, a high number of plants were killed due to *Fusarium spec.* infection. From 4,855 M₁ plants after vernalization, only 1,945 flowered, meaning that 2,910 M₁ plants probably died of the infection. The infections were noticeable through *Fusarium* typical root lesions (Figure 14A) and leaf necrosis, depicted in Figure 14B.

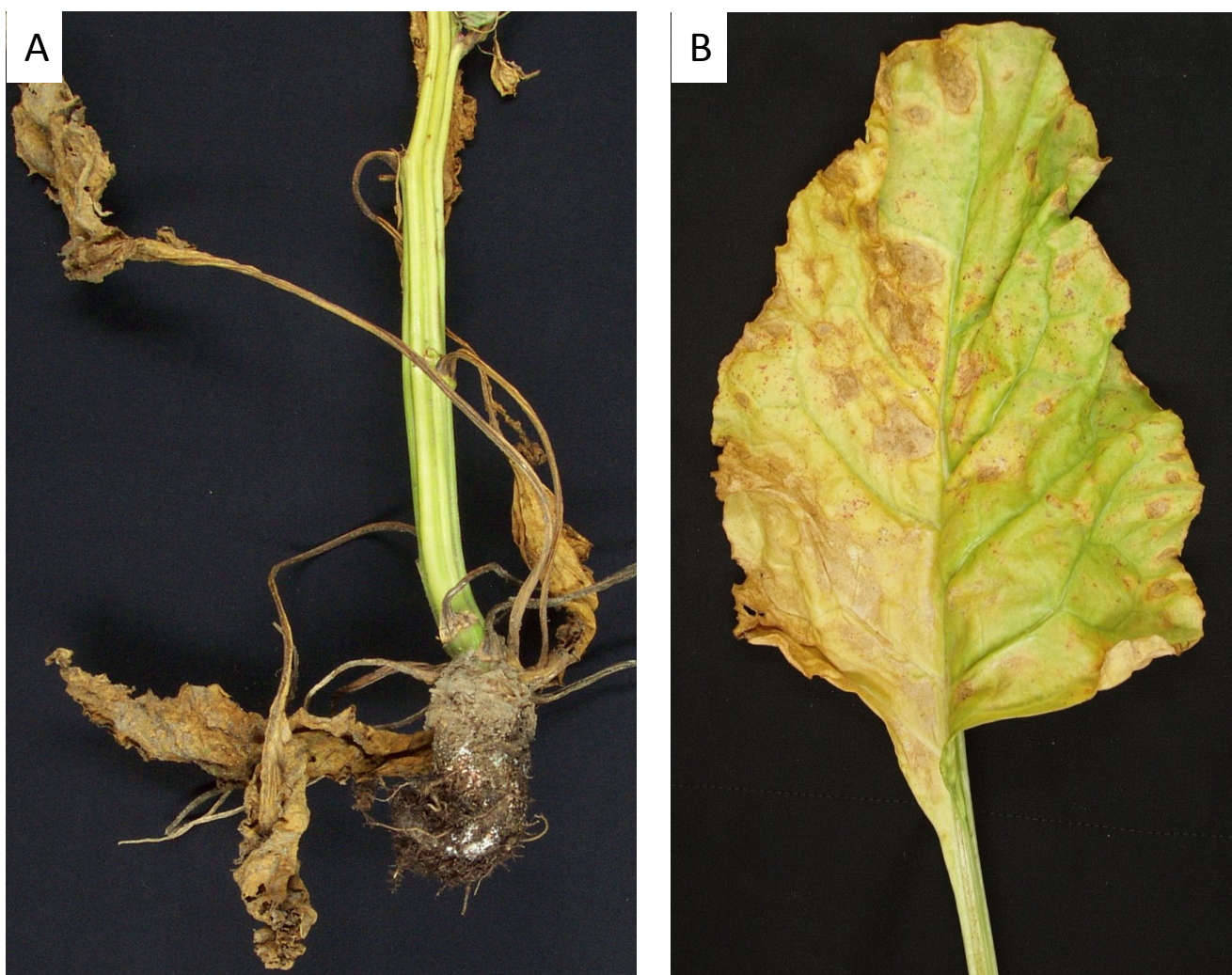


Figure 14: *Fusarium spec.* infection on M₁ plants of SynDH1. A) *Fusarium spec.* caused root. B) *Fusarium spec.* caused leaf necrosis.

Unexpectedly, after performing TILLING screens, identical mutations in different M₂ families were also observed in the SynDH1 mutant population, i.e., a mutation in the *BTC1* gene at position 7,201 (exon 5) was found in the M₂ family 070852 and also in three other M₂ families (070851, 070962, and 070850) indicating prevalence of the same phenomenon like in population 930190. Furthermore, one M₃ family with a green hypocotyl was observed in the phenotypic screening. All other M₃ families exhibited a red hypocotyl. This gives rise to an improper propagation of plants. A possible explanation is cross contamination during bag isolation. Since screening of one target sequence was a continuous process as the population size increased over time, the heterogeneity of the SynDH1 population was not apparent until the final phase of the project. The SynDH1 population was developed in consecutive charges from November 2007 on and was completed in August 2010. DNA pools were finished in October 2010 representing the complete population of 3,403 M₂ plants, as shown in Table 6.

EMS population 940043:

8,000 seed balls were treated with EMS as described (2.2.2 EMS mutagenesis and population propagation). Germination rate were determined for all 8,000 mutagenized seeds. The germination rate was 62.3 %. 4,983 M₁ plants were planted to the field in fall 2009. Due to bad weather conditions (rain, early frost), improper root formation of the plantlets and animals feeding on plantlets no M₁ plant survived the winter.

2.4 Discussion

In the present study, I present results from TILLING in *Beta vulgaris*. I established a TILLING protocol based on high throughput PCR and LI-COR[®] based CEL1 endonuclease detection for TILLING screens in sugar beet. Furthermore, I implemented a crash-test for primer testing to avoid mispriming and the amplification of single primer products. In addition to the annual EMS sugar beet population from Hohmann et al. (2005), I set up a biennial EMS sugar beet population, comprising 6,024 M₂ plant from 837 M₁ families. Taken both population together 6,106 M₂ plants representing 2,387 M₁ families were screened.

The efficiency of a TILLING platform relies largely on (1) the number of M₂ plants, (2) availability of DNA and costs for the extraction, (3) enzyme availability, and (4) mainly the mutation frequency. All of these points will be addressed here and compared to already published TILLING results of other plant species.

In this project a TILLING population of 3,418 M₂ plants was established. The range of published population sizes for TILLING ranges from 529 M₂ plants in *Glycine max* (soybean; Cooper et al., 2008), to 20,000 in hexaploid *Triticum aestivum* (wheat; Fitzgerald et al., 2010). The overall mean of 47 published TILLING populations was about 4,600 M₂ plants per population, thus ranking my TILLING population in the lower part of published TILLING populations. This can be assumed to be sufficient to detect truncation mutation since the similar-sized populations in *Arabidopsis* (3,712 M₂ plants (Martin et al., 2009)), *Arachis hypogaea* (peanut, 3,420 M₂ plants (Knoll et al., 2011)), and *Cucumis melo* (melon, 4,023 M₂ plants (Dahmani-Mardas et al., 2010)) led to successful mutation detection. The rather small number of individuals in the present study derives from a low germination rate of 57.9 % and an exceptional high rate of infertile M₁ plants with 62 %. This can be attributed to the usage of EMS, as EMS is known to account for a high frequency of infertile plants not only in the M₁ but also in subsequent generations (Greene et al., 2003; Le Signor et al., 2009; Perry et al., 2003; Wang et al., 2008b; Wu et al., 2005). As an extreme example for infertility caused by mutagenic treatment serves a so far unpublished study in rye, here EMS treatment of seeds led to an infertility of 100% of rye M₁ plants (Eva Bauer, personal communication). Furthermore, seed set can be negatively influenced by self-incompatibility, which in an outbreeding species like sugar beet may result in lower seed yield (Maletsky and Weisman, 1978). In addition, many plants died because of *Fusarium spec.* infection, i.e. from 11,571 grown plants only 5,817 survived (50 %). The final goal was to obtain 1,500 to 2,000 M₂ families and 6,000 to 10,000 M₂ plants. Due to the above-mentioned problems, only 56 % of the planned M₂ families and 63 % of the planned M₂ plants could be obtained.

2.4.1 DNA extraction methods

Yield and quality of genomic DNA is one of the crucial components in any PCR- based method and this is even more critical for TILLING success. The efficiency of the PCR, as with any other DNA assay, depends on DNA quality and purity. DNA quality is determined by its fragment length and the degree of damage, which is dependent on exposure to heat, low pH and/or nucleases that cause depurination by hydrolysis and/or enzymatic degradation (Anklam et al., 2002). For that reason, several genomic DNA extraction methods were tested for their performance concerning elution volume, DNA yield and resulting DNA quality for further analysis.

CTAB and two kit-based methods were evaluated for their usability and the NucleoSpin[®] Kit was regarded as most appropriate because of the following reasons: a high elution volume is essential, but also a high DNA yield is a major aim. The MagAttract[®] kit gave the poorest results with regard to elution volume and yield, which is in accordance to Pfeiffer (2008) who reported that the DNA yield with MagAttract[®] was low compared to CTAB and NucleoSpin[®] for *B. vulgaris* and *B. napus*, while in *H. vulgare* the yield was high. CTAB gave the best results for yield, but did not meet the

requirements for DNA quality, while the NucleoSpin kit performed intermediate in terms of yield and gave good results after quality check by PCR and subsequent agarose gel electrophoresis. Pfeiffer (2008) reported that DNA extracted with NucleoSpin[®] Kit gave the highest yield for sugar beets, while in barley and rapeseed CTAB method gave the highest yields. Spaniolas et al. (2008) tested five commercial kits and CTAB method for their suitability for DNA extractions in fresh green and roasted coffee beans. CTAB performed best in terms of DNA yield and quality in roasted coffee beans, while a kit based method performed best in fresh green beans followed by CTAB method.

In two other studies it was shown that the combination of CTAB method with a commercially available anion exchange matrix performed best with regard to DNA amount and quality (Csaikl et al., 1998; Sánchez-Hernández and Gaytán-Oyarzún, 2006). In the first study, eight different species were tested: *Rhododendron luteum* (Honeysuckle Azalea), *Quercus robur* (English oak), *Ulmus glabra* (scots elm), *Abies alba* (silver fir), *Pinus sylvestris* (scots pine), *Populus tremula* (aspen), *P. tremuloides* (white poplar) and *Z. mays* (maize), while in the second study *Piper* ssp., *Q. robur*, *Z. mays*, and different cacti were used. In this perspective only poplar is regarded as “easy species” for DNA extraction while the remaining are regarded as “hard species”, due to different secondary metabolites (Csaikl et al., 1998). While not testing DNA from plant material, a study testing several outputs of the dairy food chain (for example: fresh whole milk, ultra-high-temperature skimmed milk, yoghurt, cooking cream, Emmental cheese) for GMO content demonstrated that only DNA extracted with a modified CTAB method ensured end point PCR and RT-PCR positive reactions from all the matrices considered (the amplicons obtained had the expected sizes, sequences and T_m) (Pirondini et al., 2010).

Taken together, these results suggest that a certain method does not work for all species or all samples from which DNA is extracted. This is also shown by Elsanhoty et al. (2011) who tested 5 kit based and CTAB method for their usability in GMO detection in several maize related products. Here the CTAB method gave only poor results in terms of DNA yield and quality and a specialized kit performed best.

DNA yield is not the only variable being crucial for subsequent steps of molecular analysis, but also DNA of superior quality is important especially for TILLING approaches. With respect to DNA quality, the NucleoSpin[®] and MagAttract[®] kits performed best. This is not unexpected considering the underlying principles. The MagAttract[®] kit uses small beads to bind genomic DNA while washing excess proteins and RNA out of the disrupted cell lysate. The NucleoSpin[®] kit makes use of a silica membrane. DNA binds to the membrane under the control of guanidinium hydrochloride buffer and excess proteins and RNA are washed from the membrane with ethanol. In the final step, DNA is eluted from the membrane using an alkaline low salt buffer such as TE. By application of the CTAB method, the DNA is not bound to a matrix, such as a bead or a membrane and therefore cannot be washed as efficiently. Consequently, the eluate may contain protein, cell debris, and RNA contamination. Apart from yield and quality, protocols based on commercial kits required less time to obtain results, but were significantly more expensive than the non-commercial protocols and did not always give good results (Alaey et al., 2005; Elsanhoty et al., 2011; Pirondini et al., 2010). Nonetheless, the most important parameter was to obtain good quality DNA to be used in the PCR reaction.

2.4.2 Pooling depth and cost evaluation

The next step in method evaluation was a test concerning the maximal possible pooling depth for the detection of sequence variance in sugar beet. Pooling to the optimal extent has two advantages, one being the cost and the other the time needed to screen all present individuals in the two TILLING populations. Detection of digested products was strongest in the 2x pools, which was expected, but still showed sufficient resolution in the 8x pools. 8x pooling has several advantages but also suffers from some drawbacks. Cost and time for PCR amplification and screening are reduced, but the probability of missing mutations is increased at screening 8x pools. Homozygote mutations will appear at a ratio

of 1/8 and a heterozygous mutation will only appear at a 1/16 ratio, since only one strand of the DNA carries the mutation. Till et al. (2004) report that for maize there was no difference in screening in 8x pools, as they obtained almost precisely the expected 2:1 heterozygote:homozygote ratio for ~1,900 mutations in 8x pools. Suzuki et al. (2008) observed that SNPs between two rice varieties could be detected even if the DNA fragments from these two varieties were mixed at a ratio of 1:11, indicating that it is possible to detect only one heterozygote SNP in a DNA mixture containing another five homozygous non-mutated plants. In contrast, Uauy et al. (2009) report that a small percentage of mutations are not detected in a LI-COR[®] screen. They compared LI-COR[®] results with non-denaturing polyacrylamide detection results and reported that between the two methods, two at least mutants were not detected. These results suggest that detection of a heterozygous mutation is less probable in an 8x pool.

One other critical aspect in detection of mutants in pooled samples is the underrepresentation of one or more plants in a pool of eight plants, due to differences in DNA concentration. This has also been reported by Till et al. (2004) and Talamè et al. (2008), where differences in detection of mutants were attributed to difference in DNA concentration of pooled samples. Theoretically between ~ 300 and 300,000 copies of a unique target sequence are in a standard PCR as starting material (Taberlet et al., 1996). However, when one M₂ plant is underrepresented in a DNA pool by only 300 DNA molecules compared to 75,000 DNA molecules each remaining other M₂ plants, I would expect 3.2×10^9 versus 5.6×10^{14} DNA molecules in an 8x pool, respectively, resulting in a non-detectable band on the gel.. The impact of this underrepresentation could be shown by Gerloff et al. (1995). In their study on *Pan paniscus* (bonobos), a heterozygous locus was genotyped as homozygous. In a review by Pompanon et al (2005), genotyping errors increased sharply from 1989 to 2004 and some were accounted to low quantity and/or low quality of DNA. To exclude this as much as possible, all DNA samples in my study were normalized to 10 ng/μl and all samples were extracted by robot to minimize pipetting errors. However, during the analysis process, minor variations could have taken place, which eventually may have a high impact on the later screening results.

Another important objective in high throughput TILLING is the pooling design. When TILLING was first introduced by McCallum et al., (2000a) a 1D pooling strategy was used. The 1D strategy has an advantage when the population under investigation is rather small or in the case of *Brassica napus* is complex, because of the amphipolyploid character. Here a 1D strategy was used to avoid the selection of locus-specific primer combinations (Wang et al., 2008b). A disadvantage of 1D pooling will come prominent when screening 500 and more individuals, where 1D pooling comes to its limitations, as cost and labor will rise exponentially. But cost factors are not always of concern as shown by Muth et al. (2008), where 837 EMS mutagenized doubled haploid potato (*Solanum tuberosum*) lines were screened in a 1D pool by direct Sanger sequencing. The 2D design has the advantage of a positive signal on a LI-COR[®] gel indicating a SNP has to appear twice, therefore giving an extra level of reliability and a reduction of the number of PCR reactions necessary to identify a positive SNP by sequencing. In a 1D pool, each plant of the positive pool has to be sequenced in order to identify the SNP responsible for the signal. Through the double signal in the 2D pool the coordinates for the plant carrying the SNP leading to the signal are given, thus eliminating the need to sequence all the plants in the identified DNA pool. The achieved results led to the setup of a sugar beet TILLING platform with DNA extraction by Tecan Freedom EVO[®] Robot with Macherey-Nagel NucleoSpin[®] 96 Plant kit I and II, robot-based DNA normalization, and 2D 8x pool composition in 96-well format.

2.4.3 Primer design

Primer design is a central aspect of TILLING. It is mandatory to have the sequence of the genes of interest available to evaluate the most suitable region for primer annealing. Many TILLING protocols used primer3, a web-based primer design tool (Greene et al., 2003; Perry et al., 2003; Talamè et al., 2008), to generate gene-specific primers. In order to evaluate the best-suited primer for TILLING,

three different primer design tools (Perl Primer, Primer3Plus and FastPCR[®]) were tested. Primer designed with FastPCR[®] gave the best results in the practical primer test for two *Beta vulgaris* genes, *BvCO* and *BvLHPI*, and thus, subsequent primer design during this study was also performed with FastPCR.

From the primer “crash-test”, I achieved an overall success rate of 0.35, and single gene success rates ranging from 0.61 for *FL1* to 0.15 for *BvLHPI*. The obtained results were substantially lower than the calculated success rates for maize (0.78, (Till et al., 2004)) and Arabidopsis (0.93, (Greene et al., 2003)), but were still sufficient to obtain at least one functional primer pair per region of interest. In this respect, the Arabidopsis TILLING project (ATP) is distinguished from other TILLING projects, because many researchers contributed primers for regions of their interest and the ATP team tested these primers for single product amplification prior to the actual TILLING screen. Hence, a significant proportion of preparatory work was already performed in advance, which is usually not the case for TILLING populations in other plant species thus, and may explain the high success rate in Arabidopsis. The success of a primer depends on several factors, i.e. nucleotide composition at the 3'-end being essential for the control of mispriming (Kwok et al., 1990) and complementation of a primer pair (Dieffenbach et al., 1993). A homology at the 3'-end has the tendency to build primer dimers, and these dimers will compete with the template in the PCR reaction. Furthermore, the melting temperature (T_M) between the forward and reverse primer should not differ more than 4°C, as this will have a negative effect on primer binding to the template, because the optimal annealing temperature is shifted (Singh and Kumar, 2001). As a result, primer will have different success rates, because of the region chosen for amplification and the resulting nucleotide composition.

2.4.4 EMS populations

The ideal TILLING population would be homozygous and homogenic in terms of the structure of the population, but as shown in the results section, the TILLING populations were heterogenic. However, this heterogeneity is not necessarily obstructive for TILLING screens, as it just increases the rate of sequence variations detected by TILLING without masking mutations coming from the EMS treatment. There can be several reasons for the heterogeneity:

- Remaining heterozygosity in the founder inbred line
- Seed admixture in the founder seed lot
- Alien pollination during selfing of M_1 plants
- DNA admixture
- High mutation frequency

Remaining heterozygosity from the founder inbred line of population 930190 should result in heterozygous loci, segregating in a Mendelian manner. 930190 is an inbred line with supposedly low heterogeneity leading to the assumption that after an AFLP analysis the level of polymorphic bands should be low. Unfortunately during this study, the off types in the founder seed sample identified by AFLP showed that the seed lot 930190 used for EMS treatment was likely contaminated by another genotype. How this contamination took place cannot be clarified, as there are no seed samples left of the founder seed stock obtained from Dieckmann in 1996. In the founder seed lot used for TILLING, I could estimate the rate of polymorphic bands to be 18.7%, in the 930190-EMS plants it was 22.6% and for the 28 M_2 plants of the mutant family the rate was 17.6%. Hansen et al. (1999) reported of a polymorphic rate of 96.4 % for ten sugar beet breeding lines and five wild beets. That is a fivefold higher value as I estimated it to be. In contrast, De Riek et al. (2001) reported a polymorphic rate of about 21% for seven sugar beet lines, which is in the range of values I estimated. The results obtained from the AFLP screen and the results presented above lead to the assumption that the contamination of the founder seed lot seems to be the most likely explanation. An AFLP screen was not performed for *SynDH1* but here I assume the same reason for heterogeneity.

Alien pollination during M_1 and M_2 seed production as reason for off types cannot be completely ruled out, especially as sugar beet is a partially self-incompatible outbreeding species and the bags used for isolation during are not completely pollen tight. There is one other source for cross-fertilization. The bags were in some cases re-opened to slide the bags a small distance to allow for growing of the flowering stem. This, was done when the flowering stem grew substantially after the initial bagging. In addition, Hohmann reports in the final report of GABI-TILL that in 2005 1,100 M_2 plants were open-pollinated. This was done in order to assure seed formation on highly aphid invested plants. If those plants would have been bagged aphid, treatment would have been impossible and that would most likely disturb seed formation substantially. The effect of the open pollination can be a reason for the heterogeneity of 93190-EMS as presented in the result. If the seed lot was homozygous, the contamination must have occurred during propagation. Additionally from sorghum, we know that cross-fertilization rates increased dramatically after EMS treatment (Xin et al., 2008). So a likely scenario could be assumed for the sugar beet TILLING populations.

DNA admixture as a source of error is quite unlikely as the DNA for TILLING was handled and stored separately from other DNA. Also, the 3:1 segregation of the AFLP off bands in the M_3 family 054658 rather points to a heterozygous M_2 plant. Heterogeneity has been reported in other TILLING projects as well. Cooper *et al.* (2008) report that some base changes identified in *G. max* (soybean) were not the expected G/C to A/T changes from EMS mutagenesis and were therefore considered as off types to the mutant population. The same accounts for the TILLING population of *Brassica oleracea* (wild cabbage), where two uniform mutations were present in more than one individual (Himmelblau et al., 2009). Nevertheless, sequence variants were successfully detected and led to MFs of 1/140 kb for *G. max* and 1/447 kb for *B. oleracea*. These results do not differ from TILLING populations, where no contaminations were reported. Therefore suggesting that detection of sequence variants is possible. However, every detected SV has to be carefully checked for possible contaminations through off types.

With MFs of 1/1,108 kb and 1/1,016 kb in 930190 and SynDH1, respectively, the sugar beet TILLING populations are in the lower end of published MFs of other species. Only the MF of *Medicago truncatula* is lower with 1/1,290 kb. However, MFs are not easily comparable as they are dependent on many factors. One of these factors is the choice of the mutagen. It is known that diverse species and even varieties of the same species display differential responses to mutagenic treatments (Wu et al., 2005). For *Oryza sativa ssp. japonica* “Nipponbare” for instance a suitable mutation density was only achieved by choosing Az-MNU as mutagen (Till et al., 2007). The same accounts for *H. vulgare*, where a treatment of seeds with sodium azide (NaN_3) led to 22 mutations / 4,906 M_2 plants (Talamè et al., 2008) while an EMS treatment only resulted in 10 mutations / 9,216 M_2 plants (Caldwell et al., 2004). Besides the choice of the appropriate mutagen, the treated plant organs also have an effect on mutation density. Till et al. (2007) report a MF of 1/265 kb for an NaN_3 -MNU treatment of *O. sativa ssp. japonica* “Nipponbare” seeds, however, in contrast, Suzuki et al. (2008) report a MF of 1/135 kb for a MNU treatment of panicles in *O. sativa ssp. japonica* “Taichung 65”. The effects accounting for plant organs can be explained by morphology and physiology. Seeds normally consist of three tissues in the following order (from the inside to the outside):

- Embryo (with radicle and cotyledons)
- Endosperm or perisperm (depending on location)
- Testa

The proportion and location of each tissue depends on the plant species (Sitte et al., 2002) and in some species, additional structures are present as is the case for sugar beet seeds. Sugar beet seeds are characterized by an additional layer, the pericarp (Hermann et al., 2007). It is likely, that the testa serves as an additional physiological barrier for mutagens, e.g. the panicles. Another factor reportedly influencing mutation density is the ploidy of a species. The highest MFs were reported for *Brassica napus* with 1/16 kb (Harloff et al., 2011), *Triticum aestivum* with 1/24 kb (Slade and Knauf, 2005;

Slade et al., 2005), and *Avena sativa* with 1/33 kb (Chawade et al., 2010). These species are either hexaploid (*T. aestivum* and *A. sativa*) or allotetraploid (*B. napus*). The highest MF of a diploid species has been reported by Martin et al. (2009) for *A. thaliana* with a MF of 1/89 kb, which is already almost 6 times lower than the MF reported for *B. napus*, but still almost six times higher than those of *H. vulgare* at 1/500 kb (Gottwald et al., 2009).

The observed MF of SynDH1 is slightly higher compared to that resulting from 930190. This is as expected because of differences in EMS concentration and duration of EMS treatment. All 20,000 seeds of SynDH1 were treated with 1.0% EMS for 12 hours while during production of the TILLING population 930190, 0.5 or 1.0 % EMS were used for different durations (4, 6, 8, and 12 hours). Caldwell et al. (2004) reported a twofold difference in the number of phenotypic mutants observed between two EMS populations treated with concentrations of 0.25 % and 0.4 %. These results are comparable to the differences in EMS concentration applied to SynDH1 and 930190. Furthermore, different cultivars of the same species can have different MFs even when treated in the same way and with the same concentration of a mutagen. The genetic background may have an effect on the efficiency or toxicity of the mutagen, which is indicated by results from a study in rice (Wu et al., 2005). Several *O. sativa* genotypes were treated with the same amount of EMS and showed significant differences in survival and fertility. Similar results have also been reported for *G. max* (Cooper et al., 2008), where a 4-fold difference in MF could be observed between the cultivars “cv .Forrest” with 1/140 kb and “cv .William82” with 1/550 kb. Further results from Arabidopsis underline the hypothesis of environmental conditions as partially causative for the observed results, since treatment of seed batches with the same EMS concentration led to different MF from treatment to treatment (Cooper et al., 2008). Summing up, the difference in MF of SynDH1 and 931090 is probably due to difference in EMS concentration combined with the variance of the two different cultivars.

As depicted in Table 21, there are other TILLING projects where no nonsense mutations leading to a premature stop could be identified. In the case of *A. sativa* (oat) for instance, Chawade et al. (2010) report of an MF of 1/33 kb, which is a high value, but they could not detect any nonsense mutation in any of the genes investigated. The same accounts for the TILLING projects of *B. napus* (2008b) and *B. oleracea* (2009) with MF of 1/86 kb and 1/447 kb respectively. Additionally the TILLING projects in *G. max* (soybean), *H. vulgare* (barley), *Pisum sativum* (pea), *Solanum lycopersicum* (tomato), and *Z. mays* (maize), did not identify a nonsense mutation. By comparing the results, it is noticeable that a high MF does not necessarily lead to a nonsense mutation. Therefore, another factor seems to be important. Dalmais et al. (2008) report for TILLING in pea, that despite a similar GC content (34% - 36%) of the 20 genes investigated, some were much more mutated than other (gene specific MF from 1/84 kb to 1/356kb). In one case they report of a gene (*PsMet1*) where they could find 96 mutations, while on average the investigated 34 mutations per gene. These results demonstrate that the choice of genes has a tremendous effect on overall mutation frequency and the possibility of identifying nonsense mutations.

Table 21: Functional mutation spectrum and types of nucleotide change detected in different TILLING populations. The mutagen as well as the species and the respective population size are given. The mutation frequency (MF), the average number of mutations per plants, as well as the total number of mutations detected per species is given. Furthermore the type of mutations identified and their respective percentage is given.

Mutagen	Species (botanical name)	Population size (M ₂)	Mutation frequency (kb)	Average mutation per plants	Total no. of mutations detected	Type of mutation				Reference
						Missense (%)	Silent (%)	Nonsense (%)	Non-coding (%)	
EMS	<i>Arabidopsis thaliana</i>	3,072	300	233	1890	50	45	5	0	Greene et al. 2003
		6,912	170	412	1774/1063	49.7	45.8	4.5	0	Till et al. 2003
		3,712	89	787	450	38.2	36.5	4.2	21.1	Martin et al. 2009
	<i>Arachis hypogaea</i>	3,420	967	2,744	27	48.1	37	14.8	0	Knoll et al. 2011
	<i>Avena sativa</i>	2,600	33	90,215	16	50	50	0	0	Chawade et al. 2010
	<i>Beta vulgaris</i>	6,106	1,062	321	64	34.4	34.4	1.6	29.7	This study
	<i>Brassica napus</i>	1,344	86	12,514	19	63.2	36.8	0	0	Wang et al. 2008a, b
	<i>Brassica napus</i>	8,843	39	27,952	568	52.1	25.2	2.8	19.9	Harloff et al. 2011
	<i>Brassica oleracea</i>	2,263	447	1,579	25	60	40	0	n.a.	Himmelblau et al. 2009
	<i>Brassica rapa</i>	9,216	60	12,110	617/306	63.4	34.3	2.3	n.a.	Stephenson et al. 2010
	<i>Cucumis melo</i>	4,023	573	1,381	134	65.1	31.3	2.4	n.a.	Dahmani-Mardas et al. 2010
	<i>Glycine max</i>	864	140	7,254	32	66	34	0	n.a.	Cooper et al. 2008
		3,712	550	1,846	12	33	58	8	n.a.	
767		140	7,254	25	44	52	4	n.a.		

Mutagen	Species (botanical name)	Population size (M ₂)	Mutation frequency (kb)	Average mutation per plants	Total no. of mutations detected	Type of mutation				Reference
						Missense (%)	Silent (%)	Nonsense (%)	Non-coding (%)	
EMS	<i>Helianthus annuus</i>	1,152	475	4,614	9	11.1	11.1	11.1	66.7	Sabetta et al. 2011
	<i>Hordeum vulgare</i>	9,216	1,000	6,179	10	60	40	0	0	Caldwell et al. 2004
		10,279	500	12,358	81	35.8	39.5	3.7	21	Gottwald et al. 2009
	<i>Lotus japonicus</i>	4,904	502	850	576	47.7	20.5	2.8	29	Perry et al. 2009
	<i>Medicago truncatula</i>	4,500	455	846	512	67.4	27.7	4.9	n.a.	Le Signor et al. 2009
		4,350	1,290	298	34	58.8	38.2	3	n.a.	
	<i>Nicotiana tabacum</i>	1,311	53	82,929	27	55.5	22.2	22.2	0	Julio et al. 2008
	<i>Oryza sativa</i>	768	294	1,838	27	48.1	33.4	18.5	n.a.	Till et al. 2007
	<i>Pisum sativum</i>	4,717	193	21,380	50	60	34	0	6	Triques et al. 2008
	<i>Solanum lycopersicum</i>	1,926	448	902	66	62.4	37.6	0	n.a.	Minoia et al. 2010
		4,759	574	704	256	33.2	20.7	2.7	43.4	Piron et al. 2010
	<i>Solanum tuberosum</i>	864	91	8,019	19	52.6	21.1	5.3	21.1	Muth et al. 2008
	<i>Sorghum bicolor</i>	264	137	5,773	23/10	70	20	10	0	Blomstedt et al. 2012
<i>Triticum aestivum</i>	10,000	40	474,727	196	34.2	n.a.	1	n.a.	Slade et al. 2005	
	2,348	23	825,613	140	45.4	30.3	9.2	15.1	Dong et al. 2009	
	1,700	194	97,882	13	46	38.6	7.7	n.a.	Sestili et al. 2010	

Mutagen	Species (botanical name)	Population size (M ₂)	Mutation frequency (kb)	Average mutation per plants	Total no. of mutations detected	Type of mutation				Reference
						Missense (%)	Silent (%)	Nonsense (%)	Non-coding (%)	
EMS	<i>Triticum durum</i>	8,000	40	362,094	50	34	n.a.	2	n.a.	Slade et al. 2005
	<i>Zea mays</i>	750	485	5,875	17	58.8	41.2	0	0	Till et al. 2004a
MNU	<i>Glycine max</i>	768	140	7,254	47	45	51	4	n.a.	Cooper et al. 2008
	<i>Oryza sativa</i>	767	135	4,003	24	54.2	41.7	0	4.1	Suzuki et al. 2008
	<i>Hordeum vulgare</i>	1,372	486	12,714	9	55.6	22.2	0	22.2	Kurowska et al. 2011
NaN3	<i>Hordeum vulgare</i>	4,906	374	16,522	22	68.2	18.2	0	13.6	Talamè et al. 2008
		9,573	2,500	2,472	5	60	0	0	40	Lababidi et al. 2009
NaN3/MNU	<i>Hordeum vulgare</i>	10,000	235	26,295	180	44.4	36.1	1.7	17.8	Kurowska et al. 2011
	<i>Oryza sativa</i>	768	265	2,039	30	56.7	33.3	0	10	Till et al. 2007

Furthermore, in the case of the 96 identified mutations per gene, the number of screened plants had an effect. Dalmais et al. (2008) report that the high numbers of mutants was only possible by screening the whole population of 4,717 M₂ plants. These findings can now be transferred to the *Beta vulgaris* TILLING project. The main reasons for failing to detect nonsense mutation are: (1) the low mutation frequency, (2) a small population size and (3) the choice of genes.

2.4.5 Phenotypic screening of M₃ offspring

Sugar beets with alteration in bolting and flowering time are highly desired for breeding of winter beets (Jung and Müller, 2009). Therefore, the objective of this work was to identify mutation in bolting and flowering time genes. These mutants should lead to the identification of desired phenotypes. Loss of function/altered function mutations should lead to the following phenotypes:

- For *BTC1* in the case of 930190, we would expect plants that flower earlier than the non-mutated phenotype. For the biennial SynDH1 mutants, however, we will not expect a different phenotype.
- *AtFLC* the homolog of *BvFL1* is known to play a crucial role in flowering time in *A. thaliana*. Therefore, we would expect early flowering plants.
- *BvFT1* acts as a repressor in biennial plants, a loss of function mutation would lead to early flowering plants (Pin et al., 2010).
- *BvFT2* acts as a promotor in biennial plants, a loss of function mutation would lead to late flowering plants (Pin et al., 2010).
- In the case of *BvFVE*, we would expect later flowering plants as described for *AtFVE* *Arabidopsis thaliana* by Baek et al. (2008).
- The effect for *AtLHP1* has only been described in *A. thaliana* (Mylne et al., 2006), where it leads to early flowering plants. Since *AtLHP1* represses both *AtFLC* and *AtFT1*, the phenotype of *BvLHP1* biennial mutants is hard to predict.
- The *AtTON1* loss of function mutant of *A. thaliana* displays drastic defects in morphogenesis, positioning of division planes, and cellular organization; thus we would expect a similar effect for *BvTON1* in sugar beet mutants (Azimzadeh et al., 2008).

Unfortunately, the results from the phenotypic screen of SVs were disappointing. From the screen in 930190 of putative *BvLHP1* mutants, none led to a significant reduction in time to flower. The same accounts for phenotypic screens in M₃ plants of SynDH1 carrying mutations in the flowering time genes *BTC1*, *BvFLK*, *BvLHP1*, and *BvTON1*. This is not unexpected, as the mutation does not necessarily change the phenotype of the M₃ plant carrying the mutation. In the case for the splice site mutation in *BvFT1*, I can say that the nucleotide change has no influence on bolting behavior before vernalization. Pin et al. (2010) report that RNAi *BvFT1* plants flower early. The plants carrying the splice site mutation did not flower early, implying that the nucleotide change at the splice site of *BvFT1* had no effect on the correct splicing of the mRNA. Since none of the mutations identified was a nonsense mutation, the chances of observing altered phenotypes were small. Even with a mutation, leading to a premature stop codon a phenotypic change is not always observable. Harloff et al. (2011) report that from 34 promising mutants in *B. napus*, including stop mutations, for the *BnaX.SGT* gene, none showed a significantly lower sinapine or sinapic acid ester content. In the case of *B. napus*, this is not unexpected, as only one of two functional seed-expressed genes was mutated. The fact that the identified mutations did not lead to desired phenotypes is at first hand chastening, but this should have been overcome by the combination of two or more phenotypically neutral mutations. These double or triple mutants would than ideally show phenotypes that are drastically altered compared to non-mutated plants. Unfortunately, this goal could not be achieved.

When I consider the correspondence between phenotypic and genotypic screening, I have to take into account that there are several factors, which can contribute to an absent phenotype in the M_3 family derived from a M_2 with a detected mutation. First of all, the mutant allele could get lost from M_2 to M_3 , which we actually observed in one case. This could happen if the M_2 plant was heterozygous for the mutation and during M_3 seed production some form of gametic selection against the mutant allele occurred due to lower fitness. Various degrees of gametic selection have been reported for soybean (Kopisch-Obuch and Diers, 2006) rice (Guiderdoni, 1991), mustard (Cheung et al., 1997) and sugar beet (Pillen et al., 1992). If the mutation is passed to the M_3 generation and it does not show a phenotypic effect, the most prominent assumption is that the candidate gene has no effect on the trait that is phenotyped. Furthermore, when looking at a quantitative trait, a mutation can only have a minor effect that is beyond phenotypical detection. This is the case if the target gene only has a minor effect on the trait. Apart from that, effects on quantitative traits can be masked by background mutations of other genes that are affecting directly or indirectly the same trait. This can happen if the mutation has a small effect on a quantitative trait and other genes that are affecting directly or indirectly the same trait are mutated. For example, when targeting genes of the flowering pathway, it is possible that also housekeeping genes are mutated. The mutation in housekeeping genes could then delay general plant growth, development, thus indirectly affecting bolting, and flowering time. These genes could be inherited independently of the target gene leading to a genetic background variation. This in turn could decrease the statistical power for the detection of phenotypic effects. For a quantitative trait like bolting and flowering time, this is not unexpected (Bernardo, 2008; Bohuon et al., 1998).

A more rare scenario is if a background mutation is in coupling linkage with the target gene, both affecting the same trait in opposite ways. In *Arabidopsis*, Henikoff and Comai (2003) estimated the likelihood of a phenotypic misattribution by linked mutations to only 0.0005. Therefore, in general this is not an explanation for absent phenotypic effects.

The last assumption explaining why not all mutations will lead to a phenotype could be that the mutation can be compensated by other genes copies with the same function. Gene duplication provides evolutionary opportunities for organisms. If a critical function is encoded by a single locus, a mutation that changes the function of that gene is likely to have negative consequences for the organism. However, if genome or gene duplication has created several loci that encode the same function, the selective pressure on those loci is reduced. One of the duplicate loci may retain the original function while permitting duplicates to undergo loss-of function. For example in *B. oleracea* there are orthologous genes (*gly*) for the wax biosynthesis pathway. A missense mutations in the *gly2-1* allele was not capable to produce a glossy phenotype (Himmelblau et al., 2009). Recently, Pin et al. (2010) reported the duplication of the flowering time homolog *BvFT* into *BvFT1* and *BvFT2* in sugar beet as a result of diversification of the genus *Beta*. Although mutations in *BvFT1* lead to a neofunctionalization from a floral activator to a floral repressor, this provides strong evidence for gene duplication in *B. vulgaris*.

The identified mutations in the sugar beet TILLING project showed no clear or new phenotypes. The differences in flowering or bolting time were not significantly different from the control plants. This can be due to the above-mentioned criteria or to a yet undescribed mechanism in sugar beet. In conclusion, I assume that the mutation frequency was too low, the number of M_2 was too small and the choice of genes was not satisfactory to find nonsense mutations. For future TILLING studies in sugar beet, I would suggest to test several genotypes and different mutagens for their suitability of mutation induction capacity. Once a good mutagen and high mutable genotype has been identified, I would choose M_1 plants with low seed set, as those are believed to have a high density of mutations. Then a population of at least 4,500 M_2 plants should be constructed to increase the chances of finding nonsense mutations in the actual screen.

I still believe that TILLING is a good technique to identify mutations with phenotypes of interest. Many projects have shown of the potential of TILLING and therefore TILLING in sugar beet should be repeated, but with different criteria as described above.

2.5 References

- Abe, M., Y. Kobayashi, S. Yamamoto, Y. Daimon, A. Yamaguchi, Y. Ikeda, H. Ichinoki, M. Notaguchi, K. Goto, and T. Araki, 2005: FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* **309**, 1052-1056.
- Alaey, M., R. Naderi, A. Vezvaei, A. Khalighi, and A. Salami, 2005: Comparing study between four different methods of genomic DNA extraction from *Cyclamen persicum* Mill. *International Journal of Agriculture & Biology* **7**.
- Alcantara, T.P., P.W. Bosland, and D.W. Smith, 1996: Ethyl methanesulfonate-induced seed mutagenesis of *Capsicum annuum*. *Journal of Heredity* **87**, 239-241.
- Almeida, R., and R.C. Allshire, 2005: RNA silencing and genome regulation. *Trends in Cell Biology* **15**, 251-258.
- Amasino, R.M., and S.D. Michaels, 2010: The timing of flowering. *Plant Physiology* **154**, 516-520.
- Anklam, E., F. Gadani, P. Heinze, H. Pijnenburg, and G. Van Den Eede, 2002: Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. *European Food Research and Technology* **214**, 3-26.
- Ausin, I., C. Alonso-Blanco, J.A. Jarillo, L. Ruiz-Garcia, and J.M. Martinez-Zapater, 2004: Regulation of flowering time by FVE, a retinoblastoma-associated protein. *Nature Genetics* **36**, 162-166.
- Azimzadeh, J., P. Nacry, A. Christodoulidou, S. Drevensek, C. Camilleri, N. Amiour, F. Parcy, M. Pastuglia, and D. Bouchez, 2008: Arabidopsis TONNEAU1 proteins are essential for preprophase band formation and interact with centrin. *Plant Cell* **20**, 2146-2159.
- Baek, I.S., H.Y. Park, M.K. You, J.H. Lee, and J.K. Kim, 2008: Functional conservation and divergence of *FVE* genes that control flowering time and cold response in rice and Arabidopsis. *Molecules and Cells* **26**, 368-372.
- Bäurle, I., and C. Dean, 2008: Differential interactions of the autonomous pathway RRM proteins and chromatin regulators in the silencing of Arabidopsis targets. *PLoS One* **3**, e2733.
- Becker, H.C., 1993: *Pflanzenzüchtung Ulmer*.
- Bernardo, R., 2008: Molecular markers and selection for complex traits in plants: Learning from the last 20 years. *Crop Science* **48**, 1649-1664.
- Biancardi, E., L.G. Campell, G.N. Skaracis, and M. De Biaggi, 2005: *Genetics and breeding of sugar beet* Science Publishers, Inc., Enfield.
- Bohuon, E.J.R., L.D. Ramsay, J.A. Craft, A.E. Arthur, D.F. Marshall, D.J. Lydiate, and M.J. Kearsey, 1998: The association of flowering time quantitative trait loci with duplicated regions and candidate loci in *Brassica oleracea*. *Genetics* **150**, 393-401.

- Bush, S.M., and P.J. Krysan, 2010: iTILLING: A personalized approach to the identification of induced mutations in Arabidopsis. *Plant Physiology* **154**, 25-35.
- Caldwell, D.G., N. McCallum, P. Shaw, G.J. Muehlbauer, D.F. Marshall, and R. Waugh, 2004: A structured mutant population for forward and reverse genetics in Barley (*Hordeum vulgare* L.). *Plant Journal* **40**, 143-150.
- Chawade, A., P. Sikora, M. Brautigam, M. Larsson, V. Vivekanand, M. Nakash, T. Chen, and O. Olsson, 2010: Development and characterization of an oat TILLING-population and identification of mutations in lignin and beta-glucan biosynthesis genes. *BMC Plant Biology* **10**, 1-13.
- Cheung, W.Y., L. Friesen, G.F.W. Rakow, G. Séguin-Swartz, and B.S. Landry, 1997: A RFLP-based linkage map of mustard [*Brassica juncea* (L.) Czern. and Coss.]. *Theoretical and Applied Genetics* **94**, 841-851.
- Chia, T.Y.P., A. Muller, C. Jung, and E. Mutasa-Gottgens, 2008: Sugar beet contains a large CONSTANS-LIKE gene family including a CO homologue that is independent of the early-bolting (*B*) gene locus. *Journal of Experimental Botany* **59**, 2735-2748.
- Choi, K., J. Kim, H.-J. Hwang, S.Y. Kim, C. Park, S.Y. Kim, and I. Lee, 2011: The FRIGIDA complex activates transcription of *FLC*, a strong flowering repressor in Arabidopsis, by recruiting chromatin modification factors. *Plant Cell* **23**, 289-303.
- Choi, K., S. Kim, S.Y. Kim, M. Kim, Y. Hyun, H. Lee, S. Choe, S.G. Kim, S. Michaels, and I. Lee, 2005: *SUPPRESSOR OF FRIGIDA3* encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in *Arabidopsis*. *Plant Cell* **17**, 2647-2660.
- Cooper, J.L., B.J. Till, R.G. Laport, M.C. Darlow, J.M. Kleffner, A. Jamai, T. El-Mellouki, S. Liu, R. Ritchie, N. Nielsen, K.D. Bilyeu, K. Meksem, L. Comai, and S. Henikoff, 2008: TILLING to detect induced mutations in soybean. *BMC Plant Biology* **8**, 1-10.
- Corbesier, L., C. Vincent, S. Jang, F. Fornara, Q. Fan, I. Searle, A. Giakountis, S. Farrona, L. Gissot, C. Turnbull, and G. Coupland, 2007: FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *Science* **316**, 1030-1033.
- Csaikl, U.M., H. Bastian, R. Brettschneider, S. Gauch, A. Meir, M. Schauerte, F. Scholz, C. Sperisen, B. Vornam, and B. Ziegenhagen, 1998: Comparative analysis of different DNA extraction protocols: A fast, universal maxi-preparation of high quality plant DNA for genetic evaluation and phylogenetic studies. *Plant Molecular Biology Reporter* **16**, 69-86.
- Dahmani-Mardas, F., C. Troadec, A. Boualem, S. Leveque, A.A. Alsdon, A.A. Aldoss, C. Dogimont, and A. Bendahmane, 2010: Engineering melon plants with improved fruit shelf life using the TILLING approach. *PLoS One* **5**, e15776.
- Dalmais, M., J. Schmidt, S.C. Le, F. Moussy, J. Burstin, V. Savoie, G. Aubert, V. Brunaud, O.Y. de, C. Guichard, R. Thompson, and A. Bendahmane, 2008: UTILdb, a *Pisum sativum* in silico forward and reverse genetics tool. *Genome Biology* **9**, 1-12.
- De Riek, J., E. Calsyn, I. Everaert, E. Van Bockstaele, and M. De Loose, 2001: AFLP based alternatives for the assessment of distinctness, uniformity and stability of sugar beet varieties. *Theoretical and Applied Genetics* **103**, 1254-1265.

- Dennis, E.S., and W.J. Peacock, 2007: Epigenetic regulation of flowering. *Current Opinion in Plant Biology* **10**, 520-527.
- Dieffenbach, C.W., T.M. Lowe, and G.S. Dveksler, 1993: General concepts for PCR primer design. *Genome Research* **3**, S30-S37.
- El-Mezawy, A., F. Dreyer, G. Jacobs, and C. Jung, 2002: High resolution mapping of the bolting gene *B* of sugar beet. *Theoretical and Applied Genetics* **105**, 100-105.
- Elsanhoty, R.M., M.F. Ramadan, and K.D. Jany, 2011: DNA extraction methods for detecting genetically modified foods: A comparative study. *Food Chemistry* **126**, 1883-1889.
- Fitzgerald, T., K. Kazan, Z. Li, M. Morell, and J. Manners, 2010: A high-throughput method for the detection of homoeologous gene deletions in hexaploid wheat. *BMC Plant Biology* **10**, 1-15.
- Gerloff, U., C. Schlötterer, K. Rassmann, I. Rambold, G. Hohmann, B. Fruth, and D. Tautz, 1995: Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan paniscus*). *Molecular Ecology* **4**, 515-518.
- Gómez-Galera, S., R.M. Twyman, P.A.C. Sparrow, B. Van Droogenbroeck, R. Custers, T. Capell, and P. Christou, 2012: Field trials and tribulations—making sense of the regulations for experimental field trials of transgenic crops in Europe. *Plant Biotechnology Journal* **10**, 511-523.
- Gottwald, S., P. Bauer, T. Komatsuda, U. Lundqvist, and N. Stein, 2009: TILLING in the two-rowed barley cultivar 'Barke' reveals preferred sites of functional diversity in the gene *HvHox1*. *BMC Research Notes* **2**, 1-14.
- Greene, E.A., C.A. Codomo, N.E. Taylor, J.G. Henikoff, B.J. Till, S.H. Reynolds, L.C. Enns, C. Burtner, J.E. Johnson, A.R. Odden, L. Comai, and S. Henikoff, 2003: Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in Arabidopsis. *Genetics* **164**, 731-740.
- Guideroni, E., 1991: Gametic selection in anther culture of rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* **81**, 406-412.
- Hansen, M., T. Kraft, M. Christiansson, and N.O. Nilsson, 1999: Evaluation of AFLP in Beta. *Theoretical and Applied Genetics* **98**, 845-852.
- Harloff, H.J., S. Lemcke, J. Mittasch, A. Frolov, J.G. Wu, F. Dreyer, G. Leckband, and C. Jung, 2011: A mutation screening platform for rapeseed (*Brassica napus* L.) and the detection of sinapine biosynthesis mutants. *Theoretical and Applied Genetics*.
- Henikoff, S., and L. Comai, 2003: Single-nucleotide mutations for plant functional genomics. *Annual Review of Plant Biology* **54**, 375-401.
- Hermann, K., J. Meinhard, P. Dobrev, A. Linkies, B. Pesek, B. Heß, I. Macháčková, U. Fischer, and G. Leubner-Metzger, 2007: 1-Aminocyclopropane-1-carboxylic acid and abscisic acid during the germination of sugar beet (*Beta vulgaris* L.): a comparative study of fruits and seeds. *Journal of Experimental Botany* **58**, 3047-3060.
- Himelblau, E., E. Gilchrist, K. Buono, C. Bizzell, L. Mentzer, R. Vogelzang, T. Osborn, R. Amasino, I. Parkin, and G. Haughn, 2009: Forward and reverse genetics of rapid-cycling *Brassica oleracea*. *Theoretical and Applied Genetics* **118**, 953-961.

- Hohmann, U., 2007: Schlußbericht: GABI-TILL: Establishment of a central platform for testing lead gene function in crops based on the TILLING technology, Teilprojekt: Kiel, 1-26, Vol. 1. Plant Breeding Institute, Kiel, Kiel.
- Hohmann, U., G. Jacobs, and C. Jung, 2005: An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. *Plant Breeding* **124**, 317-321.
- Jaeger, K.E., and P.A. Wigge, 2007: FT protein acts as a long-range signal in Arabidopsis. *Current Biology* **17**, 1050-1054.
- Jaggard, K.W., and A.R. Werker, 1999: An evaluation of the potential benefits and costs of autumn-sown sugarbeet in NW Europe. *Journal of Agricultural Science* **132**, 91-102.
- Jung, C., and A.E. Müller, 2009: Flowering time control and applications in plant breeding. *Trends in Plant Science* **14**, 563-573.
- Kalendar, R., D. Lee, and A.H. Schulman, 2009: FastPCR software for PCR primer and probe design and repeat search. *Genes, Genomes and Genomics* **3**, 1-14.
- Keller, B., C. Feuillet, and N. Yahiaoui, 2005: Map-based isolation of disease resistance genes from bread wheat: cloning in a supersize genome. *Genetics Research* **85**, 93-100.
- Knoll, J.E., M. Ramos, Y. Zeng, C. Holbrook, M. Chow, S. Chen, S. Maleki, A. Bhattacharya, and P. Ozias-Akins, 2011: TILLING for allergen reduction and improvement of quality traits in peanut (*Arachis hypogaea* L.). *BMC Plant Biology* **11**.
- Konopka, R.J., and S. Benzer, 1971: Clock mutants of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences* **68**, 2112-2116.
- Kopisch-Obuch, F.J., and B.W. Diers, 2006: Segregation at the SCN resistance locus *rhg1* in soybean is distorted by an association between the resistance allele and reduced field emergence. *Theoretical and Applied Genetics* **112**, 199-207.
- Kwok, S., D.E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J.J. Sninsky, 1990: Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. *Nucleic Acids Research* **18**, 999-1005.
- Le Signor, C., V. Savoio, G. Aubert, J. Verdier, M. Nicolas, G. Pagny, F. Moussy, M. Sanchez, D. Baker, J. Clarke, and R. Thompson, 2009: Optimizing TILLING populations for reverse genetics in *Medicago truncatula*. *Plant Biotechnology Journal* **7**, 430-441.
- Lee, J., and I. Lee, 2010: Regulation and function of *SOCI*, a flowering pathway integrator. *Journal of Experimental Botany* **61**, 2247-54.
- Lim, M.H., J. Kim, Y.S. Kim, K.S. Chung, Y.H. Seo, I. Lee, C.B. Hong, H.J. Kim, and C.M. Park, 2004: A new Arabidopsis gene, FLK, encodes an RNA binding protein with K homology motifs and regulates flowering time via FLOWERING LOCUS C. *Plant Cell* **16**, 731-740.
- Liu, L.J., Y.C. Zhang, Q.H. Li, Y. Sang, J. Mao, H.L. Lian, L. Wang, and H.Q. Yang, 2008: COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in Arabidopsis. *Plant Cell* **20**, 292-306.

- Maletsky, S.I., and N.J. Weisman, 1978: A population genetic analysis of self- and cross-incompatibility in sugar beet (*Beta Vulgaris* L.). *Theoretical and Applied Genetics* **52**, 21-28.
- Marshall, O.J., 2004: PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* **20**, 2471-2472.
- Martin, B., M. Ramiro, J.M. Martinez-Zapater, and C. Alonso-Blanco, 2009: A high-density collection of EMS-induced mutations for TILLING in Landsberg erecta genetic background of Arabidopsis. *BMC Plant Biology* **9**, 1-9.
- McCallum, C.M., L. Comai, E.A. Greene, and S. Henikoff, 2000: Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant Physiology* **123**, 439-442.
- Meier, U.L., L. Bachmann, E. Buhtz, H. Hack, R. Klöse, B. Märlander, and E. Weber, 1993: Phänologische Entwicklungsstadien der Beta Rübe (*Beta vulgaris* L. spp.). *Nachrichtenblatt Deutscher Pflanzenschutzdienst* **45**, 37-41.
- Michaels, S.D., and R.M. Amasino, 1999: *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949-956.
- Michaels, S.D., and R.M. Amasino, 2001: Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* **13**, 935-942.
- Muth, J., S. Hartje, R.M. Twyman, H.-R. Hofferbert, E. Tacke, and D. Prüfer, 2008: Precision breeding for novel starch variants in potato. *Plant Biotechnology Journal* **6**, 576-584.
- Mylne, J.S., L. Barrett, F. Tessadori, S. Mesnage, L. Johnson, Y.V. Bernatavichute, S.E. Jacobsen, P. Franz, and C. Dean, 2006: *LHP1*, the Arabidopsis homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of *FLC*. *Proceedings of the National Academy of Sciences* **103**, 5012-5017.
- Nakamichi, N., T. Kiba, R. Henriques, T. Mizuno, N.-H. Chua, and H. Sakakibara, 2010: Pseudo-response regulators 9, 7, and 5 are transcriptional repressors in the Arabidopsis circadian clock. *The Plant Cell Online* **22**, 594-605.
- Nakamichi, N., M. Kita, K. Niinuma, S. Ito, T. Yamashino, T. Mizoguchi, and T. Mizuno, 2007: Arabidopsis clock-associated pseudo-response regulators *PRR9*, *PRR7* and *PRR5* coordinately and positively regulate flowering time through the canonical *CONSTANS*-dependent photoperiodic pathway. *Plant and Cell Physiology* **48**, 822-832.
- Pastink, A., E. Heemskerk, M.J. Nivard, C.J. van Vliet, and E.W. Vogel, 1991: Mutational specificity of ethyl methanesulfonate in excision-repair-proficient and -deficient strains of *Drosophila melanogaster*. *Molecular Genetics and Genomics* **229**, 213-8.
- Perry, J.A., T.L. Wang, T.J. Welham, S. Gardner, J.M. Pike, S. Yoshida, and M. Parniske, 2003: A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus*. *Plant Physiology* **131**, 866-871.

- Pfeiffer, N., 2008: Vergleich verschiedener Methoden zur schnellen DNA-Extraktion aus Pflanzen für die markergestützte Selektion in der Pflanzenzüchtung. Bachelor Thesis, Christian-Albrechts-Universität, Kiel.
- Pin, P.A., R. Benlloch, D. Bonnet, E. Wremerth-Weich, T. Kraft, J.J. Gielen, and O. Nilsson, 2010: An antagonistic pair of *FT* homologs mediates the control of flowering time in sugar beet. *Science* **330**, 1397-400.
- Pin, P.A., W. Zhang, S.H. Vogt, N. Dally, B. Büttner, G. Schulze-Buxloh, N.S. Jelly, T.Y.P. Chia, E.S. Mutasa-Göttgens, J.C. Dohm, H. Himmelbauer, B. Weisshaar, J. Kraus, J.J.L. Gielen, M. Lommel, G. Weyens, B. Wahl, A. Schechert, O. Nilsson, C. Jung, T. Kraft, and A.E. Müller, 2012: The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. *Current Biology* **22**, 1095-1101.
- Pirondini, A., U. Bonas, E. Maestri, G. Visioli, M. Marmiroli, and N. Marmiroli, 2010: Yield and amplifiability of different DNA extraction procedures for traceability in the dairy food chain. *Food Control* **21**, 663-668.
- Pompanon, F., A. Bonin, E. Bellemain, and P. Taberlet, 2005: Genotyping errors: causes, consequences and solutions. *Nature Review Genetics* **6**, 847-846.
- Reeves, P.A., Y.H. He, R.J. Schmitz, R.M. Amasino, L.W. Panella, and C.M. Richards, 2007: Evolutionary conservation of the FLOWERING LOCUS C-mediated vernalization response: Evidence from the sugar beet (*Beta vulgaris*). *Genetics* **176**, 295-307.
- Rogers, S.O., and A.J. Bendich, 1985: Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant-tissues. *Plant Molecular Biology* **5**, 69-76.
- Sánchez-Hernández, C., and J.C. Gaytán-Oyarzún, 2006: Two mini-preparation protocols to DNA extraction from plants with high polysaccharide and secondary metabolites. *African Journal of Biotechnology* **5**, 1864-1867.
- Shortle, D., D. DiMaio, and D. Nathans, 1981: Directed mutagenesis. *Annual Review of Genetics* **15**, 265-294.
- Simpson, G.G., 2004: The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of *Arabidopsis* flowering time. *Current Opinion in Plant Biology* **7**, 570-574.
- Singh, V.K., and A. Kumar, 2001: PCR primer design. *Molecular Biology Today* **2**, 27-32.
- Sitte, P., E.W. Weiler, J.W. Kadereit, A. Bresinsky, and C. Körner, 2002: Strasburger - Lehrbuch der Botanik für Hochschulen Spektrum Akademischer Verlag GmbH, Heidelberg.
- Slade, A.J., and V.C. Knauf, 2005: TILLING moves beyond functional genomics into crop improvement. *Transgenic Research* **14**, 109-115.
- Slade, A.J., S.I. Fuerstenberg, D. Loeffler, M.N. Steine, and D. Facciotti, 2005: A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nature Biotechnology* **23**, 75-81.
- Spaniolas, S., M. Tsachaki, M.J. Bennett, and G.A. Tucker, 2008: Evaluation of DNA extraction methods from green and roasted coffee beans. *Food Control* **19**, 257-262.

- Springer, N.M., K. Ying, Y. Fu, T. Ji, C.-T. Yeh, Y. Jia, W. Wu, T. Richmond, J. Kitzman, H. Rosenbaum, A.L. Iniguez, W.B. Barbazuk, J.A. Jeddelloh, D. Nettleton, and P.S. Schnable, 2009: Maize inbreds exhibit high levels of copy number variation (CNV) and presence/absence variation (PAV) in genome content. *PLoS Genetics* **5**, e1000734.
- Srikanth, A., and M. Schmid, 2011: Regulation of flowering time: all roads lead to Rome. *Cellular and Molecular Life Sciences* **68**, 2013-2037.
- Stephenson, P., D. Baker, T. Girin, A. Perez, S. Amoah, G.J. King, and L. Ostergaard, 2010: A rich TILLING resource for studying gene function in *Brassica rapa*. *BMC Plant Biology* **10**, 1-10.
- Sung, S., and R.M. Amasino, 2004: Vernalization and epigenetics: how plants remember winter. *Current Opinion in Plant Biology* **7**, 4-10.
- Suzuki, T., M. Eiguchi, T. Kumamaru, H. Satoh, H. Matsusaka, K. Moriguchi, Y. Nagato, and N. Kurata, 2008: MNU-induced mutant pools and high performance TILLING enable finding of any gene mutation in rice. *Molecular Genetics and Genomics* **279**, 213-223.
- Taberlet, P., S. Griffin, B. Goossens, S. Questiau, V. Manceau, N. Escaravage, L.P. Waits, and J. Bouvet, 1996: Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* **24**, 3189-3194.
- Talamè, V., R. Bovina, M.C. Sanguineti, R. Tuberosa, U. Lundqvist, and S. Salvi, 2008: TILLMore, a resource for the discovery of chemically induced mutants in barley. *Plant Biotechnology Journal* **6**, 477-485.
- Till, B.J., T. Zerr, L. Comai, and S. Henikoff, 2006: A protocol for TILLING and EcoTILLING in plants and animals. *Nature Protocols* **1**, 2465-2477.
- Till, B.J., J. Cooper, T.H. Tai, P. Colowit, E.A. Greene, S. Henikoff, and L. Comai, 2007: Discovery of chemically induced mutations in rice by TILLING. *BMC Plant Biology* **7**, 1-12.
- Till, B.J., S.H. Reynolds, C. Weil, N. Springer, C. Burtner, K. Young, E. Bowers, C.A. Codomo, L.C. Enns, A.R. Odden, E.A. Greene, L. Comai, and S. Henikoff, 2004: Discovery of induced point mutations in maize genes by TILLING. *BMC Plant Biology* **4**, 1-8.
- Turck, F., F. Fornara, and G. Coupland, 2008: Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. *Annual Review of Plant Biology* **59**, 573-594.
- Uauy, C., F. Paraiso, P. Colasuonno, R. Tran, H. Tsai, S. Berardi, L. Comai, and J. Dubcovsky, 2009: A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant Biology* **9**, 1-14.
- Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J.A.M. Leunissen, 2007: Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* **35**, W71-W74.
- Valverde, F., A. Mouradov, W. Soppe, D. Ravenscroft, A. Samach, and G. Coupland, 2004: Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science* **303**, 1003-1006.
- van Harten, A.M., 1998: Mutation breeding: theory and practical applications Cambridge University Press, Cambridge, United Kingdom.

- Vos, P., R. Hogers, M. Bleeker, M. Reijans, T.v.d. Lee, M. Hornes, A. Friters, J. Pot, J. Paleman, M. Kuiper, and M. Zabeau, 1995: AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407-4414.
- Wang, N., Y. Wang, F. Tian, G.J. King, C. Zhang, Y. Long, L. Shi, and J. Meng, 2008: A functional genomics resource for *Brassica napus*: development of an EMS mutagenized population and discovery of FAE1 point mutations by TILLING. *New Phytologist* **180**, 751-765.
- Wang, R., S. Farrona, C. Vincent, A. Joecker, H. Schoof, F. Turck, C. Alonso-Blanco, G. Coupland, and M.C. Albani, 2009: *PEP1* regulates perennial flowering in *Arabidopsis thaliana*. *Nature* **459**, 423-427.
- Wang, T.L., C. Uauy, F. Robson, and B.J. Till, 2012: TILLING in extremis. *Plant Biotechnology Journal*, no-no.
- Weil, C.F., and R.A. Monde, 2007: Getting the point - Mutations in maize. *Crop Science* **47**, S60-S67.
- Wu, J.L., C.A. Wu, C. Lei, M. Baraoidan, A. Bordeos, M. Madamba, M. Ramos-Pamplona, R. Mauleon, A. Portugal, V. Ulat, R. Bruskiewich, G. Wang, J. Leach, G.S. Khush, and H. Leung, 2005: Chemical- and irradiation-induced mutants of Indica rice IR64 for forward and reverse genetics. *Plant Molecular Biology* **59**, 85-97.
- Xin, Z., M.L. Wang, N.A. Barkley, G. Burow, C. Franks, G. Pederson, and J. Burke, 2008: Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biology* **8**, 1-14.
- Zeevaart, J.A.D., 2008: Leaf-produced floral signals. *Current Opinion in Plant Biology* **11**, 541-547.
- Zerr, T., and S. Henikoff, 2005: Automated band mapping in electrophoretic gel images using background information. *Nucleic Acids Research* **33**, 2806-2812.
- Zhang, X., S. Germann, B.J. Blus, S. Khorasanizadeh, V. Gaudin, and S.E. Jacobsen, 2007: The Arabidopsis LHP1 protein colocalizes with histone H3 Lys27 trimethylation. *Nature Structural and Molecular Biology* **14**, 869-871.

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

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Published in BMC Plant Biology, 2013, 13:52

3.1 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 *BvBTC1*, *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*.

3.2 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 Lesion In Genomes) technique that is used to detect point mutations in mutant populations (McCallum et al., 2000a). 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 conferring 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).

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*) (Letschert et al., 1994). 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., 2012).

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 Whitlam, 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 *CBF* genes showing an increase in frost tolerance (Gilmour et al., 2004) and elevated levels of *FLC* expression (Seo et al., 2009). Deng et al. (2011) reported that *FLC* plays a dual role in flowering time control and cold stress response. 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 *COLD AIR*, from intron 1 of *FLC* (Heo and Sung, 2011).

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 survival rates after winter (Kirchhoff et al., 2012). As candidate genes we chose (i) *BTC1* 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 survival rate 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.

3.3 Methods

3.3.1 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. Recording time was before the first frost (2 December 2009). 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).

3.3.2 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 Saatzzucht Dieckmann.

Oligonucleotide primers amplifying conserved domains of the genes *BvFT1*, *BvFL1* and *BTC1* were designed from genomic sequences with 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/> website). 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 (700 nm absorption) and DY-781 (800 nm

absorption), respectively. PCR amplification was done in a 20 μ l volume containing 1 ng pooled DNA, 1 \times Taq buffer, 1.5 mM MgCl₂, 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 labelled versus non labelled 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 μ l CCE and 5.4 μ l CCE buffer for each 20 μ l reaction. The CCE buffer was prepared according to Till et al. (2006a). The reaction was stopped with 4 μ l 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.

3.3.3 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 H_t (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 *B. vulgaris* form.

Selected accessions with significant haplotypes associated with bolting rate and survival rate 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.

3.3.4 AFLP analyses and population structure analysis

The population structure of the 268 *B. vulgaris* 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 STRUCTURE HARVESTER (Earl and von Holdt, 2012).

3.3.5 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 bolting rate, survival rate, or survival rate with bolting rate 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 bolting rate or survival rate, 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) separately for each *B. vulgaris* form.

3.4 Results

3.4.1 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 that 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 15). Variation for survival rate 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. On average, sugar beet accessions performed best (0.39) while fodder beet and garden beet performed worst (0.24 and 0.19, respectively). The largest variation for survival rate was found in *B. vulgaris* ssp. *maritima* 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 (0.81) fell in the first group, most of the fodder beets (0.61) and garden beets (0.86) in the second group, and most of the leaf beets and *B. vulgaris* ssp. *maritima* in the third group (Figure 16). 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 12).

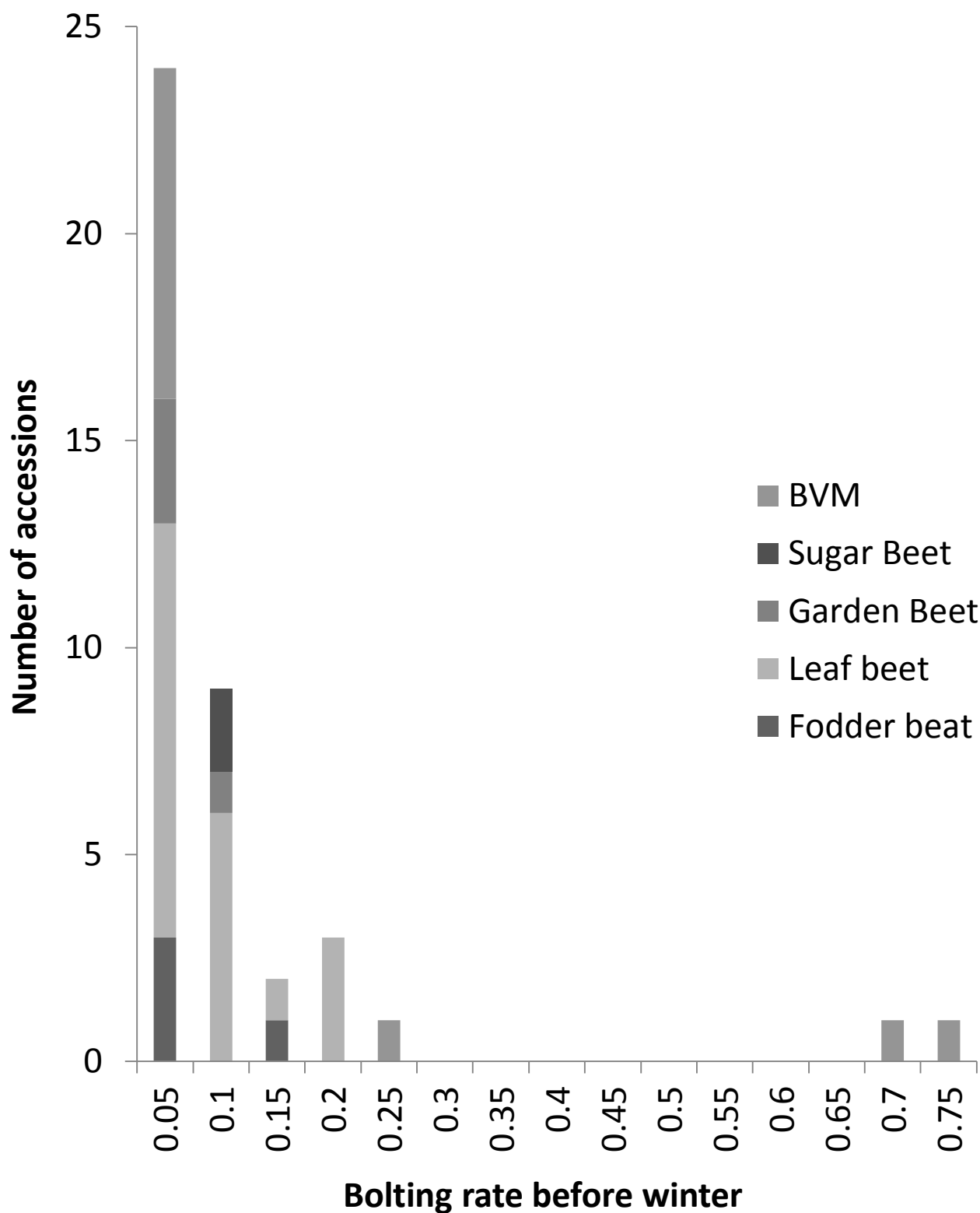


Figure 15: Distribution of bolting rate (BR) before winter among a subset of 41 *Beta vulgaris* of all accessions tested in four different environments. BVM = *Beta vulgaris* ssp. *maritima*.

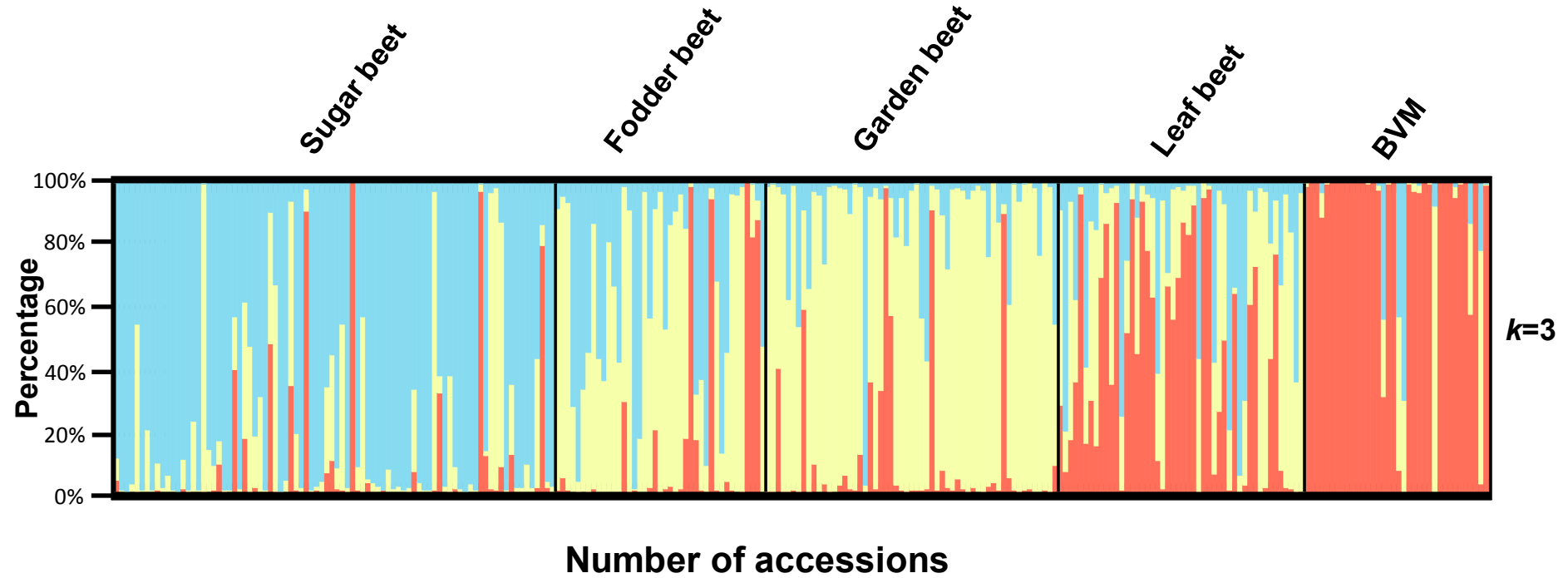


Figure 16: Population structure of 268 *B. vulgaris* accessions based on 40 AFLP markers under the assumption of $k = 3$ subpopulation. Each *B. vulgaris* accession is represented by one bar that is divided in up to k segments, each proportional to the inferred subpopulation. Accessions are grouped by their respective *B. vulgaris* group. BVM = *Beta vulgaris* ssp. *maritima*.

3.4.2 Amplification of candidate genes

For the three candidate genes *BvFLI*, *BvFTI* and *BTCI* 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 *BvFLI* [GenBank: DQ189214.1 and DQ189215.1] was taken from Reeves et al. (2007) while the genomic sequence of *BvFTI* [GenBank: HM448909.1] and *BTCI* [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 22). 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).

Table 22: The gene names, genomic sizes in kilo base pairs (kb), protein domains, sizes of amplicons in base pairs (bp) per gene, 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 *BvFLI* refers to the known portion of the sequence. A large part of intron 1 was not sequenced (Reeves et al. 2007) and is not considered here. Furthermore, the length of the investigated genomic sequence and the percentage of the open reading frame that 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
<i>BvFLI</i>	7.45 kb	MADS box/K box	FL1a: 977 bp FL1b: 632 bp	FL1a-fw tcggactttccctataagct FL1a-rv cacgtgaatcggtacagaca FL1b-fw gctgatagtctgtccctttgtc FL1b-rv tgactccaacaccacgatgca	1,609 bp (22%)	62%
<i>BvFTI</i>	6.47 kb	PBP	FT1a: 916 bp FT1b: 713 bp	FT1a-fw tggtagctgtatgaacagaagctg FT1a-rv catcaactccatattgggggtg FT1b-fw accatctatactgtcgtgacc FT1b-rv caatggggaagtgggtcacact	1,629 bp (14%)	93%
<i>BTCI</i>	9.68 kb	PRR	BTC1: 996 bp	BTC1-fw cagctgtaggatgttatcgtgctgag BTC1-rv agtagtgataaggacaagacattgc	996 bp (5%)	15%

For *BvFLI* we could amplify two regions (Figure 17). 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 part 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 *BvFLI* 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 *BvFTI* 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 17). Both regions together cover 92% of the ORF and were chosen because they contain parts of the promoter and the PEB domain (Table 22). In *BTC1* we amplified a 992 bp fragment ('BTC1') of the conserved response regulator receiver (REC) domain region (Figure 17), which covered 15% of the ORF (Table 22).

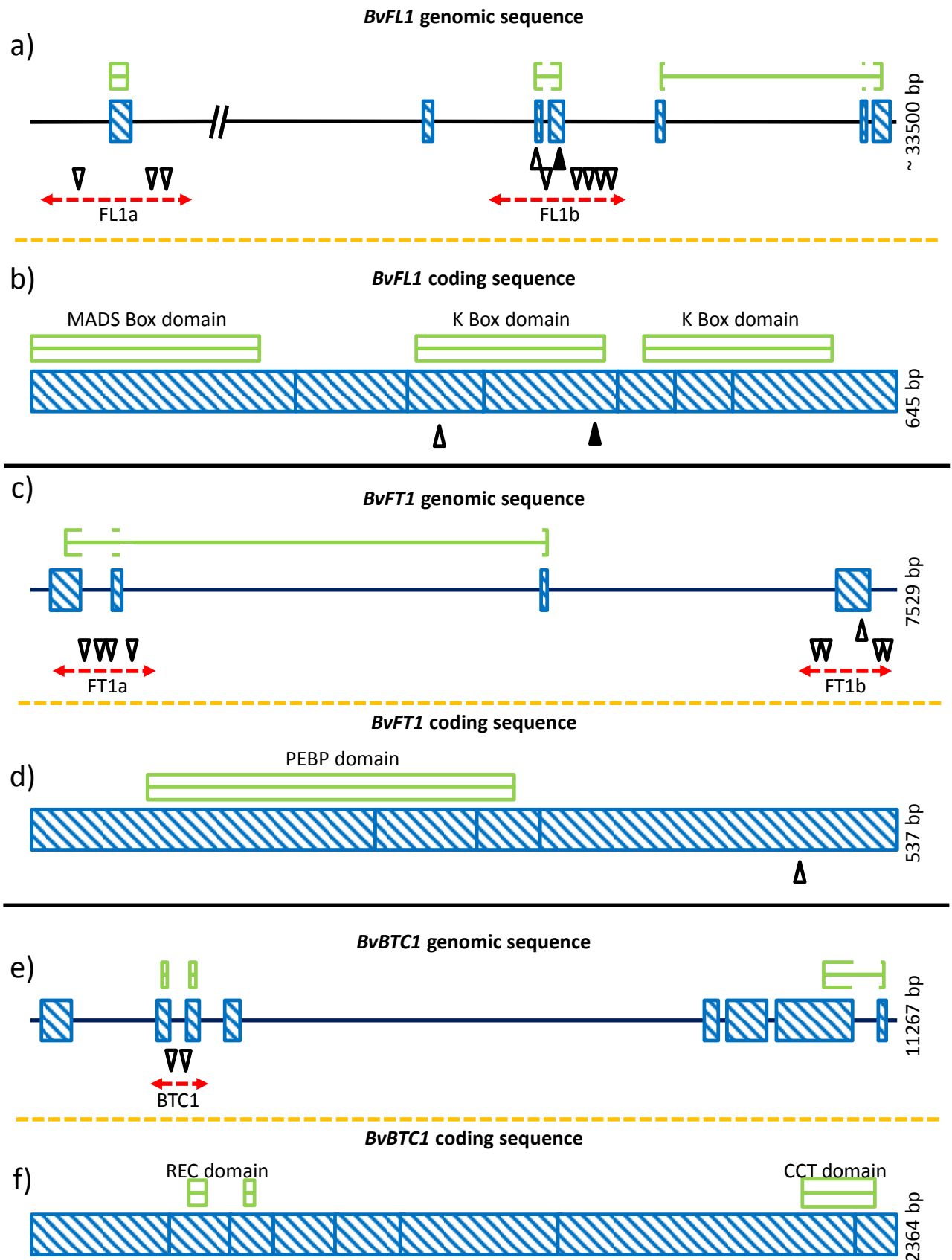


Figure 17: Flowering time genes *BvFL1*, *BvFT1* and *BTC1* and distribution of polymorphisms. Shown are graphical outputs from PARSESNP. a), c), e) Genomic sequences with intron/exon structure. b), d), f) Coding sequences. Red dotted arrowed lines indicate the amplicons investigated by EcoTILLING. Blue 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. Green boxes mark important protein domains.

3.4.3 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. Eighteen SNPs were located in introns, while the remaining three SNPs were located in exon 4 of *BvFT1* and in exon 3 and exon 4 of *BvFL1*, the latter SNP 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 *BTC1* (2.01 SNP/kb), whereas the highest SNP density occurred in FL1b (9.82 SNP/kb) (Supplementary Table 13). The SNP allocations and gene structures are shown in Figure 17. 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 14). 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 18).

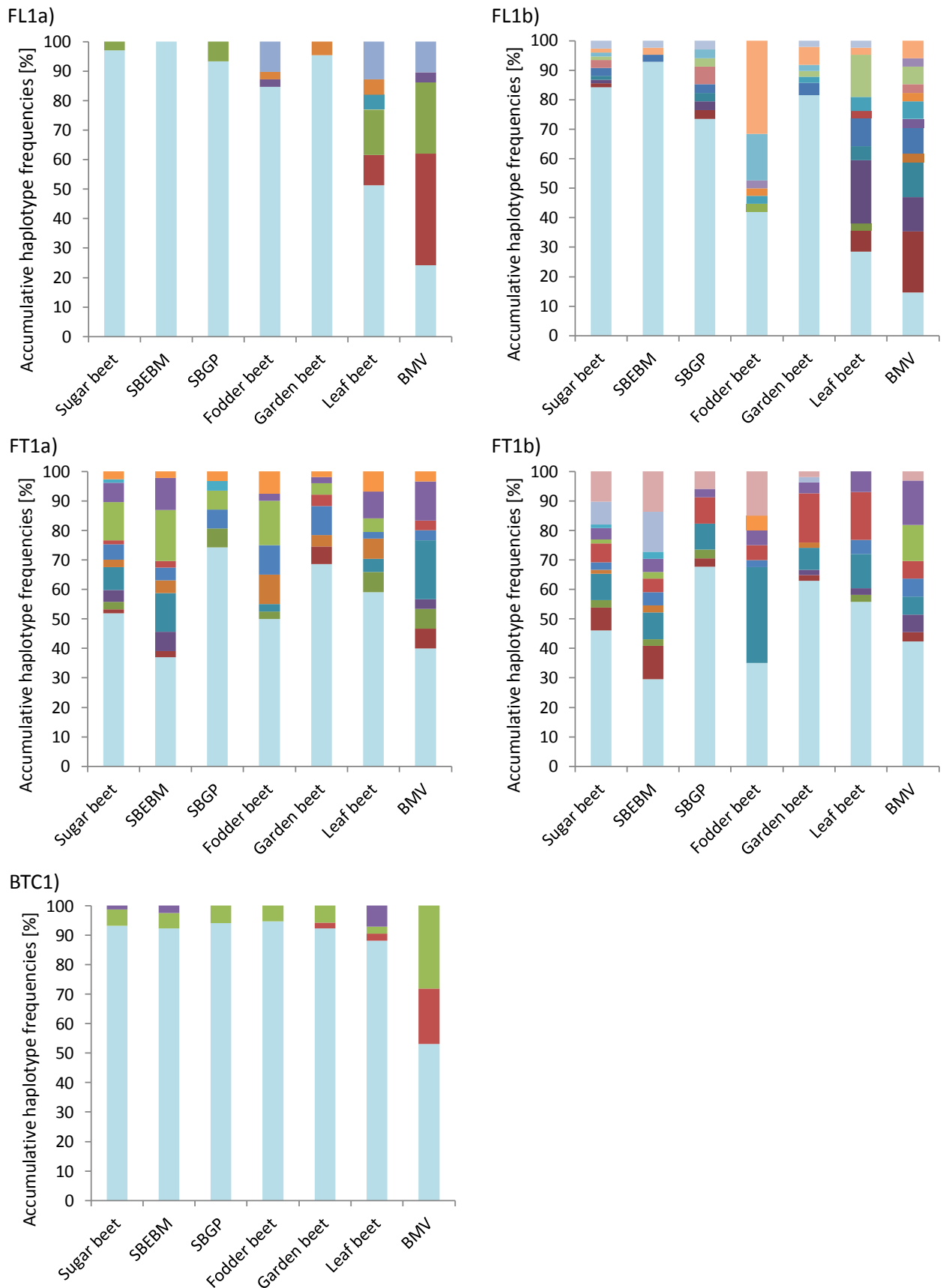


Figure 18: Haplotype diversity diagram. Shown is the haplotype distribution for the amplicons FL1a, FL1b, FT1a, FT1b and BTC1. The percentage of haplotypes for each amplicon is given for each *B. vulgaris* form. Haplotypes are colour coded. The reference haplotype H0 is always shown in light blue, while 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*.

The non-reference haplotype frequencies (NHF) ranged from 0.01 to 0.38 across the amplicons within the distinct *B. vulgaris* forms (Figure 18). 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 Figure 2. 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 (Supplementary Table 15).

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

Based on the Q matrix for $k = 3$, associations with bolting rate, survival rate, and survival rate with bolting rate as cofactor were each significant ($P \leq 0.05$) for the amplicon FL1a and for the amplicon FL1b (Table 23). Dunnet comparisons revealed that *B. vulgaris ssp. maritima* accessions with haplotype FL1a_H6, FL1b_H5 and FL1b_H10 had a significantly higher bolting rate of 55% ($P < 0.0001$), 75% ($P < 0.0001$) and 11% ($P < 0.05$), respectively, compared to 1% for FL1a_H0 and 2% for FL1b_H0 (Supplementary Table 15).

Table 23: Association analysis of bolting and survival rate with amplicons. Significant association between amplicons of *BvFL1* and bolting (BR) and survival rate (SR) of Beta accessions as revealed by association mapping with TASSEL v. 3.0, using the general linear model (GLM). A corrected p-value (Bonferroni multiple test correction) is given to account for the experiment-wise error rate.

Trait	Amplicon	F ^{d)}	P ^{e)}	R ² 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) Bonferroni corrected p value, f) R² is the fraction of the total variation explained by the amplicon. **($P < 0.001$) indicates the amplicon is highly significantly associated with trait

Furthermore, garden beet accessions with haplotype FL1b_H6 bolted before winter with a bolting rate of 6% ($P < 0.0001$) compared to 1% for accessions with the reference haplotype FL1b_H0 (Figure 19). Dunnet comparisons for survival rate revealed that *B. vulgaris ssp. maritima* accessions with haplotype FL1a_H6 had a significantly lower survival rate of 13% ($P = 0.015$) compared to 39% observed for accessions with the reference haplotype FL1a_H0 (Supplementary Table 16). By contrast, leaf beet accessions with the haplotype FL1b_H3 had a significantly higher survival rate of 37% ($P = 0.012$) compared to 19% of accessions with the reference haplotype FL1b_H0 (Figure 20). DNA sequences of significant haplotypes of *BvFL1* are shown in Supplementary Figure 3.

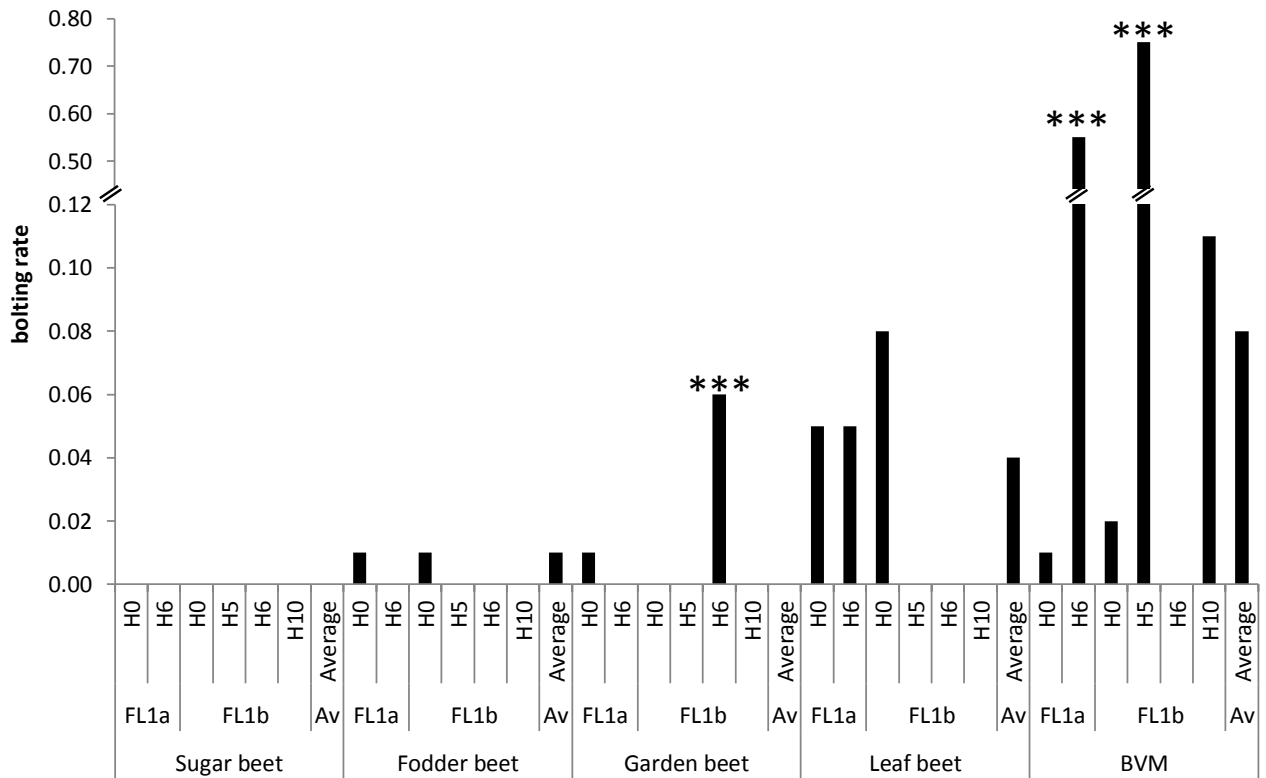


Figure 19: *BvFLI* haplotype effect on bolting rate before winter. Graphic representation of the effect of *BvFLI* haplotypes of the amplicons FL1a and FL1b on bolting rate before winter in five *B. vulgaris* forms. * or *** mark non-reference haplotypes with a significantly different bolting rate from reference haplotypes FL1a_H0 or FL1b_H0 ($P < 0.05$ or $P < 0.0001$, respectively); BVM = *B. vulgaris* ssp. *maritima*.

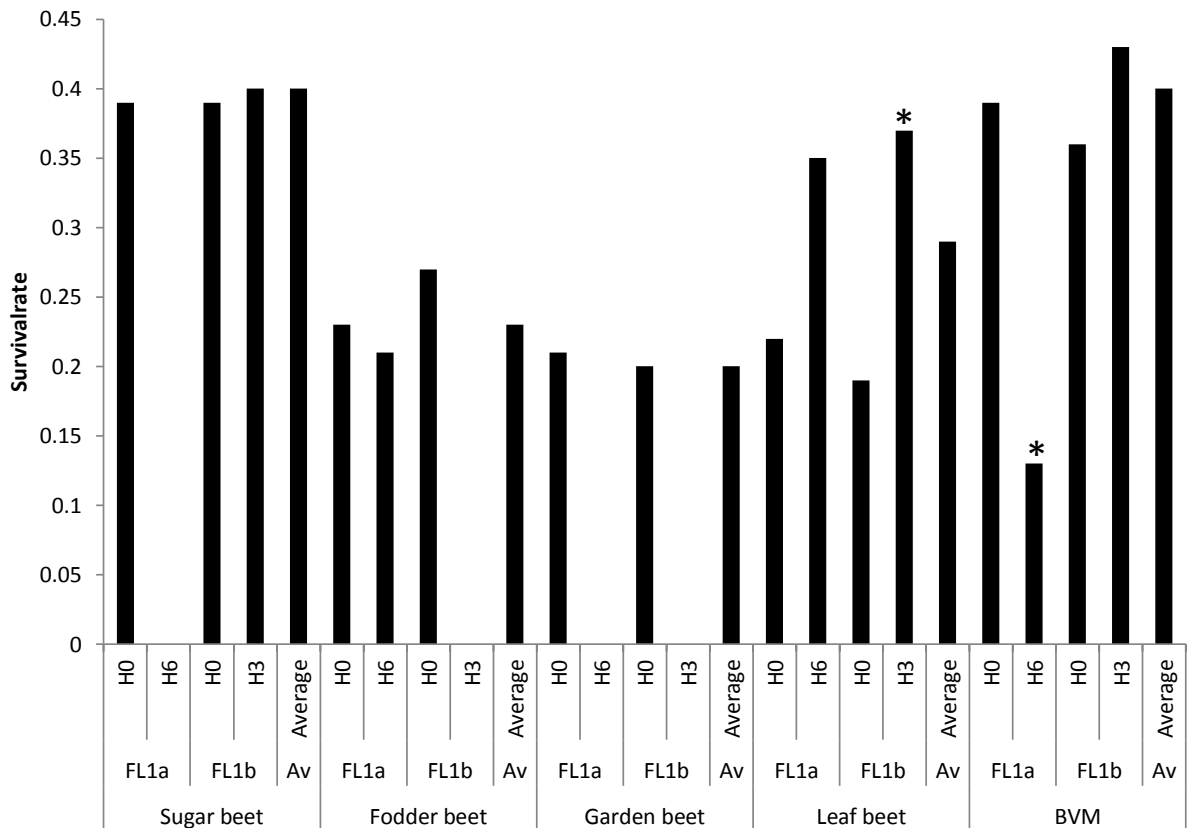


Figure 20: *BvFLI* haplotype effect on survival rate after winter. Graphic representation of the effect of *BvFLI* haplotypes of the amplicons FL1a and FL1b on survival rate after winter in five *B. vulgaris* forms. * marks non-reference haplotypes with a significantly different survival rate from the reference haplotypes FL1a_H0 or FL1b_H0 ($\alpha = 0.05$); BVM = *B. vulgaris* ssp. *maritima*.

3.5 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). In addition, 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, this increases the cost efficiency of EcoTILLING. We were further 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. However, it has to be considered 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% which is similar to rates reported in human (Till et al., 2006b) and banana (Till et al., 2010) EcoTILLING.

The population structure analysis by AFLP markers indicates that the *B. vulgaris* accessions can be grouped into three groups ($k = 3$). Under consideration of the phenotypic classification of the panel (gene bank information as well as our field observations on plant habitus), the three groups can be referred to as a sugar beet group, a fodder beet and garden beet group, and a group comprising leaf beets and wild sea beets. This reflects 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 (see Figure 16). 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% (see Figure 16). 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 (Biancardi et al., 2002; Lewellen, 1992; Savitsky). 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 occurrences of non-reference nucleotides in the heterozygous or homozygous state. Nevertheless, gene diversity in our study is lower compared to Li et al. (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. This is especially the case for *BvFL1* where we estimated Ht values of 0.03 and 0.12 for FL1a and FL1b, respectively, and 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* (see also Supplementary Figure 2). 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 *BvFL1*, we were able to detect an association with bolting. Four haplotypes of this gene (FL1a_H6, FL1b_H5, FL1b_H6 and FL1b_H10) had a significant effect on bolting rate before winter in *B. vulgaris ssp. maritima* 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 *B. vulgaris* forms, which in part may be due to the absence of the divergent haplotypes that affect bolting rate in *B. vulgaris ssp. maritima* 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 *BTC1* 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 22), 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 (see Supplementary Table 16). Similar to bolting rate, the absence of haplotype effects on survival rate in some *B. vulgaris* forms might also be due to the polygenic inheritance of survival rate.

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* (Choi et al., 2011; Sheldon et al., 2002). Also, Heo and Sung (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.

3.6 Acknowledgements

The authors thank Michaela Jahn for her excellent technical assistance, and Monika Bruisch, and Erwin Danklefsen for substantial help and support in field and greenhouse work. Financial support of the project was provided by the Federal Ministry of Education and Research (BMBF) within the GABI program (FKZ: 0315052C and 0315058A). We thank Dr. Axel Schechert from Strube Research GmbH & Co.KG for providing seed material, and Gretel Schulze-Buxloh and Sebastian Vogt for providing the full-length genomic sequence of *BvFTI*. We are indebted to Sonja Hollmer from the Zoological Institute, Department of Structural Biology for preparing the crude celery extract, and Dr. Mario Hassler for support in statistical analysis. We also acknowledge the helpful comments of four unknown reviewers.

3.7 References

- Abegg, F., 1936: A genetic factor for the annual habit in beets and linkage relationships. *Journal Agricultural Research* **53**, 493-511.
- Abou-Elwafa, S.F., B. Büttner, T.Y.P. Chia, G. Schulze-Buxloh, U. Hohmann, E. Mutasa-Göttgens, C. Jung, and A.E. Müller, 2012: Conservation and divergence of autonomous pathway genes in the flowering regulatory network of *Beta vulgaris*. *Journal of Experimental Botany* **62**, 3359-3374.
- Barkley, N.A., M. Wang, A. Gillaspie, R. Dean, G. Pederson, and T. Jenkins, 2008: Discovering and verifying DNA polymorphisms in a mung bean [*V. radiata* (L.) R. Wilczek] collection by EcoTILLING and sequencing. *BMC Research Notes* **1**, 28.
- Biancardi, E., R.T. Lewellen, M. De Biaggi, A. Erichsen, and P. Stevanato, 2002: The origin of rhizomania resistance in sugar beet. *Euphytica* **127**, 383-397.
- Biancardi, E., J.M. McGrath, L. Panella, R.T. Lewellen, and P. Stevanato, 2010: Sugar beet, In: J. E. Bradshaw, (ed.) *Handbook of plant breeding: Root and tuber crops*, 173-219. Springer, New York.
- Bieniawska, Z., C. Espinoza, A. Schlereth, R. Sulpice, D.K. Hinch, and M.A. Hannah, 2008: Disruption of the *Arabidopsis* circadian clock is responsible for extensive variation in the cold-responsive transcriptome. *Plant Physiology* **147**, 263-279.
- Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler, 2007: TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics* **23**, 2633-2635.
- Büttner, B., S.F. Abou-Elwafa, W. Zhang, C. Jung, and A.E. Müller, 2010: A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*. *Theoretical and Applied Genetics* **121**, 1117-1131.
- Chia, T.Y.P., A. Muller, C. Jung, and E. Mutasa-Gottgens, 2008: Sugar beet contains a large CONSTANS-LIKE gene family including a CO homologue that is independent of the early-bolting (*B*) gene locus. *Journal of Experimental Botany* **59**, 2735-2748.
- Choi, K., J. Kim, H.-J. Hwang, S.Y. Kim, C. Park, S.Y. Kim, and I. Lee, 2011: The FRIGIDA complex activates transcription of *FLC*, a strong flowering repressor in *Arabidopsis*, by recruiting chromatin modification factors. *Plant Cell* **23**, 289-303.

Comai, L., K. Young, B.J. Till, S.H. Reynolds, E.A. Greene, C.A. Codomo, L.C. Enns, J.E. Johnson, C. Burtner, A.R. Odden, and S. Henikoff, 2004: Efficient discovery of DNA polymorphisms in natural populations by EcoTILLING. *Plant Journal* **37**, 778-786.

Deng, W., H. Ying, C.A. Helliwell, J.M. Taylor, W.J. Peacock, and E.S. Dennis, 2011: FLOWERING LOCUS C (FLC) regulates development pathways throughout the life cycle of *Arabidopsis*. *Proceedings of the National Academy of Sciences* **108**, 6680-6685.

Earl, D., and B. von Holdt, 2012: STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* **4**, 359-361.

Elias, R., B.J. Till, C. Mba, and B. Al-Safadi, 2009: Optimizing TILLING and EcoTILLING techniques for potato (*Solanum tuberosum* L). *BMC Research Notes* **2**, 1-5.

Eriksson, M.E., and A.A.R. Webb, 2011: Plant cell responses to cold are all about timing. *Current Opinion in Plant Biology* **14**, 731-737.

Evanno, G., S. Regnaut, and J. Goudet, 2005: Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology* **14**, 2611-2620.

Falush, D., M. Stephens, and J.K. Pritchard, 2007: Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes* **7**, 574-578.

FAO, 2012: FAOSTAT - Food and agricultural commodities production world wide 2010, Vol. 2012.

Fenart, S., J.F. Arnaud, I. De Cauwer, and J. Cuguen, 2008: Nuclear and cytoplasmic genetic diversity in weed beet and sugar beet accessions compared to wild relatives: new insights into the genetic relationships within the *Beta vulgaris* complex species. *Theoretical and Applied Genetics* **116**, 1063-1077.

Fowler, D.B., A.E. Limin, A.J. Robertson, and L.V. Gusta, 1993: Breeding for low-temperature tolerance in field crops *International Crop Science I.*, 357-362. *Crops Science Society of America*, Ames, Iowa.

Franklin, K.A., and G.C. Whitelam, 2007: Light-quality regulation of freezing tolerance in *Arabidopsis thaliana*. *Nature Genetics* **39**, 1410-1413.

Galeano, C.H., M. Gomez, L.M. Rodriguez, and M.W. Blair, 2009: CEL I nuclease digestion for SNP discovery and marker development in common bean (*Phaseolus vulgaris* L.). *Crop Science* **49**, 381-394.

Gilchrist, E.J., G.W. Haughn, C.C. Ying, S.P. Otto, J. Zhuang, D. Cheung, B. Hamberger, F. Aboutorabi, T. Kalynyak, L. Johnson, J. Bohlmann, B. Ellis, C.J. Douglas, and Q.C.B. Bronk, 2006: Use of Ecotilling as an efficient SNP discovery tool to survey genetic variation in wild populations of *Populus trichocarpa*. *Molecular Ecology* **15**, 1367-1378.

Gilmour, S.J., S.G. Fowler, and M.F. Thomashow, 2004: *Arabidopsis* transcriptional activators *CBF1*, *CBF2*, and *CBF3* have matching functional activities. *Plant Molecular Biology* **54**, 767-781.

Greene, E.A., C.A. Codomo, N.E. Taylor, J.G. Henikoff, B.J. Till, S.H. Reynolds, L.C. Enns, C. Burtner, J.E. Johnson, A.R. Odden, L. Comai, and S. Henikoff, 2003: Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in *Arabidopsis*. *Genetics* **164**, 731-740.

Heo, J.B., and S. Sung, 2011: Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. *Science* **331**, 76-9.

Hoffmann, C.M., and S. Kluge-Severin, 2010: Light absorption and radiation use efficiency of autumn and spring sown sugar beets. *Field Crops Research* **119**, 238-244.

Ibiza, V., J. Canizares, and F. Nuez, 2010: EcoTILLING in *Capsicum* species: searching for new virus resistances. *BMC Genomics* **11**, 1-15.

Ietswaart, R., Z. Wu, and C. Dean, 2012: Flowering time control: another window to the connection between antisense RNA and chromatin. *Trends Genet* **28**, 445-53.

Johanson, U., J. West, C. Lister, S.D. Michaels, R.M. Amasino, and C. Dean, 2000: Molecular analysis of *FRIGIDA*, a major determinant of natural variation in *Arabidopsis* flowering time. *Science* **290**, 344-347.

Jung, C., and A.E. Müller, 2009: Flowering time control and applications in plant breeding. *Trends in Plant Science* **14**, 563-573.

Jung, C., K. Pillen, L. Frese, and A. Melchinger, 1993: Phylogenetic relationships between cultivated and wild species of the genus *Beta* revealed by DNA "fingerprinting". *Theoretical and Applied Genetics* **86**, 449-457.

Kadaru, S., A. Yadav, R. Fjellstrom, and J. Oard, 2006: Alternative ecotilling protocol for rapid, cost-effective single-nucleotide polymorphism discovery and genotyping in rice (*Oryza sativa* L.). *Plant Molecular Biology Reporter* **24**, 3-22.

Kadereit, J.W., S. Hohmann, and J.W. Kadereit, 2006: A synopsis of *Chenopodiaceae* subfam. *Betoideae* and notes on the taxonomy of *Beta*. *Wildenowia* **36**, 9-19.

Kalendar, R., D. Lee, and A.H. Schulman, 2009: FastPCR software for PCR primer and probe design and repeat search. *Genes, Genomes and Genomics* **3**, 1-14.

Kirchhoff, M., A. Svirshchevskaya, C. Hoffmann, A. Schechert, C. Jung, and F. Kopsisch-Obuch, 2012: High degree of genetic variation of winter hardiness in a panel of *Beta vulgaris* L. *Crop Science* **52**, 179-188.

Kobayashi, Y., H. Kaya, K. Goto, M. Iwabuchi, and T. Araki, 1999: A pair of related genes with antagonistic roles in mediating flowering signals. *Science* **286**, 1960-1962.

Korves, T.M., K.J. Schmid, A.L. Caicedo, C. Mays, J.R. Stinchcombe, M.D. Purugganan, and J. Schmitt, 2007: Fitness effects associated with the major flowering time gene *FRIGIDA* in *Arabidopsis thaliana* in the field. *American Naturalist* **169**, E141-E157.

Lewellen, R.T., 1992: Use of plant introductions to improve populations and hybrids of sugar beet, In: H. L. Shands, (ed.) *Use of Plant Introductions in Cultivar Development*, 117-135, Vol. 2. *Crop Sci. Soc. Am.*, Madison, WI.

- Li, J., A.-K. Luhmann, K. Weißleder, and B. Stich, 2011: Genome-wide distribution of genetic diversity and linkage disequilibrium in elite sugar beet germplasm. *BMC Genomics* **12**.
- McCallum, C.M., L. Comai, E.A. Greene, and S. Henikoff, 2000: Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant Physiology* **123**, 439-442.
- McGrath, J.M., C.A. Derrico, and Y. Yu, 1999: Genetic diversity in selected, historical US sugarbeet germplasm and *Beta vulgaris* ssp. *maritima*. *Theoretical and Applied Genetics* **98**, 968-976.
- McGrath, J.M., M. Saccomani, P. Stevanato, and E. Biancardi, 2007: Beet, In: C. Kole, (ed.), 191-202. Springer-Verlag, Berlin Heidelberg.
- Mejlhede, N., Z. Kyjovska, G. Backes, K. Burhenne, S.K. Rasmussen, and A. Jahoor, 2006: EcoTILLING for the identification of allelic variation in the powdery mildew resistance genes *mlo* and *Mla* of barley. *Plant Breeding* **125**, 461-467.
- Michaels, S.D., and R.M. Amasino, 1999: *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949-956.
- Michaels, S.D., Y.H. He, K.C. Scortecci, and R.M. Amasino, 2003: Attenuation of *FLOWERING LOCUS C* activity as a mechanism for the evolution of summer-annual flowering behavior in *Arabidopsis*. *Proceedings of the National Academy of Sciences* **100**, 10102-10107.
- Negrao, S., C. Almadanim, I. Pires, K. McNally, and M. Oliveira, 2011: Use of EcoTILLING to identify natural allelic variants of rice candidate genes involved in salinity tolerance. *Plant Genetic Resources* **9**, 300-304.
- Ng, P.C., and S. Henikoff, 2003: SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Research* **31**, 3812-3814.
- Nieto, C., F. Piron, M. Dalmais, C.F. Marco, E. Moriones, M.L. Gomez-Guillamon, V. Truniger, P. Gomez, J. Garcia-Mas, M.A. Aranda, and A. Bendahmane, 2007: EcoTILLING for the identification of allelic variants of melon *eIF4E*, a factor that controls virus susceptibility. *BMC Plant Biology* **7**, 1-9.
- Ota, T., 1993: *DISPAN: Genetic distance and phylogenetic analysis.*, 1.0 ed. Institute of Molecular Evolutionary Genetics, Pennsylvania State University, University Park, PA, USA.
- Owen, F.V., 1945: Cytoplasmically inherited male-sterility in sugar beets. *Journal of Agricultural Research* **71**, 423-440.
- Pin, P.A., R. Benlloch, D. Bonnet, E. Wremmerth-Weich, T. Kraft, J.J. Gielen, and O. Nilsson, 2010: An antagonistic pair of *FT* homologs mediates the control of flowering time in sugar beet. *Science* **330**, 1397-400.
- Pin, P.A., W. Zhang, S.H. Vogt, N. Dally, B. Büttner, G. Schulze-Buxloh, N.S. Jelly, T.Y.P. Chia, E.S. Mutasa-Göttgens, J.C. Dohm, H. Himmelbauer, B. Weisshaar, J. Kraus, J.J.L. Gielen, M. Lommel, G. Weyens, B. Wahl, A. Schechert, O. Nilsson, C. Jung, T. Kraft, and A.E. Müller, 2012: The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. *Current Biology* **22**, 1095-1101.

Pritchard, J.K., M. Stephens, and P. Donnelly, 2000: Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.

R Development Core Team, 2011: R: A language and environment for statistical computing, 2.12 ed. R Foundation for Statistical Computing, Vienna, Austria.

Ramos, M., J. Huntley, S. Maleki, and P. Ozias-Akins, 2009: Identification and characterization of a hypoallergenic ortholog of *Ara h 2.01*. *Plant Molecular Biology* **69**, 325-335.

Reeves, P.A., Y.H. He, R.J. Schmitz, R.M. Amasino, L.W. Panella, and C.M. Richards, 2007: Evolutionary conservation of the FLOWERING LOCUS C-mediated vernalization response: Evidence from the sugar beet (*Beta vulgaris*). *Genetics* **176**, 295-307.

Rigola, D., J. van Oeveren, A. Janssen, A. Bonne, H. Schneiders, H.J. van der Poel, N.J. van Orsouw, R.C. Hogers, M.T. de Both, and M.J. van Eijk, 2009: High-throughput detection of induced mutations and natural variation using KeyPoint technology. *PLoS One* **4**, e4761-.

Savitsky, V.F., 1952: A genetic study of monogerm and multigerm characters in beets Proceedings American Society of Sugar Beet Technologists, 824. The American Society of Sugar Beet Technologists, Salt Lake City, Utah, USA.

Schmitz, R.J., and R.M. Amasino, 2007: Vernalization: A model for investigating epigenetics and eukaryotic gene regulation in plants. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1769**, 269-275.

Seo, E., H. Lee, J. Jeon, H. Park, J. Kim, Y.S. Noh, and I. Lee, 2009: Crosstalk between cold response and flowering in *Arabidopsis* is mediated through the flowering-time gene *SOC1* and its upstream negative regulator *FLC*. *Plant Cell* **21**, 3185-3197.

Sheldon, C.C., A.B. Conn, E.S. Dennis, and W.J. Peacock, 2002: Different regulatory regions are required for the vernalization-induced repression of FLOWERING LOCUS C and for the epigenetic maintenance of repression. *Plant Cell* **14**, 2527-37.

Sheldon, C.C., J.E. Burn, P.P. Perez, J. Metzger, J.A. Edwards, W.J. Peacock, and E.S. Dennis, 1999: The FLF MADS box gene: A repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. *Plant Cell* **11**, 445-458.

Taylor, N.E., and E.A. Greene, 2003: PARSESNP: A tool for the analysis of nucleotide polymorphisms. *Nucleic Acids Research* **31**, 3808-3811.

Till, B.J., T. Zerr, L. Comai, and S. Henikoff, 2006a: A protocol for TILLING and EcoTILLING in plants and animals. *Nature Protocols* **1**, 2465-2477.

Till, B.J., T. Zerr, E. Bowers, E.A. Greene, L. Comai, and S. Henikoff, 2006b: High-throughput discovery of rare human nucleotide polymorphisms by EcoTILLING. *Nucleic Acids Research* **34**, e9901-12.

Till, B.J., J. Jankowicz-Cieslak, L. Sigi, O. Huynh, H. Utsushi, R. Swennen, R. Terauchi, and C. Mba, 2010: Discovery of nucleotide polymorphisms in the *Musa* gene pool by EcoTILLING. *Theoretical and Applied Genetics* **121**, 1381-1389.

Till, B.J., T. Colbert, C. Codomo, L. Enns, J. Johnson, S.H. Reynolds, J.G. Henikoff, E.A. Greene, M.N. Steine, L. Comai, and S. Henikoff, 2006c: High-throughput TILLING for *Arabidopsis*. *Methods in Molecular Biology* **323**, 127-135.

Trap-Gentil, M.-V., C. Hébrard, C. Lafon-Placette, A. Delaunay, D. Hagège, C. Joseph, F. Brignolas, M. Lefebvre, S. Barnes, and S. Maury, 2011: Time course and amplitude of DNA methylation in the shoot apical meristem are critical points for bolting induction in sugar beet and bolting tolerance between genotypes. *Journal of Experimental Botany* **62**, 2585-2597.

Turck, F., F. Fornara, and G. Coupland, 2008: Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. *Annual Review of Plant Biology* **59**, 573-594.

Van Dijk, H., and P. Boudry, 1991: Genetic variation for life histories in *Beta maritima*. *International Board for Plant Genetic Resources* **7**, 44-55.

Van Dijk, H., P. Boudry, H. McCombre, and P. Vernet, 1997: Flowering time in wild beet (*Beta vulgaris* ssp. *maritima*) along a latitudinal cline. *Acta Oecologica* **18**, 47-60.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T.v.d. Lee, M. Hornes, A. Friters, J. Pot, J. Paleman, M. Kuiper, and M. Zabeau, 1995: AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407-4414.

Wang, N., L. Shi, F. Tian, H. Ning, X. Wu, Y. Long, and J. Meng, 2010: Assessment of *FAEI* polymorphisms in three *Brassica* species using EcoTILLING and their association with differences in seed erucic acid contents. *BMC Plant Biology* **10**, 1-11.

Wang, R., S. Farrona, C. Vincent, A. Joecker, H. Schoof, F. Turck, C. Alonso-Blanco, G. Coupland, and M.C. Albani, 2009: *PEP1* regulates perennial flowering in *Arabis alpina*. *Nature* **459**, 423-427.

Weigel, D., 2012: Natural variation in *Arabidopsis*: From molecular genetics to ecological genomics. *Plant Physiology* **158**, 2-22.

Weil, C.F., and R.A. Monde, 2007: Getting the Point - Mutations in Maize. *Crop Science* **47**, S60-S67.

Zerr, T., and S. Henikoff, 2005: Automated band mapping in electrophoretic gel images using background information. *Nucleic Acids Research* **33**, 2806-2812.

4 Closing Discussion

The main aim of this study was to establish a sugar beet TILLING platform for routine screens in candidate genes within the bolting and flowering pathway as well as genes involved in virus resistance and storage root formation. Although this aim was not reached, a number of important goals have been achieved. A sugar beet doubled haploid EMS population (SynDH1) of 3,418 M₂ plants has been developed. Further, a protocol for the setup of 2-dimensional 8x pooling of sugar beet DNA and a protocol for the extraction and activity testing of an alternative endonuclease from crude celery extract (CCE). A primer pre-test called “crash-test” for the suitability of IRDye tagged PCR primer for TILLING screens, and furthermore a TILLING protocol based on endonuclease digestion and PAGE on a LI-COR[®] 4300 has been adapted for sugar beet.

In the second part of the work, an EcoTILLING protocol has been successfully adapted for the identification of natural sequence variation in a diversity panel of *Beta vulgaris* including the cultivated forms sugar beet (var. *altissima*), garden beet (var. *conditiva*), leaf beet (var. *flavescens*), fodder beet (var. *crassa*) and wild beet (*B. vulgaris* ssp. *maritima* = BVM) (chapter 3). EcoTILLING is a well-suited method for the identification of sequence variants and in this respect can be regarded as a valuable tool for the development of a winter beet, which is a long-term goal in sugar beet breeding. Together with the study by Kirchhoff et al. (2012), showing sugar beets to be the most winter hard among the cultivated *Beta vulgaris* types while the wild beet displayed the largest genetic variation, we came closer to the identification of genes regulating winter hardiness in *B. vulgaris*. The results presented in chapter 3 clearly give evidence that nucleotide polymorphism in *BvFL1* are associated with bolting before winter in garden beets and wild beets, as well as winter hardiness in leaf beets and wild beets.

4.1 Reasons for low mutant detection rates in sugar beet

The goal in mutagenesis based techniques is to cause maximal genomic variation with a minimum decrease in viability (Kurowska et al., 2011). The efficiency of a TILLING platform or a TILLING project relies on the mutation frequency (MF).

$$MF = \frac{(total\ number\ of\ bp\ screened\ \times\ population\ size)}{total\ number\ of\ mutations}$$

Looking at the formula above, MF is a direct measure of mutagen efficiency in the given species/cultivar. The overall mutation frequency of 1 mutation in 1,1062 kb in the two TILLING populations (1/1,1108 kb for 930190 and 1/1,016 kb for SynDH1) is comparably low and only three other TILLING projects came up with an equally low MF using LI-COR[®] machines and EMS as mutagenic agent. Knoll et al. (2011) screened 3 genes responsible to elicit hypersensitivity in a population of 3,420 peanut M₂ plants and estimated a MF of 1 mutation every 967 kb. Wu et al. (2005) screened 10 genes in a population of 2,000 rice M₂ plants and estimated a MF of 1 mutation every 1,000 kb. Furthermore, Le Signor et al. (2009) produced two *Medicago truncatula* EMS TILLING populations and in one population of 4,350 M₂ plants they report a MF of 1/1,290 kb by screening 10 genes. These results lead to the assumption that the concentration of mutagen was probably not high enough to induce a desired amount of mutations. Till et al. (2007) stated, that efficient TILLING requires a population with a mutation frequency of $\geq 1/500$ kb to ensure at least one mutation per gel run on the LI-COR[®]

Before producing a large scale TILLING population, most researchers start by establishing a LD50 (lethal dose of 50%) analysis for their desired species/cultivar with the mutagen of choice. Here, the concentration of mutagen is plotted against seed viability/germination rate. A good rate is in the range of 30% to 70 % (Wang et al., 2010b). For the investigated sugar beet cultivars, I have calculated a LD50 value accordingly, which resulted in a survival rate of 50% and treated as a result the seeds with 1% EMS. Therefore, the obtained low mutation frequency in this study should not be due to a low mutagen dosage. Wu et al. (2005) report from a mutagenic study in rice, where lower survival rates were associated with higher mutation frequencies. This leads to the assumption that a survival rate of 30 % after mutagen treatment would probably have led to a higher MF in the beet population. However, such a set up would have meant treatment of at least 40,000 instead of 20,000 seeds, to ensure an appropriate number of plants for further analysis (from 20,000 seeds I was only able to obtain 11,571 viable M₁ plants). Doubling the number of plants would have meant also a doubling of the space requirement, or a split of the treatment over consecutive years. From other TILLING projects, it is known that a high number of M₂ plants and a high mutation frequency do not guarantee to find nonsense mutations, as TILLING results from barley shows. Talamé et al. (2008) screened a population of 4,906 M₂ plants and yielded an average MF of 1 mutation in 376 kb. In total, they found 22 mutations, 3 of which were intronic and 19 exonic (18.2 % silent, 68.2 % missense, no nonsense mutation).

Looking at amplicon specific mutation frequencies, another picture emerges. In two cases (*BvLHP1* and *BvTON1*), I calculated the MF to be below 1/400 kb and in six other cases (*BTCl*, *BvFL1*, *BvFT1*, and *BvFT2*) the MF ranged between 1/466 kb and 1/570 kb. These results are comparable to amplicon specific MFs in other crop species, e.g. melon (Dahmani-Mardas et al., 2010) with a range from 1/327 kb to 1/1,415 kb and an overall MF of 1/573 kb. Another example is described for barley (Talamé et al., 2008). Here the amplicon specific MF ranged between 1/229 and 1/703 kb. Therefore, the amplicon specific MFs in my work are well within the range of other TILLING projects. Taking these results together, it can be concluded, that overall MF alone may not be a sufficient value for measuring the quality of a TILLING project.

Many projects also give the average number of mutations per plant. Since this value is estimated from the overall mutation frequency as well as the genome size and the G/C content in the coding region it is likewise not considered to be satisfactory for comparison of the results from different TILLING projects. This is illustrated by comparing my results to the results of *Arabidopsis*. I estimated the average number of mutations per beet plant to be 375. Till et al. (2003) estimated the average number of mutations per *Arabidopsis* plant to be 412. Regarding the overall mutation frequency, I obtained for sugar beet a MF of 1/1,062 kb whereas for *Arabidopsis* 1/170 kb was calculated. Since the genome size differs by 1/5 and the G/C content by 1.2 % these results are not really comparable. Thus, it is only legitimate to compare results from within the same species, which can be shown by comparing the results from TILLING experiments within *Arabidopsis*, barley, rice and wheat (Table 24). There is a clear correlation of MF with average number of mutations per plant.

Table 24: Overview of selected TILLING projects and their respective results. The species, the respective genome size in mega base pairs (Mb) as well as the ploidy of the species is shown. Further the mutagen and its concentration are given. The number of investigated M₂ plants and the identified number of mutations per plants as well as the mutation frequency is given.

Species (botanical name)	Genome size	Ploidy level	Mutagen, concentration	Number of M ₂ plants investigated	Number of mutations/plant	Mutation frequency	Reference
Arabidopsis (<i>Arabidopsis thaliana</i>)	157 Mb	2x	20 – 40 mM EMS	3,712	787	1/89 kb	Martin et al., 2009 Till et al., 2003 Greene et al., 2003
				6,912	412	1/170 kb	
				3,072	233	1/300 kb	
Sugar beet (<i>Beta vulgaris</i>)	758 Mb	2x	77/88 mM EMS	6,106	375	1/1,062 kb	This study
Barley (<i>Hordeum vulgare</i>)	10,856 Mb	2x	10mM MNU	4,906	16,522	1/374 kb	Talamè et al., 2008 Kurowska et al., 2011
			n.a.	1,753	1,874	1/3,297 kb	
Rice (<i>Oryza sativa</i>)	978 Mb	2x	1 mM MNU	767	4,003	1/135 kb	Suzuki et al., 2008 Sato et al., 2006
			500 Gy gamma rays	2,130	87	1/6,190 kb	
Wheat (<i>Triticum aestivum</i>)	3,3897 Mb	6x	48/88 mM EMS	2,348	97,822	1/23 kb	Dong et al., 2009b Sestili et al., 2010
			48/73 mM EMS	1,700	825,613	1/194 kb	

The main goal in TILLING is to identify mutations that alter the function of a desired gene, by (1) interfering with the correct splicing of the messenger RNA, or (2) cause a premature start or a premature stop. An alteration of gene function will most likely be introduced by a missense mutation. In this case, the point mutation introduced by the mutagen, in the case of the sugar beet TILLING project by EMS, will change the nucleotide triplet so that it will translate for a different amino acid. This amino acid substitution can then alter the function of the protein. The substitution can have different effects, depending on where the mutation took place and which amino acid is substituted. Missense mutations are favored when the protein should still be functional, but acts in a different way. A mutation at a splice site can change the splice acceptor or donor site, which then will probably result in a failure to remove the intron. Through this failure to remove the intron, the intron will be likely translated into amino acid code as well. Depending on the DNA bases in the intron, two cases are possible. (1) The open reading frame will be extended by the DNA base information of the intron (read-through), which can alter protein function, but could also have no effect. (2) A stop triplet can be introduced, resulting in a stop of protein synthesis before the actually translation stop point. Mutations that change the amino acid triplet to a stop codon are called nonsense mutations. These mutations are favored when the investigated candidate gene should be knocked out completely, resulting in the absence of protein function. If the protein is essential for a desired trait, e.g. flowering, the plant carrying this mutation will most likely show a different phenotype compared to non-mutated plants.

There is one other result to be discussed regarding the success of TILLING projects. When comparing the results from different TILLING projects (Table 19 (chapter 2)), it is noticeable that although in some projects a high MF was achieved, no nonsense mutations were identified, which introduces a premature stop codon. This is the case in the oat TILLING project (Chawade et al., 2010), where screening of two genes in 2,600 M₂ plants produced a MF of 1/33 kb without detection of any

nonsense mutation. The same accounts for population A of the soybean TILLING project (Cooper et al., 2008). Here screening of five genes in 529 M₂ plants resulted in a MF of 1/140 kb, but no nonsense mutation could be detected. The opposite has also been reported. For TILLING in peanut, Knoll et al. (2011) report two nonsense mutations leading to a disruption of the start codon and another mutation leading to a premature stop codon, which is noticeable because of the observed low MF (1/967 kb) compared to other studies. These results lead to the assumption that the MF is not the only factor determining a successful TILLING project.

The question arises why there is such a discrepancy between MF and nonsense mutations, as the expectation would be to find more nonsense mutations the higher the MF. The probability of obtaining nonsense mutations leading to a premature stop codon or disruption of a start codon are depending on the number of triplets coding for tryptophane (TGG), glutamine (CAA, CAG), arginine (CGA), and methionine (ATG), as here a G/C → A/T transition will have the desired effect. Therefore, genes/amplicons with low numbers of the above-mentioned triplets will most likely not contribute to a nonsense mutation. For the ten genes investigated in sugar beet the calculated overall rate of finding a nonsense mutation leading to a premature stop was 2.3% and for the disruption of a start codon 1.4%. Together with the low obtained MF, the chances of obtaining a nonsense mutation were limited. The ideal amplicon would have an expected possibility of at least 5% for a nonsense mutation (Till et al., 2006a; Till et al., 2007). Harloff et al. (2011) could demonstrate that a high MF together with a high expected value of 6% for obtaining a stop codon through EMS mutagenesis, will lead to the successful identification of such mutants in *Brassica napus*.

Besides the composition of triplets in amplicons, there is one other factor influencing the MF. When evaluating the results from 48 TILLING projects (Chapter 2) it becomes evident, that the ploidy level of a species has a high influence on mutation frequency (Figure 21). The results from *B. napus* (Harloff et al., 2011), *T. aestivum*, *T. durum* (Slade et al., 2005; Uauy et al., 2009), and *A. sativa* (Chawade et al., 2010) clearly underline this fact. The mean MF in 6x species is 1/45kb, whereas the MF in 4x species is 1/51 kb. The highest MF in a diploid species was obtained with 1/60 kb in *B. rapa* (Stephenson et al., 2010) followed by *Arabidopsis* with a MF of 1/89 kb (Martin et al., 2009), whereas the mean MF in diploid species is 1/555 kb.

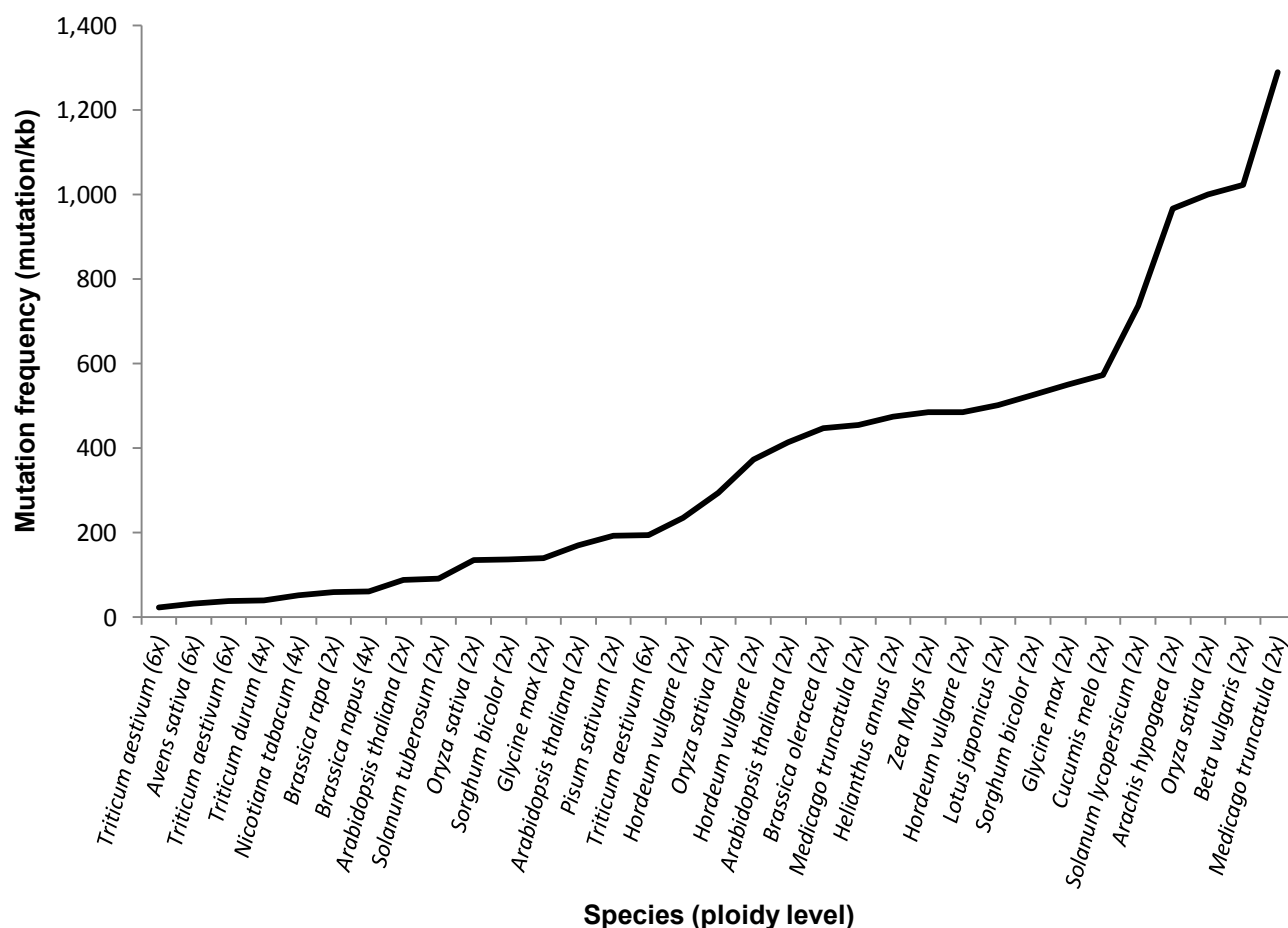


Figure 21: Mutation frequency versus ploidy level. Shown are the mutation frequencies in dependence of the ploidy level of the given species.

In polyploid species most genes are represented by multiple homologous copies, which share a high sequence identity (Wang et al., 2012). This is presumably the explanation for the higher tolerance against the mutagen and consequently, the higher MF. However, there are two exceptions: *B. rapa* is a diploid species, but it went through an ancient triplication event, leaving some level of functional redundancy (Stephenson et al., 2010). And in the case of *Arabidopsis*, Martin et al. (2009) selected M_1 plants with a low seed fertility to maximize the mutation frequency, as it is known that seed fertility is correlated with EMS concentration (Henikoff and Comai, 2003). One problem of polyploid species merits mention. Due to the multiple homologous copies of each gene, it is extremely important to increase the probability of identifying at least one nonsense mutation per amplicon. Otherwise, a single mutant may be masked by wild-type homologues present in another genome (Wang et al., 2012). Another point is primer design in polyploid species. The exact sequence of each homologous copy is needed to evaluate the best target region (Fitzgerald et al., 2010). Otherwise, a primer will lead to false positive mutants.

The last point influencing the MF is the mutagen itself. Different mutagens have a different effect on the species/cultivar treated. As described for soybean (Cooper et al., 2008), two mutagens (EMS and MNU) leading to the formation of O^6 -alkylguanine (O^6 -Gua) were used to create two different mutant populations of equal size from the same cultivar “Williams 82”. Environmental and mutational conditions were also equal, but they varied in MF by almost 90% (MNU = 1/140 kb; EMS = 1/250 kb). All alkylating agents have a certain affinity towards a specific position at the DNA bases (Wyatt and Pittman, 2006). EMS for instance preferentially alkylates adenine at the N^3 position (7%), while it only alkylates guanine at the O^6 position with a frequency of 3.4%. N^3 -alkyladenine is thought to block replication and there is evidence that low levels of A-T \rightarrow T-A transversions can occur following

treatment with methyl methanesulfonate (MMS; Wyatt and Pittman, 2006). Boysen et al. (2009) report that MNU has a 3-fold higher relative reactivity towards the formation of O⁶-Gua than EMS. They further report that ethyl-nitrosourea (ENU) has the highest relative reactivity towards the formation of O⁶-Gua with 66%. O⁶-Gua does not induce cell cycle arrest until a second round of replication, which implies that the biological consequences are not elicited until O⁶-Gua is paired opposite thymine (Wyatt and Pittman, 2006). Now instead of cytosine an adenine base is inserted and this leads to a transition, (Knippers, 2006). Therefore, O⁶-Gua is the main cause for transition mutations, and the higher the relative reactivity towards O⁶-Gua the higher the probability of obtaining transition mutations. This further leads to the assumption that organisms treated with MNU or ENU will have a higher MF.

In summary, five criteria can be regarded to have an influence on the mutation frequency. (1) The mutagen of choice, (2) the cultivar/species together with the level of ploidy, (3) the initial mutation experiment with the evaluation of the LD50 value, (4) the genome size, and (5) the G/C content of the investigated genes.

For the two sugar beet TILLING populations used, I can conclude that three approaches should have been taken for improvement of the mutation frequency. The first one would have been to choose a higher EMS dosage, although this would have meant more material and space requirements. The second step involves the same strategy as Martin et al. (2009) applied for Arabidopsis. Only M₁ plants with low seed fertility should have been chosen to maximize the number of mutations per plant. Third, for future studies another mutagen or combination of two mutagens such as NMU or EMU is recommended, as they are known to alkylate O⁶-Gua positions at a higher frequency than EMS does (Uchiyama et al., 2004).

Taking every possible reason for the low mutation frequency observed in sugar beet into account, it can be assumed that some mutations have been repaired by endogenous DNA repair mechanisms, as mutations can ultimately cause DNA damage. Toxicity and mutagenicity of the incurred damage will depend on the efficiency of the cellular mechanism that evolved to sense, recognize and eliminate the damage and the accuracy of the pathway available to the cell to repair the lesion (Bray and West, 2005).

DNA repair can already take place directly after mutagen treatment in the seed, as the DNA of seeds experiences a different chemical environment from that in the nucleus of actively metabolizing cells. At this stage DNA repair is very efficient, giving rise to the hypothesis that point mutations arisen from EMS treatment have been repaired to some extent. In mutation experiments carried out with EMS, MMS, and MNU on barley seeds Velemínský and Gichner (1978) demonstrated that a higher number of DNA single strand breaks (SSB) were repaired when the seeds were hydrated to about 30% compared to control treatments. The breaks were determined by sedimentation analyses in alkaline sucrose gradients, with a subsequent spectrophotometric assay of isolated DNA. They further reported that SSB were favorably repaired when the seeds were washed extensively with water after treatment with alkylating agents. Additionally they presented results where MNU treated seeds were rinsed with sodium azide (SA) pH 7. The SA treated seeds showed fewer SSB than non-treated control seeds and the extent of repair increased with rising azide concentration. This was not expected, as sodium azide is a mutagen itself when applied at pH 3. Chen and Gao (1988) reported that damage and frequency of chimeras, chlorophyll mutations and morphological mutations were consistently reduced with a longer time of hydrating the seeds prior to SA plus EMS treatments. After hydrating for 24 hours, the observed damage was reduced to 65% compared to the non-hydrated seeds. In our mutagen treatments we imbibed our seeds to make sure that mitosis is initiated, as hydrated enzymes become active and metabolic activity is resumed (Bewley, 1997). Furthermore, we also treated our seeds with a de-alkylating agent, sodium thiosulfate (STS) which should neutralize EMS. Moreover, we extensively rinsed the seeds with running tap water after STS treatment, to remove any extent EMS and STS. On

the basis of data from Velemínský and Gichner (1978) and Chen and Gao (1988), I assume that one of the three steps or a combination had a negative effect on the mutation frequency favoring dark repair of DNA damage. In the case of the pre-soaking, it could be possible, that the seeds were hydrated to a favorable humidity for DNA repair, as I chose a period of 24 hours. From cancer research it is known that STS is used in chemotherapy as a potent chemoprotective agent against alkylating chemotherapeutics (Muldoon et al., 2001). In that respect, STS may not only neutralize the EMS on the seed surface, but also the EMS within the seed, giving rise to a low mutation frequency. Furthermore, the tap water may have had the same effect as STS but in a reduced manner. I assume that the low obtained MF resulted from a combined action of imbibing and the use of STS.

If the thiosulfate and the mutagenesis procedure are not the causes for the low MF, there are more hypotheses underlying an interaction of DNA repair. In *E. coli* there is evidence that EMS caused mutations are preferably repaired at specific sites, therefore arguing for a nearest-neighbor effect (Burns et al., 1988). The same accounts for *Saccharomyces cerevisiae*, where mutated bases in neighborhood of purine bases are more likely to be repaired (Marsischky and Kolodner, 1999). These nearest neighbor effects have also been described for *A. thaliana* (Greene et al., 2003), and are thought to limit the mutation frequency. The mechanism is equal to prokaryotes, where a specialized enzyme recognizes the mismatch and excises the damaged region, which is later repaired by a special DNA polymerase (Kolodner and Marsischky, 1999). The recognition depends on the identities of several base pairs on each site (Marsischky and Kolodner, 1999). Although I did not perform any experiments to support and prove this hypothesis, a likely mechanism possibly exists in sugar beet as DNA repair mechanisms are highly conserved among diverse organisms, as DNA repair is essential for the survival of an organism (Tuteja et al., 2001b).

4.2 Alternative mutation detection methods

During the propagation and construction of the *Beta vulgaris* TILLING platform several alternative techniques have been described for the mutation detection in mutagenized populations, e.g. HRM (high resolution melting curve analysis), capillary sequencing on ABI3730, MALDI-TOF (Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer) and a TaqMan qPCR-based method. A new technique for the detection of mutations is next generation sequencing (NGS), which enables sequencing of entire genomes at high coverage with small read lengths. All techniques have their benefits but also suffer from some drawbacks. HRM has been used for the evaluation of SVF in *Solanum lycopersicum* (tomato; Gady et al., 2009) and *A. thaliana* (Bush and Krysan, 2010) resulting in SVFs of 1/737 kb and 1/415 kb, respectively. HRM eliminates the need for endonuclease digestion, which is a critical step during detection on LI-COR[®] machines. During endonuclease digestion, *Cell* has the tendency to digest not only heteroduplexes, but also exhibits a 5'-3' exonuclease activity, which may lead to a truncation of the IR-dye and thus the reduced detection of digested fragments on the LI-COR[®] machine (Rigola et al., 2009). Disadvantages of HRM include a comparably high detection of false positives, thus requiring a careful re-screening and re-assessment of the data (Bush and Krysan, 2010; Gady et al., 2009). Another alternative method, MALDI-TOF, also eliminates the need for endonuclease digestion and was reported to give reasonable results for the screening of induced mutations in *Avena sativa* (oat) with a SVF of 1/33 kb (Chawade et al., 2010). Another potential benefit is, that MALDI-TOF does not rely on heteroduplex formation, allowing for accurate detection of homozygous mutations without the need to pool samples (Sikora et al., 2011). However, applying MALDI-TOF, a proportion of mutations will be unnoticed, as the investigated fragments are too long or short after applying MassCLEAVE[™] (van den Boom and Ehrich, 2007) to deliver satisfactory resolution (Chawade et al., 2010). Furthermore, this technique is only interesting to laboratories where MALDI-TOF equipment already exists, as the equipment is rather expensive. TaqMan qPCR was used to screen deletions in homologous copies of four genes in wheat in a high throughput approach. TaqMan qPCR can be carried out as long as there are sufficient polymorphisms for specific probe design between the homologous copies (Fitzgerald et al., 2010). With this method it

was possible to identify 53 deletion mutations. The big drawback of this technique is that it can be only reliably used for the presence/ absence detection of homologous candidate genes copies. It therefore is a remarkably good method for mutant screening in translocation lines induced by radiation or heavy ion bombardment (Fitzgerald et al., 2010). NGS has accelerated the prospects of identifying mutations at the whole genome level and has already been used for the identification of mutants in *S. lycopersicum* (Rigola et al., 2009), *Triticum aestivum* (wheat), *Triticum durum* (durum wheat), and *O. sativa* (rice; Tsai et al., 2011).

With NGS it is possible to screen up to 12,000 samples for mutations in one to several genes using KeyPoint technology (Rigola et al., 2009). So far, the only limitations for NGS are the costs and the amplicon size. While the average read length for 454 GenomeSequencer FLX Ti (Roche Applied Science) is 750 bases, Illumina (Solexa) Genome Analyzer only gives up to 250 bases per read but in turn generates much higher sequence coverage. The limitation in amplicon length requires the development of a two-to three-fold higher number of specific primers to obtain the same gene coverage as in classical LI-COR[®] TILLING. Another important aspect that accounts for the use of NGS in the discovery of novel sequence variance in mutant population is the fact, that mutation detection in pools deeper than eight individuals is possible (Tsai et al., 2011). Finally, with application of NGS no additional sequencing has to be performed after identification of the novel identified sequence variants, which is a major advantage compared to the classical identification methods such as HRM, MALDI-TOF, and endonuclease based identification by gel analysis. By technical innovations in the NGS platforms, a further increase in output data per run in terms of sequence length and sequencing depth and thus resolution can be expected, and thus it can be assumed, that NGS-based analysis will replace the classical methods as standard technique for mutation detection in TILLING populations in the near future.

4.3 Prospects for TILLING in *Beta vulgaris*

A future goal in sugar beet research is the breeding of a winter beet (Jung and Müller, 2009; Kirchhoff et al., 2012). To breed a winter beet, substantial knowledge of all underlying principles to control bolting and consequently flowering have to be known. As stated in Chapters 2 and 3 TILLING and EcoTILLING are two useful tools to accomplish such a goal. Unfortunately, TILLING in sugar beet has not led to the identification of mutants useful to gain knowledge in bolting and flowering pathways. These results indicate that mutations have been induced and that I was just not able to find a nonsense mutation. Analyses of triplet usage (Chapter 2) showed that nonsense mutations could possibly be induced by EMS in the ten candidate genes chosen, but only to a very slight percentage.

In combination with both, a moderate mutation frequency and a moderate population size this explains why TILLING in sugar beet has not been as successful as for instance in *Brassica napus* (oilseed rape; Harloff et al., 2011), *A. sativa* (oat; Chawade et al., 2010), *T. aestivum* (wheat; Slade et al., 2012), or *Solanum tuberosum* (potato; Muth et al., 2008). Therefore, in order to obtain useful mutants the two TILLING populations have to be re-mutagenized or increased in size. For the mutagenesis, I would use Az-MNU or a combined mutagenesis of SA and Az-MNU as there is indication that those mutagens are more tolerated by plants compared to EMS treatment (Cooper et al., 2008; Till et al., 2007).

Regarding mutant screening, a TILLING by sequencing approach as done for wheat and rice (Tsai et al., 2011), tomato (Rigola et al., 2009) and tobacco (Reddy et al., 2012) could be carried out. This approach could be useful, as (1) all plants could be screened for a handful of candidate genes at once without a prior re-mutagenesis or (2) all plants could be sequenced completely. However, this would also mean a substantial investment of money. Wang et al. (2012) calculated the costs of re-sequencing 2,000 *B. rapa* M₂ plants by NGS with a 20x genome coverage to be 1.4 Mill. US Dollar (USD) in late 2011. Sequencing the same number of *B. rapa* M₂ plants with a 20x genome coverage at the Center for

Molecular Biosciences (ZMB) would cost 3.3 Mill. Euro (EU), while sequencing of 2,000 *B. vulgaris* M₂ plants with a 20x genome coverage would cost 1.6 Mill. EU (Schilhabel, personal communication). To establish TILLING by sequencing for candidate genes in sugar beet new DNA pools would have to be established, as for NGS screening 3D pools are needed to allow for SV identification. In the case of tomato, barcoded gene specific primers were used (KeyPoint™). With KeyPoint™ it is possible to screen at least four amplicons in about 3,000 M₂ plants per GS FLX run, therefore making this technique highly suitable for sugar beet re-TILLING. Rigola et al. (2009) could further show that it was possible to identify SNPs in 2D pools, therefore eliminating the need to re-pool the already pooled sugar beet DNA samples. In summary, the resource can still be used with substantial investments in terms of time and money. TILLING by sequencing in the current sugar beet EMS populations would lead to the identification of mutations, but it cannot be concluded if it would lead to the identification of nonsense mutations.

Before this study, no sugar beet TILLING protocol was available and the identification and analysis of sugar beet mutants was being done by forward genetic approaches (Abou-Elwafa et al., 2011; Büttner et al., 2010; Hohmann et al., 2005). In these forward genetic screens of population 930190 five non-bolting mutants were identified with a clear phenotype of non-bolting before vernalization. However, the identified non-bolting mutants were not included by Uwe Hohmann in the TILLING population that was eventually used for this study. These mutants had been investigated by Sanger sequencing for the candidate genes used for TILLING, but no mutations were discovered (Müller, personal communication). Therefore it can be excluded, that in this population (930190) mutations are present with a drastic impact on bolting time at least in the genes *BTC1* and *BvFT1* where stop codons should lead to an early flowering phenotype (Pin et al., 2010; Pin et al., 2012). Recently, Nadine Dally (personal communication) was able to identify two independent mutations within the same candidate gene, each leading to a non-bolting phenotype. There are two likely explanations why the forward genetic approach was successful in finding mutants with a phenotype while the TILLING approach was not. (1) By using forward genetics an altered or interesting phenotype is chosen and the plants carrying this phenotype and the respective offspring are investigated for the causative nucleotide change, which makes this approach more direct in case of phenotypes. In the TILLING approach, a couple of genes are chosen and investigated for a likely nucleotide change caused by mutagen application. As written earlier, EMS has been shown to cause mutations randomly. And the chance of identifying a mutation rises with mutation frequency and population size (Wang et al., 2012). Assuming the possibility of introducing a nonsense mutation by EMS is 2% for a given gene, and 50 mutations have been identified for the gene, then the number of nonsense mutations should be one. However because the mutations are introduced randomly, it would be not unsurprising to not find a stop mutation in the given amplicon. If less than 50 mutations are recovered, the changes are even smaller. This means for the TILLING approach, the changes of obtaining a nonsense mutation at the given MF of 1/1,062 kb and a population size of only 2,387 M₂ families was very low. (2) The choice of genes investigated has also a great influence of the identification of nonsense mutation, as can be seen in the studies of Dalmais et al. (2008) in pea (*Pisum sativum*) and Gottwald et al. (2009) in barley (*H. vulgare*). Dalmais et al. (2008) report of gene specific MF ranging from as low as 1/84 kb to 1/356 kb, while Gottwald et al. (2009) report of gene specific MF ranging from as low as 1/215 kb to 1/874 kb. This can have according to Dalmais et al. (2008) two reasons, (a) the gene is important for the organism and mutations leading to truncation are most likely to cause the death of the organism and (b) the GC content of the gene investigated. As a higher GC level will have a higher MF as results, because most alkylating agents favor guanine for the alkylation (Wyatt and Pittman, 2006). The possibility of obtaining nonsense mutations can be increased with a higher number of genes screened (B. Till, personal communication), which can be shown by the Arabidopsis TILLING project (Greene et al., 2003). Here more than 190 genes have been analyzed and 1890 mutations have been identified. Concluding I have to say that even though TILLING has not been successful in sugar beet, it is a technique, which can be powerful in finding nonsense mutations when the conditions are right. To find the right conditions is the most important part before starting a TILLING project. For sugar beet that

would mean to start new from scratch, with the evaluation of mutagens, genotypes and mutagen application condition. Once those three goals have been reached, I believe that TILLING in sugar beet can be as successful as described for other crops.

4.4 EcoTILLING in *Beta vulgaris*

Regarding EcoTILLING another picture can be drawn. The results in Chapter 3 demonstrate that EcoTILLING is a fast and efficient way of screening for genetic variation in *B. vulgaris*. With the results obtained in Chapter 3 I could provide a snapshot of the species-wide genetic diversity that underlies the phenotypic variation. This phenotypic variation has important biological consequences as well as practical implication for beet improvement, allowing to introgress interesting phenotypes in elite breeding material to produce beets with desired traits. The Beta panel used in my study exhibits a wide spectrum of phenotypic differences including annuality, bolting time, growth type, and frost hardiness (Kirchhoff et al., 2012). I chose to take a look at a diverse panel of different Beta accessions (e.g. sugar beets, garden beets, leaf beets, fodder beets, and wild sea beets), rather than examine the diversity within, for instance just sugar beets in order to obtain an as broad as possible picture of the variation that exists in this species.

The established EcoTILLING protocol is optimized for the fast identification of natural sequence variations in a high throughput manner. Once a sequence variant (SV) was identified I could use it to assign the specific plant to a haplotype. When all the plants were screened and all haplotypes assigned only a small portion of plants had to be sequenced, as only one to two plants per haplotype were needed to identify the underlying SVs. That reduced the sequencing costs tremendously as discussed in Chapter 3, therefore EcoTILLING turned out to be a fast and convenient technique. To even reduce sequencing costs it may also be advantageous to perform a first screening round to detect individuals that are homozygous for all SVs in an amplicon. Further, EcoTILLING can also be used for genotyping applications without subsequent sequencing when only the presence or absence of a band on the gel is needed as a marker.

In my study, I used a combination of coding and noncoding sequences and conclude that both types of DNA can provide useful data. I could show that for *BvFL1* more variations were detected in noncoding regions (Figure 17, Chapter 3). It is not clear whether these sequence variations are the crucial factor for the phenotypes observed. However, I can say that the SVs identified have an effect on annuality and survival after winter in leaf beets, garden beets and wild sea beets. To clarify whether the identified SVs are the causative elements for the observed phenotypes more experiments need to be conducted. For instance, the whole gene for the significant haplotypes including the UTR regions as well as the promoter would need to be sequenced, as from the results obtained, it cannot be concluded that the identified SVs are causative for the phenotype observed. From studies in *Arabidopsis* it is known that e.g. frost hardiness is caused by a combined effect of *FRI* and *FLC* (Caicedo et al., 2004) and further that regions in the promoter and the first intron of *FLC* are needed for the interaction and regulation of the combined *FRI/FLC* action (Korves et al., 2007). In the case of coding regions it is well documented that a SV which leads to an amino acid exchange or even to a triplet change, which results in a premature stop codon, will have a tremendous effect on the function of the gene (Rashid et al., 2011).

Concluding, I can say that EcoTILLING is an accurate and robust method for the discovery of nucleotide polymorphisms in different Beta accessions. The technique is highly adaptable and many applications can be considered. It can be used to measure heterozygosity as a selection criterion in breeding programs, for studies of chromosomal inheritance and for functional genomic analysis. According to Gilchrist and Haughn (2005) EcoTILLING is “the technique of choice for medium-to-high throughput reverse genetics in many organisms”. Furthermore, EcoTILLING can be used as a functional marker system. Every SV detected can be used as a marker because it results in a change in the overall fragment pattern (Mejlhede et al., 2006).

5 Summary

Induced mutations have been used in plant breeding since the early 1960s. The traditional way (forward genetics) for identifying new traits was to analyze phenotypic changes caused by mutations and to link these to a gene. This approach has been complemented by a reverse genetic strategy, in which a gene of interest and its alteration in structure or activity is analyzed for an impact on phenotype. Targeting Induced Local Lesions in Genomes (TILLING) is a reverse genetic technique to identify point mutations in mutant populations, which has been successfully applied for a variety of crop species as a valuable tool for the manipulation of genes underlying traits of agronomic importance.

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima*) is the only crop plant used for sugar production in Europe. Due to the narrow genetic variability of the sugar beet gene pool, the introduction of new traits is of high interest to breeders. The aims of this work were (1) to identify flowering time mutants among EMS treated offspring and (2) to identify natural sequence variants by genotyping (EcoTILLING).

In the first part, 20,000 seeds of the doubled haploid (DH) sugar beet line SynDH1 were mutagenized with 1% ethyl methanesulfonate (EMS) and M₂ seeds were produced from 837 M₁ plants after bag isolation. From 3,418 M₂ plants, M₃ seed was produced in the greenhouse. A second pre-existing mutant population derived from the annual sugar beet line 930190 was examined as well. This population included 2688 M₂-plants, which were obtained from the seed of 1550 M₁-plants. DNA samples were taken from all M₂ plants and pooled by a two dimensional 8x pooling scheme. A TILLING protocol for sugar beet has been established based on heteroduplex digestion with the restriction endonuclease (CEL I) from celery stalks. The following genes from the sugar beet bolting and flowering time pathway were examined for induced mutations with 19 primer combinations: *BTC1*, *BvCOL1*, *BvFLI*, *BvFLK*, *BvFT1*, *BvFT2*, *BvFVE*, *BvLHP1*, *BvSWP*, and *BvTON1*. In ten of these genes 72 sequence variants (SV) were found, including 52 (58%) within exon regions. Twenty-four (32%) of the mutations in exon regions were non-synonymous mutations that cause an amino acid exchange. One other mutation was located within a splice site. No nonsense (stop codon) mutation could be found. Sixty-four SVs were transition mutations (G/C → A/T) likely to result from EMS mutagenesis. The overall frequency of SVs was 1/816 kb, whereas a transition frequency of 1/1,062 kb could be determined. M₃ progenies from 28 M₂ mutants of the genes *BTC1*, *BvFLK*, *BvFT1*, *BvLHP1*, and *BvTON1* were grown in the greenhouse and scored with respect to time to bolting and time to flower. However, no statistically significant effects of the mutations were found. Based on the low mutation frequency and the low phenotypic variation the here presented resource appears to be not suitable for further TILLING screens. In addition, an unexpected high heterogeneity (8.6%) was detected among the non-EMS treated plants of the 930190 population after AFLP analysis of 104 M₂ plants. It is likely that this heterogeneity has already existed before EMS treatment, and / or occurred during selfing of M₁ plants through cross-pollination.

In the second part of this work, natural SVs were identified by the same genotyping technique (referred as EcoTILLING) from 268 accessions in a diversity panel of *Beta vulgaris* accessions including sugar beet, garden beet (var. *conditiva*), leaf beet (var. *flavescens*), and fodder beet (var. *crassa*) as well as the wild beet (*B. vulgaris* ssp. *maritima*, BVM). This diversity panel had already been tested in overwintering field trials with respect to bolting before winter and survival after winter. For both traits, a large variation was determined. With the aim to analyze the population structure of the diversity panel with the software, STRUCTURE an AFLP analysis was carried out. This analysis suggested the presence of three subgroups (k = 3), in which most of the sugar beets accessions were assigned to the first group, while the examined accessions of fodder beet and garden beet were assigned to the second group. The third group comprised primarily of leaf beet and BVM-accessions. EcoTILLING in the three bolting and flowering time genes *BTC1*, *BvFLI*, and *BvFT1* resulted in the

identification of 20 silent SNPs and one non-synonymous SNP, which could be grouped in 55 haplotypes. The haplotypes were tested for an association with bolting before winter (BR), survival rate after winter (SR), and SR with BR as cofactor. The association study revealed that haplotypes for *BvFL1* are significantly associated with BR, SR, and SR with BR as cofactor. The data give further evidence that the *FLC*-like gene may affect flowering time and winter survival in Beta species.

6 Zusammenfassung

Seit den 1960er Jahren werden induzierte Mutationen in der Pflanzenzüchtung eingesetzt, um neue Eigenschaften zu generieren. Dies erforderte bisher meist aufwändige phänotypische Analysen umfassender Mutantenpopulationen, um interessante Mutantenphänotypen zu detektieren. Diese Mutanten dienen zugleich der Identifikation der zugrunde liegenden Gene („forward genetics“). Im Gegensatz dazu erfolgt beim „reverse genetics“-Ansatz zunächst eine Sequenz/Gen-Identifikation mit nachfolgender phänotypischer Analyse. Die Technik des „Targeting Induced Local Lesions in Genomes“ (TILLING) zählt zu den „reverse genetic“-Ansätzen. TILLING ermöglicht die Detektion sowie Identifikation von Punktmutationen in Mutantenpopulationen. In der Pflanzenzüchtung wurde diese Technik bereits erfolgreich eingesetzt, um Mutationen in Genen verschiedener Nutzpflanzen zu finden, welche für wichtige züchterische Eigenschaften kodieren.

Die Zuckerrübe (*Beta vulgaris* L.) ist die einzige Nutzpflanze, welche in Europa zur Zuckergewinnung angebaut wird. Aufgrund der geringen genetischen Variabilität des Zuckerrüben-Genpools ist die Integration neuer Merkmale von besonderem züchterischem Interesse. Ziele dieser Arbeit waren (1) die Identifikation von Blühzeitpunkt-Mutanten in der Nachkommenschaft (M₂) einer mit einem chemischen Mutagen (Ethylmethansulfonat, EMS) behandelten Zuckerrüben-Population (TILLING) sowie (2) die Ermittlung natürlicher Sequenzvarianten durch Genotypisierung (EcoTILLING).

Im ersten Teil der Arbeit wurden 20.000 Samen der doppelthaploiden (DH) Zuckerrübenlinie SynDH1 mit 1% (EMS) mutagenisiert und von 837 M₁-Pflanzen M₂-Samen nach Tüten-Isolation produziert. Im Folgenden konnte von 3.418 M₂-Pflanzen M₃ Saatgut erstellt werden. Weiterhin wurde eine zweite, bereits existierende Mutatenpopulation aus einer annualen Zuckerrübenlinie (930190) untersucht. Diese M₂ Population umfasste 2.688 M₂-Pflanzen, welche aus dem Saatgut von 1.550 M₁-Pflanzen erhalten wurden. Von allen M₂-Pflanzen wurden DNA Proben genommen und in sogenannte zweidimensionale Achtfachpools überführt. Ein TILLING-Protokoll für die Zuckerrübe wurde basierend auf einem Heteroduplex-Restriktionsverdau mit der Endonuklease CEL1 aus Stangensellerie etabliert. Die folgenden Gene aus dem Schoss- und Blüh-Signalweg der Zuckerrübe wurden hinsichtlich induzierter Mutationen mit 19 Primerkombinationen untersucht: *BTC1*, *BvCOL1*, *BvFL1*, *BvFLK*, *BvFT1*, *BvFT2*, *BvFVE*, *BvLHP1*, *BvSWP*, und *BvTON1*. In zehn dieser Gene konnten insgesamt 72 Sequenzvarianten (SV) gefunden werden, darunter 52 (67%) in Exon-Regionen. Vierundzwanzig (32%) der Mutationen in Exon-Regionen waren nicht-synonyme Mutationen, welche einen Aminosäure-Austausch bewirken. Eine weitere Mutation befand sich innerhalb einer Spleißstelle. Es konnte keine nonsense Mutationen gefunden werden. 64 SVs wiesen eine Transition (G/C → A/T) auf, welche eine Folge der EMS-Behandlung darstellt. Die Frequenz aller 72 SV betrug 1/816 kb, wohingegen eine Transitionsfrequenz von 1/1.062 kb ermittelt wurde. M₃ Nachkommen von 28 M₂ Mutanten der Gene *BTC1*, *BvFLK*, *BvFT1*, *BvLHP1* und *BvTON1* wurden im Gewächshaus angebaut und hinsichtlich ihres Schoss- und Blühzeitpunktes bonitiert. Es konnten jedoch keine statistisch signifikanten Effekte der Mutationen gefunden werden. Aufgrund der geringen Mutationsrate und der geringen phänotypischen Variation scheint die hier vorgestellte Ressource nicht für weitere TILLING Untersuchungen geeignet zu sein. Darüber hinaus wurde mit Hilfe einer AFLP-Analyse von 104 M₂ Pflanzen der Populationen 930190 eine unerwartet hohe Heterogenität (8,6%) der nicht mit EMS behandelten Pflanzen gefunden. Es ist anzunehmen, dass diese Heterogenität bereits vor der EMS Behandlung vorhanden war und/oder dass Fremdbestäubung während der Selbstbefruchtung der M₁ Pflanzen die Ursache dafür ist.

Im zweiten Teil dieser Arbeit wurden natürliche SVs mit dem gleichen methodischen Ansatz (als sogenanntes EcoTILLING) in einem Diversitäts-Sortiment von 268 *Beta vulgaris* Akzessionen identifiziert. Dieses Sortiment umfasste die Kulturformen Zuckerrübe, Rote Bete (var. *conditiva*), Mangold (var. *flavescens*) und Futterrübe (var. *crassa*) sowie die Wildrübe (*B. vulgaris* subsp. *maritima* L., BVM) und war bereits zuvor in Überwinterungsfeldversuchen hinsichtlich des

Schossverhaltens vor dem Winter und der Überlebensrate nach dem Winter untersucht worden. Für beide Merkmale wurde eine große Variation ermittelt. Mit dem Ziel der Erfassung der Populationsstruktur des Sortiments mit der Software-STRUCTURE wurde eine AFLP-Analyse durchgeführt. Es konnten drei Untergruppen ($k = 3$) detektiert werden, welche in der ersten Gruppe hauptsächlich Vertreter der Zuckerrüben-Akzessionen aufwies, während die untersuchten Akzessionen von Futterrüben und Roter Bete der zweiten Gruppe zugeordnet wurden. Die dritte Gruppe enthielt vorrangig Mangold und BVM-Akzessionen. Das hier durchgeführte EcoTILLING in den drei Schoss- und Blühzeitpunkt-Genen *BTC1*, *BvFL1* und *BvFT1* führte zur Identifikation von 20 synonymen Einzelnukleotid-Polymorphismen (Single Nucleotide Polymorphisms, SNPs) sowie einem nicht-synonymen SNP, welche in 55 Haplotypen gruppiert wurden. Eine Assoziation mit den Parametern Schossrate, Überlebensrate, Überlebensrate in Abhängigkeit von Schossrate als Co-Faktor konnte für die untersuchten *BvFL1* Haplotypen nachgewiesen werden. Diese Ergebnisse liefern weitere Hinweise darauf, dass das *FLC*-ähnliche Gen eventuell die Blühzeit und auch die Winterhärte in Beta Arten beeinflusst.

7 Supplementary material

7.1 Supplementary Tables

Supplementary Table 1: List of transition mutations identified via TILLING and sequencing. Given are the EMS populations, the M₂ plant number (seed code for 930190, Syngenta-code for SynDHI, as the M₂ plants were propagated at Landskrona, Sweden), the gene, and the bp position of the mutation as well as the effect of the mutation. Furthermore the effect on aa and the respective aa position is given. In addition the position of the mutation in an intron or exon region is given, as well as the state in the M₂.

Population	M ₂ -plant	gene	bp position	triplet-change	effect on aa	aa position	remark	exon/intron	state in M ₂
930190	041859	<i>BTC1</i>	7,079	-	-	-	-	intron	heterozygous
	040959		7,132	TCG-TCA	silent	128	-	exon	heterozygous
	041660		7,366	-	-	-	-	intron	heterozygous
	041661		12,391	-	-	-	-	intron	heterozygous
	054993		12,876	AGT-AAT	Ser-Asn	625	-	exon	homozygous
	054661		12,917	CCC-TCC	Pro-Ser	638	-	exon	homozygous
	040838		13,072	GTG-GTA	silent	689	-	exon	heterozygous
	051473		<i>BvCOL1</i>	2,009	-	-	-	-	intron
	045274	2,833		TCC-TCT	silent	318	-	exon	homozygous
	044542	<i>BvFL1</i>	400	-	-	-	-	UTR	homozygous
	054732		533	-	-	-	-	UTR	heterozygous
	044591		550	-	-	-	-	UTR	heterozygous
	045136		813	TCT-TTT	Ser-Phe	26	-	exon	heterozygous
	054922		892	GGC-GGT	silent	52	-	exon	heterozygous
	055245		<i>BvFLK</i>	1,426	GTA-GTT	silent	140	-	exon
	044595	1,632		-	-	-	-	intron	homozygous
	055798	2,963		GGA-GAA	Gly-Glu	132	-	exon	heterozygous
	042496	<i>BvFT1</i>	3,135	-	-	-	-	UTR	homozygous
	055926		8,457	-	-	-	-	Intron	heterozygous
	044771		8,576	GCT-GTT	Ala-Val	136	-	exon	homozygous

Population	M ₂ -plant	gene	bp position	triplet-change	effect on aa	aa position	remark	exon/intron	state in M ₂
930190	051773	<i>BvFT2</i>	1,110	-	-	-	-	UTR	homozygous
	054728		1,492	-	-	-	-	UTR	heterozygous
	043285		4,622	AGA-AAA	Arg-Lys	116	-	Exon	heterozygous
	051179		4,676	AGA-AAA	Arg-Lys	134	-	exon	homozygous
	044606	<i>BvFVE</i>	7,599	-	-	-	-	intron	heterozygous
	044818	<i>BvLHPI</i>	1,420	GTC-ATC	Val-Ile	52	-	exon	heterozygous
	041253		1,495	GAG-AAG	Glu-Lys	77	-	exon	heterozygous
	044824		1,497	GAG-GAA	silent	77	-	exon	heterozygous
	054658		1,728	-	-	-	-	intron	heterozygous
	054877		1,864	-	-	-	-	intron	heterozygous
	042748	<i>BvSWP</i>	1,031	GTG-GAT	Gly-Asp	154	-	exon	homozygous
	055904	<i>BvTON1</i>	9,727	-	-	-	-	intron	heterozygous
SynDH1	08775251	<i>BTC1</i>	7,201	AAC-AAT	silent	205	-	exon	homozygous
	08775114		7,202	GAA-AAA	Lys-Glu	206	-	exon	heterozygous
	08775248		7,274	-	-	-	-	intron	homozygous
	08774900		7,520	-	-	-	-	intron	homozygous
	09776243		7,633	GAT-AAT	Asp-Asn	250	-	exon	heterozygous
	08775033		12,376	-	-	-	-	intron	heterozygous
	08774925		12,621	CCT-CTT	Pro-Leu	588	-	exon	heterozygous
	08775257		12,758	GTA-ATA	Val-Ile	634	-	exon	heterozygous
	08775253		12,800	CTG-TTG	silent	648	-	exon	heterozygous
	08775242		12,843	CGC-CAC	Arg-Asp	659	-	exon	homozygous
	08775256		12,983	GAA-AAA	Lys-Glu	706	-	exon	homozygous
	08774873	<i>BvFLI</i>	388	-	-	-	-	UTR	heterozygous
	09776348		701	-	-	-	-	UTR	heterozygous
	09776346		872	CTT-TTT	Leu-Phe	46	-	exon	homozygous
	08775306		1,022	-	-	-	-	intron	homozygous
09773613	<i>BvFLK</i>	2,961	CAG-CAA	silent	131	-	exon	homozygous	

Population	M ₂ -plant	gene	bp position	triplet-change	effect on aa	aa position	remark	exon/intron	state in M ₂
SynDH1	09773836	<i>BvFT1</i>	3,051	-	-	-	-	intron	homozygous
	09775781		8,395	-	-	-	-	intron	homozygous
	09775049		8,477	GGA-GAA	Gly-Glu	106	Splice site	exon	heterozygous
	09775095		8,732	-	-	-	-	UTR	heterozygous
	09775758		8,736	-	-	-	-	UTR	heterozygous
	09773767	<i>BvFT2</i>	1,121	-	-	-	-	UTR	heterozygous
	09777844		1,239	GTT-ATT	Val-Ile	37	-	exon	heterozygous
	09775933		4,647	TTC-TTT	silent	124	-	exon	homozygous
	08775365	<i>BvLHP1</i>	1,466	GGC-GAC	Gly-Asp	67	-	exon	heterozygous
	08775169		1,479	GAC-GAT	silent	71	-	exon	homozygous
	08775036		1,865	-	-	-	-	intron	homozygous
	08774902	<i>BvTON1</i>	9,865	-	-	-	-	intron	homozygous
	08776584		9,874	-	-	-	-	intron	heterozygous
	09775781		9,932	GAG-GAA	silent	159	-	exon	heterozygous
	08775150		10,186	-	-	-	-	intron	heterozygous
08771543	10,229		-	-	-	-	intron	homozygous	

Supplementary Table 2: Codon usage of *BTC1*. The coding sequence (CDS) of *BTC1* is 2,367 base pairs long and consists of 789 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BTC1</i>			CDS ¹⁾ → 2,367 bp 789 AA ²⁾																
#	Codon	AA	per triplet	per CDS	#	Codon	AA	per triplet	per CDS	#	Codon	AA	per triplet	per CDS	#	Codon	AA	per triplet	per CDS
7	TTT	Phe	0.89	0.30	17	TCT	Ser	2.15	0.72	5	TAT	Tyr	0.63	0.21	8	TGT	Cys	1.01	0.34
6	TTC	Phe	0.76	0.25	10	TCC	Ser	1.27	0.42	3	TAC	Tyr	0.38	0.13	5	TGC	Cys	0.63	0.21
9	TTA	Leu	1.14	0.38	17	TCA	Ser	2.15	0.72	1	TAA	Stop	0.13	0.04	0	TGA	Stop	0.00	0.00
9	TTG	Leu	1.14	0.38	5	TCG	Ser	0.63	0.21	0	TAG	Stop	0.00	0.00	8	TGG	Trp	1.01	0.34
11	CTT	Leu	1.39	0.46	14	CCT	Pro	1.77	0.59	21	CAT	His	2.66	0.89	7	CGT	Arg	0.89	0.30
7	CTC	Leu	0.89	0.30	5	CCC	Pro	0.63	0.21	6	CAC	His	0.76	0.25	2	CGC	Arg	0.25	0.08
4	CTA	Leu	0.51	0.17	17	CCA	Pro	2.15	0.72	20	CAA	Gln	2.53	0.84	5	CGA	Arg	0.63	0.21
7	CTG	Leu	0.89	0.30	2	CCG	Pro	0.25	0.08	25	CAG	Gln	3.17	1.06	3	CGG	Arg	0.38	0.13
18	ATT	Ile	2.28	0.76	16	ACT	Thr	2.03	0.68	45	AAT	Asn	5.70	1.90	32	AGT	Ser	4.06	1.35
1	ATC	Ile	0.13	0.04	3	ACC	Thr	0.38	0.13	18	AAC	Asn	2.28	0.76	19	AGC	Ser	2.41	0.80
8	ATA	Ile	1.01	0.34	9	ACA	Thr	1.14	0.38	26	AAA	Lys	3.30	1.10	10	AGA	Arg	1.27	0.42
19	ATG	Start	2.41	0.80	1	ACG	Thr	0.13	0.04	24	AAG	Lys	3.04	1.01	14	AGG	Arg	1.77	0.59
25	GTT	Val	3.17	1.06	17	GCT	Ala	2.15	0.72	31	GAT	Asp	3.93	1.31	25	GGT	Gly	3.17	1.06
11	GTC	Val	1.39	0.46	5	GCC	Ala	0.63	0.21	15	GAC	Asp	1.90	0.63	7	GGC	Gly	0.89	0.30
12	GTA	Val	1.52	0.51	18	GCA	Ala	2.28	0.76	29	GAA	Glu	3.68	1.23	21	GGA	Gly	2.66	0.89
14	GTG	Val	1.77	0.59	0	GCG	Ala	0.00	0.00	19	GAG	Glu	2.41	0.80	11	GGG	Gly	1.39	0.46

1) Coding sequence; 2) Amino acid

Supplementary Table 3: Codon usage of *BvCOLI*. The coding sequence (CDS) of *BvCOLI* is 1,104 base pairs (bp) long and consists of 559 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BvCOLI</i>		CDS ¹⁾ → 1,104 bp				559 AA ²⁾													
#	Codon	AA	per triplet	per CDS	#	Codon	AA	per triplet	per CDS	#	Codon	AA	per triplet	per CDS	#	Codon	AA	per triplet	per CDS
6	TTT	Phe	1.63	0.54	5	TCT	Ser	1.36	0.45	6	TAT	Tyr	1.63	0.54	10	TGT	Cys	2.72	0.91
5	TTC	Phe	1.36	0.45	4	TCC	Ser	1.09	0.36	7	TAC	Tyr	1.90	0.63	6	TGC	Cys	1.63	0.54
3	TTA	Leu	0.82	0.27	11	TCA	Ser	2.99	1.00	1	TAA	Stop	0.27	0.09	0	TGA	Stop	0.00	0.00
6	TTG	Leu	1.63	0.54	1	TCG	Ser	0.27	0.09	0	TAG	Stop	0.00	0.00	3	TGG	Trp	0.82	0.27
1	CTT	Leu	0.27	0.09	3	CCT	Pro	0.82	0.27	4	CAT	His	1.09	0.36	5	CGT	Arg	1.36	0.45
1	CTC	Leu	0.27	0.09	3	CCC	Pro	0.82	0.27	4	CAC	His	1.09	0.36	2	CGC	Arg	0.54	0.18
4	CTA	Leu	1.09	0.36	13	CCA	Pro	3.53	1.18	8	CAA	Gln	2.17	0.72	1	CGA	Arg	0.27	0.09
4	CTG	Leu	1.09	0.36	1	CCG	Pro	0.27	0.09	9	CAG	Gln	2.45	0.82	2	CGG	Arg	0.54	0.18
3	ATT	Ile	0.82	0.27	4	ACT	Thr	1.09	0.36	8	AAT	Asn	2.17	0.72	7	AGT	Ser	1.90	0.63
6	ATC	Ile	1.63	0.54	6	ACC	Thr	1.63	0.54	9	AAC	Asn	2.45	0.82	3	AGC	Ser	0.82	0.27
1	ATA	Ile	0.27	0.09	8	ACA	Thr	2.17	0.72	5	AAA	Lys	1.36	0.45	8	AGA	Arg	2.17	0.72
13	ATG	Start	3.53	1.18	1	ACG	Thr	0.27	0.09	11	AAG	Lys	2.99	1.00	7	AGG	Arg	1.90	0.63
12	GTT	Val	3.26	1.09	11	GCT	Ala	2.99	1.00	17	GAT	Asp	4.62	1.54	7	GGT	Gly	1.90	0.63
3	GTC	Val	0.82	0.27	5	GCC	Ala	1.36	0.45	7	GAC	Asp	1.90	0.63	4	GGC	Gly	1.09	0.36
3	GTA	Val	0.82	0.27	16	GCA	Ala	4.35	1.45	13	GAA	Glu	3.53	1.18	6	GGA	Gly	1.63	0.54
6	GTG	Val	1.63	0.54	1	GCG	Ala	0.27	0.09	14	GAG	Glu	3.80	1.27	4	GGG	Gly	1.09	0.36

1) Coding sequence; 2) Amino acid

Supplementary Table 4: Codon usage of *BvFLK*. The coding sequence (CDS) of *BvFLK* is 1,677 base pairs (bp) long and consists of 559 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BvFLK</i>			CDS ¹⁾ → 1,677 bp		559 AA ²⁾														
#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS
10	TTT	Phe	1.79	0.60	9	TCT	Ser	1.61	0.54	13	TAT	Tyr	2.33	0.78	1	TGT	Cys	0.18	0.06
2	TTC	Phe	0.36	0.12	1	TCC	Ser	0.18	0.06	5	TAC	Tyr	0.89	0.30	0	TGC	Cys	0.00	0.00
2	TTA	Leu	0.36	0.12	8	TCA	Ser	1.43	0.48	1	TAA	Stop	0.18	0.06	0	TGA	Stop	0.00	0.00
13	TTG	Leu	2.33	0.78	3	TCG	Ser	0.54	0.18	0	TAG	Stop	0.00	0.00	3	TGG	Trp	0.54	0.18
8	CTT	Leu	1.43	0.48	16	CCT	Pro	2.86	0.95	11	CAT	His	1.97	0.66	4	CGT	Arg	0.72	0.24
2	CTC	Leu	0.36	0.12	2	CCC	Pro	0.36	0.12	6	CAC	His	1.07	0.36	2	CGC	Arg	0.36	0.12
1	CTA	Leu	0.18	0.06	26	CCA	Pro	4.65	1.55	20	CAA	Gln	3.58	1.19	1	CGA	Arg	0.18	0.06
2	CTG	Leu	0.36	0.12	3	CCG	Pro	0.54	0.18	23	CAG	Gln	4.11	1.37	1	CGG	Arg	0.18	0.06
15	ATT	Ile	2.68	0.89	8	ACT	Thr	1.43	0.48	19	AAT	Asn	3.40	1.13	9	AGT	Ser	1.61	0.54
6	ATC	Ile	1.07	0.36	6	ACC	Thr	1.07	0.36	8	AAC	Asn	1.43	0.48	4	AGC	Ser	0.72	0.24
7	ATA	Ile	1.25	0.42	6	ACA	Thr	1.07	0.36	7	AAA	Lys	1.25	0.42	5	AGA	Arg	0.89	0.30
15	ATG	Start	2.68	0.89	2	ACG	Thr	0.36	0.12	10	AAG	Lys	1.79	0.60	6	AGG	Arg	1.07	0.36
23	GTT	Val	4.11	1.37	28	GCT	Ala	5.01	1.67	22	GAT	Asp	3.94	1.31	19	GGT	Gly	3.40	1.13
3	GTC	Val	0.54	0.18	8	GCC	Ala	1.43	0.48	8	GAC	Asp	1.43	0.48	6	GGC	Gly	1.07	0.36
6	GTA	Val	1.07	0.36	19	GCA	Ala	3.40	1.13	18	GAA	Glu	3.22	1.07	24	GGA	Gly	4.29	1.43
6	GTG	Val	1.07	0.36	1	GCG	Ala	0.18	0.06	25	GAG	Glu	4.47	1.49	11	GGG	Gly	1.97	0.66

1) Coding sequence; 2) Amino acid

Supplementary Table 5: Codon usage of *BvFLI*. The coding sequence (CDS) of *BvFLI* is 645 base pairs (bp) long and consists of 215 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BvFLI</i> CDS ¹⁾ → 645 bp					215 AA ²⁾														
#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS
1	TTT	Phe	0.47	0.16	10	TCT	Ser	4.65	1.55	1	TAT	Tyr	0.47	0.16	3	TGT	Cys	1.40	0.47
3	TTC	Phe	1.40	0.47	2	TCC	Ser	0.93	0.31	3	TAC	Tyr	1.40	0.47	0	TGC	Cys	0.00	0.00
1	TTA	Leu	0.47	0.16	4	TCA	Ser	1.86	0.62	1	TAA	Stop	0.47	0.16	0	TGA	Stop	0.00	0.00
4	TTG	Leu	1.86	0.62	0	TCG	Ser	0.00	0.00	0	TAG	Stop	0.00	0.00	0	TGG	Trp	0.00	0.00
11	CTT	Leu	5.12	1.71	1	CCT	Pro	0.47	0.16	1	CAT	His	0.47	0.16	4	CGT	Arg	1.86	0.62
2	CTC	Leu	0.93	0.31	1	CCC	Pro	0.47	0.16	0	CAC	His	0.00	0.00	2	CGC	Arg	0.93	0.31
5	CTA	Leu	2.33	0.78	3	CCA	Pro	1.40	0.47	7	CAA	Gln	3.26	1.09	2	CGA	Arg	0.93	0.31
5	CTG	Leu	2.33	0.78	0	CCG	Pro	0.00	0.00	5	CAG	Gln	2.33	0.78	1	CGG	Arg	0.47	0.16
3	ATT	Ile	1.40	0.47	2	ACT	Thr	0.93	0.31	5	AAT	Asn	2.33	0.78	7	AGT	Ser	3.26	1.09
3	ATC	Ile	1.40	0.47	2	ACC	Thr	0.93	0.31	3	AAC	Asn	1.40	0.47	1	AGC	Ser	0.47	0.16
3	ATA	Ile	1.40	0.47	5	ACA	Thr	2.33	0.78	7	AAA	Lys	3.26	1.09	5	AGA	Arg	2.33	0.78
5	ATG	Start	2.33	0.78	0	ACG	Thr	0.00	0.00	8	AAG	Lys	3.72	1.24	6	AGG	Arg	2.79	0.93
7	GTT	Val	3.26	1.09	7	GCT	Ala	3.26	1.09	9	GAT	Asp	4.19	1.40	3	GGT	Gly	1.40	0.47
3	GTC	Val	1.40	0.47	1	GCC	Ala	0.47	0.16	2	GAC	Asp	0.93	0.31	1	GGC	Gly	0.47	0.16
3	GTA	Val	1.40	0.47	4	GCA	Ala	1.86	0.62	11	GAA	Glu	5.12	1.71	3	GGA	Gly	1.40	0.47
1	GTG	Val	0.47	0.16	0	GCG	Ala	0.00	0.00	10	GAG	Glu	4.65	1.55	2	GGG	Gly	0.93	0.31

1) Coding sequence; 2) Amino acid

Supplementary Table 6: Codon usage of *BvFTI*. The coding sequence (CDS) of *BvFTI* is 540 base pairs (bp) long and consists of 180 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BvFTI</i> CDS ¹⁾ → 540 bp					180 AA ²⁾														
#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS
9	TTT	Phe	5.00	1.67	0	TCT	Ser	0.00	0.00	3	TAT	Tyr	1.67	0.56	1	TGT	Cys	0.56	0.19
2	TTC	Phe	1.11	0.37	1	TCC	Ser	0.56	0.19	3	TAC	Tyr	1.67	0.56	2	TGC	Cys	1.11	0.37
3	TTA	Leu	1.67	0.56	4	TCA	Ser	2.22	0.74	0	TAA	Stop	0.00	0.00	0	TGA	Stop	0.00	0.00
6	TTG	Leu	3.33	1.11	0	TCG	Ser	0.00	0.00	1	TAG	Stop	0.56	0.19	1	TGG	Trp	0.56	0.19
2	CTT	Leu	1.11	0.37	4	CCT	Pro	2.22	0.74	2	CAT	His	1.11	0.37	0	CGT	Arg	0.00	0.00
2	CTC	Leu	1.11	0.37	2	CCC	Pro	1.11	0.37	1	CAC	His	0.56	0.19	1	CGC	Arg	0.56	0.19
0	CTA	Leu	0.00	0.00	8	CCA	Pro	4.44	1.48	9	CAA	Gln	5.00	1.67	2	CGA	Arg	1.11	0.37
0	CTG	Leu	0.00	0.00	1	CCG	Pro	0.56	0.19	0	CAG	Gln	0.00	0.00	1	CGG	Arg	0.56	0.19
3	ATT	Ile	1.67	0.56	4	ACT	Thr	2.22	0.74	9	AAT	Asn	5.00	1.67	3	AGT	Ser	1.67	0.56
1	ATC	Ile	0.56	0.19	2	ACC	Thr	1.11	0.37	4	AAC	Asn	2.22	0.74	2	AGC	Ser	1.11	0.37
1	ATA	Ile	0.56	0.19	3	ACA	Thr	1.67	0.56	1	AAA	Lys	0.56	0.19	6	AGA	Arg	3.33	1.11
2	ATG	Start	1.11	0.37	1	ACG	Thr	0.56	0.19	0	AAG	Lys	0.00	0.00	7	AGG	Arg	3.89	1.30
7	GTT	Val	3.89	1.30	5	GCT	Ala	2.78	0.93	7	GAT	Asp	3.89	1.30	4	GGT	Gly	2.22	0.74
3	GTC	Val	1.67	0.56	0	GCC	Ala	0.00	0.00	2	GAC	Asp	1.11	0.37	2	GGC	Gly	1.11	0.37
6	GTA	Val	3.33	1.11	3	GCA	Ala	1.67	0.56	5	GAA	Glu	2.78	0.93	9	GGA	Gly	5.00	1.67
1	GTG	Val	0.56	0.19	1	GCG	Ala	0.56	0.19	4	GAG	Glu	2.22	0.74	1	GGG	Gly	0.56	0.19

1) Coding sequence; 2) Amino acid

Supplementary Table 7: Codon usage of *BvFT2*. The coding sequence (CDS) of *BvFT2* is 528 base pairs (bp) long and consists of 176 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BvFT2</i> CDS ¹⁾ → 528 bp					176 AA ²⁾														
#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS
4	TTT	Phe	2.27	0.76	2	TCT	Ser	1.14	0.38	5	TAT	Tyr	2.84	0.95	2	TGT	Cys	1.14	0.38
4	TTC	Phe	2.27	0.76	0	TCC	Ser	0.00	0.00	2	TAC	Tyr	1.14	0.38	1	TGC	Cys	0.57	0.19
2	TTA	Leu	1.14	0.38	2	TCA	Ser	1.14	0.38	1	TAA	Stop	0.57	0.19	0	TGA	Stop	0.00	0.00
7	TTG	Leu	3.98	1.33	0	TCG	Ser	0.00	0.00	0	TAG	Stop	0.00	0.00	2	TGG	Trp	1.14	0.38
5	CTT	Leu	2.84	0.95	4	CCT	Pro	2.27	0.76	1	CAT	His	0.57	0.19	1	CGT	Arg	0.57	0.19
0	CTC	Leu	0.00	0.00	1	CCC	Pro	0.57	0.19	2	CAC	His	1.14	0.38	0	CGC	Arg	0.00	0.00
1	CTA	Leu	0.57	0.19	9	CCA	Pro	5.11	1.70	6	CAA	Gln	3.41	1.14	2	CGA	Arg	1.14	0.38
1	CTG	Leu	0.57	0.19	1	CCG	Pro	0.57	0.19	1	CAG	Gln	0.57	0.19	0	CGG	Arg	0.00	0.00
1	ATT	Ile	0.57	0.19	5	ACT	Thr	2.84	0.95	7	AAT	Asn	3.98	1.33	2	AGT	Ser	1.14	0.38
1	ATC	Ile	0.57	0.19	2	ACC	Thr	1.14	0.38	4	AAC	Asn	2.27	0.76	3	AGC	Ser	1.70	0.57
2	ATA	Ile	1.14	0.38	1	ACA	Thr	0.57	0.19	0	AAA	Lys	0.00	0.00	11	AGA	Arg	6.25	2.08
2	ATG	Start	1.14	0.38	0	ACG	Thr	0.00	0.00	0	AAG	Lys	0.00	0.00	5	AGG	Arg	2.84	0.95
13	GTT	Val	7.39	2.46	4	GCT	Ala	2.27	0.76	8	GAT	Asp	4.55	1.52	6	GGT	Gly	3.41	1.14
2	GTC	Val	1.14	0.38	0	GCC	Ala	0.00	0.00	2	GAC	Asp	1.14	0.38	2	GGC	Gly	1.14	0.38
2	GTA	Val	1.14	0.38	3	GCA	Ala	1.70	0.57	7	GAA	Glu	3.98	1.33	5	GGA	Gly	2.84	0.95
4	GTG	Val	2.27	0.76	0	GCG	Ala	0.00	0.00	0	GAG	Glu	0.00	0.00	3	GGG	Gly	1.70	0.57

1) Coding sequence; 2) Amino acid

Supplementary Table 8: Codon usage of *BvFVE*. The coding sequence (CDS) of *BvFVE* is 1,416 base pairs (bp) long and consists of 472 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BvFVE</i> CDS ¹⁾ → 1,416 bp					472 AA ²⁾														
#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS
10	TTT	Phe	2.12	0.71	15	TCT	Ser	3.18	1.06	3	TAT	Tyr	0.64	0.21	4	TGT	Cys	0.85	0.28
4	TTC	Phe	0.85	0.28	3	TCC	Ser	0.64	0.21	5	TAC	Tyr	1.06	0.35	6	TGC	Cys	1.27	0.42
4	TTA	Leu	0.85	0.28	10	TCA	Ser	2.12	0.71	1	TAA	Stop	0.21	0.07	0	TGA	Stop	0.00	0.00
7	TTG	Leu	1.48	0.49	1	TCG	Ser	0.21	0.07	0	TAG	Stop	0.00	0.00	13	TGG	Trp	2.75	0.92
10	CTT	Leu	2.12	0.71	8	CCT	Pro	1.69	0.56	15	CAT	His	3.18	1.06	3	CGT	Arg	0.64	0.21
7	CTC	Leu	1.48	0.49	3	CCC	Pro	0.64	0.21	6	CAC	His	1.27	0.42	2	CGC	Arg	0.42	0.14
2	CTA	Leu	0.42	0.14	12	CCA	Pro	2.54	0.85	3	CAA	Gln	0.64	0.21	5	CGA	Arg	1.06	0.35
7	CTG	Leu	1.48	0.49	1	CCG	Pro	0.21	0.07	11	CAG	Gln	2.33	0.78	1	CGG	Arg	0.21	0.07
10	ATT	Ile	2.12	0.71	11	ACT	Thr	2.33	0.78	18	AAT	Asn	3.81	1.27	8	AGT	Ser	1.69	0.56
5	ATC	Ile	1.06	0.35	3	ACC	Thr	0.64	0.21	7	AAC	Asn	1.48	0.49	7	AGC	Ser	1.48	0.49
10	ATA	Ile	2.12	0.71	8	ACA	Thr	1.69	0.56	12	AAA	Lys	2.54	0.85	8	AGA	Arg	1.69	0.56
4	ATG	Start	0.85	0.28	3	ACG	Thr	0.64	0.21	12	AAG	Lys	2.54	0.85	2	AGG	Arg	0.42	0.14
18	GTT	Val	3.81	1.27	18	GCT	Ala	3.81	1.27	27	GAT	Asp	5.72	1.91	8	GGT	Gly	1.69	0.56
4	GTC	Val	0.85	0.28	3	GCC	Ala	0.64	0.21	8	GAC	Asp	1.69	0.56	6	GGC	Gly	1.27	0.42
4	GTA	Val	0.85	0.28	10	GCA	Ala	2.12	0.71	12	GAA	Glu	2.54	0.85	16	GGA	Gly	3.39	1.13
8	GTG	Val	1.69	0.56	3	GCG	Ala	0.64	0.21	12	GAG	Glu	2.54	0.85	5	GGG	Gly	1.06	0.35

1) Coding sequence; 2) Amino acid

Supplementary Table 9: Codon usage of *BvLHP1*. The coding sequence (CDS) of *BvLHP1* is 1,230 base pairs (bp) long and consists of 410 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BvLHP1</i> CDS ¹⁾ → 1,230 bp					410 AA ²⁾														
#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS
8	TTT	Phe	1.95	0.65	15	TCT	Ser	3.66	1.22	10	TAT	Tyr	2.44	0.81	5	TGT	Cys	1.22	0.41
2	TTC	Phe	0.49	0.16	4	TCC	Ser	0.98	0.33	3	TAC	Tyr	0.73	0.24	4	TGC	Cys	0.98	0.33
5	TTA	Leu	1.22	0.41	5	TCA	Ser	1.22	0.41	0	TAA	Stop	0.00	0.00	1	TGA	Stop	0.24	0.08
4	TTG	Leu	0.98	0.33	2	TCG	Ser	0.49	0.16	0	TAG	Stop	0.00	0.00	2	TGG	Trp	0.49	0.16
5	CTT	Leu	1.22	0.41	10	CCT	Pro	2.44	0.81	6	CAT	His	1.46	0.49	6	CGT	Arg	1.46	0.49
3	CTC	Leu	0.73	0.24	1	CCC	Pro	0.24	0.08	3	CAC	His	0.73	0.24	1	CGC	Arg	0.24	0.08
2	CTA	Leu	0.49	0.16	4	CCA	Pro	0.98	0.33	11	CAA	Gln	2.68	0.89	4	CGA	Arg	0.98	0.33
4	CTG	Leu	0.98	0.33	2	CCG	Pro	0.49	0.16	4	CAG	Gln	0.98	0.33	1	CGG	Arg	0.24	0.08
4	ATT	Ile	0.98	0.33	6	ACT	Thr	1.46	0.49	11	AAT	Asn	2.68	0.89	8	AGT	Ser	1.95	0.65
2	ATC	Ile	0.49	0.16	6	ACC	Thr	1.46	0.49	12	AAC	Asn	2.93	0.98	6	AGC	Ser	1.46	0.49
2	ATA	Ile	0.49	0.16	6	ACA	Thr	1.46	0.49	9	AAA	Lys	2.20	0.73	9	AGA	Arg	2.20	0.73
8	ATG	Start	1.95	0.65	5	ACG	Thr	1.22	0.41	23	AAG	Lys	5.61	1.87	7	AGG	Arg	1.71	0.57
12	GTT	Val	2.93	0.98	4	GCT	Ala	0.98	0.33	23	GAT	Asp	5.61	1.87	9	GGT	Gly	2.20	0.73
5	GTC	Val	1.22	0.41	2	GCC	Ala	0.49	0.16	8	GAC	Asp	1.95	0.65	3	GGC	Gly	0.73	0.24
8	GTA	Val	1.95	0.65	5	GCA	Ala	1.22	0.41	32	GAA	Glu	7.80	2.60	5	GGA	Gly	1.22	0.41
4	GTG	Val	0.98	0.33	7	GCG	Ala	1.71	0.57	18	GAG	Glu	4.39	1.46	4	GGG	Gly	0.98	0.33

1) Coding sequence; 2) Amino acid

Supplementary Table 10: Codon usage of *BvSWP*. The coding sequence (CDS) of *BvSWP* is 2,490 base pairs (bp) long and consists of 830 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BvSWP</i> CDS ¹⁾ → 2,490 bp					830 AA ²⁾														
#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS
26	TTT	Phe	3.30	1.10	15	TCT	Ser	1.90	0.63	15	TAT	Tyr	1.90	0.63	10	TGT	Cys	1.27	0.42
10	TTC	Phe	1.27	0.42	18	TCC	Ser	2.28	0.76	5	TAC	Tyr	0.63	0.21	4	TGC	Cys	0.51	0.17
18	TTA	Leu	2.28	0.76	18	TCA	Ser	2.28	0.76	1	TAA	Stop	0.13	0.04	0	TGA	Stop	0.00	0.00
27	TTG	Leu	3.42	1.14	5	TCG	Ser	0.63	0.21	0	TAG	Stop	0.00	0.00	5	TGG	Trp	0.63	0.21
20	CTT	Leu	2.53	0.84	18	CCT	Pro	2.28	0.76	6	CAT	His	0.76	0.25	7	CGT	Arg	0.89	0.30
12	CTC	Leu	1.52	0.51	14	CCC	Pro	1.77	0.59	6	CAC	His	0.76	0.25	10	CGC	Arg	1.27	0.42
2	CTA	Leu	0.25	0.08	13	CCA	Pro	1.65	0.55	8	CAA	Gln	1.01	0.34	4	CGA	Arg	0.51	0.17
6	CTG	Leu	0.76	0.25	4	CCG	Pro	0.51	0.17	9	CAG	Gln	1.14	0.38	7	CGG	Arg	0.89	0.30
17	ATT	Ile	2.15	0.72	12	ACT	Thr	1.52	0.51	26	AAT	Asn	3.30	1.10	10	AGT	Ser	1.27	0.42
12	ATC	Ile	1.52	0.51	33	ACC	Thr	4.18	1.39	10	AAC	Asn	1.27	0.42	6	AGC	Ser	0.76	0.25
6	ATA	Ile	0.76	0.25	11	ACA	Thr	1.39	0.46	12	AAA	Lys	1.52	0.51	7	AGA	Arg	0.89	0.30
18	ATG	Start	2.28	0.76	2	ACG	Thr	0.25	0.08	25	AAG	Lys	3.17	1.06	19	AGG	Arg	2.41	0.80
35	GTT	Val	4.44	1.48	24	GCT	Ala	3.04	1.01	39	GAT	Asp	4.94	1.65	22	GGT	Gly	2.79	0.93
8	GTC	Val	1.01	0.34	5	GCC	Ala	0.63	0.21	16	GAC	Asp	2.03	0.68	5	GGC	Gly	0.63	0.21
8	GTA	Val	1.01	0.34	18	GCA	Ala	2.28	0.76	18	GAA	Glu	2.28	0.76	15	GGA	Gly	1.90	0.63
10	GTG	Val	1.27	0.42	5	GCG	Ala	0.63	0.21	30	GAG	Glu	3.80	1.27	23	GGG	Gly	2.92	0.97

1) Coding sequence; 2) Amino acid

Supplementary Table 11: Codon usage of *BvTONI*. The coding sequence (CDS) of *BvTONI* is 798 base pairs (bp) long and consists of 266 amino acids (AA). The total number of each triplet as well as the corresponding amino acid is given. The percentage (%) per triplet and per CDS is shown.

<i>BvTONI</i> CDS ¹⁾ → 798 bp					266 AA ²⁾														
#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS	#	Codon	AA	per triplet %	per CDS
3	TTT	Phe	1.13	0.38	8	TCT	Ser	3.01	1.00	3	TAT	Tyr	1.13	0.38	2	TGT	Cys	0.75	0.25
2	TTC	Phe	0.75	0.25	2	TCC	Ser	0.75	0.25	4	TAC	Tyr	1.50	0.50	1	TGC	Cys	0.38	0.13
7	TTA	Leu	2.63	0.88	8	TCA	Ser	3.01	1.00	1	TAA	Stop	0.38	0.13	0	TGA	Stop	0.00	0.00
7	TTG	Leu	2.63	0.88	2	TCG	Ser	0.75	0.25	0	TAG	Stop	0.00	0.00	4	TGG	Trp	1.50	0.50
7	CTT	Leu	2.63	0.88	4	CCT	Pro	1.50	0.50	2	CAT	His	0.75	0.25	2	CGT	Arg	0.75	0.25
5	CTC	Leu	1.88	0.63	0	CCC	Pro	0.00	0.00	2	CAC	His	0.75	0.25	1	CGC	Arg	0.38	0.13
5	CTA	Leu	1.88	0.63	6	CCA	Pro	2.26	0.75	3	CAA	Gln	1.13	0.38	3	CGA	Arg	1.13	0.38
0	CTG	Leu	0.00	0.00	3	CCG	Pro	1.13	0.38	2	CAG	Gln	0.75	0.25	2	CGG	Arg	0.75	0.25
2	ATT	Ile	0.75	0.25	6	ACT	Thr	2.26	0.75	7	AAT	Asn	2.63	0.88	5	AGT	Ser	1.88	0.63
1	ATC	Ile	0.38	0.13	2	ACC	Thr	0.75	0.25	5	AAC	Asn	1.88	0.63	3	AGC	Ser	1.13	0.38
1	ATA	Ile	0.38	0.13	1	ACA	Thr	0.38	0.13	7	AAA	Lys	2.63	0.88	9	AGA	Arg	3.38	1.13
6	ATG	Start	2.26	0.75	2	ACG	Thr	0.75	0.25	7	AAG	Lys	2.63	0.88	6	AGG	Arg	2.26	0.75
7	GTT	Val	2.63	0.88	10	GCT	Ala	3.76	1.25	15	GAT	Asp	5.64	1.88	6	GGT	Gly	2.26	0.75
1	GTC	Val	0.38	0.13	1	GCC	Ala	0.38	0.13	5	GAC	Asp	1.88	0.63	2	GGC	Gly	0.75	0.25
1	GTA	Val	0.38	0.13	3	GCA	Ala	1.13	0.38	10	GAA	Glu	3.76	1.25	7	GGA	Gly	2.63	0.88
6	GTG	Val	2.26	0.75	2	GCG	Ala	0.75	0.25	14	GAG	Glu	5.26	1.75	5	GGG	Gly	1.88	0.63

1) Coding sequence; 2) Amino acid

Supplementary Table 12: 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 13: Results of EcoTILLING screens in the three genes *BTC1*, *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	

Supplementary Table 14: Non-reference nucleotide frequencies (NNFs) in three flowering time genes *BvFL1*, *BvFT1*, and *BTC1* 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
<i>BvFL1</i>	0.05	0.11	0.04	0.36	0.55	0.18
<i>BvFT1</i>	0.14	0.21	0.12	0.15	0.23	0.17
<i>BTC1</i>	0.05	0.03	0.04	0.06	0.3	0.07

Supplementary Table 15: Statistics of haplotypes for the two amplicons of *BvFL1* with significant differences in bolting rate 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 bolting rate, 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	B. v. maritima^{b)}
All	average Bolting Rate before winter	0%	1%	0%	4%	8%
FL1a_H0	n ^{a)}	66	32	42	20	7
	average Bolting Rate before winter	0%	1%	1%	5%	1%
FL1a_H6	n	-	4	-	5	3
	average Bolting Rate before winter	-	0%	-	5%	55%
	p-Value	-	0.93	-	1	<1e-06
FL1b_H0	n	64	16	40	12	5
	average Bolting Rate before winter	0%	1%	0%	8%	2%
FL1b_H5	n	-	-	-	-	1
	average Bolting Rate before winter	-	-	-	-	75%
	p-Value	-	-	-	-	<1e-04
FL1b_H6	n	-	-	2	4	2
	average Bolting Rate before winter	-	-	6%	0%	0%
	p-Value	-	-	<1e-09	0.461	1
FL1b_H10	n	-	1	1	2	2
	average Bolting Rate 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*

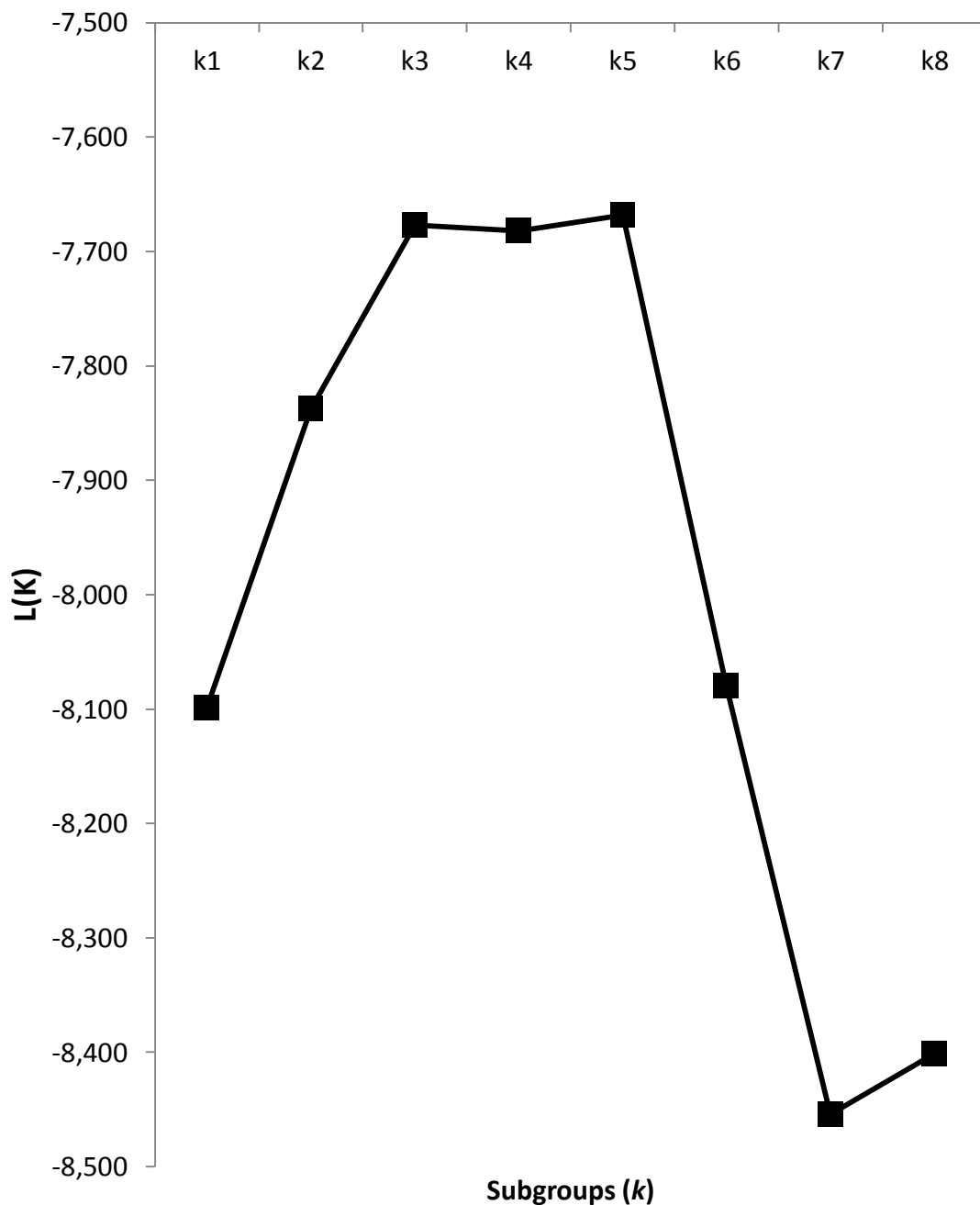
Supplementary Table 16: Statistical analysis of haplotypes on survival rate. Statistics of haplotypes for the two amplicons of *BvFL1* with significant differences in survival rate compared with the respective reference haplotypes (FL1a_H0 or FL1b_H0). Shown are the observed haplotypes, their occurrence (n) in each *B. vulgaris* form, the average survival rate, 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	B. v. maritima^{b)}
All	average Survival Rate	40%	23%	20%	29%	40%
FL1a_H0	n ^{a)}	66	32	42	20	7
	average Survival Rate	39%	23%	21%	22%	39%
FL1a_H6	n	-	4	-	5	3
	average Survival Rate	-	21%	-	35%	13%
	p-Value	-	1	-	0.339	0.0143
FL1b_H0	n	64	16	40	12	5
	average Survival Rate	39%	27%	20%	19%	36%
FL1b_H3	n	1	-	-	9	4
	average Survival Rate	40%	-	-	37%	43%
	p-Value	1	-	-	0.0116	1

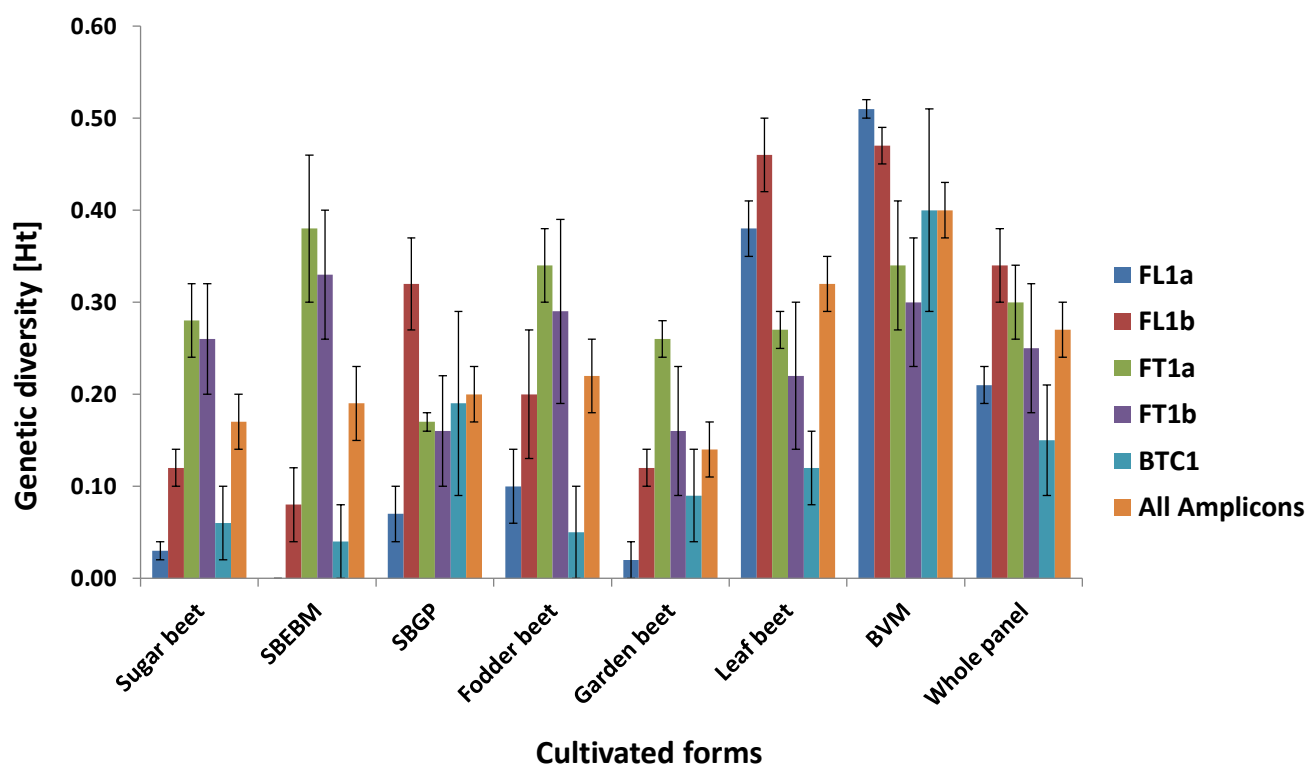
a) n: Number of accessions carrying the given haplotype

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

7.2 Supplementary Figures



Supplementary Figure 1: Diagram of mean Log probability for subpopulation calculation. 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 $L(K)$.



Supplementary Figure 2: Average gene diversity in divergent *B. vulgaris* forms. Comparison of average gene diversity (Ht) among divergent *B. vulgaris* forms for EcoTILLING amplicons of *BvFL1*, *BvFT1* and *BTC1*. Shown are the gene diversity (Ht) and the standard error (SE) value for each amplicon and across all amplicons within each *B. vulgaris* form as well as across the whole panel. Gene diversity was estimated with the genetic distance and phylogenetic analysis package DISPAN (Ota, 1993).

(A)

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FL1a_H0      TCGGACTTTCCTATAAGCTTAAGAAAATAAATGAATGATACTATAAATAGATAAAAAATA
FL1a_H6      TCGGACTTTCCTATAAGCTTAAGAAAATAAATGAATGATACTATAAATAGATAAAAAATA

FL1a_H0      GCCTAAATAGGGCATCCACAGCTAGTAAGGAGATAAACTGATAAACTAAAAGTCTAAAAC
FL1a_H6      GCCTAAATAGGGCATCCACAGCTAGTAAGGAGATAAACTGATAAACTAAAAGTCTAAAAC

FL1a_H0      TAAAACGAAAACGATAAGTTGCACTAAAATCTAATACTTATCTAACATTTCCACGTGTAA
FL1a_H6      TAAAACGAAAACGATAAGTTGCACTAAAATCTAATACTTATCTAACATTTCCACGTGTAA

FL1a_H0      TAAATAAAACATGGGGCCCAATGATTAATCTTCAACTGACCTCTCCCTCCAATCGCGTAT
FL1a_H6      TAAATAAAACATGGGGCCCAATGATTAATCTTCAACTGACCTCTCCCTCCAATCGCGTAT

FL1a_H0      TCTAAAAATCATTTCACATCTCTTTCATCTTTTTTTTTTCCGGCATTTTTTTTTCATTTT
FL1a_H6      TCTAAAAATCATTTCACATCTCTTTCATCTTTTTTTTTTCCGGCATTTTTTTTTCATTTT

FL1a_H0      CTCTCTCTTACTCTATACTACTCTAGGGTCTATATGATACTAGTATTTCAGTAGTAGCA
FL1a_H6      CTCTCTCTTACTCTATACTACTCTAGGGTCTATATGATACTAGTATTTCAGTAGTAGCA

FL1a_H0      GTAATAGAAGTACTATCAGTTTTCTCTCCTTTTGAAATAAAATAGTATTCTATTCAAT
FL1a_H6      GTAATAGAAGTACTATCAGTTTTCTCTCCTTTTGAAATAAAATAGTATTCTATTCAAT

FL1a_H0      TAAAATCGATCCACTAGTCTAAATACTAGTACTATTATCAGAGGAGAAGAAAGGACAGAG
FL1a_H6      TAAAATCGATCCACTAGTCTAAATACTAGTACTATTATCAGAGGAGAAGAAAGGACAGAG

FL1a_H0      AGAGTGAGAGAAATGCAGCGCAGAAAGACAGAGAAAGGTATTTGGATAAGGATGGGAAGA
FL1a_H6      AGAGTGAGAGAAATGCAGCGCAGAAAGACAGAGAAAGGTATTTGGATAAGGATGGGAAGA

FL1a_H0      AGGAAGATAGAGATGAAAAGAATTGAAGATAAAAGTAGTCGTCARGTTACATTTTCAAAG
FL1a_H6      AGGAAGATAGAGATGAAAAGAATTGAAGATAAAAGTAGTCGTCARGTTACATTTTCAAAG

FL1a_H0      CGGCGTTCTGGTCTTATCAAAAAGCTCGCGAAGTCTCTATCCTTTGTGATGTCGATGTT
FL1a_H6      CGGCGTTCTGGTCTTATCAAAAAGCTCGCGAAGTCTCTATCCTTTGTGATGTCGATGTT

FL1a_H0      GCTGTTCTTGTTTTCTCTAATCGTGGCCGTCTTTACGAATTCGTCAATAGTTCTTCTTCT
FL1a_H6      GCTGTTCTTGTTTTCTCTAATCGTGGCCGTCTTTACGAATTCGTCAATAGTTCTTCTTCT

FL1a_H0      TCCAGGTTTTTCTTCTATCTCTTTCCTTGTATTAGTTTTTTCTTTTCAATTTTCAAGTT
FL1a_H6      TCCAGGTTTTTCTTCTATCTCTTTCCTTGTATTAGTTTTTTCTTTTCAATTTTCAAGTT

FL1a_H0      TAATTTTAAATGGTATGCTTTGTTGAATAGTTTCGGTGTAAATGGCGGAATTTGTTGTT
FL1a_H6      TAATTTTAAATGGTATGCTTTGTTGAATAGTTTCGGTGTAAATGGCGGAATTTGTTGTT
                                     *

FL1a_H0      TAGTTTTTTTCGGTGTGTTTTGTTGTTTTGTGATTCTAGGTTTTAGATGATTTTGCTTGAT
FL1a_H6      TAGTTTTTTTCGGTGTGTTTTGTTGTTTTGTGATTCTAGGTTTTAGATGATTTTGCTTGAT

FL1a_H0      TTCATGAATTTTGAATTTTGGAGGTTTTTGGTTTTACCGTATAAAATGAATGATTTTGT
FL1a_H6      TTCATGAATTTTGAATTTTGGAGGTTTTTGGTTTTACCGTATAAAATGAATGATTTTGT

FL1a_H0      CTGTAACGATTCACGTG
FL1a_H6      CTGTAACGATTCACGTG
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(B)

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FL1b_H0      GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTTATT
FL1b_H03    GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTTATT
FL1b_H05    GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTTATT
FL1b_H06    GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTTATT
FL1b_H10    GCTGATAGTCTGTCCCTTTTGTCAATCAGAGAGTTCTTTGTCTCCAATGTCATTCTTTATT

FL1b_H0      CAAGGCCATGAATAAATTTTCTACTTTGAATATTCATAATTCATATCCTTCATGCTTC
FL1b_H03    CAAGGCCATGAATAAATTTTCTACTTTGAATATTCATAATTCATATCCTTCATGCTTC
FL1b_H05    CAAGGCCATGAATAAATTTTCTACTTTGAATATTCATAATTCATATCCTTCATGCTTC
FL1b_H06    CAAGGCCATGAATAAATTTTCTACTTTGAATATTCATAATTCATATCCTTCATGCTTC
FL1b_H10    CAAGGCCATGAATAAATTTTCTACTTTGAATATTCATAATTCATATCCTTCATGCTTC

FL1b_H0      TGTCCGTACAACCTTTTCGTTCTATTCTCTGACAATTCGGAGTTCTTTTGTCTTTTGATA
FL1b_H03    TGTCCGTACAACCTTTTCGTTCTATTCTCTGACAATTCGGAGTTCTTTTGTCTTTTGATA
FL1b_H05    TGTCCGTACAACCTTTTCGTTCTATTCTCTGACAATTCGGAGTTCTTTTGTCTTTTGATA
FL1b_H06    TGTCCGTACAACCTTTTCGTTCTATTCTCTGACAATTCGGAGTTCTTTTGTCTTTTGATA
FL1b_H10    TGTCCGTACAACCTTTTCGTTCTATTCTCTGACAATTCGGAGTTCTTTTGTCTTTTGATA

FL1b_H0      GCAGAGTTCACCTTCTAGTTGTGCAGAAGTCAAACATGTGGTGAGCTAGTAAAATCAGT
FL1b_H03    GCAGAGTTCACCTTCTAGTTGTGCAGAAGTCAAACATGTGGTGAGCTAGTAAAATCAGT
FL1b_H05    GCAGAGTTCACCTTCTAGTTGTGCAGAAGTCAAACATGTGGTGAGCTAGTAAAATCAGT
FL1b_H06    GCAGAGTTCACCTTCTAGTTGTGCAGAAGTCAAACATGTGGTGAGCTAGTAAAATCAGT
FL1b_H10    GCAGAGTTCACCTTCTAGTTGTGCAGAAGTCAAACATGTGGTGAGCTAGTAAAATCAGT
*

FL1b_H0      TGAAGGGCAAGTACTCAATTTTCTATTTACTTCTGACGATGACTTCTCCATGTTCCATAA
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    TTCATGAGGCTGGAGAGGCAACTAGCTGATGCCCTTATACAGACCAGAACCCGAAAGGTT
FL1b_H05    TTCATGAGGCTGGAGAGGCAACTAGCTGATGCCCTTATACAGACCAGAACCCGAAAGGTT
FL1b_H06    TTCATGAGGCTGGAGAGGCAACTAGCTGATGCCCTTATACAGACCAGAACCCGAAAGGTT
FL1b_H10    TTCATGAGGCTGGAGAGGCAACTAGCTGATGCCCTTATACAGACCAGAACCCGAAAGGTT
*

FL1b_H0      CTCTTCTCCCTTGTAACCATTAGTTTGTATGATCTACCACGTGGTTGGGTTGGAAGCT
FL1b_H03    CTCTTCTCCCTTGTAACCATTAGTTTGTATGATCTACCACGTGGTTGGGTTGGAAGCT
FL1b_H05    CTCTTCTCCCTTGTAACCATTAGTTTGTATGATCTACCACGTGGTTGGGTTGGAAGCT
FL1b_H06    CTCTTCTCCCTTGTAACCATTAGTTTGTATGATCTACCACGTGGTTGGGTTGGAAGCT
FL1b_H10    CTCTTCTCCCTTGTAACCATTAGTTTGTATGATCTACCACGTGGTTGGGTTGGAAGCT
*

FL1b_H0      GGATAGTTTATATGCTTTACCCTTTCTCTAGCTTACACTTTGCATATTTGATGGATGG
FL1b_H03    GGATAGTTTATATGCTTTACCCTTTCTCTAGCTTACACTTTGCATATTTGATGGATGG
FL1b_H05    GGATAGTTTATATGCTTTACCCTTTCTCTAGCTTACACTTTGCATATTTGATGGATGG
FL1b_H06    GGATAGTTTATATGCTTTACCCTTTCTCTAGCTTACACTTTGCATATTTGATGGATGG
FL1b_H10    GGATAGTTTATATGCTTTACCCTTTCTCTAGCTTACACTTTGCATATTTGATGGATGG
* * *

FL1b_H0      AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG
FL1b_H03    AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG
FL1b_H05    AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG
FL1b_H06    AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG
FL1b_H10    AGGTTCACTAATTTAAATTTGCAAGATCAAAGAGCTACTGTTCTCTGAAGCAAAGATGCG

FL1b_H0      ACTGTATTTTATGCATCGTGGTGTGGAGTCA
FL1b_H03    ACTGTATTTTATGCATCGTGGTGTGGAGTCA
FL1b_H05    ACTGTATTTTATGCATCGTGGTGTGGAGTCA
FL1b_H06    ACTGTATTTTATGCATCGTGGTGTGGAGTCA
FL1b_H10    ACTGTATTTTATGCATCGTGGTGTGGAGTCA

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Supplementary Figure 3: 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.

8 References

- Abe, J., G.P. Guan, and Y. Shimamoto, 1997: A gene complex for annual habit in sugar beet (*Beta vulgaris* L). *Euphytica* **94**, 129-135.
- Abe, M., Y. Kobayashi, S. Yamamoto, Y. Daimon, A. Yamaguchi, Y. Ikeda, H. Ichinoki, M. Notaguchi, K. Goto, and T. Araki, 2005: FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* **309**, 1052-1056.
- Abegg, F., 1936: A genetic factor for the annual habit in beets and linkage relationships. *Journal Agricultural Research* **53**, 493-511.
- Abou-Elwafa, S.F., B. Büttner, F. Kopsisch-Obuch, C. Jung, and A.E. Müller, 2011: Genetic identification of a novel bolting locus in *Beta vulgaris* which promotes annuality independently of the bolting gene *B*. *Molecular Breeding*, 1-10.
- Abou-Elwafa, S.F., B. Büttner, T.Y.P. Chia, G. Schulze-Buxloh, U. Hohmann, E. Mutasa-Göttgens, C. Jung, and A.E. Müller, 2012: Conservation and divergence of autonomous pathway genes in the flowering regulatory network of *Beta vulgaris*. *Journal of Experimental Botany* **62**, 3359-3374.
- Adrian, J., S. Farrona, J.J. Reimer, M.C. Albani, G. Coupland, and F. Turck, 2010: Cis-regulatory elements and chromatin state coordinately control temporal and spatial expression of *FLOWERING LOCUS T* in Arabidopsis. *Plant Cell* **22**, 1425-1440.
- Alaey, M., R. Naderi, A. Vezvaei, A. Khalighi, and A. Salami, 2005: Comparing study between four different methods of genomic DNA extraction from *Cyclamen persicum* Mill. *International Journal of Agriculture & Biology* **7**.
- Alcantara, T.P., P.W. Bosland, and D.W. Smith, 1996: Ethyl methanesulfonate-induced seed mutagenesis of *Capsicum annuum*. *Journal of Heredity* **87**, 239-241.
- Almeida, R., and R.C. Allshire, 2005: RNA silencing and genome regulation. *Trends in Cell Biology* **15**, 251-258.
- Alonso, J.M., and J.R. Ecker, 2006: Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in Arabidopsis. *Nature Review Genetics* **7**, 524-536.
- Amasino, R.M., and S.D. Michaels, 2010: The timing of flowering. *Plant Physiology* **154**, 516-520.
- An, H.L., C. Roussot, P. Suarez-Lopez, L. Corbesler, C. Vincent, M. Pineiro, S. Hepworth, A. Mouradov, S. Justin, C. Turnbull, and G. Coupland, 2004: CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. *Development* **131**, 3615-3626.
- Andres, F., and G. Coupland, 2012: The genetic basis of flowering responses to seasonal cues. *Nature Review Genetics* **13**, 627-639.
- Anklam, E., F. Gadani, P. Heinze, H. Pijnenburg, and G. Van Den Eede, 2002: Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. *European Food Research and Technology* **214**, 3-26.

- Arumuganathan, K., and E. Earle, 1991: Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* **9**, 208-218.
- Auerbach, C., and J.M. Robson, 1944: Production of mutations by allyl isothiocyanate. *Nature* **154**, 81.
- Auerbach, C., and J.M. Robson, 1946: Chemical production of mutations. *Nature* **157**, 302.
- Ausin, I., C. Alonso-Blanco, J.A. Jarillo, L. Ruiz-Garcia, and J.M. Martinez-Zapater, 2004: Regulation of flowering time by FVE, a retinoblastoma-associated protein. *Nature Genetics* **36**, 162-166.
- Azimzadeh, J., P. Nacry, A. Christodoulidou, S. Drevensek, C. Camilleri, N. Amiour, F. Parcy, M. Pastuglia, and D. Bouchez, 2008: Arabidopsis TONNEAU1 proteins are essential for preprophase band formation and interact with centrin. *Plant Cell* **20**, 2146-2159.
- Baek, I.S., H.Y. Park, M.K. You, J.H. Lee, and J.K. Kim, 2008: Functional conservation and divergence of *FVE* genes that control flowering time and cold response in rice and Arabidopsis. *Molecules and Cells* **26**, 368-372.
- Barkley, N.A., M. Wang, A. Gillaspie, R. Dean, G. Pederson, and T. Jenkins, 2008: Discovering and verifying DNA polymorphisms in a mung bean [*V. radiata* (L.) R. Wilczek] collection by EcoTILLING and sequencing. *BMC Research Notes* **1**, 28.
- Barzen, E., W. Mechelke, E. Ritter, J. Seitzer, and F. Salamini, 1992: Rflp markers for sugar beet breeding - chromosomal linkage maps and location of major genes for rhizomania resistance, monogerm and hypocotyl color. *Plant Journal* **2**, 601-611.
- Barzen, E., W. Mechelke, E. Ritter, E. Schultekappert, and F. Salamini, 1995: An extended map of the sugar beet genome containing rflp and rapd loci. *Theoretical and Applied Genetics* **90**, 189-193.
- Bäurle, I., and C. Dean, 2008: Differential interactions of the autonomous pathway RRM proteins and chromatin regulators in the silencing of Arabidopsis targets. *PLoS One* **3**, e2733.
- Bennet, M.D., I.J. Leitch, H.J. Price, and J.S. Johnston, 2003: Comparisons with *Caenorhabditis* (~100 Mb) and *Drosophila* (~175 Mb) using flow cytometry show genome size in Arabidopsis to be ~157 Mb and thus ~25 % larger than the Arabidopsis genome initiative estimate of ~125 Mb. *Annals of Botany* **91**, 547-557.
- Bernardo, R., 2008: Molecular markers and selection for complex traits in plants: Learning from the last 20 years. *Crop Science* **48**, 1649-1664.
- Bewley, J.D., 1997: Seed germination and dormancy. *The Plant Cell* **9**, 1055-1066.
- Biancardi, E., L.G. Campell, G.N. Skaracis, and M. De Biaggi, 2005: Genetics and breeding of sugar beet Science Publishers, Inc., Enfield.
- Biancardi, E., R.T. Lewellen, M. De Biaggi, A. Erichsen, and P. Stevanato, 2002: The origin of rhizomania resistance in sugar beet. *Euphytica* **127**, 383-397.
- Biancardi, E., J.M. McGrath, L. Panella, R.T. Lewellen, and P. Stevanato, 2010: Sugar beet, In: J. E. Bradshaw, (ed.) *Handbook of plant breeding: Root and tuber crops*, 173-219. Springer, New York.

- Bieniawska, Z., C. Espinoza, A. Schlereth, R. Sulpice, D.K. Hinch, and M.A. Hannah, 2008: Disruption of the *Arabidopsis* circadian clock is responsible for extensive variation in the cold-responsive transcriptome. *Plant Physiology* **147**, 263-279.
- Bohuon, E.J.R., L.D. Ramsay, J.A. Craft, A.E. Arthur, D.F. Marshall, D.J. Lydiate, and M.J. Kearsey, 1998: The association of flowering time quantitative trait loci with duplicated regions and candidate loci in *Brassica oleracea*. *Genetics* **150**, 393-401.
- Bossoutrot, D., and D. Hosemans, 1985: Gynogenesis in *Beta vulgaris* L.: From in vitro culture of unpollinated ovules to the production of doubled haploid plants in soil. *Plant Cell Reports* **4**, 300-303.
- Boualem, A., M. Fergany, R. Fernandez, C. Troadec, A. Martin, H. Morin, M.A. Sari, F. Collin, J.M. Flowers, M. Pitrat, M.D. Purugganan, C. Dogimont, and A. Bendahmane, 2008: A conserved mutation in an ethylene biosynthesis enzyme leads to andromonoecy in melons. *Science* **321**, 836-838.
- Boubriak, I.I., D.M. Grodzinsky, V.P. Polischuk, V.D. Naumenko, N.P. Gushcha, A.N. Micheev, S.J. McCready, and D.J. Osborne, 2008: Adaptation and impairment of DNA repair function in pollen of *Betula verrucosa* and seeds of *Oenothera biennis* from differently radionuclide-contaminated sites of Chernobyl. *Annals of Botany* **101**, 267-276.
- Boysen, G., B.F. Pachkowski, J. Nakamura, and J.A. Swenberg, 2009: The formation and biological significance of N7-guanine adducts. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **678**, 76-94.
- Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler, 2007: TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics* **23**, 2633-2635.
- Bray, C.M., and C.E. West, 2005: DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. *New Phytologist* **168**, 511-528.
- Britt, A.B., 1996: DNA damage and repair in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 75-100.
- Britt, A.B., 1999: Molecular genetics of DNA repair in higher plants. *Trends in Plant Science* **4**, 20-25.
- Burns, P.A., A.J.E. Gordon, K. Kunsmann, and B.W. Glickman, 1988: Influence of neighboring base sequence on the distribution and repair of N-ethyl-N-nitrosourea-induced lesions in *Escherichia coli*. *Cancer Research* **48**, 4455-4458.
- Burrell, M.M., 2003: Starch: the need for improved quality or quantity - an overview. *Journal of Experimental Botany* **54**, 451-456.
- Bush, S.M., and P.J. Krysan, 2010: iTILLING: A personalized approach to the identification of induced mutations in *Arabidopsis*. *Plant Physiology* **154**, 25-35.
- Büttner, B., S.F. Abou-Elwafa, W. Zhang, C. Jung, and A.E. Müller, 2010: A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*. *Theoretical and Applied Genetics* **121**, 1117-1131.

- Caicedo, A.L., J.R. Stinchcombe, K.M. Olsen, J. Schmitt, and M.D. Purugganan, 2004: Epistatic interaction between *Arabidopsis FRI* and *FLC* flowering time genes generates a latitudinal cline in a life history trait. *Proceedings of the National Academy of Sciences* **101**, 15670-15675.
- Caldwell, D.G., N. McCallum, P. Shaw, G.J. Muehlbauer, D.F. Marshall, and R. Waugh, 2004: A structured mutant population for forward and reverse genetics in Barley (*Hordeum vulgare* L.). *Plant Journal* **40**, 143-150.
- Causier, B., Z. Schwarz-Sommer, and B. Davies, 2010: Floral organ identity: 20 years of ABCs. *Seminars in Cell & Developmental Biology* **21**, 73-79.
- Ceccarelli, S., E.P. Guimarães, and E. Weltzien, 2009: Plant breeding and farmer participation Food and Agriculture Organization of the United Nations (FAO), Rome.
- Chawade, A., P. Sikora, M. Brautigam, M. Larsson, V. Vivekanand, M. Nakash, T. Chen, and O. Olsson, 2010: Development and characterization of an oat TILLING-population and identification of mutations in lignin and beta-glucan biosynthesis genes. *BMC Plant Biology* **10**, 1-13.
- Cheng, X., and M. Gao, 1988: Biological and genetic effects of combined treatments of sodium azide, gamma rays and EMS in barley. *Environmental and Experimental Botany* **28**, 281-288.
- Cheung, W.Y., L. Friesen, G.F.W. Rakow, G. Séguin-Swartz, and B.S. Landry, 1997: A RFLP-based linkage map of mustard [*Brassica juncea* (L.) Czern. and Coss.]. *Theoretical and Applied Genetics* **94**, 841-851.
- Chia, T.Y.P., A. Muller, C. Jung, and E. Mutasa-Gottgens, 2008: Sugar beet contains a large CONSTANS-LIKE gene family including a CO homologue that is independent of the early-bolting (*B*) gene locus. *Journal of Experimental Botany* **59**, 2735-2748.
- Choi, K., J. Kim, H.-J. Hwang, S.Y. Kim, C. Park, S.Y. Kim, and I. Lee, 2011: The FRIGIDA complex activates transcription of *FLC*, a strong flowering repressor in *Arabidopsis*, by recruiting chromatin modification factors. *Plant Cell* **23**, 289-303.
- Choi, K., S. Kim, S.Y. Kim, M. Kim, Y. Hyun, H. Lee, S. Choe, S.G. Kim, S. Michaels, and I. Lee, 2005: *SUPPRESSOR OF FRIGIDA3* encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in *Arabidopsis*. *Plant Cell* **17**, 2647-2660.
- Colbert, T., B.J. Till, R. Tompa, S. Reynolds, M.N. Steine, A.T. Yeung, C.M. McCallum, L. Comai, and S. Henikoff, 2001: High-throughput screening for induced point mutations. *Plant Physiology* **126**, 480-484.
- Comai, L., K. Young, B.J. Till, S.H. Reynolds, E.A. Greene, C.A. Codomo, L.C. Enns, J.E. Johnson, C. Burtner, A.R. Odden, and S. Henikoff, 2004: Efficient discovery of DNA polymorphisms in natural populations by EcoTILLING. *Plant Journal* **37**, 778-786.
- Cooper, J.L., B.J. Till, R.G. Laport, M.C. Darlow, J.M. Kleffner, A. Jamai, T. El-Mellouki, S. Liu, R. Ritchie, N. Nielsen, K.D. Bilyeu, K. Meksem, L. Comai, and S. Henikoff, 2008: TILLING to detect induced mutations in soybean. *BMC Plant Biology* **8**, 1-10.
- Corbesier, L., C. Vincent, S. Jang, F. Fornara, Q. Fan, I. Searle, A. Giakountis, S. Farrona, L. Gissot, C. Turnbull, and G. Coupland, 2007: FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science* **316**, 1030-1033.

- Córdoba-Cañero, D., T. Morales-Ruiz, T. Roldán-Arjona, and R.R. Ariza, 2009: Single-nucleotide and long-patch base excision repair of DNA damage in plants. *The Plant Journal* **60**, 716-728.
- Csaikl, U.M., H. Bastian, R. Brettschneider, S. Gauch, A. Meir, M. Schauerte, F. Scholz, C. Sperisen, B. Vornam, and B. Ziegenhagen, 1998: Comparative analysis of different DNA extraction protocols: A fast, universal maxi-preparation of high quality plant DNA for genetic evaluation and phylogenetic studies. *Plant Molecular Biology Reporter* **16**, 69-86.
- Dahmani-Mardas, F., C. Troadec, A. Boualem, S. Leveque, A.A. Alsadon, A.A. Aldoss, C. Dogimont, and A. Bendahmane, 2010: Engineering melon plants with improved fruit shelf life using the TILLING approach. *PLoS One* **5**, e15776.
- Dalmais, M., J. Schmidt, S.C. Le, F. Moussy, J. Burstin, V. Savoie, G. Aubert, V. Brunaud, O.Y. de, C. Guichard, R. Thompson, and A. Bendahmane, 2008: UTILdb, a *Pisum sativum* in silico forward and reverse genetics tool. *Genome Biology* **9**, 1-12.
- Dany, A.-L., T. Douki, C. Triantaphylides, and J. Cadet, 2001: Repair of the main UV-induced thymine dimeric lesions within *Arabidopsis thaliana* DNA: evidence for the major involvement of photoreactivation pathways. *Journal of Photochemistry and Photobiology B: Biology* **65**, 127-135.
- Darwin, C., 1859: *On the origin of species by means of natural selection* J. Murray, London, UK.
- de Laat, W.L., N.G.J. Jaspers, and J.H.J. Hoeijmakers, 1999: Molecular mechanism of nucleotide excision repair. *Genes & Development* **13**, 768-785.
- De Riek, J., E. Calsyn, I. Everaert, E. Van Bockstaele, and M. De Loose, 2001: AFLP based alternatives for the assessment of distinctness, uniformity and stability of sugar beet varieties. *Theoretical and Applied Genetics* **103**, 1254-1265.
- de Vries, H., 1901: *Die Entstehung der Arten durch Mutationen* Veit &Comp., Leipzig.
- de Wind, N., and J.B. Hays, 2001: Mismatch repair: praying for genome stability. *Current Biology* **11**, R545-R548.
- Del-Favero, J., M. Vauterin, G. Weyens, K.E. Edwards, and M. Jacobs, 1994: Construction and characterisation of a yeast artificial chromosome library containing five haploid sugarbeet (*Beta vulgaris* L.) genome equivalents. *Theoretical and Applied Genetics* **88**, 449-453.
- Deng, W., H. Ying, C.A. Helliwell, J.M. Taylor, W.J. Peacock, and E.S. Dennis, 2011: FLOWERING LOCUS C (FLC) regulates development pathways throughout the life cycle of *Arabidopsis*. *Proceedings of the National Academy of Sciences* **108**, 6680-6685.
- Dennis, E.S., and W.J. Peacock, 2007: Epigenetic regulation of flowering. *Current Opinion in Plant Biology* **10**, 520-527.
- Dertinger, H., and H. Jung, 1970: *Molecular radiation biology* Springer Verlag, New York.
- Dhondt, S., H. Vanhaeren, D. Van Loo, V. Cnudde, and D. Inzé, 2010: Plant structure visualization by high-resolution X-ray computed tomography. *Trends in Plant Science* **15**, 419-422.

- Dieffenbach, C.W., T.M. Lowe, and G.S. Dveksler, 1993: General concepts for PCR primer design. *Genome Research* **3**, S30-S37.
- Dohm, J.C., C. Lange, D. Holtgräwe, T. Rosleff Sörensen, D.C. Borchardt, B. Schulz, H. Lehrach, B. Weisshaar, and H. Himmelbauer, 2011: Palaeohexaploid ancestry for Caryophyllales inferred from extensive gene-based physical and genetic mapping of the sugar beet genome (*Beta vulgaris*). *Plant Journal* **70**, 528-540.
- Dong, C., J. Dalton-Morgan, K. Vincent, and P. Sharp, 2009: A modified TILLING method for wheat breeding. *Plant Genome* **2**, 39-47.
- Earl, D., and B. von Holdt, 2012: STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* **4**, 359-361.
- El-Mezawy, A., F. Dreyer, G. Jacobs, and C. Jung, 2002: High resolution mapping of the bolting gene *B* of sugar beet. *Theoretical and Applied Genetics* **105**, 100-105.
- Elias, R., B.J. Till, C. Mba, and B. Al-Safadi, 2009: Optimizing TILLING and EcoTILLING techniques for potato (*Solanum tuberosum* L). *BMC Research Notes* **2**, 1-5.
- Elsanhoty, R.M., M.F. Ramadan, and K.D. Jany, 2011: DNA extraction methods for detecting genetically modified foods: A comparative study. *Food Chemistry* **126**, 1883-1889.
- Engelbergs, J., J. Thomale, and M.F. Rajewsky, 2000: Role of DNA repair in carcinogen-induced ras mutation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **450**, 139-153.
- Eriksson, M.E., and A.A.R. Webb, 2011: Plant cell responses to cold are all about timing. *Current Opinion in Plant Biology* **14**, 731-737.
- Evanno, G., S. Regnaut, and J. Goudet, 2005: Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology* **14**, 2611-2620.
- Evans, L.T., 1993: *Crop evolution, adaption and yield* Cambridge University Press, NY, USA, New York.
- Eyers, M., K.E. Edwards, and W. Schuch, 1992: Construction and characterisation of a yeast artificial chromosome library containing two haploid *Beta vulgaris* L. genome equivalents. *Gene* **121**, 195-201.
- Falush, D., M. Stephens, and J.K. Pritchard, 2007: Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes* **7**, 574-578.
- Fang, X.H., S.H. Gu, Z.Y. Xu, F. Chen, D.D. Guo, H.B. Zhang, and N.H. Wu, 2004: Construction of a binary BAC library for an apomictic monosomic addition line of *Beta corolliflora* in sugar beet and identification of the clones derived from the alien chromosome. *Theoretical and Applied Genetics* **108**, 1420-1425.
- FAO, 2012a: FAOSTAT - Production - Crops - Sugar beet 2010, Vol. 2012.
- FAO, 2012b: FAOSTAT - Food and agricultural commodities production world wide 2010, Vol. 2012.

- FAO/IAEA, 1989: 25 years plant breeding and genetics section of the joint FAO/IAEA division, Vienna. Mutation Breeding Newsletter **34**, 2-3.
- Fenart, S., J.F. Arnaud, I. De Cauwer, and J. Cuguen, 2008: Nuclear and cytoplasmic genetic diversity in weed beet and sugar beet accessions compared to wild relatives: new insights into the genetic relationships within the *Beta vulgaris* complex species. Theoretical and Applied Genetics **116**, 1063-1077.
- Fischer, H.E., 1989: Origin of the 'Weisse Schlesische Rübe' (white Silesian beet) and resynthesis of sugar beet. Euphytica **41**, 75-80.
- Fitzgerald, T., K. Kazan, Z. Li, M. Morell, and J. Manners, 2010: A high-throughput method for the detection of homoeologous gene deletions in hexaploid wheat. BMC Plant Biology **10**, 1-15.
- Formosa, T., and B.M. Alberts, 1986: DNA synthesis dependent on genetic recombination: Characterization of a reaction catalyzed by purified bacteriophage T4 proteins. Cell **47**, 793-806.
- Fowler, D.B., A.E. Limin, A.J. Robertson, and L.V. Gusta, 1993: Breeding for low-temperature tolerance in field crops International Crop Science I., 357-362. Crops Science Society of America, Ames, Iowa.
- Franklin, K.A., and G.C. Whitelam, 2007: Light-quality regulation of freezing tolerance in *Arabidopsis thaliana*. Nature Genetics **39**, 1410-1413.
- Freisleben, R., and A. Lein, 1942: Über die Auffindung einer mehlttauresistenten Mutante nach Röntgenbestrahlung einer auffälligen Linie von Sommergerste. Die Naturwissenschaften **30**, 608.
- Frese, L., B. Desprez, and D. Ziegler, 2001: Potential of genetic resources and breeding strategies for base-broadening in *Beta*, -.
- Futsuhara, Y., K. Toriyama, and K. Tsunoda, 1967: On the breeding of rice variety reimei by irradiation of gamma rays. Japanese Journal of Breeding **17**, 85-90.
- Gady, A., F. Hermans, M. Van de Wal, E. van Loo, R. Visser, and C. Bachem, 2009: Implementation of two high through-put techniques in a novel application: detecting point mutations in large EMS mutated plant populations. Plant Methods **5**, 1-14.
- Gady, A., W. Vriezen, M. Van de Wal, P. Huang, A. Bovy, R. Visser, and C. Bachem, 2012: Induced point mutations in the phytoene synthase 1 gene cause differences in carotenoid content during tomato fruit ripening. Molecular Breeding **29**, 801-812.
- Galeano, C.H., M. Gomez, L.M. Rodriguez, and M.W. Blair, 2009: CEL I nuclease digestion for SNP discovery and marker development in common bean (*Phaseolus vulgaris* L.). Crop Science **49**, 381-394.
- Gepts, P., 2002: A comparison between crop domestication, classical plant breeding, and genetic engineering. Crop Science **42**, 1780-1790.
- Gerloff, U., C. Schlötterer, K. Rassmann, I. Rambold, G. Hohmann, B. Fruth, and D. Tautz, 1995: Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan paniscus*). Molecular Ecology **4**, 515-518.

- Gidner, S., B.L. Lennefors, N.O. Nilsson, J. Bensefelt, E. Johansson, U. Gyllenspetz, and T. Kraft, 2005: QTL mapping of BNYVV resistance from the WB41 source in sugar beet. *Genome* **48**, 279-285.
- Gilchrist, E.J., and G.W. Haughn, 2005: TILLING without a plough: a new method with applications for reverse genetics. *Current Opinion in Plant Biology* **8**, 211-215.
- Gilchrist, E.J., G.W. Haughn, C.C. Ying, S.P. Otto, J. Zhuang, D. Cheung, B. Hamberger, F. Aboutorabi, T. Kalynyak, L. Johnson, J. Bohlmann, B. Ellis, C.J. Douglas, and Q.C.B. Bronk, 2006: Use of Ecotilling as an efficient SNP discovery tool to survey genetic variation in wild populations of *Populus trichocarpa*. *Molecular Ecology* **15**, 1367-1378.
- Gilmour, S.J., S.G. Fowler, and M.F. Thomashow, 2004: *Arabidopsis* transcriptional activators *CBF1*, *CBF2*, and *CBF3* have matching functional activities. *Plant Molecular Biology* **54**, 767-781.
- Gindullis, F., D. Dechyeva, and T. Schmidt, 2001: Construction and characterization of a BAC library for the molecular dissection of a single wild beet centromere and sugar beet (*Beta vulgaris*) genome analysis. *Genome* **44**, 846-855.
- Goff, S.A., D. Ricke, T.-H. Lan, G. Presting, R. Wang, M. Dunn, J. Glazebrook, A. Sessions, P. Oeller, H. Varma, D. Hadley, D. Hutchison, C. Martin, F. Katagiri, B.M. Lange, T. Moughamer, Y. Xia, P. Budworth, J. Zhong, T. Miguel, U. Paszkowski, S. Zhang, M. Colbert, W.-I. Sun, L. Chen, B. Cooper, S.H. Park, T.C. Wood, L. Mao, P. Quail, R. Wing, R. Dean, Y. Yu, A. Zharkikh, R. Shen, S. Sahasrabudhe, A. Thomas, R. Cannings, A. Gutin, D. Pruss, J. Reid, S. Tavtigian, J. Mitchell, G. Eldredge, T. Scholl, R.M. Miller, S. Bhatnagar, N. Adey, T. Rubano, N. Tusneem, R. Robinson, J. Feldhaus, T. Macalma, A. Oliphant, and S. Briggs, 2002: A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science* **296**, 92-100.
- Gómez-Galera, S., R.M. Twyman, P.A.C. Sparrow, B. Van Droogenbroeck, R. Custers, T. Capell, and P. Christou, 2012: Field trials and tribulations—making sense of the regulations for experimental field trials of transgenic crops in Europe. *Plant Biotechnology Journal* **10**, 511-523.
- Gorbunova, V., and A.A. Levy, 1999: How plants make ends meet: DNA double-strand break repair. *Trends in Plant Science* **4**, 263-269.
- Gottwald, S., P. Bauer, T. Komatsuda, U. Lundqvist, and N. Stein, 2009: TILLING in the two-rowed barley cultivar 'Barke' reveals preferred sites of functional diversity in the gene *HvHox1*. *BMC Research Notes* **2**, 1-14.
- Greene, E.A., C.A. Codomo, N.E. Taylor, J.G. Henikoff, B.J. Till, S.H. Reynolds, L.C. Enns, C. Burtner, J.E. Johnson, A.R. Odden, L. Comai, and S. Henikoff, 2003: Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in *Arabidopsis*. *Genetics* **164**, 731-740.
- Greilhuber, J., T. Borsch, K. Müller, A. Worberg, S. Porembski, and W. Barthlott, 2006: Smallest angiosperm genomes found in *Lentibulariaceae*, with chromosomes of bacterial size. *Plant Biology* **8**, 770-777.
- Grimmer, M.K., K.M.R. Bean, and M.J.C. Asher, 2007: Mapping of five resistance genes to sugar-beet powdery mildew using AFLP and anchored SNP markers. *Theoretical and Applied Genetics* **115**, 67-75.
- Guiderdoni, E., 1991: Gametic selection in anther culture of rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* **81**, 406-412.

- Gundry, C.N., J.G. Vandersteen, G.H. Reed, R.J. Pryor, J. Chen, and C.T. Wittwer, 2003: Amplicon melting analysis with labeled primers: A closed-tube method for differentiating homozygotes and heterozygotes. *Clinical Chemistry* **49**, 396-406.
- Gustafson, Å., 1941: Mutation experiments in barley. *Hereditas* **27**, 225-242.
- Gustafson, Å., 1954: Mutations, viability, and population structure. *Acta Agriculturae Scandinavica* **4**, 601-632.
- Gustafson, Å., 1963: Productive mutations induced in barley by ionizing radiations and chemical mutagens. *Hereditas* **50**, 211-263.
- Hagihara, E., H. Matsuhira, M. Ueda, T. Mikami, and T. Kubo, 2005: Sugar beet BAC library construction and assembly of a contig spanning *Rfl*, a restorer-of-fertility gene for Owen cytoplasmic male sterility. *Molecular Genetics and Genomics* **274**, 316-323.
- Hansen, M., T. Kraft, M. Christiansson, and N.O. Nilsson, 1999: Evaluation of AFLP in Beta. *Theoretical and Applied Genetics* **98**, 845-852.
- Harloff, H.J., S. Lemcke, J. Mittasch, A. Frolov, J.G. Wu, F. Dreyer, G. Leckband, and C. Jung, 2011: A mutation screening platform for rapeseed (*Brassica napus* L.) and the detection of sinapine biosynthesis mutants. *Theoretical and Applied Genetics*.
- Harlow, G.R., M.E. Jenkins, T.S. Pittalwala, and D.W. Mount, 1994: Isolation of *uvh1*, an *Arabidopsis* mutant hypersensitive to ultraviolet light and ionizing radiation. *The Plant Cell Online* **6**, 227-35.
- Hartmann, A., T. Czauderna, R. Hoffmann, N. Stein, and F. Schreiber, 2011: HTPPheno: An image analysis pipeline for high-throughput plant phenotyping. *BMC Bioinformatics* **12**, 148.
- Heeraman, D., J. Hopmans, and V. Clausnitzer, 1997: Three dimensional imaging of plant roots in situ with X-ray computed tomography. *Plant and Soil* **189**, 167-179.
- Henikoff, S., and L. Comai, 2003: Single-nucleotide mutations for plant functional genomics. *Annual Review of Plant Biology* **54**, 375-401.
- Heo, J.B., and S. Sung, 2011: Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. *Science* **331**, 76-9.
- Hermann, K., J. Meinhard, P. Dobrev, A. Linkies, B. Pesek, B. Heß, I. Macháčková, U. Fischer, and G. Leubner-Metzger, 2007: 1-Aminocyclopropane-1-carboxylic acid and abscisic acid during the germination of sugar beet (*Beta vulgaris* L.): a comparative study of fruits and seeds. *Journal of Experimental Botany* **58**, 3047-3060.
- Hillman, G.C., and M.S. Davies, 1990: 6. Domestication rates in wild-type wheats and barley under primitive cultivation. *Biological Journal of the Linnean Society* **39**, 39-78.
- Himmelblau, E., E. Gilchrist, K. Buono, C. Bizzell, L. Mentzer, R. Vogelzang, T. Osborn, R. Amasino, I. Parkin, and G. Haughn, 2009: Forward and reverse genetics of rapid-cycling *Brassica oleracea*. *Theoretical and Applied Genetics* **118**, 953-961.

- Hjerdin, A., T. Sall, S. Tuvešson, and C. Hallden, 1994: RFLP markers in the genus *Beta* - characterization of DNA-sequences from a *Beta vulgaris* library. *Genetica* **92**, 91-99.
- Hoffmann, C.M., and S. Kluge-Severin, 2010: Light absorption and radiation use efficiency of autumn and spring sown sugar beets. *Field Crops Research* **119**, 238-244.
- Hoffmann, C.M., and S. Kluge-Severin, 2011: Growth analysis of autumn and spring sown sugar beet. *European Journal of Agronomy* **34**, 1-9.
- Hohmann, U., 2007: Schlußbericht: GABI-TILL: Establishment of a central platform for testing lead gene function in crops based on the TILLING technology, Teilprojekt: Kiel, 1-26, Vol. 1. Plant Breeding Institute, Kiel, Kiel.
- Hohmann, U., G. Jacobs, and C. Jung, 2005: An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. *Plant Breeding* **124**, 317-321.
- Hohmann, U., G. Jacobs, A. Telgmann, R. Gaafar, S. Alam, and C. Jung, 2003: A bacterial artificial chromosome (BAC) library of sugar beet and a physical map comprising the bolting gene *B*. *Molecular and General Genomics* **269**, 126-136.
- Hutton, W., J. Garbow, and T. Hayes, 1999: Nondestructive NMR determination of oil composition in transformed canola seeds. *Lipids* **34**, 1339-1346.
- IAEA, 2011: Joint FAO/IAEA programme database of mutant variety and genetic stocks, Vol. 2011.
- Jacob, F., and J. Monod, 1961: Genetic regulatory mechanisms in the synthesis of proteins. *Journal of Molecular Biology* **3**, 318-356.
- Jacobs, G., D. Dechyeva, T. Wenke, B. Weber, and T. Schmidt, 2009: A BAC library of *Beta vulgaris* L. for the targeted isolation of centromeric DNA and molecular cytogenetics of *Beta* species. *Genetica* **135**, 157-167.
- Jaeger, K.E., and P.A. Wigge, 2007: FT protein acts as a long-range signal in Arabidopsis. *Current Biology* **17**, 1050-1054.
- Jaggard, K.W., and A.R. Werker, 1999: An evaluation of the potential benefits and costs of autumn-sown sugarbeet in NW Europe. *Journal of Agricultural Science* **132**, 91-102.
- Jaggard, K.W., R. Wickens, D.J. Webb, and R.K. Scott, 1983: Effects of sowing date on plant establishment and bolting and the influence of these factors on yields of sugar beet. *Journal of Agricultural Science* **101**, 147-161.
- Johnston, J.S., A.E. Pepper, A.E. Hall, Z.J. Chen, G. Hodnett, J. Drabek, R. Lopez, and H.J. Price, 2005: Evolution of genome size in brassicaceae. *Annals of Botany* **95**, 229-235.
- Jones-Rhoades, M.W., D.P. Bartel, and B. Bartel, 2006: MicroRNAs and their regulatory roles in plants. *Annual Review of Plant Biology* **57**, 19-53.
- Jung, C., and A.E. Müller, 2009: Flowering time control and applications in plant breeding. *Trends in Plant Science* **14**, 563-573.

- Jung, C., K. Pillen, L. Frese, and A. Melchinger, 1993: Phylogenetic relationships between cultivated and wild species of the genus *Beta* revealed by DNA "fingerprinting". *Theoretical and Applied Genetics* **86**, 449-457.
- Kadaru, S., A. Yadav, R. Fjellstrom, and J. Oard, 2006: Alternative ecotilling protocol for rapid, cost-effective single-nucleotide polymorphism discovery and genotyping in rice (*Oryza sativa* L.). *Plant Molecular Biology Reporter* **24**, 3-22.
- Kadereit, J.W., S. Hohmann, and J.W. Kadereit, 2006: A synopsis of *Chenopodiaceae* subfam. *Betoideae* and notes on the taxonomy of *Beta*. *Willdenowia* **36**, 9-19.
- Kalendar, R., D. Lee, and A.H. Schulman, 2009: FastPCR software for PCR primer and probe design and repeat search. *Genes, Genomes and Genomics* **3**, 1-14.
- Kawai, T., and E. Amano, 1991: Plant mutation breeding for crop improvement, In: FAO/IAEA, (ed.) FAO/IAEA Symposium, 47-66, Vol. 1, Vienna.
- Keller, B., C. Feuillet, and N. Yahiaoui, 2005: Map-based isolation of disease resistance genes from bread wheat: cloning in a supsize genome. *Genetics Research* **85**, 93-100.
- Kimura, S., Y. Tahira, T. Ishibashi, Y. Mori, T. Mori, J. Hashimoto, and K. Sakaguchi, 2004: DNA repair in higher plants; photoreactivation is the major DNA repair pathway in non-proliferating cells while excision repair (nucleotide excision repair and base excision repair) is active in proliferating cells. *Nucleic Acids Research* **32**, 2760-2767.
- Kirchhoff, M., A. Svirshchevskaya, C. Hoffmann, A. Schechert, C. Jung, and F. Kopsisch-Obuch, 2012: High degree of genetic variation of winter hardiness in a panel of *Beta vulgaris* L. *Crop Science* **52**, 179-188.
- Klein-Lankhorst, R.M., E.M.J. Salentijn, W.G. Dirkse, M. Arens-de Reuver, and W.J. Stiekema, 1994: Construction of a YAC library from a *Beta vulgaris* fragment addition and isolation of a major satellite DNA cluster linked to the beet cyst nematode resistance locus *Hs1*. *Theoretical and Applied Genetics* **89**, 426-434.
- Kleine, M., D. Cai, C. Elbl, R.G. Herrmann, and C. Jung, 1995: Physical mapping and cloning of a translocation in sugar beet (*Beta vulgaris* L) carrying a gene for nematode (*Heterodera schachtii*) resistance from *B. procumbens*. *Theoretical and Applied Genetics* **90**, 399-406.
- Knippers, R., 2006: *Molekulare Genetik* Georg Thieme Verlag, Stuttgart.
- Knoll, J.E., M. Ramos, Y. Zeng, C. Holbrook, M. Chow, S. Chen, S. Maleki, A. Bhattacharya, and P. Ozias-Akins, 2011: TILLING for allergen reduction and improvement of quality traits in peanut (*Arachis hypogaea* L.). *BMC Plant Biology* **11**.
- Koernicke, M., 1904a: Die Wirkung der Radiumstrahlen auf die Keimung und das Wachstum. *Berichte der Deutschen Botanischen Gesellschaft* **22**, 155-166.
- Koernicke, M., 1904b: Über die Wirkung von Röntgenstrahlen auf die Keimung und das Wachstum. *Berichte der Deutschen Botanischen Gesellschaft* **22**, 148-155.
- Kolodner, R.D., and G.T. Marsischky, 1999: Eukaryotic DNA mismatch repair. *Current Opinion in Genetics & Development* **9**, 89-96.

- Konopka, R.J., and S. Benzer, 1971: Clock mutants of *Drosophila melanogaster*. Proceedings of the National Academy of Sciences **68**, 2112-2116.
- Kopisch-Obuch, F.J., and B.W. Diers, 2006: Segregation at the SCN resistance locus *rhg1* in soybean is distorted by an association between the resistance allele and reduced field emergence. Theoretical and Applied Genetics **112**, 199-207.
- Korves, T.M., K.J. Schmid, A.L. Caicedo, C. Mays, J.R. Stinchcombe, M.D. Purugganan, and J. Schmitt, 2007: Fitness effects associated with the major flowering time gene *FRIGIDA* in *Arabidopsis thaliana* in the field. American Naturalist **169**, E141-E157.
- Kurowska, M., A. Daszkowska-Golec, D. Gruszka, M. Marzec, M. Szurman, I. Szarejko, and M. Maluszynski, 2011: TILLING - a shortcut in functional genomics. Journal of Applied Genetics **52**, 371-390.
- Kwok, S., D.E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson, and J.J. Sninsky, 1990: Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. Nucleic Acids Research **18**, 999-1005.
- Lander, E.S., L.M. Linton, B. Birren, C. Nusbaum, M.C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczkzy, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J.P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J.C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R.H. Waterston, R.K. Wilson, L.W. Hillier, J.D. McPherson, M.A. Marra, E.R. Mardis, L.A. Fulton, A.T. Chinwalla, K.H. Pepin, W.R. Gish, S.L. Chissoe, M.C. Wendl, K.D. Delehaunty, T.L. Miner, A. Delehaunty, J.B. Kramer, L.L. Cook, R.S. Fulton, D.L. Johnson, P.J. Minx, S.W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J.F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, R.A. Gibbs, D.M. Muzny, S.E. Scherer, J.B. Bouck, E.J. Sodergren, K.C. Worley, C.M. Rives, J.H. Gorrell, M.L. Metzker, S.L. Naylor, R.S. Kucherlapati, D.L. Nelson, G.M. Weinstock, Y. Sakaki, A. Fujiyama, M. Hattori, T. Yada, A. Toyoda, T. Itoh, C. Kawagoe, H. Watanabe, Y. Totoki, T. Taylor, J. Weissenbach, R. Heilig, W. Saurin, F. Artiguenave, P. Brottier, T. Bruls, E. Pelletier, C. Robert, P. Wincker, D.R. Smith, L. Doucette-Stamm, M. Rubenfield, K. Weinstock, H.M. Lee, J. Dubois, A. Rosenthal, M. Platzer, G. Nyakatura, S. Taudien, A. Rump, H. Yang, J. Yu, J. Wang, G. Huang, J. Gu, L. Hood, L. Rowen, A. Madan, S. Qin, R.W. Davis, N.A. Federspiel, A.P. Abola, M.J. Proctor, R.M. Myers, J. Schmutz, M. Dickson, J. Grimwood, D.R. Cox, M.V. Olson, R. Kaul, N. Shimizu, K. Kawasaki, S. Minoshima, G.A. Evans, M. Athanasiou, R. Schultz, B.A. Roe, F. Chen, H. Pan, J. Ramser, H. Lehrach, R. Reinhardt, W.R. McCombie, M. de la Bastide, N. Dedhia, H. Blocker, K. Hornischer, G. Nordsiek, R. Agarwala, L. Aravind, J.A. Bailey, A. Bateman, S. Batzoglou, E. Birney, P. Bork, D.G. Brown, C.B. Burge, L. Cerutti, H.C. Chen, D. Church, M. Clamp, R.R. Copley, T. Doerks, S.R. Eddy, E.E. Eichler, T.S. Furey, J. Galagan, J.G. Gilbert, C. Harmon, Y. Hayashizaki, D. Haussler, H. Hermjakob, K. Hokamp, W. Jang, L.S. Johnson, T.A. Jones, S. Kasif, A. Kasprzyk, S. Kennedy, W.J. Kent, P. Kitts, E.V. Koonin, I. Korf, D. Kulp, D. Lancet, T.M. Lowe, A. McLysaght, T. Mikkelsen, J.V. Moran, N. Mulder, V.J. Pollara, C.P. Ponting, G. Schuler, J. Schultz, G. Slater, A.F. Smit, E. Stupka, J. Szustakowski, D. Thierry-Mieg, J. Thierry-Mieg, L. Wagner, J. Wallis, R. Wheeler, A. Williams, Y.I. Wolf, K.H. Wolfe, S.P. Yang, R.F. Yeh, F. Collins, M.S. Guyer, J. Peterson, A. Felsenfeld, K.A.

- Wetterstrand, A. Patrinos, M.J. Morgan, P. de Jong, J.J. Catanese, K. Osoegawa, H. Shizuya, S. Choi, and Y.J. Chen, 2001: Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921.
- Lange, C., D. Holtgräwe, B. Schulz, B. Weisshaar, and H. Himmelbauer, 2008: Construction and characterization of a sugar beet (*Beta vulgaris*) fosmid library. *Genome* **51**, 948-951.
- Le Signor, C., V. Savoio, G. Aubert, J. Verdier, M. Nicolas, G. Pagny, F. Moussy, M. Sanchez, D. Baker, J. Clarke, and R. Thompson, 2009: Optimizing TILLING populations for reverse genetics in *Medicago truncatula*. *Plant Biotechnology Journal* **7**, 430-441.
- Lee, H., S.J. Yoo, J.H. Lee, W. Kim, S.K. Yoo, H. Fitzgerald, J.C. Carrington, and J.H. Ahn, 2010: Genetic framework for flowering-time regulation by ambient temperature-responsive miRNAs in *Arabidopsis*. *Nucleic Acids Research* **38**, 3081-3093.
- Lee, J., and I. Lee, 2010: Regulation and function of *SOCL1*, a flowering pathway integrator. *Journal of Experimental Botany* **61**, 2247-54.
- Lein, J.C., K. Asbach, Y.Y. Tian, D. Schulte, C.Y. Li, G. Koch, C. Jung, and D.G. Cai, 2007: Resistance gene analogues are clustered on chromosome 3 of sugar beet and cosegregate with QTL for rhizomania resistance. *Genome* **50**, 61-71.
- Leonard, J.M., S.R. Bollmann, and J.B. Hays, 2003: Reduction of stability of *Arabidopsis* genomic and transgenic DNA-repeat sequences (microsatellites) by inactivation of *AtMSH2* mismatch-repair function. *Plant Physiology* **133**, 328-338.
- Letschert, J., W. Lange, L. Frese, and R. Van Den Berg, 1994: Taxonomy of Beta section Beta. *Journal of sugar beet research* **31**, 69-85.
- Lewellen, R.T., 1992: Use of plant introductions to improve populations and hybrids of sugar beet, In: H. L. Shands, (ed.) *Use of Plant Introductions in Cultivar Development*, 117-135, Vol. 2. Crop Sci. Soc. Am., Madison, WI.
- Li, J., A.-K. Luhmann, K. Weißleder, and B. Stich, 2011: Genome-wide distribution of genetic diversity and linkage disequilibrium in elite sugar beet germplasm. *BMC Genomics* **12**.
- Lim, M.H., J. Kim, Y.S. Kim, K.S. Chung, Y.H. Seo, I. Lee, C.B. Hong, H.J. Kim, and C.M. Park, 2004: A new *Arabidopsis* gene, *FLK*, encodes an RNA binding protein with K homology motifs and regulates flowering time via *FLOWERING LOCUS C*. *Plant Cell* **16**, 731-740.
- Lin, F.L., K. Sperle, and N. Sternberg, 1984: Model for homologous recombination during transfer of DNA into mouse L cells: role for DNA ends in the recombination process. *Molecular and Cellular Biology* **4**, 1020-1034.
- Lindgren, D., 1972: The temperature influence on the sponateous mutation rate I. Literature review. *Hereditas* **70**, 165-178.
- Liu, L.J., Y.C. Zhang, Q.H. Li, Y. Sang, J. Mao, H.L. Lian, L. Wang, and H.Q. Yang, 2008: COP1-mediated ubiquitination of *CONSTANS* is implicated in cryptochrome regulation of flowering in *Arabidopsis*. *Plant Cell* **20**, 292-306.

- Mackay, I.J., J.P. Gibson, and P.D.S. Caligari, 1999: The genetics of selfing with concurrent backcrossing in breeding hybrid sugar beet (*Beta vulgaris altissima* L.). *Theoretical and Applied Genetics* **98**, 1156-1162.
- Magori, S., A. Tanaka, and M. Kawaguchi, 2010: Physically induced mutations: Ion beam mutagenesis, In: G. Kahl and K. Meksem, (eds.) *The Handbook of Plant Mutation Screening*, 1-14. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.
- Maletsky, S.I., and N.J. Weisman, 1978: A population genetic analysis of self- and cross-incompatibility in sugar beet (*Beta Vulgaris* L.). *Theoretical and Applied Genetics* **52**, 21-28.
- Marshall, O.J., 2004: PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* **20**, 2471-2472.
- Marsischky, G.T., and R.D. Kolodner, 1999: Biochemical characterization of the interaction between the *Saccharomyces cerevisiae* MSH2-MSH6 complex and mispaired bases in DNA. *Journal of Biological Chemistry* **274**, 26668-26682.
- Martin, B., M. Ramiro, J.M. Martinez-Zapater, and C. Alonso-Blanco, 2009: A high-density collection of EMS-induced mutations for TILLING in Landsberg erecta genetic background of Arabidopsis. *BMC Plant Biology* **9**, 1-9.
- McCallum, C.M., L. Comai, E.A. Greene, and S. Henikoff, 2000a: Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant Physiology* **123**, 439-442.
- McCallum, C.M., L. Comai, E.A. Greene, and S. Henikoff, 2000b: Targeted screening for induced mutations. *Nature Biotechnology* **18**, 455-457.
- McClintock, B., 1950: The origin and behavior of mutable loci in maize. *Proceedings of the National Academy of Sciences* **36**, 344-355.
- McClintock, B., 1953: Induction of instability at selected loci in maize. *Genetics* **38**, 579-599.
- McGrath, J.M., C.A. Derrico, and Y. Yu, 1999: Genetic diversity in selected, historical US sugarbeet germplasm and *Beta vulgaris* ssp. *maritima*. *Theoretical and Applied Genetics* **98**, 968-976.
- McGrath, J.M., R.S. Shaw, B.G. de los Reyes, and J.J. Weiland, 2004: Construction of a sugar beet BAC library from a hybrid with diverse traits. *Plant Molecular Biology Reporter* **22**, 23-28.
- McGrath, J.M., M. Saccomani, P. Stevanato, and E. Biancardi, 2007: Beet, In: C. Kole, (ed.), 191-202. Springer-Verlag, Berlin Heidelberg.
- Meier, U.L., L. Bachmann, E. Buhtz, H. Hack, R. Klose, B. Märlander, and E. Weber, 1993: Phänologische Entwicklungsstadien der Beta Rübe (*Beta vulgaris* L. ssp.). *Nachrichtenblatt Deutscher Pflanzenschutzdienst* **45**, 37-41.
- Mejlhede, N., Z. Kyjovska, G. Backes, K. Burhenne, S.K. Rasmussen, and A. Jahoor, 2006: EcoTILLING for the identification of allelic variation in the powdery mildew resistance genes *mlo* and *Mla* of barley. *Plant Breeding* **125**, 461-467.
- Michaels, S.D., and R.M. Amasino, 1999: *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949-956.

- Michaels, S.D., and R.M. Amasino, 2001: Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* **13**, 935-942.
- Michaels, S.D., Y.H. He, K.C. Scortecci, and R.M. Amasino, 2003: Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in *Arabidopsis*. *Proceedings of the National Academy of Sciences* **100**, 10102-10107.
- Muldoon, L.L., S.L. Walker-Rosenfeld, C. Hale, S.E. Purcell, L.C. Bennett, and E.A. Neuwelt, 2001: Rescue from enhanced alkylator-induced cell death with low molecular weight sulfur-containing chemoprotectants. *Journal of Pharmacology and Experimental Therapeutics* **296**, 797-805.
- Muller, H.J., 1927: Artificial transmutation of the gene. *Science* **66**, 84-87.
- Munerati, O., 1931: L'eredità della tendenza alla annualità nella comune barbabietola, coltivata. *Zeitschrift Züchtung, Reihe A, Pflanzenzüchtung* **17**, 84-89.
- Mutasa-Gottgens, E., A. Qi, A. Mathews, S. Thomas, A. Phillips, and P. Hedden, 2008: Modification of gibberellin signalling (metabolism & signal transduction) in sugar beet: analysis of potential targets for crop improvement. *Transgenic Research*.
- Muth, J., S. Hartje, R.M. Twyman, H.-R. Hofferbert, E. Tacke, and D. Prüfer, 2008: Precision breeding for novel starch variants in potato. *Plant Biotechnology Journal* **6**, 576-584.
- Mylne, J.S., L. Barrett, F. Tessoro, S. Mesnage, L. Johnson, Y.V. Bernatavichute, S.E. Jacobsen, P. Franz, and C. Dean, 2006: *LHP1*, the *Arabidopsis* homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of *FLC*. *Proceedings of the National Academy of Sciences* **103**, 5012-5017.
- Nakamichi, N., T. Kiba, R. Henriques, T. Mizuno, N.-H. Chua, and H. Sakakibara, 2010: Pseudo-response regulators 9, 7, and 5 are transcriptional repressors in the *Arabidopsis* circadian clock. *The Plant Cell Online* **22**, 594-605.
- Nakamichi, N., M. Kita, K. Niinuma, S. Ito, T. Yamashino, T. Mizoguchi, and T. Mizuno, 2007: *Arabidopsis* clock-associated pseudo-response regulators *PRR9*, *PRR7* and *PRR5* coordinately and positively regulate flowering time through the canonical CONSTANS-dependent photoperiodic pathway. *Plant and Cell Physiology* **48**, 822-832.
- Negrao, S., C. Almadanim, I. Pires, K. McNally, and M. Oliveira, 2011: Use of EcoTILLING to identify natural allelic variants of rice candidate genes involved in salinity tolerance. *Plant Genetic Resources* **9**, 300-304.
- Ng, P.C., and S. Henikoff, 2003: SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Research* **31**, 3812-3814.
- Nieto, C., F. Piron, M. Dalmais, C.F. Marco, E. Moriones, M.L. Gomez-Guillamon, V. Truniger, P. Gomez, J. Garcia-Mas, M.A. Aranda, and A. Bendahmane, 2007: EcoTILLING for the identification of allelic variants of melon *eIF4E*, a factor that controls virus susceptibility. *BMC Plant Biology* **7**, 1-9.

- Oehlkers, F., 1946: Weitere Versuche zur Mutationsauslösung durch Chemikalien. Biologisches Zentralblatt **65**, 176-186.
- Oehlkers, F., 1956: Die Auslösung von Mutationen durch Chemikalien bei *Antirrhinum majus*. Zeitschrift für induktive Abstammungs- und Vererbungslehre **87**, 584-589.
- Ota, T., 1993: DISPAN: Genetic distance and phylogenetic analysis., 1.0 ed. Institute of Molecular Evolutionary Genetics, Pennsylvania State University, University Park, PA, USA.
- Owen, F.V., 1945: Cytoplasmically inherited male-sterility in sugar beets. Journal of Agricultural Research **71**, 423-440.
- Panella, L., and R.T. Lewellen, 2007: Broadening the genetic base of sugar beet: introgression from wild relatives. Euphytica **154**, 383-400.
- Pareek, C., R. Smoczynski, and A. Tretyn, 2011: Sequencing technologies and genome sequencing. Journal of Applied Genetics **52**, 413-435.
- Pastink, A., E. Heemskerk, M.J. Nivard, C.J. van Vliet, and E.W. Vogel, 1991: Mutational specificity of ethyl methanesulfonate in excision-repair-proficient and -deficient strains of *Drosophila melanogaster*. Molecular Genetics and Genomics **229**, 213-8.
- Perry, J.A., T.L. Wang, T.J. Welham, S. Gardner, J.M. Pike, S. Yoshida, and M. Parniske, 2003: A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus*. Plant Physiology **131**, 866-871.
- Peters, J.L., F. Cnudde, and T. Gerats, 2003: Forward genetics and map-based cloning approaches. Trends in Plant Science **8**, 484-491.
- Pfeiffer, N., 2008: Vergleich verschiedener Methoden zur schnellen DNA-Extraktion aus Pflanzen für die markergestützte Selektion in der Pflanzenzüchtung. Bachelor Thesis, Christian-Albrechts-Universität, Kiel.
- Pillen, K., G. Steinrücken, G. Wricke, R.G. Herrmann, and C. Jung, 1992: A linkage map of sugar beet (*Beta vulgaris* L.). Theoretical and Applied Genetics **84**, 129-135.
- Pin, P.A., R. Benlloch, D. Bonnet, E. Wremmerth-Weich, T. Kraft, J.J. Gielen, and O. Nilsson, 2010: An antagonistic pair of *FT* homologs mediates the control of flowering time in sugar beet. Science **330**, 1397-400.
- Pin, P.A., W. Zhang, S.H. Vogt, N. Dally, B. Büttner, G. Schulze-Buxloh, N.S. Jelly, T.Y.P. Chia, E.S. Mutasa-Göttgens, J.C. Dohm, H. Himmelbauer, B. Weisshaar, J. Kraus, J.J.L. Gielen, M. Lommel, G. Weyens, B. Wahl, A. Schechert, O. Nilsson, C. Jung, T. Kraft, and A.E. Müller, 2012: The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. Current Biology **22**, 1095-1101.
- Pirondini, A., U. Bonas, E. Maestri, G. Visioli, M. Marmioli, and N. Marmioli, 2010: Yield and amplifiability of different DNA extraction procedures for traceability in the dairy food chain. Food Control **21**, 663-668.
- Pompanon, F., A. Bonin, E. Bellemain, and P. Taberlet, 2005: Genotyping errors: causes, consequences and solutions. Nature Review Genetics **6**, 847-846.

- Powell, W., R. Waugh, J.E. Bradshaw, J. Russell, L.D. Ramsay, and B.P. Forster, 2004: Introduction to classical genetics and plant breeding, In: R. B. Flavell, (ed.) Handbook of Plant Biotechnology, Vol. 1, 3-30, Vol. 1. John Wiley & Sons Ltd, Chichester, GB.
- Pritchard, J.K., M. Stephens, and P. Donnelly, 2000: Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.
- Puchta, H., 2005: The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *Journal of Experimental Botany* **56**, 1-14.
- R Development Core Team, 2011: R: A language and environment for statistical computing, 2.12 ed. R Foundation for Statistical Computing, Vienna, Austria.
- Rae, S.J., C. Aldam, I. Dominguez, M. Hoebrechts, S. Barnes, and K.J. Edwards, 2000: Development and incorporation of microsatellite markers into the linkage map of sugar beet (*Beta vulgaris* spp.). *Theoretical and Applied Genetics* **100**, 1240-1248.
- Ramos, M., J. Huntley, S. Maleki, and P. Ozias-Akins, 2009: Identification and characterization of a hypoallergenic ortholog of *Ara h 2.01*. *Plant Molecular Biology* **69**, 325-335.
- Rashid, M., G.Y. He, G.X. Yang, and Z. Khurram, 2011: Relevance of tilling in plant genomics. *Australian Journal of Crop Science* **5**, 411-420.
- Reddy, T.V., S. Dwivedi, and N.K. Sharma, 2012: Development of TILLING by sequencing platform towards enhanced leaf yield in tobacco. *Industrial Crops and Products* **40**, 324-335.
- Reeves, P.A., Y.H. He, R.J. Schmitz, R.M. Amasino, L.W. Panella, and C.M. Richards, 2007: Evolutionary conservation of the FLOWERING LOCUS C-mediated vernalization response: Evidence from the sugar beet (*Beta vulgaris*). *Genetics* **176**, 295-307.
- Rigola, D., J. van Oeveren, A. Janssen, A. Bonne, H. Schneiders, H.J. van der Poel, N.J. van Orsouw, R.C. Hogers, M.T. de Both, and M.J. van Eijk, 2009: High-throughput detection of induced mutations and natural variation using KeyPoint technology. *PLoS One* **4**, e4761-.
- Rogers, S.O., and A.J. Bendich, 1985: Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant-tissues. *Plant Molecular Biology* **5**, 69-76.
- Rutger, J.N., and D.J. McKill, 2001: Application of mendelian genetics in rice breeding, In: G. S. Khush, D. S. Brar and B. Hardy, (eds.) Rice Genetics IV, 38-49. Science Publishers, Inc., Enfield, NH, USA.
- Sadeghian, S.Y., and E. Johansson, 1992: Genetic study of bolting and stem length in sugar beet (*Beta vulgaris* L.) using a factorial cross design. *Euphytica* **65**, 177-185.
- Sánchez-Hernández, C., and J.C. Gaytán-Oyarzún, 2006: Two mini-preparation protocols to DNA extraction from plants with high polysaccharide and secondary metabolites. *African Journal of Biotechnology* **5**, 1864-1867.
- Sanger, F., and A.R. Coulson, 1975: A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology* **94**, 441-448.

- Santerre, A., and A.B. Britt, 1994: Cloning of a 3-methyladenine-DNA glycosylase from *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences **91**, 2240-2244.
- Sato, Y., K. Shirasawa, Y. Takahashi, M. Nishimura, and T. Nishio, 2006: Mutant selection from progeny of gamma-ray-irradiated rice by DNA heteroduplex cleavage using Brassica petiole extract. Breeding Science **56**, 179-183.
- Savitsky, V.F., 1952: A genetic study of monogerm and multigerm characters in beets Proceedings American Society of Sugar Beet Technologists, 824. The American Society of Sugar Beet Technologists, Salt Lake City, Utah, USA.
- Schafer-Pregl, R., D.C. Borchardt, E. Barzen, C. Glass, W. Mechelke, J.F. Seitzer, and F. Salamini, 1999: Localization of QTLs for tolerance to *Cercospora beticola* on sugar beet linkage groups. Theoretical and Applied Genetics **99**, 829-836.
- Schiemann, E., 1912: Mutationen bei *Aspergillus niger* van Tieghem. Zeitschrift für induktive Abstammungs- und Vererbungslehre **8**, 1-35.
- Schmitz, R.J., and R.M. Amasino, 2007: Vernalization: A model for investigating epigenetics and eukaryotic gene regulation in plants. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression **1769**, 269-275.
- Schneider, K., D. Kulosa, T. Rosleff Sørensen, S. Möhring, M. Heine, G. Durstewitz, A. Polley, E. Weber, Jamsari, J. Lein, U. Hohmann, E. Tahiro, B. Weisshaar, B. Schulz, G. Koch, C. Jung, and M. Ganai, 2007: Analysis of DNA polymorphisms in sugar beet (*Beta vulgaris* L.) and development of an SNP-based map of expressed genes. Theoretical and Applied Genetics **115**, 601-615.
- Schondelmaier, J., and C. Jung, 1997: Chromosomal assignment of the nine linkage groups of sugar beet (*Beta vulgaris* L.) using primary trisomics. Theoretical and Applied Genetics **95**, 590-596.
- Schulte, D., D.G. Cai, M. Kleine, L.J. Fan, S. Wang, and C. Jung, 2006: A complete physical map of a wild beet (*Beta procumbens*) translocation in sugar beet. Molecular Genetics and Genomics **275**, 504-511.
- Seo, E., H. Lee, J. Jeon, H. Park, J. Kim, Y.S. Noh, and I. Lee, 2009: Crosstalk between cold response and flowering in *Arabidopsis* is mediated through the flowering-time gene *SOCl* and its upstream negative regulator *FLC*. Plant Cell **21**, 3185-3197.
- Sestili, F., E. Botticella, Z. Bedo, A. Phillips, and D. Lafiandra, 2010: Production of novel allelic variation for genes involved in starch biosynthesis through mutagenesis. Molecular Breeding **25**, 145-154.
- Sheldon, C.C., A.B. Conn, E.S. Dennis, and W.J. Peacock, 2002: Different regulatory regions are required for the vernalization-induced repression of FLOWERING LOCUS C and for the epigenetic maintenance of repression. Plant Cell **14**, 2527-37.
- Sheldon, C.C., J.E. Burn, P.P. Perez, J. Metzger, J.A. Edwards, W.J. Peacock, and E.S. Dennis, 1999: The FLF MADS box gene: A repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. Plant Cell **11**, 445-458.
- Shortle, D., D. DiMaio, and D. Nathans, 1981: Directed mutagenesis. Annual Review of Genetics **15**, 265-294.

- Sigurbjörnsson, B., and A. Micke, 1969: Plant mutation breeding for crop improvement Proceedings FAO/IAEA Symposium, 673-698. Vienna: IAEA, Pullman, USA.
- Sikora, P., A. Chawade, M. Larsson, J. Olsson, and O. Olsson, 2011: Mutagenesis as a tool in plant genetics, functional genomics, and breeding. *International Journal of Plant Genomics* **2011**, 13.
- Simpson, G.G., 2004: The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of *Arabidopsis* flowering time. *Current Opinion in Plant Biology* **7**, 570-574.
- Singh, V.K., and A. Kumar, 2001: PCR primer design. *Molecular Biology Today* **2**, 27-32.
- Sitte, P., E.W. Weiler, J.W. Kadereit, A. Bresinsky, and C. Körner, 2002: Strasburger - Lehrbuch der Botanik für Hochschulen Spektrum Akademischer Verlag GmbH, Heidelberg.
- Slade, A.J., and V.C. Knauf, 2005: TILLING moves beyond functional genomics into crop improvement. *Transgenic Research* **14**, 109-115.
- Slade, A.J., S.I. Fuerstenberg, D. Loeffler, M.N. Steine, and D. Facciotti, 2005: A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nature Biotechnology* **23**, 75-81.
- Slade, A.J., C. McGuire, D. Loeffler, J. Mullenberg, W. Skinner, G. Fazio, A. Holm, K. Brandt, M.N. Steine, J. Goodstal, and V. Knauf, 2012: Development of high amylose wheat through TILLING. *BMC Plant Biology* **12**, 69.
- Spaniolas, S., M. Tsachaki, M.J. Bennett, and G.A. Tucker, 2008: Evaluation of DNA extraction methods from green and roasted coffee beans. *Food Control* **19**, 257-262.
- Srikanth, A., and M. Schmid, 2011: Regulation of flowering time: all roads lead to Rome. *Cellular and Molecular Life Sciences* **68**, 2013-2037.
- Stadler, L.J., 1928a: Mutations in barley induced by X-rays and radium. *Science* **68**, 186-187.
- Stadler, L.J., 1928b: Genetic effects of X-rays in maize. *Proceedings of the National Academy of Sciences* **14**, 69-75.
- Stephenson, P., D. Baker, T. Girin, A. Perez, S. Amoah, G.J. King, and L. Ostergaard, 2010: A rich TILLING resource for studying gene function in *Brassica rapa*. *BMC Plant Biology* **10**, 1-10.
- Sterck, L., S. Rombauts, K. Vandepoele, P. Rouzé, and Y. Van de Peer, 2007: How many genes are there in plants (... and why are they there)? *Current Opinion in Plant Biology* **10**, 199-203.
- Stubbe, H., 1930a: Untersuchungen über experimentelle Auslösung von Mutationen bei *Antirrhinum majus* L. II. *Zeitschrift für induktive Abstammungs- und Vererbungslehre* **56**, 202-232.
- Stubbe, H., 1930b: Untersuchungen über experimentelle Auslösung von Mutationen bei *Antirrhinum majus* L. I. *Zeitschrift für induktive Abstammungs- und Vererbungslehre* **56**, 1-56.
- Stubbe, H., 1930c: Untersuchungen über experimentelle Auslösung von Mutationen bei *Antirrhinum majus* L. III. *Zeitschrift für induktive Abstammungs- und Vererbungslehre* **60**, 474-513.

- Stubbe, H., 1935: Samenalter und Genmutabilität bei *Anthriscus majus* L. (Nebst einigen Beobachtungen über den Zeitpunkt des Mutierens während der Entwicklung) Biologisches Zentralblatt **55**, 209-214.
- Sung, S., and R.M. Amasino, 2004a: Vernalization and epigenetics: how plants remember winter. *Current Opinion in Plant Biology* **7**, 4-10.
- Sung, S., and R.M. Amasino, 2004b: Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. *Nature* **427**, 159-164.
- Sung, S., Y. He, T.W. Eshoo, Y. Tamada, L. Johnson, K. Nakahigashi, K. Goto, S.E. Jacobsen, and R.M. Amasino, 2006: Epigenetic maintenance of the vernalized state in *Arabidopsis thaliana* requires LIKE HETEROCHROMATIN PROTEIN 1. *Nature Genetics* **38**, 706-710.
- Suzuki, T., M. Eiguchi, T. Kumamaru, H. Satoh, H. Matsusaka, K. Moriguchi, Y. Nagato, and N. Kurata, 2008: MNU-induced mutant pools and high performance TILLING enable finding of any gene mutation in rice. *Molecular Genetics and Genomics* **279**, 213-223.
- Swarbreck, D., C. Wilks, P. Lamesch, T.Z. Berardini, M. Garcia-Hernandez, H. Foerster, D. Li, T. Meyer, R. Muller, L. Ploetz, A. Radenbaugh, S. Singh, V. Swing, C. Tissier, P. Zhang, and E. Huala, 2008: The arabidopsis information resource (TAIR): gene structure and function annotation. *Nucleic Acids Research* **36**, D1009-D1014.
- Taberlet, P., S. Griffin, B. Goossens, S. Questiau, V. Manceau, N. Escaravage, L.P. Waits, and J. Bouvet, 1996: Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* **24**, 3189-3194.
- Talamè, V., R. Bovina, M.C. Sanguineti, R. Tuberosa, U. Lundqvist, and S. Salvi, 2008: TILLMore, a resource for the discovery of chemically induced mutants in barley. *Plant Biotechnology Journal* **6**, 477-485.
- Taylor, N.E., and E.A. Greene, 2003: PARSESNP: A tool for the analysis of nucleotide polymorphisms. *Nucleic Acids Research* **31**, 3808-3811.
- Till, B.J., T. Zerr, L. Comai, and S. Henikoff, 2006a: A protocol for TILLING and EcoTILLING in plants and animals. *Nature Protocols* **1**, 2465-2477.
- Till, B.J., T. Zerr, E. Bowers, E.A. Greene, L. Comai, and S. Henikoff, 2006b: High-throughput discovery of rare human nucleotide polymorphisms by EcoTILLING. *Nucleic Acids Research* **34**, e99 01-12.
- Till, B.J., J. Cooper, T.H. Tai, P. Colowit, E.A. Greene, S. Henikoff, and L. Comai, 2007: Discovery of chemically induced mutations in rice by TILLING. *BMC Plant Biology* **7**, 1-12.
- Till, B.J., J. Jankowicz-Cieslak, L. Sigi, O. Huynh, H. Utsushi, R. Swennen, R. Terauchi, and C. Mba, 2010: Discovery of nucleotide polymorphisms in the *Musa* gene pool by EcoTILLING. *Theoretical and Applied Genetics* **121**, 1381-1389.
- Till, B.J., T. Colbert, C. Codomo, L. Enns, J. Johnson, S.H. Reynolds, J.G. Henikoff, E.A. Greene, M.N. Steine, L. Comai, and S. Henikoff, 2006c: High-throughput TILLING for *Arabidopsis*. *Methods in Molecular Biology* **323**, 127-135.

- Till, B.J., S.H. Reynolds, E.A. Greene, C.A. Codomo, L.C. Enns, J.E. Johnson, C. Burtner, A.R. Odden, K. Young, N.E. Taylor, J.G. Henikoff, L. Comai, and S. Henikoff, 2003: Large-scale discovery of induced point mutations with high-throughput TILLING. *Genome Research* **13**, 524-530.
- Till, B.J., S.H. Reynolds, C. Weil, N. Springer, C. Burtner, K. Young, E. Bowers, C.A. Codomo, L.C. Enns, A.R. Odden, E.A. Greene, L. Comai, and S. Henikoff, 2004: Discovery of induced point mutations in maize genes by TILLING. *BMC Plant Biology* **4**, 1-8.
- Tollenaar, D., 1934: Untersuchungen über Mutation bei Tabak. *Genetica* **16**, 111-152.
- Tollenaar, D., 1938: Untersuchungen über Mutation bei Tabak. *Genetica* **20**, 285-294.
- Triques, K., E. Piednoir, M. Dalmais, J. Schmidt, S.C. Le, M. Sharkey, M. Caboche, B. Sturbois, and A. Bendahmane, 2008: Mutation detection using ENDO1: application to disease diagnostics in humans and TILLING and Eco-TILLING in plants. *BMC Molecular Biology* **9**, 1-9.
- Triques, K., B. Sturbois, S. Gallais, M. Dalmais, S. Chauvin, C. Clepet, S. Aubourg, C. Rameau, M. Caboche, and A. Bendahmane, 2007: Characterization of *Arabidopsis thaliana* mismatch specific endonucleases: application to mutation discovery by TILLING in pea. *Plant Journal* **51**, 1116-1125.
- Tsai, H., T. Howell, R. Nitcher, V. Missirian, B. Watson, K. Ngo, M. Lieberman, J. Fass, C. Uauy, R. Tran, A.A. Khan, V. Filkov, T.H. Tai, J. Dubcovsky, and L. Comai, 2011: Discovery of rare mutations in populations: TILLING by sequencing. *Plant Physiology*.
- Turck, F., and G. Coupland, 2011: Plant science. When vernalization makes sense. *Science* **331**, 36-7.
- Turck, F., F. Fornara, and G. Coupland, 2008: Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. *Annual Review of Plant Biology* **59**, 573-594.
- Tuteja, N., P. Ahmad, B.B. Panda, and R. Tuteja, 2009: Genotoxic stress in plants: Shedding light on DNA damage, repair and DNA repair helicases. *Mutation Research/Reviews in Mutation Research* **681**, 134-149.
- Tuteja, N., M.B. Singh, M.K. Misra, P.L. Bhalla, and R. Tuteja, 2001a: Molecular mechanisms of DNA damage and repair: progress in plants. *Critical Reviews in Biochemistry and Molecular Biology* **36**, 337-397.
- Tuteja, N., M.B. Singh, M.K. Misra, P.L. Bhalla, and R. Tuteja, 2001b: Molecular mechanisms of DNA damage and repair: progress in plants. *Critical reviews in biochemistry and molecular biology* **36**, 337-97.
- Tzfira, T., and C. White, 2005: Towards targeted mutagenesis and gene replacement in plants. *Trends in Biotechnology* **23**, 567-569.
- Uauy, C., F. Paraiso, P. Colasuonno, R. Tran, H. Tsai, S. Berardi, L. Comai, and J. Dubcovsky, 2009: A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant Biology* **9**, 1-14.
- Uchiyama, Y., S. Kimura, T. Yamamoto, T. Ishibashi, and K. Sakaguchi, 2004: Plant DNA polymerase λ , a DNA repair enzyme that functions in plant meristematic and meiotic tissues. *European Journal of Biochemistry* **271**, 2799-2807.

- Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J.A.M. Leunissen, 2007: Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research* **35**, W71-W74.
- Uphoff, H., and G. Wricke, 1995: A genetic-map of sugar beet (*Beta vulgaris*) based on rapd markers. *Plant Breeding* **114**, 355-357.
- Valverde, F., A. Mouradov, W. Soppe, D. Ravenscroft, A. Samach, and G. Coupland, 2004: Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science* **303**, 1003-1006.
- van den Boom, D., and M. Ehrlich, 2007: Discovery and identification of sequence polymorphisms and mutations with MALDI-TOF MS. *Methods in Molecular Biology* **366**, 287-306.
- Van Dijk, H., and P. Boudry, 1991: Genetic variation for life histories in *Beta maritima*. *International Board for Plant Genetic Resources* **7**, 44-55.
- Van Dijk, H., P. Boudry, H. McCombre, and P. Vernet, 1997: Flowering time in wild beet (*Beta vulgaris* ssp. *maritima*) along a latitudinal cline. *Acta Oecologica* **18**, 47-60.
- van Harten, A.M., 1998: Mutation breeding: theory and practical applications Cambridge University Press, Cambridge, United Kingdom.
- Velasco, L., C. Möllers, and H.C. Becker, 1999: Estimation of seed weight, oil content and fatty acid composition in intact single seeds of rapeseed (*Brassica napus* L.) by near-infrared reflectance spectroscopy. *Euphytica* **106**, 79-85.
- Velemínský, J., and T. Gichner, 1978: DNA repair in mutagen-injured higher plants. *Mutation Research/Reviews in Genetic Toxicology* **55**, 71-84.
- Venter, J.C., M.D. Adams, E.W. Myers, P.W. Li, R.J. Mural, G.G. Sutton, H.O. Smith, M. Yandell, C.A. Evans, R.A. Holt, J.D. Gocayne, P. Amanatides, R.M. Ballew, D.H. Huson, J.R. Wortman, Q.F. Zhang, C.D. Kodira, X.H. Zheng, L. Chen, M. Skupski, G. Subramanian, P.D. Thomas, J. Zhang, G.L. Gabor Miklos, C.D. Nelson, S. Broder, A.G. Clark, J. Nadeau, V.A. McKusick, N. Zinder, A.J. Levine, R.J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V.D. Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A.E. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T.J. Heiman, M.E. Higgins, R.-R. Ji, Z. Ke, K.A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G.V. Merkulov, N. Milshina, H.M. Moore, A.K. Naik, V.A. Narayan, B. Neelam, D. Nusskern, D.B. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z.Y. Wang, A. Wang, X. Wang, J. Wang, M.-H. Wei, R. Wides, C. Xiao, C. Yan, A. Yao, J. Ye, M. Zhan, W. Zhang, H. Zhang, Q. Zhao, L. Zheng, F. Zhong, W. Zhong, S.C. Zhu, S. Zhao, D. Gilbert, S. Baumhueter, G. Spier, C. Carter, A. Cravchik, T. Woodage, F. Ali, H. An, A. Awe, D. Baldwin, H. Baden, M. Barnstead, I. Barrow, K. Beeson, D. Busam, A. Carver, A. Center, M.L. Cheng, L. Curry, S. Danaher, L. Davenport, R. Desilets, S. Dietz, K. Dodson, L. Doup, S. Ferreira, N. Garg, A. Gluecksmann, B. Hart, J. Haynes, C. Haynes, C. Heiner, S. Hladun, D. Hostin, J. Houck, T. Howland, C. Ibegwam, J. Johnson, F. Kalush, L. Kline, S. Koduru, A. Love, F. Mann, D. May, S. McCawley, T. McIntosh, I. McMullen, M. Moy, L. Moy, B. Murphy, K. Nelson, C. Pfannkoch, E. Pratts, V. Puri, H. Qureshi, M. Reardon, R. Rodriguez, Y.-H. Rogers, D. Romblad, B. Ruhfel, R. Scott, C. Sitter, M. Smallwood, E. Stewart, R. Strong, E. Suh, R. Thomas, N.N. Tint, S. Tse, C. Vech, G. Wang, J. Wetter, S. Williams, M. Williams, S. Windsor, E. Winn-Deen, K. Wolfe, J. Zaveri, K. Zaveri, J.F. Abril, R. Guigó, M.J. Campbell, K.V. Sjolander, B. Karlak, A. Kejariwal, H. Mi, B. Lazareva, T. Hatton, A. Narechania, K. Diemer, A. Muruganujan, N. Guo, S. Sato, V. Bafna, S.

- Istrail, R. Lippert, R. Schwartz, B. Walenz, S. Yooseph, D. Allen, A. Basu, J. Baxendale, L. Blick, M. Caminha, J. Carnes-Stine, P. Caulk, Y.-H. Chiang, M. Coyne, C. Dahlke, A.D. Mays, M. Dombroski, M. Donnelly, D. Ely, S. Esparham, C. Fosler, H. Gire, S. Glanowski, K. Glasser, A. Glodek, M. Gorokhov, K. Graham, B. Gropman, M. Harris, J. Heil, S. Henderson, J. Hoover, D. Jennings, C. Jordan, J. Jordan, J. Kasha, L. Kagan, C. Kraft, A. Levitsky, M. Lewis, X. Liu, J. Lopez, D. Ma, W. Majoros, J. McDaniel, S. Murphy, M. Newman, T. Nguyen, N. Nguyen, M. Nodell, S. Pan, J. Peck, M. Peterson, W. Rowe, R. Sanders, J. Scott, M. Simpson, T. Smith, A. Sprague, T. Stockwell, R. Turner, E. Venter, M. Wang, M. Wen, D. Wu, M. Wu, A. Xia, A. Zandieh, and X. Zhu, 2001: The Sequence of the human genome. *Science* **291**, 1304-1351.
- Vos, P., R. Hogers, M. Bleeker, M. Reijans, T.v.d. Lee, M. Hornes, A. Friters, J. Pot, J. Paleman, M. Kuiper, and M. Zabeau, 1995: AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407-4414.
- Wang, G., M. Tan, S. Rakshit, H. Saitoh, R. Terauchi, T. Imaizumi, T. Ohsako, and T. Tominaga, 2007: Discovery of single-nucleotide mutations in acetolactate synthase genes by EcoTILLING. *Pesticide Biochemistry and Physiology* **88**, 143-148.
- Wang, J., J. Sun, D. Liu, W. Yang, D.M. Wang, Y. Tong, and A. Zhang, 2008a: Analysis of *Pina* and *Pinb* alleles in the micro-core collections of Chinese wheat germplasm by Ecotilling and identification of a novel *Pinb* allele. *Journal of Cereal Science* **48**, 836-842.
- Wang, N., L. Shi, F. Tian, H. Ning, X. Wu, Y. Long, and J. Meng, 2010a: Assessment of *FAE1* polymorphisms in three *Brassica* species using EcoTILLING and their association with differences in seed erucic acid contents. *BMC Plant Biology* **10**, 1-11.
- Wang, N., Y. Wang, F. Tian, G.J. King, C. Zhang, Y. Long, L. Shi, and J. Meng, 2008b: A functional genomics resource for *Brassica napus*: development of an EMS mutagenized population and discovery of FAE1 point mutations by TILLING. *New Phytologist* **180**, 751-765.
- Wang, R., S. Farrona, C. Vincent, A. Joecker, H. Schoof, F. Turck, C. Alonso-Blanco, G. Coupland, and M.C. Albani, 2009: *PEP1* regulates perennial flowering in *Arabidopsis thaliana*. *Nature* **459**, 423-427.
- Wang, T., C. Uauy, B.J. Till, and C.M. Liu, 2010b: TILLING and associated technologies. *Journal of Integrative Plant Biology* **52**, 1027-1030.
- Wang, T.L., C. Uauy, F. Robson, and B.J. Till, 2012: TILLING in extremis. *Plant Biotechnology Journal*, no-no.
- Watson, J.D., and F.H.C. Crick, 1953: Molecular structure of nucleic acids: A structure for deoxyribose nucleic acid. *Nature* **171**, 737-738.
- Weil, C.F., and R.A. Monde, 2007: Getting the point - Mutations in maize. *Crop Science* **47**, S60-S67.
- Weisshaar, B., J.C. Dohm, A. Minoche, B. Schulz, T. Kraft, M. Wolf, D. Holtgraewe, and H. Himmelbauer, 2011: The draft genome sequence of sugar beet (*Beta vulgaris*) Plant & Animal Genomes XIX Conference, Town & Country Convention Center San Diego, CA.
- Wolf, F., 1909: Über Modifikationen und experimentell ausgelöste Mutationen von *Bacillus prodigiosus* und anderen Schizomyzeten. *Zeitschrift für induktive Abstammungs- und Vererbungslehre* **2**, 90-132.

- Wu, J.L., C.A. Wu, C. Lei, M. Baraoidan, A. Bordeos, M. Madamba, M. Ramos-Pamplona, R. Mauleon, A. Portugal, V. Ulat, R. Bruskiwich, G. Wang, J. Leach, G.S. Khush, and H. Leung, 2005: Chemical- and irradiation-induced mutants of Indica rice IR64 for forward and reverse genetics. *Plant Molecular Biology* **59**, 85-97.
- Wyatt, M.D., and D.L. Pittman, 2006: Methylating agents and DNA repair responses: Methylated bases and sources of strand breaks. *Chemical Research in Toxicology* **19**, 1580-1594.
- Xin, Z., M.L. Wang, N.A. Barkley, G. Burow, C. Franks, G. Pederson, and J. Burke, 2008: Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biology* **8**, 1-14.
- Xu, Y., and J.H. Crouch, 2008: Marker-assisted selection in plant breeding: from publications to practice. *Crop Science* **48**, 391-407.
- Yu, J., S. Hu, J. Wang, G.K.-S. Wong, S. Li, B. Liu, Y. Deng, L. Dai, Y. Zhou, X.W. Zhang, M. Cao, J. Liu, J. Sun, J. Tang, Y. Chen, X. Huang, W. Lin, C. Ye, W. Tong, L. Cong, J. Geng, Y. Han, L. Li, W. Li, G. Hu, X. Huang, W. Li, J. Li, Z. Liu, L. Li, J. Liu, Q. Qi, J. Liu, L. Li, T. Li, X. Wang, H. Lu, T. Wu, M. Zhu, P. Ni, H. Han, W. Dong, X. Ren, X. Feng, P. Cui, X. Li, H. Wang, X. Xu, W. Zhai, Z. Xu, J. Zhang, S. He, J. Zhang, J. Xu, K. Zhang, X. Zheng, J. Dong, W. Zeng, L. Tao, J. Ye, J. Tan, X. Ren, X. Chen, J. He, D. Liu, W. Tian, C. Tian, H. Xia, Q. Bao, G. Li, H. Gao, T. Cao, J. Wang, W. Zhao, P. Li, W. Chen, X. Wang, Y. Zhang, J. Hu, J. Wang, S. Liu, J. Yang, G. Zhang, Y. Xiong, Z. Li, L. Mao, C. Zhou, Z. Zhu, R. Chen, B. Hao, W. Zheng, S. Chen, W. Guo, G. Li, S. Liu, M. Tao, J. Wang, L. Zhu, L. Yuan, and H. Yang, 2002: A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). *Science* **296**, 79-92.
- Zeevaart, J.A.D., 2008: Leaf-produced floral signals. *Current Opinion in Plant Biology* **11**, 541-547.
- Zerr, T., and S. Henikoff, 2005: Automated band mapping in electrophoretic gel images using background information. *Nucleic Acids Research* **33**, 2806-2812.
- Zhang, X., S. Germann, B.J. Blus, S. Khorasanizadeh, V. Gaudin, and S.E. Jacobsen, 2007: The Arabidopsis LHP1 protein colocalizes with histone H3 Lys27 trimethylation. *Nature Structural and Molecular Biology* **14**, 869-871.

9 Supplementary Data on DVD

File name	Content	Format
AFLP_gel_images	AFLP from 930190 with eight primer pairs	.pptx
TILLING_Primer	Primer used for primer evaluation and TILLING	.xlsx

10 Curriculum Vitae

Personal data

Name	Sebastian Leo Max Frerichmann
Date of birth	27.10.1978 in Hamburg
Marital status	married
Children	two daughters (8 and 6 years)

Education

07/85 – 06/89	Elementary School Waldenau
08/89 – 06/95	Geschwister Scholl Gesamtschule Hamburg
08/95 – 06/96	North Miami High School, Denver, Indiana, USA
08/96 – 06/99	Gymnasium Schenefeld
Degree:	Abitur

Apprenticeship

10/ 00 –10/07	Study of molecular biology at the University of Hamburg Major: Genetics/Molecular biology Minor: Biochemistry und Evolutionary biology Degree: Diploma Diploma thesis: Cloning und characterization of a deoxyhypusine synthase from <i>Triticum aestivum</i> L.
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11/ 07 – 01/13	Doctoral candidate at the Christian-Albrechts University of Kiel, Plant breeding Institute Thesis: TILLING and EcoTILLING for flowering and bolting time genes in <i>Beta vulgaris</i> :
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Internships	Institute for Immunology and transfusion medicine at the UKSH Lübeck Molecular parasitology department of the Bernhard Nocht Institute for tropical medicine Hamburg Virology department of the Bernhard Nocht Institute for tropical medicine Hamburg
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Military service

01/00 –03/00	Basic training List Sylt
03/00 –10/00	Emergency medical technician Rank: Hauptgefreiter

Community service

01/91 - present	Volunteer fire department Qualification: 97 Truppmann 98 Atemschutzgeräteträger 02 Maschinist 03 Truppführer 04 Sprechfunk Rank: Hauptfeuerwehrmann
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11 Publications and declaration of own contribution

Chapter 2 of this thesis is not published. Chapter 3 has been published in BMC Plant Biology. The doctoral student Sebastian Leo Max Frerichmann contributed to the chapters as follows:

Chapter 2: TILLING in *Beta vulgaris* ssp. *vulgaris* L. - Detection of new sequence variants for flowering and bolting time genes

- Designing the experiments: 75 %
- Results: 100 %
- Writing the manuscript: 100 %
- Discussion and revision: 100 %

Chapter 3: EcoTILLING in *Beta vulgaris* reveals polymorphisms in the *FLC*-like gene *BvFL1* that are associated with annuality and winter hardiness

- Designing the experiments: 50 %
- Results: 65 %
- Writing the manuscript: 75 %
- Discussion and revision: 75 %

12 Acknowledgements

I would like to express my gratitude to Prof. Dr. Christian Jung for the opportunity to conduct my doctoral thesis at the Plant Breeding Institute, for scientific support, advice, comments and discussion. He always pushed further and sought for the best. Thank you.

Dr. Friedrich Kopisch I would like to thank for his never-ending patience, even when he was on the verge of losing it. He helped to get this thesis into shape with his ability to set me back on track and focus on the important things. Dr. Friedrich Kopisch-Obuch showed me that even so the time is limited; there is always a thing I can do, to perform best.

Without the technical support of Verenna Kowalewski, Birgit Defant and mostly Micheal Jahn this thesis would have been impossible. Their PCR strength, organizing talents and good humour got me going every day. It has been a pleasure working with you.

There would never be a day without my Ladies. I cannot thank Nadine Dally, Nina Pfeifer, Sarah Jäger, Steffi Fritsche and Gina Capistrano-Gossmann enough for all the support they gave me. I will miss you bunches, but their will always be our memories.

Further, I would like to thank the whole Plant Breeding Institute who has made contribution to this thesis, especially Antje Jakobeit, Hilke Jensen, Moni and Erwin, Gisling Bräcker and Cay Kruse.

I would like to give a special thank to my cooperation partner Dr. Annika Hjerdin-Panagopoulou, and Maïke Hinze from Syngenta Seeds AB for their help in plant propagation, leaf harvest and moral support.

The BMBF is kindly acknowledged for financial support.

I thank my parents Joachim and Heidemarie Frerichmann for their never-ending patience, their believe in me and for all the opportunities that they made possible to me. I cannot thank you enough.

Finally, I love to express my endless gratitude to my wife Judith and my girls Mailin and Mette for given me the strength, calmness and believe to finish this thesis. Without your unlimited patience, love, and joy this thesis would have been impossible.

Do not part with your illusions. When they are gone you may still exist, but you have ceased to live.

Mark Twain (1835 – 1910)

13 Erklärung

Ich versichere an Eides statt, dass ich bis zum heutigen Tage weder an der Christian-Albrechts-Universität zu Kiel noch an einer anderen Hochschule ein Promotionsverfahren endgültig nicht bestanden habe oder mich in einem entsprechenden Verfahren befinde.

Kiel, _____
(Datum)

(Unterschrift Kandidat/in)

Ich versichere an Eides statt, dass ich die Inanspruchnahme fremder Hilfen aufgeführt habe, sowie, dass ich die wörtlich oder inhaltlich aus anderen Quellen entnommen Stellen als solche gekennzeichnet habe.

Kiel, _____
(Datum)

(Unterschrift Kandidat/in)

