Life Cycle and Flowering Time Control in Beet

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Cover: close-up view of developing flowers of a transgenic *35S::BvFT2* sugar beet plant. Biennial sugar beet plant (*Beta vulgaris*) overexpressing the *Beta FLOWERING LOCUS T* gene, *BvFT2*, succeeds to bolt and flower without vernalization requirement.
(Photo: P. Pin)

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Abstract

Flowering plants switch from vegetative growth to flowering at specific points in time. This biological process is triggered by the integration of endogenous stimuli and environmental cues such as changes in day length and temperature. The first sign of the flowering transition is sometimes marked by the formation and the elongation of the stem in a process known as “bolting” that precedes flower development.

Flowering plants have developed different life cycles to ensure optimal reproductive success depending on their habitat. Annual species complete their life cycle in one year whereas biennial species typically fulfill their life cycle in two years and need to overwinter. Perennial species, which can exhibit long juvenile periods, typically flower for several years or even decades rather than just once.

This thesis describes research in which sugar beet (*Beta vulgaris* ssp. *vulgaris*) was used as a new model for experimental studies of the floral transition. Sugar beet is an attractive organism for plant biologists studying life cycle control because of its biennial growth habit and its strict vernalization- and long-day-dependent flowering. Moreover, beets belong to the caryophyllids, which is a core-eudicot clade that is distinct from the rosids and the asterids and for which no molecular-scale investigations into flowering control have previously been reported.

I isolated a pair of *FLOWERING LOCUS T* homologs, named *BvFT1* and *BvFT2*, which have surprisingly evolved antagonistic transcriptional regulation capabilities and functions. I show that synchronized regulation of these two genes is essential to ensure flowering in beets. In addition, by using a map-based cloning approach, I isolated the bolting gene *B* – a dominant promoter of bolting and flowering that can bypass the need for vernalization in annual wild beets (*Beta vulgaris* ssp. *maritima*). I show that *B* encodes a pseudo-response regulator protein, BOLTING TIME CONTROL1 (BTC1), which acts upstream of the *BvFT1* and *BvFT2* genes, and that the biennial habit results from a partial loss of function of *BvBTC1*. My data illustrate how evolutionary changes at strategic molecular layers have shaped life cycle adaptation in plants.

Keywords: bolting, *BTC1*, flowering, *FT*, neofunctionalization, photoperiod, pseudo-response regulator, subfunctionalization, sugar beet, vernalization

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To Yvonne,
Thomas,
Jan,
and my family

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Pin, P.A.**, Benlloch, R., Bonnet, D., Wremerth-Weich, E., Kraft, T., Gielen, J.J.L. & Nilsson, O. (2010) An antagonistic pair of *FT* homologs mediates flowering time control in sugar beet. *Science* 330, 1397-1400.

II **Pin, P.A.**, Zhang, W., Vogt, S.H., Dally, N., Büttner, B., Schulze-Buxloh, G., Jelly, N.S., Chia, T.Y., Mutasa-Göttgens, E.S., Dohm, J.C., Himmelbauer, H., Weisshaar, B., Kraus, J., Gielen, J.J.L., Lommel, M, Weyens, G., Wahl, B., Schechert, A., Nilsson, O., Jung, C., Kraft, T. & Müller, A.E. (2012) The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. *Current Biology* 22, 1095-1101.

III **Pin, P.A.** & Nilsson, O. (2012) The multifaceted roles of FLOWERING LOCUS T in plant development. *Plant, Cell & Environment*, in press.

IV Klintenäs, M., **Pin, P.A.**, Benlloch, R., Ingvarsson, P.K. & Nilsson, O. (2012) Analysis of conifer *FT/TFL1*-like genes provides evidence for dramatic biochemical evolution in the angiosperm *FT* lineage. *New Phytologist*, accepted.

The contribution of Pin, P.A. to the papers included in this thesis was as follows:

I designed and performed experiments, analyzed data and co-wrote the paper

II designed and performed experiments, analyzed data and co-wrote the paper

III co-wrote the paper

IV performed experiments, analyzed data and co-wrote the paper

Abbreviations

AGL24	*AGAMOUS-LIKE24*
AP1	*APETALA1*
AP2Ls	AP2-likes
BAC	Bacterial Artificial Chromosome
BFT	*BROTHER OF FT AND TFL1*
BLAST	Basic Local Alignment Search Tool
BSA	Bulked Segregant Analysis
BTC1	*BOLTING TIME CONTROL1*
CAM	Crassulacean Acid Metabolism
CaMV	Cauliflower Mosaic Virus
CCA1	*CIRCADIAN CLOCK ASSOCIATED1*
CCT	CONSTANS, CONSTANS-LIKE, TOC1 domain
CDF	CYCLING DOF FACTOR
CEN	*CENTRORADIALIS*
cM	centiMorgans
CMS	Cytoplasmic Male Sterility
CO	*CONSTANS*
COLDAIR	*COLD ASSISTED INTRONIC NONCODING RNA*
COOLAIR	*cold induced long antisense intragenic RNA*
DNA	Deoxyribonucleic Acid
EAM8	*EARLY MATURITY8*
ELF	EARLY FLOWERING
Ehd1	*Early heading date1*
EST	Expressed Sequence Tag
FA	*FALSIFLORA*
FLC	*FLOWERING LOCUS C*
FRI	*FRIGIDA*
FUL	*FRUITFULL*

FT	*FLOWERING LOCUS T*
GA	Gibberellic Acid
GA20ox	*Gibberellic Acid 20-oxidase*
GAI	*Gibberellic Acid Insensitive*
Ghd7	*Grain number, plant height, heading date7*
ha	hectare
Hd	Heading-date
InDel	Insertion-Deletion
LDs	Long Days
LHY	*LATE ELONGATED HYPOCOTYL*
LUX	*LUX ARRHYTHMO*
MADS	MCM1, AGAMOUS, DEFICIENS and SRF domain
MFT	*MOTHER OF FT AND TFL1*
PEBP	PhosphatidylEthanolamine-Binding Protein
Ppd-1	*Photoperiod-1*
PHD	Plant HomeoDomain
PIF4	Phytochrome-Interacting Factor4
PRC2	Polycomb Repressive Complex2
PRR	Pseudo-Response Regulator
qPCR	quantitative Polymerase Chain Reaction
REC	response regulator receiver domain
RFT1	*RICE FLOWERING LOCUS T1*
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
RT-qPCR	Reverse Transcription-qPCR
SDs	Short Days
SFT	*SINGLE FLOWER TRUSS*
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CO1*
SP	*SELF-PRUNING*
SPL	*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*
TEM	TEMPRANILLO
TFL1	*TERMINAL FLOWER1*
TOC1	*TIMING OF CAB2 EXPRESSION1*
TSF	*TWIN SISTER OF FT*
VIN3	*VERNALIZATION INSENSITIVE3*
VRN	VERNALIZATION
ZCN8	*Zea CENTRORADIALIS8*
ZT	Zeitgeber Time
5’UTR	5’ untranslated region

1 Introduction

1.1 Aim and Objectives

The aim of this thesis was to investigate the core molecular mechanism controlling bolting and flowering initiation in beets (*Beta vulgaris*). This was accomplished using an empirical approach based on observing and dissecting natural variation in flowering time in beet populations, in conjunction with a deductive approach based on knowledge acquired from model plants such as *Arabidopsis thaliana*. The second strategy relied on the general assumption that information obtained by studying a model organism can be applied to understand the behavior of a related organism, which is equivalent to the assumption that developmental pathways have been maintained over the course of evolution.

The first objective was to identify the major locus controlling life cycle decisions in native and cultivated beet populations. This was successfully achieved using forward genetics by developing a large mapping population in segregation for annuality, positional cloning and functional validation (**Paper II**).

The second objective was to isolate flowering-time-control genes in beets by means of reverse genetics using the *Arabidopsis* flowering model as a "blueprint". Two *Beta* homologs of a major floral integrator gene in *Arabidopsis* were isolated and characterized using transgenic approaches (**Paper I**).

By performing these experiments, we tested and confirmed the presence of key features among plant species in controlling flowering induction, but also falsified the hypothesis of a conserved and unique molecular layer governing flowering in all living flowering plants.

The data obtained on the molecular mechanisms controlling growth habits and flowering time in beets will have direct applications in sugar beet breeding and seed production.

1.2 The sugar beet plant

1.2.1 Economic importance

The sugar beet (*Beta vulgaris*) is essentially cultivated for its large taproot which accumulates a high concentration of sucrose (18-20% of its total fresh weight) during the vegetative growing period of its biennial life cycle. It represents one of the major crops for sugar production, being second only to sugar cane (*Saccharum officinarum*). Sugar beet became a major crop in Europe after Napoleon's decision, in 1811, to substitute imported cane sugar with beet sugar in response to the English continental blockade (**Fig. 1**).

Two hundred years later, sugar beet was the eighth most heavily produced crop in the world: 227 million tons were produced in 2011, representing 30-35% of the world's sugar production (FAOSTAT, 2011). This is partly due to growing demand from producers of sustainable energy sources such as bioethanol and biogas. Today, sugar beets are mainly grown in Europe and North America, but they are also grown in tropical countries, which produce so-called "tropical beets".

Figure 1. French cartoon from 1811 showing Napoleon I squeezing the sweet juice out of a sugar beet root and adding it to his coffee (modified illustration from: The sugar beet crop. Science into practice, Cooke and Scott, 1993).

1.2.2 Origin

Cultivated beets (*Beta vulgaris* ssp. *vulgaris*) are eudicots from the Amaranthaceae family (caryophyllids, order of the Caryophyllales). There are four agriculturally-important groups within the sub-species *vulgaris*: sugar beet

(*B. v.* ssp. *vulgaris* convar. *vulgaris* var. *altissima*), garden beet (table or red beet; *B. v.* ssp. *vulgaris* convar. *vulgaris* var. *vulgaris*), fodder beet (*B. v.* ssp. *vulgaris* convar. *vulgaris* var. *crassa*) and leaf beet (mangold, chard or silver beet; *B. v.* ssp. *vulgaris* convar. *cicla*). All of these are cultivated descendents of the sea beet plant (*Beta vulgaris* ssp. *maritima*) (**Fig. 2**), which is commonly found in Europe on the Mediterranean, Atlantic, North and Baltic coastlines. The common ancestor is thought to have emerged from weeds growing on the shores of Ancient Greece (Cooke and Scott, 1993). Cultivated beets and sea beet are diploid with nine pairs of chromosomes and are cross-compatible.

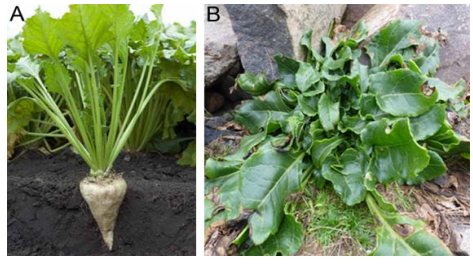

Figure 2. Cultivated sugar beet A) and its ancestor, the sea beet B)[1].

1.2.3 Sugar beet breeding

The main objective in sugar beet breeding is to develop varieties with high sugar contents. Sugar yield is dependent on the length of the vegetative growing period (which typically runs from April to November) and the degree of environmental stress, which depends on where the plant is grown. Beets are inherently very resistant to drought and salinity, and breeders continuously attempt to develop varieties that are also resistant to diseases (e.g. rhizomania, rhizoctonia, cercospora, etc...) and pests (e.g. cyst nematodes, root knot nematodes, etc...). Early sowing in February or March can extend the growing period. However, the low temperatures at this time of year cause thermal induction (also known as vernalization) and bolting (i.e. the onset of the reproductive phase) in bolting-sensitive varieties, especially in temperate climates. Bolting causes the development of a thick and highly lignified stem and reduces the sugar content of the beet. Resistance to bolting is therefore another important agronomic trait that needs to be bred for. Other traits of interest to growers and the sugar industry include various seed quality traits (e.g. high seed emergence, high seed loculi filling) and processing quality traits (e.g. low-tare roots, low sodium and potassium contents, and alpha-amino

[1] Sources for images shown in Fig. 2:
A) http://www.umu.se; B) private picture (P. Pin)

nitrogen content), as well as root yield (which correlates negatively with sugar content).

Although triploid sugar beet hybrids have been grown in the 90's, most current commercial sugar beets are diploid hybrids produced by three-way crossing. Hybrid sugar beet seed production relies on cytoplasmic male sterility (CMS) where a sugar beet male-sterile (MS) line is used as mother plant and crossed with a sugar beet line called O-type. The offspring, which is referred to as an F1MS line, is also male-sterile and is used as a mother plant in a second cross with a third line that is referred to as a Pollinator (**Fig. 3**). The crosses are only possible once the sugar beet lines enter their second, reproductive, growing phase which takes place after overwintering or artificial exposure to cold temperatures.

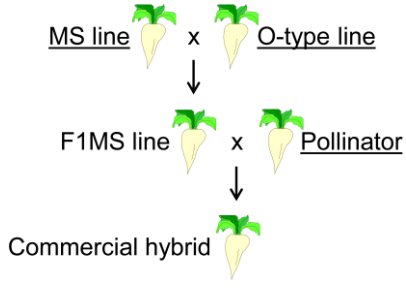

Figure 3. Hybrid sugar beet production. Three parental lines are used in crossing: a male-sterile (MS), an O-type and a Pollinator. Commercial hybrid seed production is performed in open fields, where the Pollinator and F1MS lines are autumn-sown next to each other. Pollination occurs in the following year once the parental lines have overwintered. This thermal induction is an essential process in beets in the transition from the vegetative to the reproductive stage.

There are separate breeding programs for the MS/O-type lines and the Pollinator line. Seed companies make heavy use of molecular markers in the early stages of these breeding programs to pre-select plants with the most useful traits and to eliminate those that are unlikely to satisfy the agronomical requirements of subsequent phenotypic breeding tests. Marker-assisted trait selection (MATS) has proven to be very powerful for tracing single (or monogenic) traits and is increasingly popular for use in selecting quantitative trait locus (QTL) regions (quantitative MATS). The use of molecular markers substantially reduces costs and the need for space during phenotypic evaluation and also makes it possible to implement back-crossing programs more quickly and precisely. The current sugar beet breeding at Syngenta Seeds uses more than 3000 SNP-based markers and this number is expected to increase following the sequencing of the sugar beet genome and the re-sequencing of genomes of elite lines.

1.2.4 Why use beets as a new model organism?

The core-eudicot angiosperms consist of three major clades: the asterids, the caryophyllids and the rosids (**Fig. 4**). The mechanisms that control the time of flowering in both asterids and rosids have been characterized in some detail. At present, the most extensively studied asterids are species from the Solanaceae (e.g. potato and tomato), while the rosid *Arabidopsis thaliana* has been and is still intensively used by molecular biologists. However, very few of the caryophyllids have been studied at the molecular level. The ice plant (*Mesembryanthemum crystallinum*) and the sugar beet (*Beta vulgaris*) are probably the two most attractive model species from this clade due to their evolutionary divergence (with the ice plant and sugar beet being Crassulacean Acid Metabolism (CAM) and C3 photosynthetic plants, respectively) and the availability of genetic tools (e.g. expressed sequence tag (EST) libraries, mutant collection/tilling population, established transformation protocols, etc…). Moreover, the recent sequencing of the sugar beet genome will facilitate map-based cloning of genes of interest and enable comparative genomic analysis.

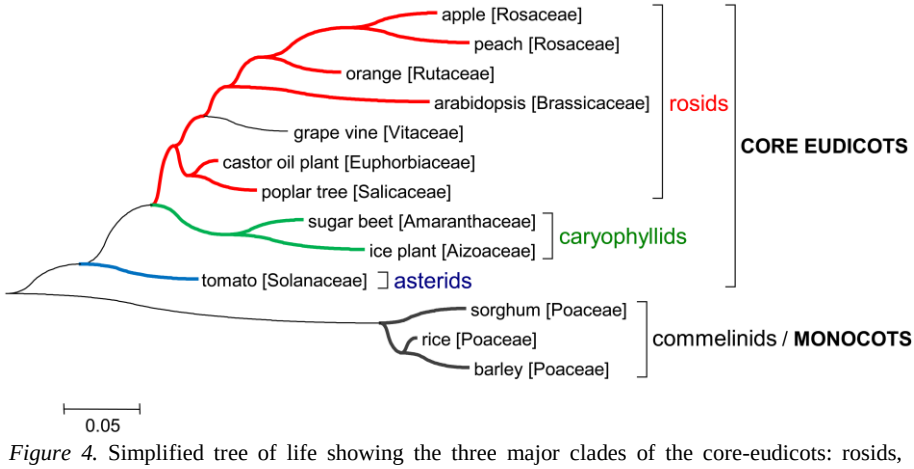

Figure 4. Simplified tree of life showing the three major clades of the core-eudicots: rosids, caryophyllids and asterids. The phylogenetic tree was constructed in MEGA5 (Tamura *et al.*, 2011) from a multiple alignment of the response regulator receiver domain (REC) domain of the TIMING OF CAB2 EXPRESSION1 (TOC1) proteins. For each entry, the common name is given followed by the plant family in brackets. The poaceae (monocots) were used as an outgroup. The evolutionary history was inferred using the Maximum Likelihood method based on the JTT matrix-based model (Jones *et al.*, 1992). The relevant accession numbers (GenBank) and Gene IDs (Phytozome) are: apple, MDP0000453272; peach, ppa015394m; orange, orange1.1g008761m; arabidopsis, NM_125531; grape vine, XM_002281721; castor oil plant, XM_002514679; poplar tree, XM_002330094; sugar beet, BI543444; ice plant, AY371288; tomato, Solyc03g115770; sorghum, XM_002452417; rice, NM_001053983; barley, AK376384.

1.3 Life cycle and flowering control in model plants

The following sub-chapters give a short summary of the key molecular mechanisms involved in flowering time and life cycle control in the most widely used flowering model plants. The aim of this section is to illustrate the common layers of regulation and also the different machineries that have developed over time across the plant species.

1.3.1 The *Arabidopsis* model

Arabidopsis thaliana has been and is still today by far the most heavily studied plant species (Somerville and Koornneef, 2002), particularly with respect to flowering control. Consequently, its properties are only briefly reviewed herein (**Fig. 5**). *Arabidopsis* responds to two essential environmental stimuli – the variation in day length (or photoperiod signal) and prolonged exposure to cold temperatures (or vernalization).

Arabidopsis is a facultative long day (LD) plant based on its ability to flower more rapidly in LDs than in short days (SDs). The integration of the photoperiod is controlled in the leaf through the transcriptional activation of the mobile flowering promoter (or *florigen*) *FLOWERING LOCUS T (FT)*[1] via CONSTANS (CO) (reviewed in Kobayashi and Weigel, 2007; Turck *et al.*, 2008). This mechanism is tightly controlled via the circadian clock, which coordinates the diurnal oscillation in *CO* expression (Suarez-Lopez *et al.*, 2001), and is only possible in LDs when nuclear CO protein activity is stabilized (Valverde *et al.*, 2004). CO-mediated *FT* expression is balanced by the repressing action of TEMPRANILLO (TEM) proteins (Castillejo and Pelaz, 2008). The FT protein moves through the vascular tissues to the shoot apex (Corbesier *et al.*, 2007; Jaeger and Wigge, 2007; Mathieu *et al.*, 2007) where it activates the transcription of floral meristem identity genes (Abe *et al.*, 2005; Wigge *et al.*, 2005) (**Fig. 5**). In addition, the *FT* messenger RNA (mRNA) itself has been shown to move independently of its protein to the shoot apical meristem (Li *et al.*, 2009) and to be directly involved in the long-distance florigenic signaling (Li *et al.*, 2011; Lu *et al.*, 2012).

A vernalization period facilitates flowering in the winter-annual *Arabidopsis* accessions via the epigenetic silencing of the major flowering repressor gene *FLOWERING LOCUS C (FLC)* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999).

[1] Normal upper case names are proteins, uppercase italic names refer to genes, lower case italics to mutants

This involves the activation of two *FLC* non-coding ribonucleic acids (ncRNAs), *cold induced long antisense intragenic RNA (COOLAIR)* (Swiezewski *et al.*, 2009) and *COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR)* (Heo and Sung, 2011), which transiently silence *FLC* transcription.

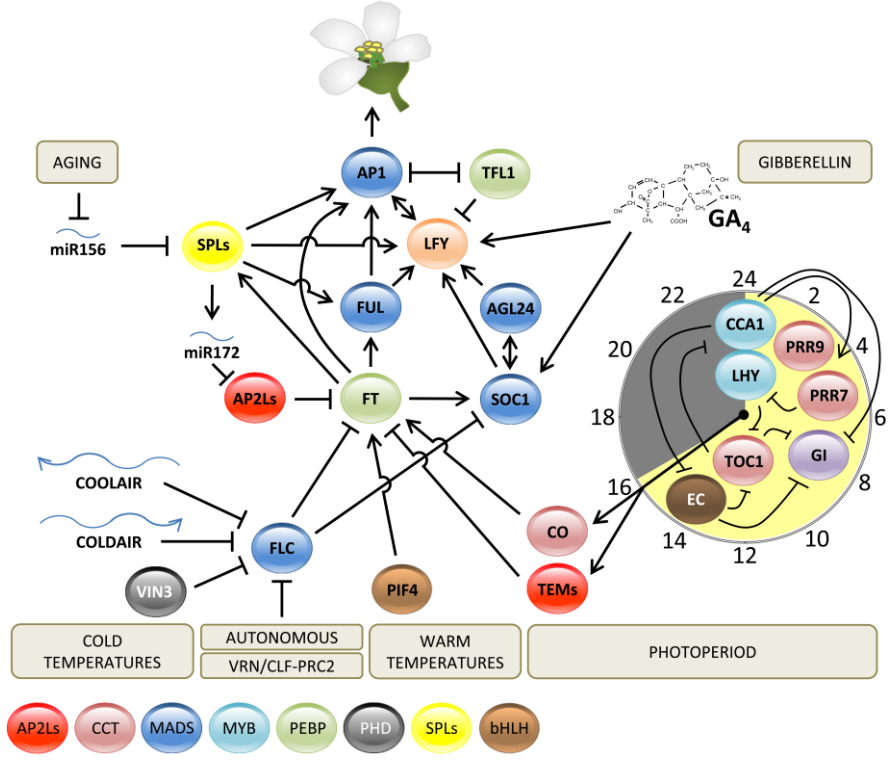

Figure 5. Simplified synopsis of the molecular mechanisms that underpin flowering time control in *Arabidopsis*. Factors that affect the flowering transition in winter-annual accessions include aging, exposure to cold temperatures (that is, vernalization), exposure to warm temperatures, day-length sensing (that is, photoperiod) and gibberellic acid concentrations. Endogenous and exogenous stimuli are integrated through the two major flowering integrators, FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1), which in turn orchestrate the transcriptional regulation of meristem identity genes such as *LEAFY (LFY)*, *AGAMOUS-LIKE24 (AGL24)*, *FRUITFULL (FUL)* and *APETALA1 (AP1)*. Icons represent individual genes or group of genes encoding similar protein motifs. The different classes of protein motifs encoded are shown in different colors. LFY and GIGANTEA (GI) represent two unique classes of proteins. EC stands for Evening Complex that is composed of EARLY FLOWERING3 (ELF3), ELF4 and LUX ARRHYTMO (LUX) proteins. AP2Ls stands for *APETALA2*-like proteins. SPLs stands for SQUAMOSA PROMOTER BINDING PROTEIN-LIKE proteins. VERNALIZATION (VRN), Polycomb Repressive Complex2 (PRC2) and AUTONOMOUS consist of several components from different protein classes that are involved in the transcriptional repression of the flowering repressor gene *FLOWERING LOCUS C (FLC)*.

This is followed by transcriptional activation of *VERNALIZATION INSENSITIVE3 (VIN3)* (Sung and Amasino, 2004) which, together with VERNALIZATION1 (VRN1) (Levy *et al.*, 2002), VRN2 (Gendall *et al.*, 2001) and VRN5 (Greb *et al.*, 2007) induce the stable repression of *FLC* by histone methylation (Bastow *et al.*, 2004). Mutations at *FLC* and at its upstream regulator FRIGIDA (FRI) (Johanson *et al.*, 2000; Shindo *et al.*, 2005; Werner *et al.*, 2005), account for much of the natural variation in *Arabidopsis* growth habits. The autonomous pathway acts in parallel with vernalization through different layers of regulation involving RNA-mediated chromatin silencing of *FLC* (Simpson, 2004) (**Fig. 5**).

By contrast, warm temperatures promote *FT* transcription via the transcription factor Phytochrome-Interacting Factor4 (PIF4) (Kumar *et al.*, 2012).

Aging is another important factor that affects flowering initiation. As the plant ages, there is a gradual deregulation of the highly conserved micro ribonucleic acid (miRNA) miR156, which represses the transcriptional regulation of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* genes (Wang *et al.*, 2009; Wu *et al.*, 2009). *SPL*s can both act independently of *FT*, by promoting the expression of floral meristem identity genes, and via *FT*, by relieving the repressive action of *AP2*-like genes on *FT* via the intermediacy of another miRNA, miR172 (Wu *et al.*, 2009) (**Fig. 5**).

Finally, gibberellin signaling also affects the flowering transition in *Arabidopsis* (as reviewed by Mutasa-Göttgens and Hedden, 2009). The extent of its control over flowering under LD conditions is currently unclear, but gibberellic acids (GAs) have been shown to be essential in flowering promotion under SD conditions. The active compound GA_4 mediates flowering (Eriksson *et al.*, 2006) by activating *LFY* (Blázquez *et al.*, 1998) and *SOC1* (Moon *et al.*, 2003) (**Fig. 5**).

1.3.2 The rice model

Rice (*Oryza sativa*) is a facultative SD plant that starts flowering (also known as heading in cereals) once the day length falls below a critical threshold. Rice has not developed molecular machinery that would respond to vernalization, which is probably due to the climate of its natural habitats. A large number of genes involved in the control of flowering have now been identified and the core mechanism that integrates day-length stimuli is somewhat similar to that observed in *Arabidopsis* and features a *CO* (named *Heading-date1 (Hd1)*)/*FT* (named *Heading-date3a (Hd3a)*) regulon (Yano *et al.*, 2000; Kojima *et al.*, 2002; Tamaki *et al.*, 2007). A major difference is that *Hd1* plays a dual role in promoting and inhibiting the transcription of the *florigen Hd3a* in SDs and LDs

respectively (**Fig. 6**). Interestingly a paralog of *Hd3a*, *RICE FLOWERING LOCUS T1 (RFT1)*, also functions as a flowering promoter but unlike *Hd3a*, it acts under LD conditions (Komiya *et al.*, 2009). The expression of *RFT1* is controlled by a molecular layer that is unique to rice and involves a CCT [CONSTANS, CONSTANS-LIKE, TOC1 domain] protein, Ghd7 [Grain number, plant height, heading date7]. Other key constituents of this molecular layer include a MADS [MCM1, AGAMOUS, DEFICIENS and SRF domain] gene, *MADS50*, the *SOC1* ortholog in rice, which differs from the *Arabidopsis SOC1* gene in that it acts in the leaf and upstream of the *FT* ortholog *RFT1*, and a B-type response regulator gene named *Early heading date1 (Ehd1)* (Doi *et al.*, 2004; Komiya *et al.*, 2009; Itoh *et al.*, 2010). Transgenic lines down-regulated for both *Hd3a* and *RFT1 FT* orthologs exhibit continuous vegetative growth, suggesting that flowering in rice is fully dependent on the tandem activity of the *Hd3a* and *RFT1* florigens (Komiya *et al.*, 2008). The rice example nicely illustrates how sub-functionalization between two paralogs can contribute to plant plasticity. Variation in the sequence of the *Hd3a* promoter, the expression of *Edh1* and the activity of the Hd1 protein account for most of the diversity in flowering time observed in different cultivated varieties of rice (Takahashi *et al.*, 2009).

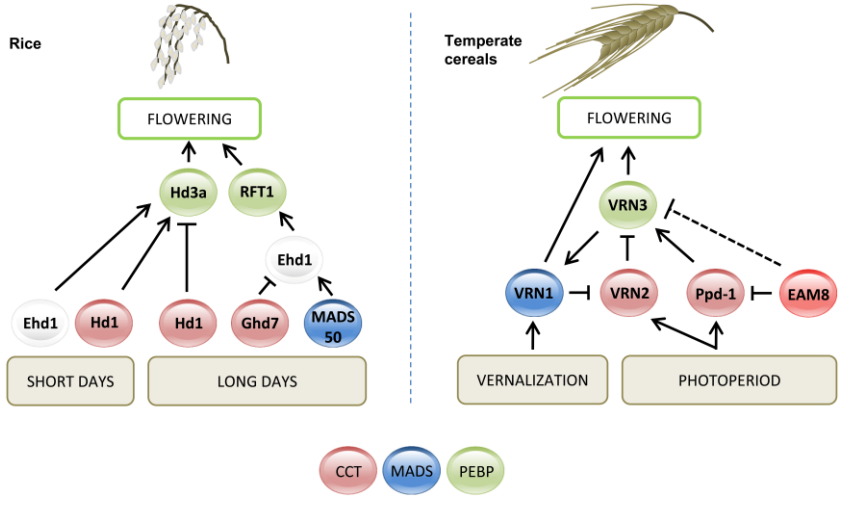

Figure 6. Simplified flowering model for rice and temperate cereals. Colored ovals represent genes or groups of genes encoding similar protein motifs. The classes of protein motifs are shown with different icon color.

1.3.3 The temperate cereal model

In contrast to rice, temperate cereals such as barley and wheat respond to vernalization. Map-based cloning approaches identified three major genes, *VRN1*, *VRN2* and *VRN3*, which mediate life cycle control in cereals (Yan *et al.*, 2003; Yan *et al.*, 2004; Yan *et al.*, 2006). It is important to note that *VRN1* and *VRN2* do not encode the same proteins as the *VRN1* and *VRN2* genes in *Arabidopsis*. VRN1 is a FRUITFULL (FUL)/APETALA1 (AP1) homolog and promotes heading whereas VRN2 is a new class of CCT protein that prevents flowering by repressing the cereal *FT* ortholog, *VRN3*. Vernalization induces *VRN1* transcription. VRN1 inhibits *VRN2* transcription which relieves the repression of VRN3. Once induced, VRN3 promotes inflorescence initiation and also enhances *VRN1* transcription through a positive feedback loop (**Fig. 6**).

In addition to the above mechanism, temperate cereals also respond to photoperiod variation. The master switch responsible for the integration of the LD signal is a pseudo-response regulator (PRR) gene called *Photoperiod-1 (Ppd-1)*, which is an upstream regulator of *VRN3* (**Fig. 6**). *Ppd-1* was also isolated via positional cloning using a mapping population derived from two spring barley varieties in which one of the parents is insensitive to LDs (*ppd-1*) (Turner *et al.*, 2005). *Ppd-1* corresponds best to the *Arabidopsis PRR7* gene, however, unlike *Ppd-1*, *PRR7* does not play a major role in flowering control or the regulation of *FT* (Nakamichi *et al.*, 2007). *EARLY MATURITY8 (EAM8)*, also known as *Praematurum-a (Mat-a)*, is a second component involved in the photoperiodic signaling through activation of *VRN3* (Faure *et al.*, 2012; Zakhrabekova *et al.*, 2012). *EAM8* is ortholog of the *Arabidopsis* circadian-clock gene *ELF3*. In contrast to *ppd-1*, *eam8* mutations severely affect the expression of core clock genes and lead to increased *Ppd-1* and *VRN3* expressions. Interestingly, the elevation of *VRN3* expression in *eam8* mutants is independent of the *Ppd-1* allelic forms (i.e. *Ppd-1* or *ppd-1*) suggesting the presence of a possible *Ppd-1*-independent *VRN3* mediation pathway (**Fig. 6**). *CO* homologs are found in barley and wheat but, unlike *CO* in *Arabidopsis* and *Hd1* in rice, their role in the photoperiodic signaling pathway in temperate cereals (in contrast to *Ppd-1*) seems to be of less importance. Recent work in wheat suggests that during early development *TaCO1* could contribute to the flowering promotion, via *Ppd-1*, but that a feedback mechanism would down-regulate its expression once *TaFT1* (the wheat ortholog of *VRN3*) is activated (Shaw *et al.*, 2012). The current data do not preclude an activation of *TaFT1* via a direct action of *Ppd-1*, or through *TaCO1*, or via an alternative pathway.

VRNs, *Ppd-1* and *EAM8* contributed to the domestication of the temperate cereals. Gain-of-function and loss-of-function mutations at *VRN1* and *VRN2*,

respectively, resulted in the development of the current spring/winter cultivated wheat and barley varieties (Hemming *et al.*, 2008). The late flowering phenotype created by the photoperiod-insensitive *ppd-H1* allele has been selected and maintained by growers cultivating barley in the northern part of Europe, where it gives higher yields than the *Ppd-H1* varieties (Cockram *et al.*, 2007). Besides, breeders selected early-flowering barley varieties carrying the recessive *eam8* mutations within the *ppd-H1* genetic pool with the scope to move barley production to high-latitude short-growing season environments in Europe (Lundqvist, 2009). In wheat, cultivation of photoperiod-insensitive varieties that flower rapidly in SDs have been widely used during the "green revolution" (Worland and Snape, 2001) allowing production in Southern Europe where early flowering avoids grain maturation during the high temperatures of the summer. The precocious flowering observed in these wheat varieties is the result of gain-of-function mutations at one or several homoeologous *Ppd-1* loci (that is, *Ppd-A1*, *Ppd-B1* and *Ppd-D1*) that yield to an elevation in *Ppd-1* expression and a subsequent *TaFT1* expression increased (Beales *et al.*, 2007; Wilhelm *et al.*, 2009; Shaw *et al.*, 2012). Breeding stack of the photoperiod-insensitive mutations *Ppd-A1a*, *Ppd-B1a* and *Ppd-D1a* demonstrated that as the number of *Ppd-1a* mutations increased, *TaFT1* expression is elevated and flowering time is accelerated (Shaw *et al.*, 2012). Another example showed that natural increase of *Ppd-B1* gene copy number is associated with the early-flowering phenotype of some photoperiod-insensitive wheat varieties (Díaz *et al.*, 2012).

1.3.4 The tomato model

In contrast to *Arabidopsis* and the cereals, the tomato plant is a day-neutral plant. Its flowering is light-dose-dependent and is not induced by changes in day length (Calvert, 1959). Despite this physiological distinction, it seems that a key molecular layer in flowering control has been conserved in both the tomato plant and photoperiod-responsive plants. Flowering is dependent on the action of an antagonistic pair of phosphatidylethanolamine-binding protein (PEBP) genes, *SINGLE FLOWER TRUSS (SFT)* (also called *SELF-PRUNING3D (SP3D)* (Carmel-Goren *et al.*, 2003)) (Molinero-Rosales *et al.*, 2004; Lifschitz *et al.*, 2006) and *SELF-PRUNING (SP)* (Pnueli *et al.*, 1998), which are orthologs of the *Arabidopsis FT* and *TERMINAL FLOWER1 (TFL1)* respectively. As in *Arabidopsis* and rice, the tomato *FT* ortholog (*SFT*) was shown to be part of the systemic signaling system that regulates flowering (Lifschitz *et al.*, 2006) and is therefore likely to be the tomato *florigen* or a component thereof. Another gene that is involved in flowering and floral meristem identity, and appears to be essential for normal floral development, is

FALSIFLORA (FA), the tomato ortholog of the *Arabidopsis LEAFY (LFY)* gene (Molinero-Rosales *et al.*, 1999).

Notably, in addition to controlling flowering, *SFT* and *SP* also regulate the characteristic sympodial growth habit of the tomato (Pnueli *et al.*, 2001; Shalit *et al.*, 2009). Elegant experiments have demonstrated that *SFT* heterozygosity causes yield overdominance (Krieger *et al.*, 2010) in the strict absence of *SP*, suggesting that the *SFT*/*SP* ratio is a critical factor in tomato development.

Mutation at the *SP* locus has huge implications in terms of the development of the tomato crop – *sp* varieties exhibit limited shoot growth (referred to as a "determinate" phenotype), which results into a bushy and compact constitution of the plant and a nearly homogeneous flower and fruit setting (Picken *et al.*, 1986; Atherton and Harris, 1986).

1.4 Flowering control in beet

1.4.1 Bolting and flowering induction

Cultivated beets are LD plants with vernalization-dependent flowering induction (Margara, 1960; Lexander, 1980). The onset of the floral transition is marked by "bolting" or the development and elongation of a stem from the primary axis. If bolting beets are exposed to suitable environmental conditions, that is, an optimal temperature and photoperiod, the stem develops into an indeterminate inflorescence with secondary shoots and flowering occurs. Bolting and flowering induction are triggered by a photothermal-sensitive process whose molecular details are currently unknown but which requires exposure to cold temperatures over an extended period ranging from a few weeks to several months (depending on the beet variety) and a certain critical day length (>12-16 hours light). Without vernalization, sugar beets remain vegetative for several years when grown under LD conditions (Ulrich, 1954) (**Fig. 7**). If beets are exposed to SD conditions rather than LDs following the vernalization period, bolting and flowering do not occur (Margara, 1960; Mutasa-Göttgens *et al.*, 2010) (**Fig. 7**).

Many studies have been conducted on stem elongation initiation using GAs. Bolting and flowering time can be accelerated in vernalized beets by GAs. GAs can also induce bolting in the absence of vernalization and independently of the photoperiod, but cannot promote flowering (Margara, 1960; Margara, 1967; Mutasa-Göttgens *et al.*, 2010) (**Fig. 7**). Consequently, and in contrast to other plant species where GAs can compensate for a lack of vernalization or photoperiod signaling (reviewed in Mutasa-Göttgens and Hedden, 2009), GA alone cannot exert full control over flowering in beets.

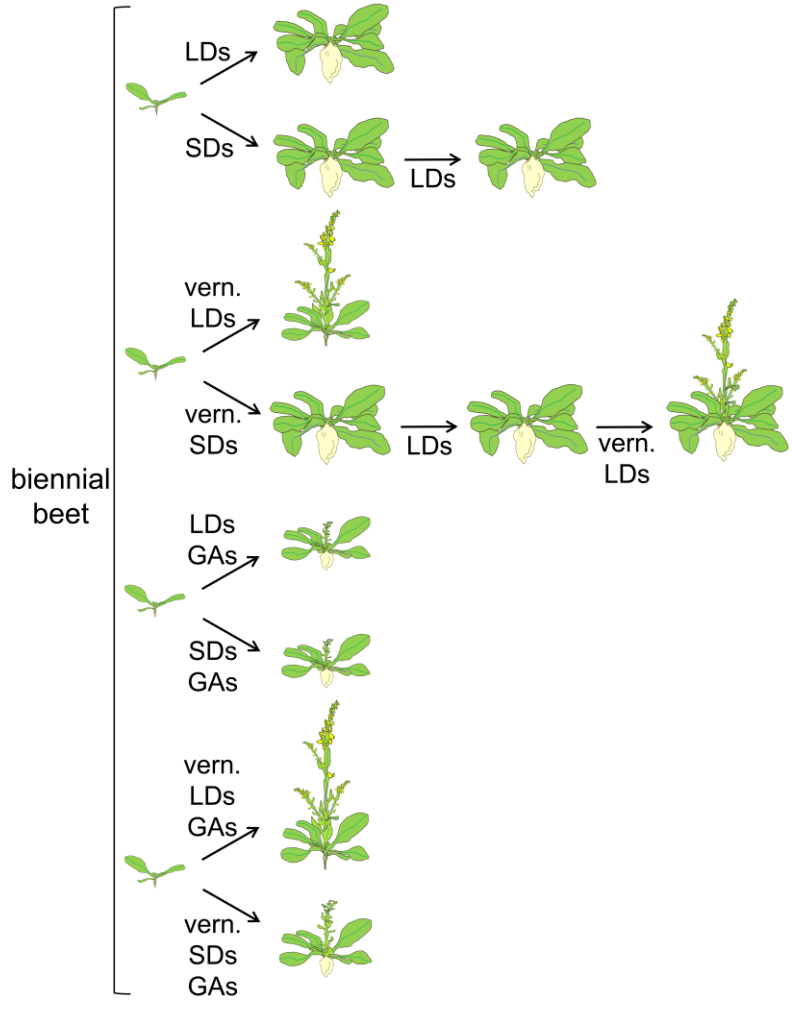

Figure 7. Effect of photoperiod and GAs on bolting and flowering time in non-vernalized and vernalized biennial beets. Biennial beets have an obligated vernalization-dependent flowering which cannot be overruled by exposure to inductive LDs or treatment with GAs alone. Post vernalization, LDs are essential for bolting and flowering. If vernalized plants are exposed to SDs for a certain time and then switched to LDs, their competence to initiate bolting is lost and they need to be re-vernalized. GAs promote bolting independently of the photoperiod, but the elongation of the stem remain limited and flowering does not occur. Under conditions that induce bolting and flowering, that is, after vernalization and with LDs, GA treatment promotes the floral transition. LDs are essential for flower development.

It has also been shown that exposure to extreme LDs with 22 hours of light following vernalization enhances bolting and flowering time (Pin, unpublished data), suggesting that a photoperiodic dose signal is involved in the bolting/flowering transition.

A *Beta Gibberellic Acid 20-oxidase (GA20ox)* ortholog has been identified and its transcription has been shown to be up-regulated after vernalization (Mutasa-Göttgens *et al.*, 2009). Heterologous expression of the *Arabidopsis Gibberellic Acid Insensitive (GAI)* gene under its own promoter, a DELLA protein that negatively regulates GA-signaling (Peng *et al.*, 1997), delays bolting and increases the required duration of vernalization, suggesting that GAs are involved in bolting transition in *Beta* (Mutasa-Göttgens *et al.*, 2009).

1.4.2 De-vernalization phenomenon

There is a distinct separation between the bolting and the flowering processes in beets, and flowering does not necessarily follow bolting. This can occur when beets have been vernalized and are subsequently exposed to non-inductive SDs or to too warm temperatures (Margara, 1960; Margara, 1967; Lexander, 1980; Van Dijk, 2009) (**Fig. 7**). In contrast to *Arabidopsis*, vernalized beets can lose the ability to initiate bolting and flowering that was acquired during vernalization. This process is called de-vernalization and remains uncharacterized at the molecular level. De-vernalization can also occur after bolting initiation, in which case stem elongation is arrested (resulting in a so-called stunted phenotype) and flowering is typically abolished. Once beets become de-vernalized, they must undergo re-vernalization in order to produce flowers and seeds (**Fig. 7**).

1.4.3 Growth habits: role of the bolting gene *B*

The sea beet is the wild ancestor of the cultivated beets and often exhibits an annual growth habit. When grown and maintained under SD conditions, annual beets cannot bolt and instead exhibit continuous vegetative growth. However, when exposed to LD conditions, annual beets start bolting and flowering rather rapidly, over a period of a few weeks to a few months, depending on the accession (**Fig. 8**). Increases in the length of the photoperiod can also greatly accelerate bolting in the annual beets, as seen in the vernalized biennial beets (Pin, unpublished data). Interestingly, bolting does not occur in vernalized annual beets that are subsequently exposed to SD conditions (Mutasa-Göttgens *et al.*, 2010). However, if the plants are exposed to LD conditions, vernalized annual beets bolt earlier than their non-vernalized counterparts (Pin, unpublished data) (**Fig. 8**). This suggests that annual beets can respond to

vernalization and therefore that the machinery involved in the vernalization integration is present and intact in annuals.

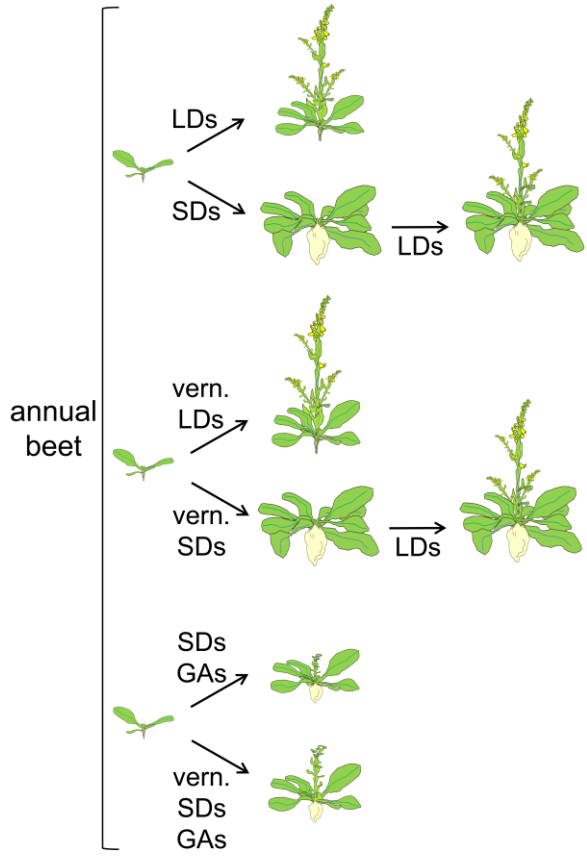

Figure 8. Effect of photoperiod and GAs on bolting and flowering time in non-vernalized and vernalized annual beets. Annual beets bolt and flower as a direct response to the inductive effects of LD conditions. Plants remain vegetative when grown under SD conditions but the flowering transition can start as soon as the plants are exposed to LDs. Annuals do respond to vernalization, which causes them to bolt more rapidly. GAs promote bolting but the elongation of the stem is limited and flowering does not occur if plants are grown under SD conditions.

Genetic studies have shown that annuality is dominant over bienniality and is governed by a single locus called the 'bolting gene' *B*, located on chromosome II (Munerati, 1931; Abegg, 1936; Abe *et al.*, 1997). Plants carrying the dominant *B* form do not require vernalization and initiate bolting and flowering as a direct response to the photoperiodic LD signal. The nature of *B* at the start of this thesis project was unknown.

Among sea beet populations, vernalization-dependent flowering promotion is strongly associated with the latitudinal cline (Van Dijk, 1997; Boudry *et al.*, 2002). Sea beet populations from the Mediterranean Basin do not require vernalization and flower rapidly under LD conditions, whereas populations from northern latitudes (the Atlantic coast, North Sea and Baltic Sea) can flower very late under LD conditions and may exhibit a latitude-dependent increase in their required vernalization period. It remains unclear whether factors other than *B* affect growth habit determinism in sea beet populations.

Box 1

The *FLOWERING LOCUS T (FT)* gene family

FLOWERING LOCUS T (FT) is a transcription factor involved in integrating the photoperiodic signal, which is crucial for the flowering transition in many flowering plant species. Recent studies have demonstrated that in addition to flowering control, *FT* genes are involved in a broad range of plant developmental processes such as leaf development, fruit setting, vegetative growth, and stomatal and tuberization regulation (reviewed in **paper III**). *FT* encodes a small mobile protein of ±175 amino acids and belongs to a small gene family called PhosphatidylEthanolamine-Binding Protein (PEBP) containing four sub-groups: FT-likes, TERMINAL FLOWER1 (TFL1)-likes, BROTHER OF FT AND TFL1 (BFT)-likes and MOTHER OF FT AND TFL1 (MFT)-likes. *Arabidopsis* has six PEBP members: *FT* and *TWIN SISTER OF FT (TSF)* (which belong to the FT-like group), the TFL1-like *TFL1* and *CENTRORADIALIS (CEN)* (or *ATC*), *BFT* and *MFT*.

TFL1 represses flowering, while both *FT* and *TSF* promote it (Ratcliffe *et al.*, 1998; Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Yamaguchi *et al.*, 2005). Elegant experiments have demonstrated that the antagonistic functions observed between FT and TFL1 are essentially due to a few amino-acid variations within the protein (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). FT and TFL1 are thought to compete for a common interacting factor at the shoot apex, FD (Abe *et al.*, 2005; Wigge *et al.*, 2005), which has some intermediate level of activity in promoting flowering in the absence of FT or TFL1. *FT* and *TFL1* orthologs have been isolated in many other flowering plant species and their activating/repressing functions in flowering control are generally conserved.

Little is known about *BFT* and *MFT*. Overexpression of *BFT* and *MFT* in *Arabidopsis* caused late and moderately early flowering, respectively. However loss-of-function mutations in these genes do not lead to obvious flowering phenotypes (Yoo *et al.*, 2010; Yoo *et al.*, 2004). *MFT* was shown to regulate seed germination (Xi *et al.*, 2010).

1.5 Study case

Bolting resistance is a challenging and major agronomic trait in sugar beet breeding. Breeders need to produce strongly bolting-resistant beet varieties without affecting the floral and seed development that are required for crossing programs and seed production. Although many experiments have been performed to better understand the environmental parameters required for the floral transition in beets, no characterization of important factors at the molecular level has so far been achieved. Insights into the molecular mechanisms controlling bolting and flowering should allow quicker, more precise and more effective strategic breeding (in terms of both conventional and also biotechnological aspects).

Numerous studies on model plants have shown that the mechanisms involved in flowering regulation have evolved over time, but that important layers of regulation appear to be conserved between species. This is the case for the integration of the photoperiodic signal, which seems to be dependent on the action of orthologs of the well described transcription factor *FT* (**Figs. 5** and **6**, and **Box 1**). On the other hand, the machineries involved in integrating the vernalization signal have diverged substantially, at least between *Arabidopsis* and the cereal models. Since the photoperiodic signal (LDs) is required to induce proper bolting and flowering in beets, it is not unlikely that *FT* genes also play a central role in their floral transitions, as is the case in *Arabidopsis*. To investigate this hypothesis, we proposed to isolate and characterize *Beta FT* homologs.

Another fundamental question is whether or not the life cycle of beets (mediated by *B*) is controlled via mechanisms similar to those previously described for other species such as *Arabidopsis* and temperate cereals. Isolating *B* would make it possible to accurately trace annuality/bienniality which can be very valuable in applied breeding for two reasons: (i) in crossing programs where no phenotypic tests have to be performed, annuality can be used to avoid the long required vernalization period necessary for the biennial plants to

flower, and therefore to speed up the breeding process. To achieve this, annual elite lines need to be developed, which require a very robust diagnostic molecular marker to select for, or against, annuality. (ii) *B*-based markers would also have applications in quality control of commercial hybrid seed lots that are produced in open fields where annual weed beets are common (Boudry *et al.*, 1993). Pollen from annual weed beets can contaminate the hybrid production, generating heterozygous annual hybrid seeds (due to the dominance of annuality). Isolating *B* would facilitate the development of specific molecular assays for annuality.

2 Methodology

2.1 Plant material and growth conditions

Cultivated beets (*Beta vulgaris* ssp. *vulgaris*) consisting of O-type and pollinator sugar beet lines, fodder beet lines and red beet lines were used in the study along with weed beets and several wild accessions from *Beta macrocarpa*, *Beta trigyna*, *Patellifolia procumbens* (formerly known as *Beta procumbens*), *Patellifolia webbiana* (formerly known as *Beta webbiana*), a large panel of sea beets (*Beta vulgaris* ssp. *maritima*) collected along the European coastlines and various species from the Amaranthaceae family outside the genus *Beta* (*Amaranthus caudatus*, *Amaranthus cruentus*, *Amaranthus paniculatus*, *Amaranthus tricolor*, *Celosia argentea*, *Chenopodium giganteum*, *Chenopodium quinoa* and *Spinacia oleracea*). *Arabidopsis* plants (Col-0, *ft-10*, *tfl1-14* and transgenic plants harboring sugar beet gene overexpressing cassette) were used for the functional validation experiments.

Two O-type sugar beet lines were used for the gene cloning, transcriptional analysis and sugar beet transformation steps: G018B0, a conventional biennial sugar beet line carrying the homozygous recessive form *b*/*b*, and G018BB, an annual near-isogenic BC2S1 sugar beet line derived from a cross between G018B0 and an annual sea beet accession. G018BB carries the homozygous dominant form *B*/*B*.

Beet plant materials were grown in controlled environment chambers at 18 °C under LD or SD conditions consisting of 18 hours light/6 hours dark and 10 hours light/14 hours dark respectively. Vernalization was induced by 15 to 20 weeks of exposure to cold temperatures varying from 4 to 6 °C, followed by a thermal buffering period of two weeks where the temperature was gradually increased from 6 °C to 18 °C. The entire vernalization treatment was applied in

controlled environment chambers under SD conditions consisting of 12 hours light/12 hours dark.

For seed production and annual habit phenotypic screening, materials were grown in a greenhouse at 20 °C under extreme LD conditions consisting of 22 hours light/2 hours dark. Weed beets and *Beta*-related species were grown under the same environmental conditions.

Arabidopsis plant materials were grown in controlled environment chambers at 22 °C under LD conditions consisting of 16 hours light/8 hours dark.

2.2 Map-based cloning

To clone the bolting gene *B*, two large independent populations segregating for annuality were developed (Syngenta and Kiel/Strube populations). In total, 8,283 F_2 plants were genotyped with two *B*-flanking markers (Gaafar *et al.*, 2005). 107 recombinant plants (i.e. plants in which a recombination event had occurred between the two flanking markers) were obtained and used for the fine mapping of the locus. A co-dominant marker co-segregating with annuality was successfully developed by means of bulked segregant analysis (BSA) and was used to screen bacterial artificial chromosome (BAC) libraries derived from annual or biennial sugar beet genotypes. Chromosome walking and sequencing using next-generation sequencing (NGS) methods was used to construct annual and biennial maps. Marker enrichment was achieved in the region by polymerase-chain-reaction (PCR) amplification and sequencing of annual and biennial genomic deoxyribonucleic acid (gDNA) fragments spanning the physical maps. Analysis of the graphical genotypes for each recombinant event made it possible to physically delimit the extent of *B*. Putative genes and repetitive elements within the identified interval were identified by homology searches based on basic local alignment search tool (BLAST) analyses of the sequence databases hosted by TAIR (http://www.arabidopsis.org) and NCBI (http://www.ncbi.nlm.nih.gov).

2.3 Gene capture and phylogenetic analysis

Sugar beet candidate homologs were identified *in silico* via homology searches using BLAST analysis of the public sugar beet EST database hosted by NCBI in conjunction with George Coupland's *Arabidopsis* gene list (http://www.mpiz-koeln.mpg.de/english/research/couplandGroup/coupland/floweringgenes/index.html). Sugar beet candidates were used as entries in a second round of BLAST

searches against the *Arabidopsis* reference protein database (RefSeq) hosted by NCBI.

For some genes, no *Beta* homologs were identified in the public sequence database. Gene capture attempts were performed using degenerate primers designed against highly conserved regions of genes of interest. Isolation was achieved using the FirstChoice® RLM-RACE kit (Ambion). The obtained complementary deoxyribonucleic acid (cDNA) fragments of putative candidates were cloned and sequenced. New specific primers were designed and used to screen a sugar beet BAC library. A BAC that gave a positive result with the existing cDNA fragment was sequenced in order to recover the full-length genomic sequence of the *Beta* gene.

Phylogenetic studies were performed using MEGA5 (Tamura *et al.*, 2011). Multiple protein or nucleotide alignments were created using ClustalW (Thompson *et al.*, 1994). A best-fit substitution model was calculated using maximum likelihood. Evolutionary reconstruction was inferred using one of the Neighbor-Joining (NJ – Saitou and Nei, 1987), Minimum Evolution (ME – Rzhetsky and Nei, 1992) or Maximum Likelihood (ML) methods**,** based on the best-fit substitution model. Nodal support was typically estimated by bootstrap analysis on the basis of 1,000 re-samplings.

2.4 Transcriptional analysis

Gene expression analysis was conducted using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Samples from various plant tissues harvested at different developmental stages and at different Zeitgeber Time (ZT) values, were dipped into RNA*later*® solution (Ambion). Total RNA was isolated using RNAqueous®-96 kits (Ambion). Deoxyribonucleic acid (DNA) was removed from the RNA samples using the DNA-*free*™ Kit (Ambion). cDNAs were synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad) starting from 1 µg of total RNA. Specific primer pairs were carefully designed for each targeted gene and, where applicable, primers spanned exon-exon boundaries. Quantitative polymerase chain reaction (qPCR) amplifications were performed on an ABI7500 Real-Time PCR System (Applied Biosystems, Inc) using the *Power* SYBR® Green PCR Master Mix (Applied Biosystems, Inc) in a final reaction volume of 20 µL, from which 5 µL of cDNA [1/10] was used as a template. All assays were performed with a final primer concentration of 125 nM. The PCR conditions were as follows: primary denaturation at 95 °C for 10 min, 40 amplification cycles of 15 seconds at 95 °C and 1 min at 60 °C, followed by a melting curve analysis. At least three biological replicates were analyzed and each sample

was assayed in triplicate. The expression was normalized to the geometric mean expression of the *Beta glyceraldehyde-3-phosphate dehydrogenase (BvGAPDH)* and *Beta isocitrate dehydrogenase (BvICDH)* genes and calculated using the comparative C_T ($\Delta\Delta C_T$) method (Schmittgen and Livak, 2008).

2.5 Functional characterization

Transgenic approaches were adopted to investigate gene function. Mis-expression of genes of interest was achieved in sugar beet by means of ribonucleic acid interference (RNAi) or overexpression using a constitutive promoter (*35S* or *Ubiquitin3*). Heterologous expressions, using the *35S* promoter of the Cauliflower Mosaic Virus (CaMV), were also performed using *Arabidopsis* as host plant with the scope to complement *Arabidopsis* mutant phenotypes by expressing putative sugar beet orthologs. Vectors were constructed either by means of cut-and-paste procedures using restriction enzymes or recombineering methods using Gateway® vectors (Invitrogen). *Agrobacterium*-mediated transformations were performed in sugar beet and *Arabidopsis* according to the multiple shoot (Chang *et al.*, 2002) and the floral dip (Clough and Bent, 1998) protocols, respectively. Sugar beet transformants were selected at the *in vitro* stage by increasing the mannose-6-phosphate concentration in the medium stepwise, up to a maximum of 12 g/l (Joersbo *et al.*, 1998). *Arabidopsis* transformants were directly selected in the greenhouse by applying Basta® to young seedlings.

3 Results and Discussion

3.1 Insights into vernalization and photoperiod integration in beets (Paper I)

3.1.1 Isolation of two *Beta FT* homologs

Two partial *Beta FT* homologs, named *BvFT1* and *BvFT2*, were isolated using degenerate primers targeting highly conserved regions of *FT*-like genes. Cloning and sequencing of the full-length genomic sequences and full-length coding regions for these genes revealed that both were organized in similar ways, with four exons similar to those previously described for the *FT* gene and other members of the PEBP family in *Arabidopsis* (**Paper I - fig. S1B**). Phylogenetic studies showed that *BvFT1* and *BvFT2* group into the flowering promoter *FT* clade, confirming that *BvFT1* and *BvFT2* are *FT*-like homologs (**Paper I - fig. S1A**).

Gene expression analyses showed that both *BvFT1* and *BvFT2* are essentially expressed in leaves; however, *BvFT1* appeared to be expressed at the juvenile stage whereas *BvFT2* transcripts were only detected at the reproductive stage (**Paper I - Fig. 1A**). Surprisingly, *BvFT1* was barely detectable in annual beets under LD conditions at any point in their lifespan (**Paper I - Fig. 1B**). Analyses of their diurnal expression patterns showed that *BvFT1* and *BvFT2* are diurnally regulated, with their expression peaking in the morning and the late stages of the illuminated period, respectively (**Paper I - Figs. 1C** and **1D**). Under SD conditions, when beets cannot flower (**Paper I - Fig. 1E**), *BvFT1* expression was high in annual, biennial and vernalized biennial beets. When grown under LD conditions, i.e. conditions that permit the flowering of annuals and vernalized biennials (**Paper I - Fig. 1E**), *BvFT1* expression was high only in non-vernalized biennials, while *BvFT2* was detected in both annuals and vernalized biennials (**Paper I - Figs. 1C** and **1D**). The contrasting transcriptional regulation of these two genes suggests that

BvFT1 and *BvFT2* act at different times during the day and at different developmental stages. The fact that *BvFT1* transcription is mainly expressed under SD conditions and in vegetative tissues suggests that *BvFT1* may not promote flowering. The gradual down-regulation of *BvFT1* expression in biennials during vernalization (**Paper I - Fig. 1F**) is intriguing for an *FT*-like gene and would suggest that *BvFT1* needs to be blocked before the flowering transition occurs.

To investigate the role of the *Beta FT* genes, the *BvFT1* and *BvFT2* coding regions were first ectopically expressed in *Arabidopsis* using the constitutive CaMV *35S* promoter. Transgenic *Arabidopsis* expressing *BvFT2* showed an extreme early-flowering phenotype, similar to that previously described for *35S::FT Arabidopsis* plants (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999), By contrast, *BvFT1* overexpressors flowered late (**Paper I - fig. S3**). The late-flowering phenotype observed in the *ft* mutant was complemented by the ectopic expression of *BvFT2* (**Paper I - fig. S3I**), suggesting that *BvFT2* is the *Beta FT* ortholog. The heterologous expression experiment showed that the sugar beet *BvFT1* and *BvFT2* genes have opposite biochemical functions in terms of flowering control in *Arabidopsis*.

3.1.2 *BvFT2* is essential for flower development in beets

To investigate the native role of the *Beta FT* genes in beets, we started by overexpressing *BvFT2* in annual and biennial beets (these overexpressors were named *BvFT2-ox*) under the constitutive CaMV *35S* promoter. Overexpression of *BvFT2* caused precocious bolting and flowering in both annual and biennial beets (**Paper I - fig. S4**). Strong transgenic events showed that floral buds were beginning to develop even during the *in vitro* stages (**Paper I - fig. S4B**). This indicates that high levels of *BvFT2* expression can bypass the need for vernalization in biennial beets. When *BvFT2* expression was down-regulated in annuals by means of RNAi, the flowering transition was abolished and transgenic plants continued in vegetative growth for up to 400 days (**Paper I - Fig. 2A** and **fig. S5A**). Once vernalized, *BvFT2 RNAi* annual plants initiated bolting but surprisingly did not develop flowers and instead formed aberrant structures that appeared to be intermediate between flowers and shoots (**Paper I - fig. S9**). These observations confirm that *BvFT2* is the true *Beta FT* ortholog in beets and suggest that a functional copy is required for floral development.

3.1.3 *BvFT1* prevents flowering during the vegetative growing period of beet

Although *BvFT1 RNAi* biennial plants were generated, transformants showed only partial down-regulation of the *BvFT1* gene (data not shown), and as a

result, no phenotypic differences were noted between the transgenic plants and the biennial controls. *BvFT1* was successfully overexpressed in annual and biennial beets (these overexpressors were named *BvFT1-ox*) under the constitutive *Ubiquitin3* (*Ubi3*) promoter from *Arabidopsis*. *BvFT1-ox* annuals did not bolt/flower and exhibited continuous vegetative growth (**Paper I - Fig. 2B** and **fig. S5B**) similar to that observed for the *BvFT2 RNAi* annual plants. Overexpression of *BvFT1* also prevented the flowering transition in biennials even after vernalization (**Paper I - Fig. 2C** and **fig. S5C**). Remarkably, *BvFT1-ox* plants exhibit very low expression of *BvFT2* (**Paper I - Figs. 2E** and **2F**), suggesting that the overexpression of *BvFT1* compromised the transcriptional activation of *BvFT2* and therefore prevent bolting/flowering. Together with the fact that *BvFT1* expression was not altered in the *BvFT2-ox* plants or the annual *BvFT2 RNAi* plants, these data suggest that *BvFT1* is upstream of *BvFT2* in the signaling pathway (**Paper I - Fig. 3**).

It is thus conceivable that *BvFT1* plays an important but also unexpected and new role in beets, preventing bolting/flowering under unfavorable environmental conditions, i.e. during SDs and before the beginning of winter. Under LD conditions, annual beets have low levels of *BvFT1* transcripts and can therefore respond directly to the LD signal by bolting and flowering via the activation of *BvFT2* (this is illustrated in **Paper I - fig. S7**). In biennial beets, *BvFT1* is strongly expressed during the vegetative growing period, when bolting is prevented, and only passage of winter enables *BvFT1* inhibition. During the second year of the biennial growth habit, *BvFT2* is induced and bolting/flowering occurs.

In conclusion, the mechanisms responsible for fine-tuning of the flowering time in beets emerged from the diversification of a paralogous pair of *FT* genes that evolved opposing functions and transcriptional responses.

3.1.4 Mutation in the P-loop domain of BvFT1 contributed to beet adaptation

The repressive function of *BvFT1* is surprising and novel since it is the only *FT*-like gene that has been observed to act as a floral repressor. While the *FT*-like sunflower gene *HaFT1* also represses flowering via dominant-negative interference with an activating paralog, *HaFT4* (Blackman *et al.*, 2010), it is unlike *BvFT1* in that it has a frame-shift mutation in its coding region and thus encodes a pseudo-*FT*-like protein. In addition, the (non *FT*-like) PEBP family member *TFL1* acts as a strong flowering repressor in *Arabidopsis* (Ratcliffe *et al.*, 1998; Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999) (**Box 1**). It has been shown that the opposing functions of *FT* and *TFL1* stem primarily from differences in the identities of only a few amino acids in their respective sequences (Hanzawa *et al.*, 2005; Ahn *et al.*, 2006). While the proteins

encoded by *BvFT1* and *BvFT2* exhibit 82% sequence identity (as much as *Arabidopsis* FT and TSF; **Paper I - table S2**), there are some slight differences between them, notably in an important region of exon 4 known as segment B, which encodes an external P-loop (Ahn *et al.*, 2006) (**Paper I - Figs. 4A** and **2B**). By ectopically expressing *BvFT1*/*BvFT2* chimeras in *Arabidopsis*, we mapped the important domains implicated in the antagonistic functions of BvFT1 and BvFT2. The results obtained suggest that variation within the P-loop domains is indeed the main reason for the functional differences between BvFT1 and BvFT2 (**Paper I - Fig. 4C**). Further experiments indicated that the substitution events primarily responsible for the opposed activities of BvFT1 and BvFT2 are N134Y and Q138W (**Paper IV - fig. S5**).

We attempted to isolate *FT1*-like genes in *Beta*-related species and other plants from the Amaranthaceae family by means of PCR amplification using *BvFT1*-specific primers. Visualization of the amplicons on agarose gel and subsequent sequencing showed that *FT1*-like genes carrying the same critical amino acids in the P-loop domain as BvFT1 were only present in *Beta*-related species (**Paper I - figs. S10A** and **S10B**). Plants outside the genus *Beta* did not give amplification products when using *BvFT1*-specific primers. Analysis of *Chenopodium rubrum* showed that this species' genome contains two *FT* paralogs, named *CrFTL1* and *CrFTL2* (Cháb *et al.*, 2008). Phylogenetic analyses indicate that *CrFTL1* and *CrFTL2* are orthologs of *BvFT2* and *BvFT1*, respectively (**Paper III - Fig. 2**). Remarkably, CrFTL2 does not carry the same amino acids as BvFT1 in its P-loop domain and does not seem to be diurnally regulated (Cháb *et al.*, 2008), suggesting that *CrFTL2* is functionally distinct from *BvFT1*. These observations imply that the amino-acid mutations in the P-loop domain of BvFT1 associated with flowering repression occurred after the evolutionary split between *Beta* and the rest of the Amaranthaceae. *Beta* species that do not require vernalization for flowering showed low expression of *BvFT1* whereas all tested *Beta* species with vernalization-dependent flowering expressed *BvFT1* strongly before being exposed to cold temperatures (**Paper I - fig. S10C**). Overall, the data suggest that in *Beta* a copy of the *FT* paralogous pair, *BvFT1*, acquired a flowering repression function due to changes in the P-loop domain. *BvFT1* is expressed in *Beta* species with vernalization-dependent flowering and prevents flowering before the winter. Conversely, in *Beta* species with annual-growth habits (e.g. *B. vulgaris* ssp. *maritima*, *B. macrocarpa* and *B. procumbens*) *BvFT1* is repressed, allowing for the rapid initiation of flowering.

3.2 Determinism of the life cycle in *Beta* (Paper II)

3.2.1 Positional cloning of *B*

The bolting gene *B* is a master key that controls growth habits in beets (**Paper II - Fig. 1A**). Using a large mapping population segregating for annuality and consisting of 16,566 gametes, we initiated the map-based cloning of *B*. 107 recombinant events were identified using markers flanking *B* (**Paper II - table S2**). Subsequent chromosome walking and marker enrichment made it possible to narrow the genetic window down from 0.6 to 0.01 centiMorgans (cM) (**Paper II - Fig. 1B** and **table S2**). Annual and biennial scaffolds spanning 0.3 and 0.8 Mb, respectively, of the new locus interval were sequenced and gene scans revealed the presence of six putative genes (**Paper II - Fig. 1C** and **table S3**), one of which was identified as a possible flowering-time-control candidate. This gene encodes a PRR protein that we named *BvBTC1* (*BOLTING TIME CONTROL1*) (**Paper II - Fig. 1D**). Although PRR-like genes have been shown to be important in the integration of the photoperiod and therefore involved in flowering control through the transcriptional regulation of *FT* orthologs, no PRR-like gene has previously been shown to control life cycle in flowering plants. While single *prr5*, *prr7* or *prr9* mutants in *Arabidopsis* show only minor late-flowering phenotypes, the flowering time increases in double *prr5prr7*, *prr7prr9* and triple *prr5prr7prr9* mutants (Nakamichi *et al.*, 2005). In temperate cereals, *Ppd-1* (a *PRR7* homolog) is essential for the integration of the LD signal, with *ppd-1* mutants being insensitive to changes in day length (Turner *et al.*, 2005). Phylogenetic analysis revealed that *BvBTC1* is a *PRR3/7* homolog (**Paper II - Fig. 1E**). Genomic sequence comparison of the *BvBTC1* annual and biennial loci revealed the presence of a large insertion in the 5' untranslated region (5'UTR) region of the biennial allele (**Paper II - Figs. 1C** and **1G**). Although several amino acids differ between the two alleles (**Paper II - Table 1**), both the annual and the biennial open reading frames appeared to be intact.

3.2.2 BvBTC1 is an upstream regulator of *BvFT1* and *BvFT2*

As with the *Beta FT* genes, *BvBTC1* is essentially expressed in leaves (**Paper II - fig. S3A**). In both annuals and biennials, *BvBTC1* transcription is diurnally regulated (**Paper II - Figs. 2A** and **2B**), however, annuals showed slightly higher expression levels at the end of the illuminated period in LDs (**Paper II - Fig. 2B**). Vernalization gradually enhanced *BvBTC1* transcription (**Paper II - fig. S3D**). Following exposure to LD conditions, *BvBTC1* remained diurnally regulated but its expression level appeared to be higher than in the non-vernalized biennial (**Paper II - Figs. 2E** and **3A**). To investigate whether

BvBTC1 is responsible for life-cycle determinism in beets, we generated *BvBTC1 RNAi* transgenic plants in an annual genetic background (**Paper II - Fig. 2**). Down-regulation of *BvBTC1* expression (**Paper II - Fig. 2B**) resulted in a continuous vegetative growth phenotype (**Paper II - Figs. 2C** and **2D**) similar to that observed in the *BvFT1-ox* and *BvFT2 RNAi* annual beets (**Paper I - Fig. 2** and **fig. S5**). Based on the genetic evidence and the loss of the annual habit phenotype of the *BvBTC1 RNAi* annual plants, our data suggest that *BvBTC1* is the bolting gene *B* (**Paper II - Figs. 1** and **2**).

Since the level of *BvFT1*/*BvFT2* expression was shown to be determinant in the transition to bolting/flowering (**Paper I**), levels of *BvFT1* and *BvFT2* expression in the *BvBTC1 RNAi* plants were assayed to see if the non-bolting phenotype is associated with changes in the expression of the *FT* genes. Strikingly, *BvFT1* expression was strong while that of *BvFT2* was comparatively weak in the *BvBTC1 RNAi* plants (**Paper II - Fig. 2B**) – an expression pattern most similar overall to the *BvFT1*/*BvFT2* ratio observed in the biennial controls. These data suggest that *BvBTC1* is an upstream regulator of the *BvFT1* and *BvFT2* genes and that the loss of the annual habit observed in the *BvBTC1 RNAi* plants is due to the de-repression of *BvFT1*, which causes the inhibition of *BvFT2* transcription and blocks the bolting/flowering transition. To investigate whether factors relating to the circadian clock act as intermediates between *BvBTC1* and *BvFT1*/*BvFT2*, we assayed the expression of various *Beta* clock-associated homologs in *BvBTC1 RNAi* plants and annual and biennial controls. However, none of the clock-associated genes exhibited any changes in expression comparable to those observed for *BvFT1* and *BvFT2* in the *BvBTC1 RNAi* plants relative to the controls (**Paper II - fig. S3B**). It is interesting to note the slight increase in *Beta LATE ELONGATED HYPOCOTYL (BvLHY)* and *Beta CYCLING DOF FACTOR1 (BvCDF1)* expression at the end of the dark period in the *BvBTC1 RNAi* plants (**Paper II - fig. S3B**). These expression profiles resemble those previously described for *Arabidopsis*, in which *LHY* and *CDF1* expression increased in *prr5prr7* and *prr7prr9* double mutants (Nakamichi *et al.*, 2007; Nakamichi *et al.*, 2010). Overall, this diurnal analysis of clock-associated genes suggests that *BvBTC1* acts downstream or in parallel to the circadian clock in mediating *BvFT1*/*BvFT2* transcription. Further studies will be required to determine whether there is any direct interaction between BvBTC1 and *BvFT1*/*BvFT2*.

After vernalization, *BvBTC1 RNAi* plants exhibited somewhat delayed bolting and varied levels of stem elongation (i.e. stunted phenotypes). In addition, none of the *BvBTC1 RNAi* plants proceeded to flower. These observations suggest that the absence of the annual *BvBTC1* perturbs the vernalization response in beets. At the end of the cold period, *BvFT1*

expression was barely detectable in the control plants whereas *BvFT2* was strongly expressed (**Paper II - Fig. 2E**). By contrast, *BvFT1* was strongly expressed in the vernalized *BvBTC1 RNAi* plants and *BvFT2* transcription was very low (**Paper II - Fig. 2E**). The data show that *BvBTC1* activity is essential in the vernalization response and the promotion of flowering in beets, most likely due to its mediation on *BvFT2* transcription.

To investigate whether the biennial *BvBTC1* allele is also functional, *BvBTC1* was down-regulated by RNAi in a biennial genetic background (named as *Bvbtc1 RNAi* plants) (**Paper II - Fig. 3**). After vernalization, *BvFT1* repression was impaired in the *Bvbtc1 RNAi* plants (**Paper II - Figs. 3B** and **3D**) in a similar way to that observed for the *BvBTC1 RNAi* plants (**Paper II - Fig. 2E**), and *BvFT2* transcription was strongly repressed (**Paper II - Figs. 3B** and **3D**). While the biennial control plants bolted six weeks after vernalization, several *Bvbtc1 RNAi* plants failed to bolt for more than thirteen weeks and did not develop flowers (**Paper II - Figs. 3C** and **3G**). A few *Bvbtc1 RNAi* plants did eventually bolt after vernalization but displayed the same stunted phenotype (**Paper II - Fig. 3F**) observed in some of the vernalized *BvBTC1 RNAi* plants. These results indicate that the *BvBTC1* allele retains some role in *BvFT1*/*BvFT2* regulation in biennial plants.

In light of these observations, a model was drawn up (**Paper II - Fig. 3H**) in which *BvBTC1* acts upstream of *BvFT1* and *BvFT2*. Plants carrying the dominant annual *BvBTC1* allele integrate the LD signal and, via the inhibition of *BvFT1* and activation of *BvFT2*, initiate rapid bolting followed by flowering. These plants do not require vernalization and exhibit an annual-growth habit. By contrast, beets carrying two copies of the recessive biennial *BvBTC1* allele (i.e. *Bvbtc1*) cannot respond to LDs and remain vegetative because of the high expression of *BvFT1*, which blocks the activation of *BvFT2*. During the vernalization period, *BvFT1* is gradually de-regulated via the action of *BvBTC1*. In turn, *BvFT2* transcription is activated and enables bolting and flowering initiation following exposure to LD conditions. Although the increase in *BvBTC1* expression observed in the vernalized biennial plants (**Paper II - Figs. 2E** and **3A**) may well contribute to the repression and activation of *BvFT1* and *BvFT2*, respectively, it is unclear today why plants carrying an annual *BvBTC1* allele can regulate the transcription of the *FT* genes before the winter but not the plants carrying a biennial *BvBTC1* allele. Further work would be required to characterize the mechanistic differences between plants having annual and biennial *BvBTC1* alleles.

Since *BvFT2 RNAi* (**Paper I**) and *BvBTC1/Bvbtc1 RNAi* (**Paper II**) plants bolted after vernalization, and because *BvFT1-ox* plants show some sign of bolting after a prolonged period of 26 weeks of vernalization (Pin, unpublished

data), additional vernalization-dependent factors are likely to act in bolting promotion, possibly through the GA-signaling pathway (Margara, 1960; Margara, 1967; Mutasa-Göttgens *et al.*, 2009; Mutasa-Göttgens *et al.*, 2010).

3.2.3 Polymorphisms at *BvBTC1* explain most of the natural growth habit variation in beets

While variations at *BvBTC1* account for the difference in life cycle between the sugar beet parental lines used in our genetic study, there is little evidence either way concerning the possibility that *B* is the only locus responsible for growth-habit control in natural populations. To determine whether or not this is the case, a large panel of sea beets collected from various coastlines in Europe (Denmark, England, France, Greece, Italy, Portugal and Sweden), was screened for annuality in a greenhouse under extreme LD conditions consisting of 22 h light/2 h dark cycles. As controls, biennial sugar beet lines, including the parental lines used in the mapping population, were screened in parallel. Plants were grown for 6 months and monitored for bolting initiation. For each individual, the allelic form of *BvBTC1* was characterized by sequencing of the 5'UTR and the coding region of *BvBTC1*. Although important variation in bolting time was noted among the wild accessions, most of the sea beets successfully bolted (**Paper II - table S4**). The genotyping analyses showed that all of these annual or late annual wild accessions carry a *BvBTC1* allele (**Paper II - Table 1**; alleles 'e' to 'k') that most closely resembles the *BvBTC1* annual allele found in the annual parental lines used in the mapping population (**Paper II - Table 1**; allele 'd'). Only a few plants from sea beet accessions originating in Denmark contained a *BvBTC1* allele (**Paper II - Table 1**; allele 'b' and 'c') that appeared to be almost identical to the *BvBTC1* biennial allele found in the biennial parental lines (**Paper II - Table 1**; allele 'a'). Plants that were homozygous for the 'b' or 'c' alleles exhibited continuous vegetative growth when grown under extreme LD conditions and required vernalization to initiate bolting. In our screen, these strict biennial wild accessions accounted for only 2.3% of the total sea beet population. There were a few exceptional plants that carried the annual 'g' and 'j' *BvBTC1* alleles and did not bolt for up to 6 months.

In conclusion, annual *BvBTC1* allelic forms were represented in more than 95% of the tested sea beet accessions, almost all of which exhibited an annual growth habit when grown under extreme LD conditions. The variation in bolting time observed among the annual accessions (from early to very late bolting) suggests that there are probably genes other than *B* involved in determining bolting time, although their influence is likely to be comparatively minor. Overall, our results indicate that only minor polymorphic changes

occurred at *B* in the natural sea beet populations. Various mutations in *BvBTC1* have emerged, including a large insertion in the 5'UTR and several amino-acid substitutions. The effects of these changes include a reduced responsiveness to inductive photoperiods before winter thus imposing a requirement for vernalization before the flowering transition can proceed. Because natural selection in northern latitudes favors a biennial growth habit, these mutations have been maintained. Based on the high degree of sequence similarity between the biennial *BvBTC1* alleles found in the sea beets (alleles 'b' and 'c') and all cultivated sugar beets (allele 'a'), the domestication of beets probably emerged from selection for these rare biennial *BvBTC1* alleles originating from northern Europe.

3.3 The role of *FT* diversification in plant evolution and adaptation (Papers III and IV)

FLOWERING LOCUS T (FT) was identified during early studies using the *Arabidopsis ft* mutant, which carries a recessive mutation at the *FT* locus, and exhibits a very late-flowering phenotype when grown under LD conditions (Koornneef *et al.*, 1991; Coupland, 1995; Koornneef *et al.*, 1998). *FT* was later cloned (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999) and shown to correspond to a PEBP protein, a transcription factor originally described in mammals (Schoentgen *et al.*, 1987). Since then, *FT* has taken center stage for many plant biologists studying flowering time control in *Arabidopsis* (**Paper III - Fig. 1**) and other flowering plant species. *FT* orthologs were first isolated in rice (Kojima et *al.*, 2002) and have since been reported in orange trees (Endo *et al.*, 2005), tomato plants (Lifschitz *et al.*, 2006), poplar (Böhlenius *et al.*, 2006; Hsu *et al.*, 2006) and barley (Yan *et al.*, 2006). In all of these cases, it had a conserved function in promoting flowering. Shortly thereafter, *FT* was found to correspond to or be part of the mobile signal *florigen* in different species (reviewed in Kobayashi and Weigel, 2007; Turck *et al.*, 2008) suggesting that *FT* may be a universal regulator of flowering in plants.

With the availability of large EST collections and genome sequences from various plant species, it becomes possible to trace the molecular evolution of *FT* through speciation. PEBPs are found in all divisions of plants (Karlgren *et al.*, 2011; **Paper IV - Fig. 1**). However, *FT*-likes (in phylogenetic terms, PEBPs that group within the *FT*-like clade; **Paper I - fig. S6**) (Karlgren *et al.*, 2011) seem to be found exclusively in flowering plants (angiosperms) (**Paper IV - Fig. 1**), in contrast to *MOTHER OF FT AND TFL1 (MFT)*-likes (**Box 1**) which are represented in all taxa (**Paper III - Fig. 2A** and **Paper IV - fig. S1**) and have been suggested to be the ancestral forms of PEBP in plants (Hedman

et al., 2009). Before the appearance of the seed-producing plants (that is, angiosperms and gymnosperms), neofunctionalization occurred after a gene duplication event leading to two PEBP types: *MFT*- and *FT/TFL1*-likes (Karlgren *et al.*, 2011). Based on current phylogenetic reconstructions, two evolutionary models for the *FT*-like and *TFL1*-like genes were drawn up: (i) the *FT/TFL1*-likes are ancestral copies of the *FT*-like and *TFL1*-like genes (**Paper IV - Fig. 7E**) or (ii) the *FT*- and *TFL1*-likes emerged from a gene duplication event that predates the common ancestor of the seed plants (**Paper IV - Fig. 7F**). In this scenario, the biochemical differentiation between FT- and TFL1-likes would have occurred in the angiosperm lineage following its divergence from the gymnosperms. All flowering plants for which extensive genomic data are available, including the basal angiosperm species *Amborella* and magnoliid species such as avocado and tuliptree, present at least one copy of an *FT*-like gene (**Paper IV - Fig. 1**). Conversely, no *FT*-like homologs are found outside the flowering plants (Karlgren *et al.*, 2011; **Paper IV - Fig. 1**) suggesting that *FT* may have emerged with the angiosperm lineage, which is consistent with its role in flowering promotion and the unique flower-producing nature of the angiosperms.

Heterologous expression of the *FT/TFL1*-like copies from conifers delays flowering in *Arabidopsis* (Karlgren *et al.*, 2011; **Paper IV - Figs. 3** and **4**) in a similar way to that observed for *TFL1* (Ratcliffe *et al.*, 1998). It is conceivable that the *FT* function evolved within the angiosperm lineage (in the case of evolutionary model 1) or that *FT*-like was lost in the gymnosperm lineage (in the case of the second evolutionary model). *BFT*-likes are likely to derive from a duplication event of the *TFL1*-like gene, as supported by the phylogeny and their common flowering repressing function (Yoo *et al.*, 2010).

New gene duplication events subsequently occurred during speciation, generating multiple copies of the *FT*-like genes (**Paper III - Fig. 2** and **Table 1**). As demonstrated by several examples, paralogous genes do not necessarily have identical functions. In *Arabidopsis*, *FT* and *TSF* redundantly promote flowering in LDs (Yamaguchi *et al.*, 2005). In contrast, subfunctionalization emerged in rice, where *Hd3a* and *RFT1* promote flowering specifically under SD and LD conditions, respectively (reviewed in Tsuji *et al.*, 2011). Neofunctionalization of FT paralogs has also occurred in some cases (reviewed in **Paper III**). For example, it seems that in poplar, *FT1* controls flowering and *FT2* regulates growth and bud set (Hsu *et al.*, 2011). In potato, *StSPD3* and *StSELF-PRUNING6A (StSP6A)* are specific regulators of flowering and tuberization, respectively (Navarro *et al.*, 2011). In tomato and maize, plasticity at single *FT*-like genes (*SFT* and *Zea CENTRORADIALIS8 (ZCN8)*, respectively) led to the acquisition of multiple functions including flowering

time control. Similarly, both *SFT* and *ZCN8* negatively regulate growth, leaf and fruit development in the tomato plant (Shalit *et al.*, 2009) and maize (Danilevskaya *et al.*, 2011), respectively. The examples of the sugar beet gene *BvFT1* (**Paper I**) and the sunflower gene *HaFT1* (Blackman *et al.*, 2010) show that in addition to new functions, *FT* diversification has also resulted in the evolution of opposing functions. Although the amino-acid composition of the P-loop domain of the FT protein was shown to control the repressive activity of BvFT1, sequence variations in this region that do not affect the ability of FT-likes to promote flowering have been identified (**Paper III - Table 1**). The N134Y and Q138W substitutions of BvFT1 seem to be unique and are not found in other FT-likes that promote flowering (**Paper III - Table 1**). It is thus conceivable that the exact sequence of the 14 amino-acid stretch constituting the P-loop domain is not as essential for the promotion of flowering by *FT* as had previously been thought, as long as the identities of certain specific residues are conserved. The diversification of *BvFT1* in *Beta*, in conjunction with the evolution of *BvBTC1*, provides a new example of plant adaptation and domestication.

4 Perspective for new applications in sugar beet breeding

4.1 Quality control of sugar beet seed production using *BvBTC1*

Seed setting is a sensitive physiological process that requires a dry and warm climate for best fitness. At present, most commercial sugar beet seed production in Europe is performed in the southern regions, whereas the sugar beet crop fields are generally found in central, eastern and northern Europe. Sugar beet seed production is performed in open fields where the F1MS and Pollinator lines (**Fig. 3**) are grown next to each other. Pollination is almost exclusively wind dependent. Hybrid seeds are harvested on the F1MS side and processed for commercialization.

To ensure optimal hybrid performance, it is vital to have a very high degree of hybrid genetic purity. However, because of the absence of crossing barriers between cultivated beets and wild accessions, crop/wild mating can occur. In southern Europe, weed beets – a ruderal form of wild beets that originally developed from hybridization between sugar beet crops and wild sea beets, are commonly found in the vicinity of the seed production fields (Boudry *et al.*, 1993; Desplanque *et al.*, 2002). If weed beets are not controlled (mainly by manual thinning), their population increases and can become permanently established (Evans and Weir, 1981). Crop/weed gene flow is a recurring event in sugar beet seed production fields that have been colonized by weed beets, resulting in sugar beet hybrid contamination (Boudry *et al.*, 1993; Desplanque *et al.*, 2002). The biggest concern for breeders and processors is the presence of *B* in the majority of the weed beet populations. Weed beets are therefore essentially annuals and because of the dominance of the annual allele, inter-hybridization between the annual weed and biennial F1MS sugar beet results in annual hybrids (Boudry *et al.*, 1993; Desplanque *et al.*, 2002) (**Fig. 9**).

Figure 9. A 'bolter' in a sugar beet field, resulting from annual-pollen contaminations from weed beets during the seed production phase (Photo: P. Pin).

Depending on the level of pollen contamination, the seed lots can contain high levels of annual hybrid seeds which will result in bolters. A contamination level of ≥1 annual/1000 biennial seeds, which corresponds to 100 bolters/hectare (ha) on average, is considered to be unacceptable by farmers.

Today, each commercial seed lot is assayed for annual contamination. Thousands of seeds per seed lot are sown and grown in greenhouses under continuous light conditions, i.e. they are illuminated for 24 h per day. Under such extreme photoperiods, annual beets typically bolt within a few weeks after germination. This phenotypic screening is labor-intensive, costly and not always reliable due to the limited number of seeds tested.

Because *B*, which governs annual growth habits, has now been isolated (**Paper II**), it is possible to conceive of a new detection strategy based on a *BvBTC1*-based molecular marker. Genotyping analysis of *BvBTC1* among various sea beet populations indicated that all tested annual beets have an annual *BvBTC1* allele (**Paper II - tables S5** and **S6**). Further genotyping analysis among weed beets collected in the vicinity of sugar beet seed production fields in south-western France suggested that all annual weed beets

have at least one copy of an annual *BvBTC1* allele (Pin, unpublished data). Consequently, by tracing the annual *BvBTC1* alleles, we would expect to identify most (if not all) annual beets. Multiple nucleotide sequence alignment of the different annual and biennial *BvBTC1* alleles revealed the presence of SNPs and Insertion-Deletions (InDels) that discriminate between annuals and biennials (**Paper II - Table 1**). The development of an SNP-based assay, preferably combining two allele-specific TaqMan® MGB probes and two common primers, would be straight forward and should provide a robust and sensitive method for detecting annual pollen contamination. Replacing the current laborious and costly greenhouse phenotypic test with *BvBTC*-based assays would greatly improve quality control in the seed lots.

4.2 From spring to autumn sowing – the development of a winter beet crop

The main factors that limit root and sugar yields in beets are the length of the vegetative growing period and the ability of the plant to capture the available solar energy, which is dependent on the leaf area index. Sugar beet is essentially grown as a spring crop – that is, it is sown in spring and harvested before the beginning of the unfavorable season. Rapid and homogenous seedling emergence in early spring is determinant for the onset of leaf development, which has a major impact on final yield. However, because of the cold temperatures at this time of the year, leaf development is slow and the optimal canopy cover necessary for radiation perception is only achieved by June and thus cannot exploit almost 40% of the total annual insolation (Jaggard *et al.*, 2009) (**Fig. 10**).

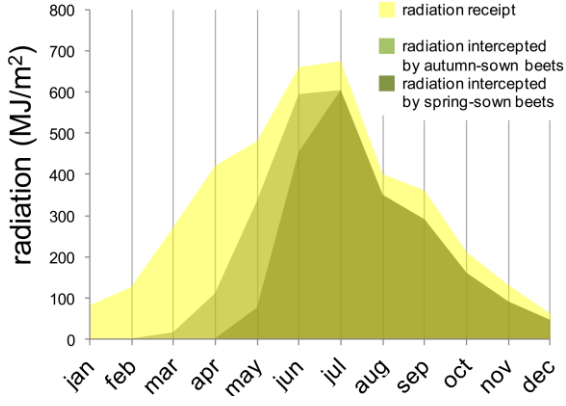

Figure 10. Total radiation receipt versus radiation intercepted by spring-sown or autumn-sown beets. Modified illustration based on data from Jaggard *et al.* (2009).

The use of winter rather than spring crops is attractive because it provides scope for avoiding this loss of energy. Varieties grown as winter crops such as winter oilseed rape and winter cereals are sown in autumn and harvested the next year. Before entering the winter period, the winter crops have already reached a significant leaf area index, which greatly facilitates the perception of light radiation during the coming spring. As a result, and although the winter cereals and oilseed rape mature earlier in the season than the spring cereals and canola varieties (that is, the spring oilseed rape type), the winter types have typically a 20-30% yield advantage over the spring types. Based on these figures, there is a huge potential for using sugar beet as a new winter crop. It has been shown that sugar beets sown in autumn have higher seedling emergence than their spring counterparts drilled in February or March (Hoffman and Kluge-Severin, 2011). As expected, the leaf area is also much higher for autumn-sowing beets, as illustrated by the finding that the dry leaf mass of autumn-sowing beets was 1-2 t/ha in December and 4-10 t/ha in June whereas that for spring-sowing beets was only 2-4 t/ha in June (Hoffman and Kluge-Severin, 2011). Consequently, autumn-sowing beets will intercept more radiation during the early phase of the growing period (Jaggard *et al.*, 2009) (**Fig. 10**). However, autumn-sowing beets exhibit changes in root yield because of the switch from the vegetative to the reproductive developmental stage, which is promoted in spring following exposure to the cold temperatures during the winter. Consequently, the only way to develop a new winter sugar beet crop will be to breed for highly bolting resistant/non-bolting sugar beet varieties.

Since transgenic sugar beet overexpressing the flowering repressor gene *BvFT1* remained vegetative after vernalization (**Paper I - Fig. 2C** and **fig. S5**), such material could be a suitable starting point for developing a winter beet. Practically, the *BvFT1-ox* sugar beet is useless because its continuous vegetative growth does not allow seed production. Consequently, the expression of *BvFT1* should be conditionally regulated and only activated in commercial hybrid plants. Methods for achieving such conditional expression have been developed in studies on transgenic organisms, allowing spatial and temporal expression to take precedence over constitutive expression. Several induction systems such as mGal4:VP16/UAS and pOp/LhG4 (Moore *et al.*, 1998) have been widely used in *Arabidopsis* (Schoof *et al.*, 2000; Benjamins *et al.*, 2001; Eshed *et al.*, 2001; Swarup *et al.*, 2005; Weijers *et al.*, 2005). Based on the schematic inductive system proposed by Moore *et al.* (2006), a *35S»BvFT1* sugar beet hybrid could be obtained with the pOp/LhG4 system, where the *BvFT1* coding region would be assembled behind the *pOp::35Sminimal* promoter (**Fig. 11**). An effector sugar beet line carrying the

pOp::35Sminimal::BvFT1 cassette would flower normally and would be crossed with an activator sugar beet line carrying a *35S::Lac1::Gal4* cassette that would cause the expression of the heterologous transcription factor Lac1/Gal4 without altering flowering. In the hybrid, Lac1/Gal4 molecules would activate the *pOp::35Sminimal* chimeric promoter, causing *BvFT1* expression and therefore bolting control (**Fig. 11**).

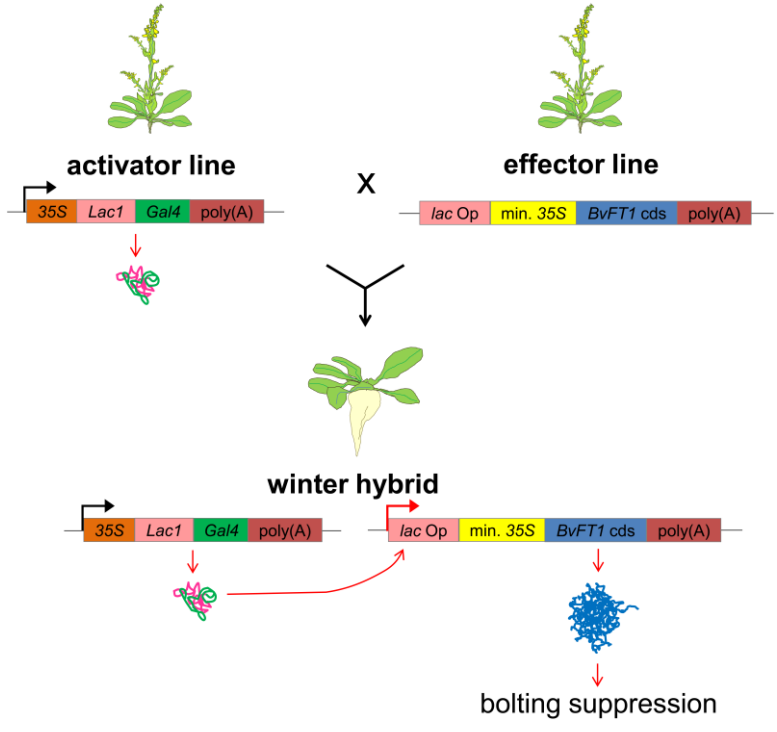

Figure 11. Development scheme for *35S»BvFT1* sugar beet hybrids using the pOp/LhG4 inductive system.

5 Conclusions

By using forward and reverse genetics approaches, I have identified three major genes, *BvFT1*, *BvFT2* and *BvBTC1*, involved in the control of life cycle and flowering time in beets. These three genes form a regulatory complex that responds to both photoperiod and vernalization cues to synchronize the time of bolting and flowering. I showed that the mis-expression of only one of these three genes caused severe changes in life cycle and flowering time. The results obtained suggest the following regulatory sequence: *BvBTC1-BvFT1-BvFT2*, where *BvBTC1* governs growth habit determinism, *BvFT1* prevents the flowering transition under unfavorable conditions (that is, under SD conditions and before the winter), and *BvFT2* mediates flowering time control and (in contrast to the situation in many other flowering plants) floral development.

This work provides the first identification of genes controlling the flowering transition in *Beta*, as well as new insights into the floral molecular mechanisms in flowering plants. Although the integration of the inductive photoperiodic signal through an *FT* ortholog turned out to be conserved in *Beta* (via *BvFT2*), the discovery that a second *FT* gene, *BvFT1*, acts as floral repressor, and a PRR gene, *BvBTC1*, controls life cycle, provides a totally new and unanticipated example of molecular regulation. Moreover, my data suggest that the cultivated beets emerged from the selection of a rare, partial loss-of-function *BvBTC1* allele that confers a biennial-growth habit. Together, these results illustrate how plant adaptation and domestication can be modulated through plasticity at different molecular layers of regulation.

My findings will have direct implications in sugar beet breeding in the short term by enabling the use of *BvBTC1* as marker for quality control in commercial seed lots. This should increase product quality and resolve a major issue in terms of costs and logistics. Moreover, in the long term, it offers the potential for using *BvFT1* to engineer and develop a new winter sugar beet crop.

Further studies will be required to elucidate (i) whether *BvBTC1* is a direct factor acting on the transcriptional regulation of *BvFT1* and *BvFT2*, (ii) how the transcriptional repression of *BvFT1* on *BvFT2* is mediated, and (iii) which vernalization-dependent factor(s) that promote bolting act in parallel with *BvFT2*.

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