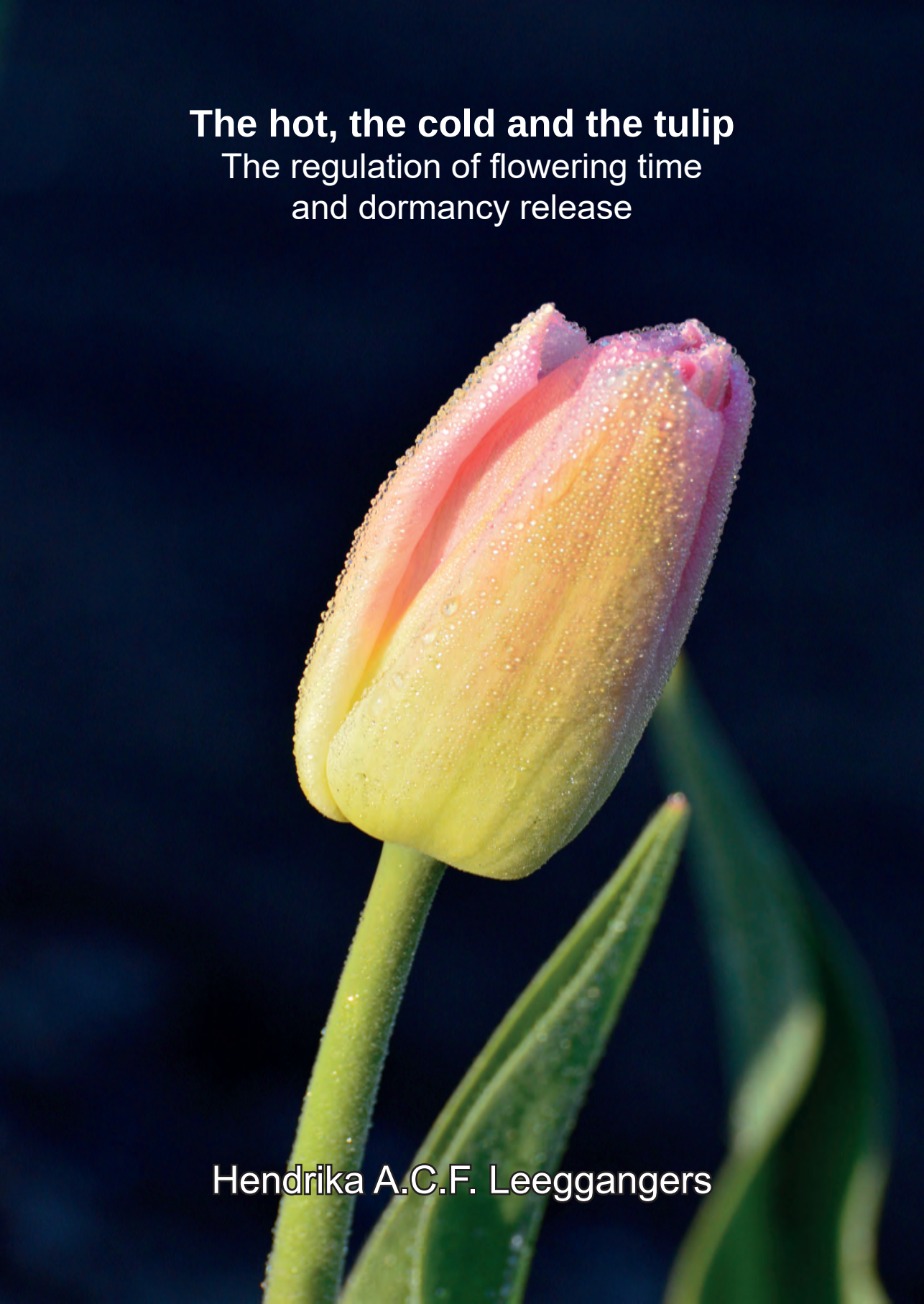
The hot, the cold and the tulip

The regulation of flowering time
and dormancy release



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Hendrika A.C.F. Leeggangers

Thesis

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Chapter 1
General Introduction

H.A.C.F. Leeggangers

History of the tulip

As then the Tulip of her morning sup
Of Heavenly Vintage lifts her chalice up
Do you, devoutly, do the like, till Heav'n
To Earth invert you like an empty cup.

In the 12th century the words above where the first mentioning of tulips in the poem collection Rubaiyat by the Persian poet Omar Khayyam and translated by Edward FitzGerald in the 19th century. The cultivation of tulips started in the Ottoman Empire where Sultan Suleiman the Great (1520-1566) grew his favourite flower, the tulip, in the gardens of the Topkapi palace. In the first half of the 16th century the tulips from Turkey were introduced in the Netherlands. Already then great diversity of cultivars could be found originating from the gardens in Persia. At the end of the 17th century hundreds of new cultivars, produced by tulip breeding, were available in the Netherlands as well as France (De Hertogh et al., 2013). Nowadays the Netherlands produces over four billion tulip bulbs per year of which 53% is exported to other countries for cut flower production (Okubo and Sochacki, 2013). Thus, tulips are economically very important to the Netherlands.

Botanical classification

Tulip, or the genus *Tulipa*, is a member of the Liliaceae family like lily and daffodils. Within this genus several species can be found, which are listed in table 1. The four species *Tulipa gesneriana*, *Tulipa kaufmanniana*, *Tulipa fosteriana* and *Tulipa greigii* have each given rise to a cultivar group. Within the different species, several classes can be distinguished based on their flowering time (e.g. Single early) or shape of the flower (e.g. Fringed). Flowering time can be divided into early, middle, and late. The time period of early ranges from mid-March to early April, middle ranges from early to the middle of April, and late ranges from late April to early May (Okubo and Sochaki, 2013). The research in this thesis has been focusing on the species *T. gesneriana*.

Table 1. Classification of Tulipa species according to the Classified List and International Register of Tulip Names (1996).

Class	Species	Flowering time	Examples cultivars
Single early (SE)	*T. gesneriana*	Early	Apricot Beauty, Baby Blue, Calgary, Christmas Marvel, Coquette
Double early (DE)	*T. gesneriana*	Early	Jan Vermeer, Murillo Maxima, Monte Carlo, Peach Blossum, Abba
Triumph (T)	*T. gesneriana*	Mid	Dynasty, Strong Gold, Purple Prince, Ile de France, Yellow Flight, Leen van der Mark
Single late (SL)	*T. gesneriana*	Late	Kingsblood, Maureen, Queen of the Night, Pink Diamond
Lily-flowered (L)	*T. gesneriana*	Late	Marilyn, Aladdin, Ballade, Ballerina, Pretty Woman
Fringed (Fr)	*T. gesneriana*	Late	Louvre, Davenport, Curly Sue, Dallas, Fancy Frills
Viridiflora (V)	*T. gesneriana*	Late	Hollywood, China Town, Golden Artist, Spring Green, Nightrider
Parrot (P)	*T. gesneriana*	Late	Destiny, Libretto, Rococo, Irene Fantasy
Double late (DL)	*T. gesneriana*	Late	Blue Diamond, Finola, Orange Princess, Angelique
Darwin hybrids (DH)	*T. gesneriana*	Mid	Apeldoorn, Golden Apeldoorn, Oxford, Ad Rem, Golden Parade
Rembrandt (R)/ Broken tulips	*T. gesneriana*	Varies	Keizerskroon, Ice Follies, Burning Heart, Sorbet, Mona Lisa
Greigii (G)	*T. greigii*	Early	Orange Toronto, Roodkapje, Pinochhio , Sweet lady, Toronto
Kaufmanniana (K)	*T. kaufmanniana*	Early	Johann Straus, Guiseppe Verdi, Scarlet Baby, Heart's Delight
Fosteriana (F)	*T. fosteriana*	Early, later than Kaufmanniana	Candela, Orange Emperor, Purissima, Sweetheart, Cantata

Morphology tulip bulb and flower

Tulip bulbs consist of two to six fleshy scales, which are modified leaves, and a protective cover on the outside known as the tunic (Fig. 1A). Inside the bulb in the axil of every scale, axillary buds (the daughter bulbs) are present, which will replace the mother bulb in the following season (Fig. 1A). In addition to the axillary buds inside the tulip bulb, the apical bud or floral bud is present at planting time, as well as the floral stem and leaf primordium (Fig. 1A; Kamenetsky and Okubo, 2013). During flowering, the floral stem and three to five leaves are completely elongated as well as the flower. The flower consists of two whorls of tepals, two whorls of stamens, and a carpel in the middle. Unlike most plants, many members of the Liliacea family have tepals instead of sepals and petals (Fig. 1B). The two outer whorls are identical, which can be explained by ectopic expression of so-called B-type genes of the ABC model in the outer floral whorl. Normally A class genes, such as *APETALA1* (*AP1*) and *SEPALATA1* (*SEP1*), are expressed in the outer whorl of sepals only. In the petals however, B class genes, such as *PISTILATA* (*PI*) and *APETALLA3* (*AP3*), are expressed. These two classes of genes give rise to the different morphological structures (Ma and dePamphilis, 2000; Soltis et al., 2007) of sepals and petals. In tulip, both A and B class genes are expressed in the first and second floral whorl, explaining the development of the same tepal morphological structures in both outer floral whorls (Fig. 1C; Kanno et al., 2003).

The growth cycle of tulip

Tulips undergo two different growth phases during their life cycle. The first one is from seed to a bulb competent to flower, where the transition is made from the juvenile vegetative to the adult vegetative phase (Rees, 1966). After pollination of a mother plant, a zygote develops into a mature embryo within a period of 12 weeks (van Tuyl and van Creij, 2006). A period of low temperature is required for the breaking of embryo dormancy, induction of germination, and initiation of the bulb primordium. The embryo produces three different tissues: one cotyledon, a primary root, and a diverticulum (dropper). After a period of low temperature,

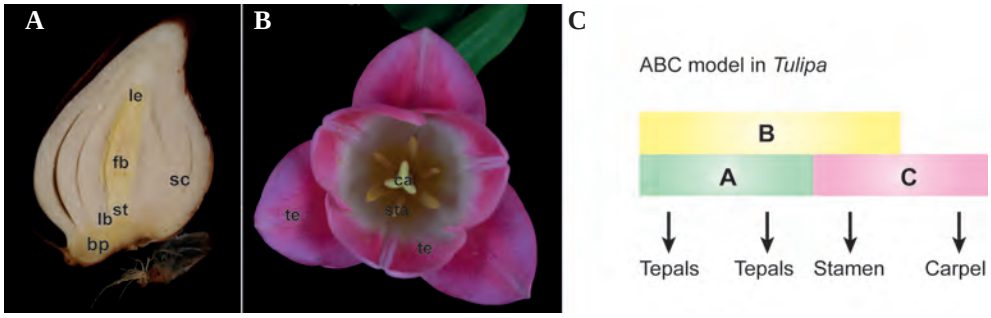

Figure 1. Overview of tulip bulb and flower morphology. (A) Cross section of a bulb at planting time in autumn, showing morphology of the different tissues and organs. sc: scales, bp: basal plate, le: leaves, fb: floral bud, st: stem and lb: lateral bud. (B) Flower morphology. te: tepals, sta: stamen and ca: carpel. (C) Modified ABC model of tulip floral organ development. In contrast to the classical ABC model, the B-function is also active in the outer floral whorl organs, causing the development of tepals in both perianth whorls.

high temperature accelerates the formation of the stolon-like dropper, which leads to the formation of the bulb primordium (Niimi, 1978; van Tuyl and van Creij, 2006). This first year bulb is not competent for sexual reproduction and requires another three to five years, depending on the cultivar, to become competent to flower. In these three to five years of the juvenile phase, the bulb gains weight, more scales are being developed and one foliage leaf is initiated inside the bulb (Rees, 1972; Botschantzeva and Varekamp, 1982). So far it is not clear why the juvenile phase is long and the bulb is not able to form a flower during this period. It is only known that a bulb which is able to produce a flower needs a minimum fresh weight of three to eight grams. Nevertheless, this characteristic is genotype dependent. In addition to weight, shortage of reserves in the scales can play a role in the duration of the juvenile phase (Le Nard and De Hertogh, 2002). The most inner scale contains reserves for growth of the sprout, which consists of the floral bud, stem and leaves (Botschantzeva and Varekamp, 1982). Another explanation can be the size of the shoot apical meristem (SAM). In *Triteleia laxa* (grassnut), it is e.g. not the reserves that determine if a flower can be formed but the size of the SAM that determines the ability to produce a flower (De Hertogh and Le Nard, 1993).

Nevertheless, once the bulb enters the adult vegetative phase, it becomes competent to flower and the next growth phase starts (Fig. 2).

The second growth phase, which is yearly repeated in the following generations of bulbs, is the development of a floral bud from the adult vegetative bud inside the bulb, finally giving rise to flowering in next spring (Fig. 2B). From October to December bulbs are planted in the field. At this stage three generations of bulbs are present: 1) the mother bulb, 2) the daughter bulbs and 3) a meristem of the granddaughter bulbs inside the daughter bulbs (Kamenetsky and Okubo, 2013). After planting, roots are formed and growth of the internal organs is slow or does not take place. This period is referred to as a period of dormancy (Rees, 1981) but is probably better described by a period of growth cessation or slow growth. In order to flower in spring, the bulbs need a period of cold for the elongation of the floral stem and outgrowth of the apical flower bud inside the mother bulb (Rietveld et al., 2000). After a period of cold, the granddaughter bulbs in the daughter bulbs start to enlarge around February. In every axil of the scale of the daughter bulb, a granddaughter bulb will be formed starting from the outside and finishing with the inner bud in July (Botschantzeva and Varekamp, 1982). In addition to enlargement of the daughter bulbs and granddaughter bulbs, growth of the floral stem, leaves and apical floral bud continues and dormancy is released. This leads to flowering in April or May, depending on the cultivar (Table 1). At this moment the mother bulb is completely consumed by the apical floral bud and daughter bulbs. Here, the daughter bulbs become themselves mother bulbs the following year. The same applies to the granddaughter bulbs that become the daughter bulbs in the new mother bulb.

Besides flowering, the enlargement of the daughter bulbs takes place and slows down around June/July, just before or after harvest (Botschantzeva and Varekamp, 1982; De Hertogh and Le Nard, 1993). Once the growth of the daughter bulbs ceases, high temperature induces the vegetative to reproductive phase change. Organogenesis of the floral bud, floral stem

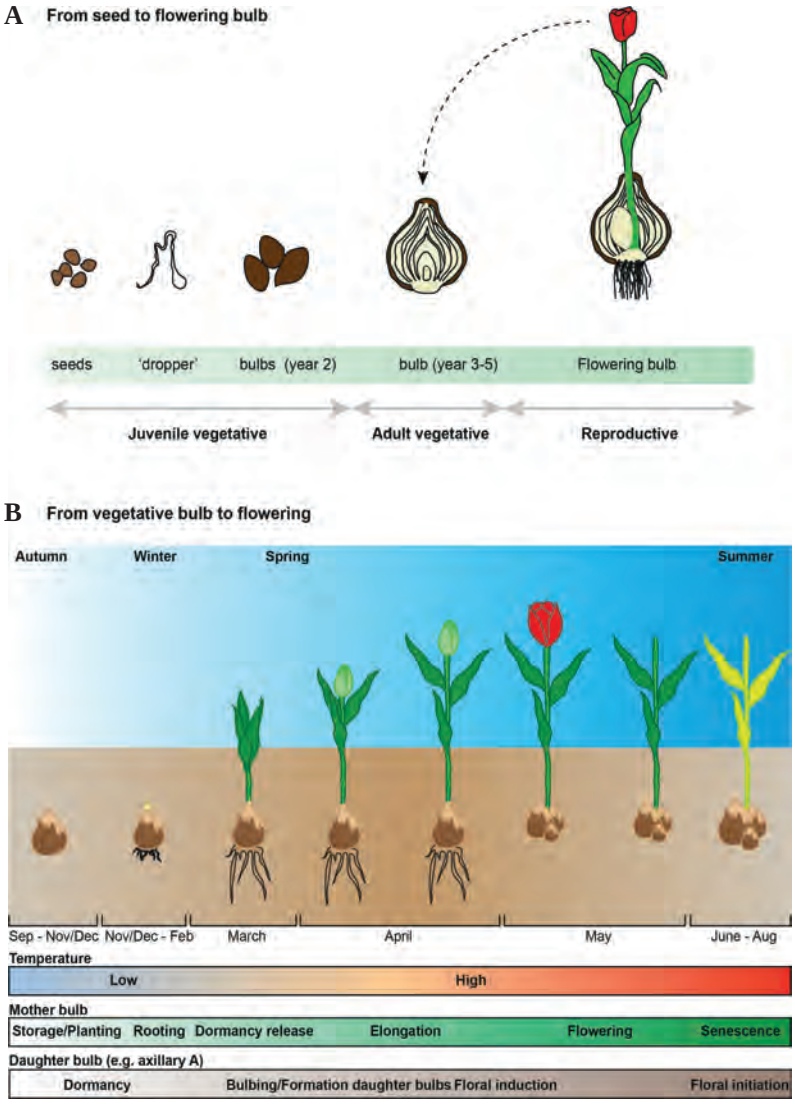

Figure 2. Schematic overview of the growth cycle of tulip. (A) From seed to flowering bulb. In the first year the embryo of the seed develops a stolon-like dropper. In the second year a proper bulb is formed. In the years thereafter the bulb gains weight and at a certain moment becomes competent to flower. This competence is impaired with the vegetative phase change from juvenile to adult. (B) Yearly cycle from an adult vegetative bulb to a flowering bulb. High temperature in summer initiates flowering, followed by the induction of dormancy. In autumn bulbs are planted, the roots start to develop and the organs inside the bulb develop slowly. After a period of low temperature in winter, dormancy is released and the growth of the organs inside the bulb is re-activated. Then in spring the flower of the mother bulb is flowering and vegetative reproduction takes place of the daughter bulbs and granddaughter bulbs.

development and initial leave growth occurs during the storage period. The development of the floral bud can be divided in seven stages starting at stage I where the SAM is still vegetative. In stage II of the SAM takes place and flower initiation. Stage P1 represents the formation of the first whorl of tepals and stage P2 indicates development of the second whorl. After formation of the two whorls of tepals, the first whorl of stamens is formed, represented by stage A1, followed by the second whorl of stamens in stage A2. The final stage is G, where the carpel is formed and the flower is completely differentiated (Beijer, 1952).

Climate change and the influence on tulips

One of the most important environmental factors for the development of tulip is temperature (De Hertogh and Le Nard, 1993). Tulips need sufficient warmth for flower initiation, but on the other hand a prolonged period of cold to break dormancy and to guarantee full stretching of the floral stem and flowering in spring. In 2008 and 2011, a phenomenon known as floral bud blasting (dehydration of the flower) was noticed in several tulip cultivars, such as Strong Gold, Purple Prince, and Cheirosa (van Dam and van Haaster, 2011). The development of the floral bud inside the bulb started most likely earlier in these years, meaning that the bulbs were in more advanced stages than normal at the moment of harvest and storage. In these years the temperature was higher than the average temperature in the months April and May (Fig. 3).

As a consequence, floral bud initiation occurs faster and this might have resulted in dehydration of the flower leading to low quality or no flowers at all in the next spring. High temperature treatments that are commonly given after harvest to boost flower induction and development could have strengthened this effect (van Dam and van Haaster, 2011). Thus, changes in climate and global warming can cause problems in the future for tulip production in the Netherlands. The last hundred years, extensive research has been done focusing on the influence of temperature on tulip development and morphological and hormonal changes (De Hertogh and Le Nard, 1993; Kamenetsky and Okubo, 2013). However, the molecular

regulation of the flowering process, from initiation to outgrowth, has been studied to a much lesser extent. Though, a better understanding of the genetic and molecular mechanisms underlying the developmental response of a tulip to temperature is essential to create 'climate-proof' tulips in the future. In contrast to the lack of knowledge on flowering time control and dormancy in tulips, some information is available from other bulbous species, such as lily (Villacorta-Martin et al., 2015) and detailed insights have been obtained from studying model dicot and monocot species (Capovilla et al., 2014; Shrestha et al., 2014; Blümel et al., 2015).

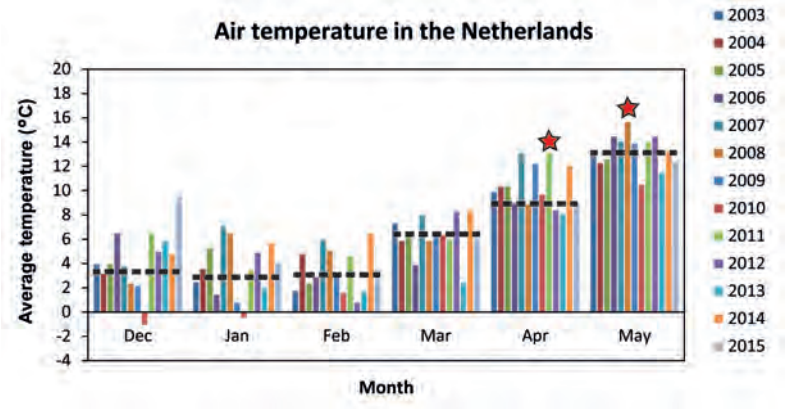

Figure 3. Air temperature in the Netherlands from 2003 to 2015. Data shown collected from the Koninklijk Nederlands Meteorologisch Instituut (KNMI). The black interrupted line represents the average temperature and the red stars indicate the year of which floral bud blasting was observed in some tulip cultivars.

Flowering time control and regulation of bud dormancy in model species

The most well studied model species in flowering time control is Arabidopsis thaliana. Five divergent pathways are involved in the regulation of the transition to flowering (Koornneef et al., 1998; Piñeiro and Coupland, 1998). The two temperature-dependent pathways are the vernalization response and thermosensory pathway. In order to flower, some Arabidopsis accessions need a period of prolonged cold (vernalization response) to repress the floral repressor *FLOWERING LOCUS C* (*FLC*) (Searle et al. 2006). The presence of *FLC* prevents the activation of the floral integrators

FLOWERING LOCUS T (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*). Once *FLC* is stably repressed by the plant homeodomain-polycomb repressive complex 2 (PHD-PRC2), *FT* and *SOC1* can be activated and this will eventually result in flowering, when other conditions are favorable (Helliwell et al., 2011). Besides winter cold, flowering can be controlled by the thermosensory pathway that senses changes in ambient temperature (Blazquez et al., 2003). So far only a small number of genes have been identified that are temperature sensitive and regulate flowering time by sensing changed ambient temperature. The most well-studied temperature responsive gene in Arabidopsis is *FLOWERING LOCUS M* (*FLM*); which can, together with *SHORT VEGETATIVE PHASE* (*SVP*), repress *FT* when the environmental conditions are not optimal to flower (Pose et al., 2013). Other genes that have shown to be regulated by temperature are *PHYTOCHROME INTERACTING FACTORS* (*PIF4* and *PIF5*), *TERMINAL FLOWER 1* (*TFL1*) and *EARLY FLOWERING 3* (*ELF3*) (Strasser et al., 2009; Thines et al., 2014).

While Arabidopsis is one of the most common used model species for studying flowering time control, perennial plants such as *Populus*, *Malus domestica* (apple), *Prunus persica* (peach) and *Vitis vinifera* (grapevine) are considered to be model plants for studying vegetative bud dormancy (Anderson et al., 2010). In perennial species dormancy is a mechanism for environmental adaptation and survival during seasonal changes (Atkinson et al., 2013). Similar to flowering time control, the environmental signals, photoperiod and ambient temperature, play a role in the regulation of bud dormancy. Not only an overlap in environmental factors controlling flowering and dormancy has been shown, but also similar genetic regulators have been identified. The *CONSTANS* (*CO*)/*FT* regulatory module is involved in photoperiodic control of flowering in Arabidopsis as well as delaying flowering time by growth cessation in Populus (Böhlenius et al., 2006). This is not the only regulatory module which is conserved, but also genes related to the Arabidopsis MADS-box transcription factors *AGAMOUS-LIKE 24* (*AGL24*) and *SVP*. Homologues genes of these MADS-box transcription factors in e.g. *Populus*

and *Euphorbia esula* (leafy spurge) are called *DORMANCY ASSOCIATED MADS-box* (*DAM*) genes and are involved the regulation of growth prevention of the meristem (endodormancy; Horvath et al., 2010; Sasaki et al., 2011). This overall knowledge of both processes can be used to understand the molecular regulation of flowering time control and dormancy release in tulip.

Scope of this thesis

Currently only small effects of global warming are seen in tulip development but in the near future it can lead to serious problems for the Dutch flower bulb industry. Therefore the aims addressed in this PhD project are: 1) To understand the effect of temperature on the vegetative to reproductive phase change in tulip at the molecular level and 2) To provide insight in the regulation of dormancy release in tulip at the molecular and metabolic level.

In **chapter 2** a review is presented on how to use knowledge of a model species, such as *Arabidopsis thaliana*, and the transfer of this knowledge to bulbous plants with a special focus on flowering time control and vegetative propagation. Two approaches, bottom-up and top-down, are described that can be followed for the transfer of knowledge.

Chapter 3 describes the elucidation and mining of the Tulipa and Lilium transcriptomes. A more complete transcriptome was created and made available through a web-based interface called '*Transcriptome Browser*'. The quality and completeness was determined using different parameters and in addition, the risks of high stringent filtering in de novo transcriptome assembly have been shown to make scientists aware of this important step in transcriptome analysis. Since hardly any molecular and sequence data was available on bulbous species at the start of this project, this activity was essential to address the main research questions of this thesis.

In **chapter 4** a first extensive transcriptome study is presented, aiming to unravel the mysteries behind high-temperature-induced vegetative to reproductive phase change in tulip. A gene ontology (GO)-enrichment analysis revealed that besides this important developmental switch, at the same time tulip bulbs also prepare for a period of rest. Based on homology with known flowering time genes and detailed expression pattern analysis, several potential novel regulators of flowering time control in tulip were identified.

Chapter 5 presents the detailed functional analysis of three PEBP genes (*TgFT1*, *TgFT2* and *TgFT3*) in tulip and one PEBP gene (*LlFT*) in lily, showing amongst others their possible role in flowering time control. Expression analysis, functional characterization, phylogeny analysis and protein-protein interaction studies were performed to understand the function of these genes. *TgFT2* and *TgFT3* had similar expression patterns in tulip, however gave different phenotypes upon ectopic expression in Arabidopsis. Therefore substitution lines were created to understand the molecular cause of their difference in function. Based on the combination of all these analyses, predictions could be done for the native functions of the various PEBP genes in tulip and lily.

In **Chapter 6** a first insight is given in the transcriptional and metabolic changes in storage and during the winter period in tulip. Morphological analysis has shown a period of no or slow growth of the floral bud inside the bulb during the autumn and winter months. Overall, after a period of ten weeks in the field all tissues accelerated growth indicating the release of dormancy. After these ten weeks, photosynthesis-related genes in the leaves increase in their expression, suggesting preparation for photosynthesis. A first analysis of the metabolic data suggests that the floral bud is preparing for elongation.

Finally, in **Chapter 7** the general discussion addresses three different

perspectives of the tulips life cycle, categorizing tulip as a perennial, biennial or annual plant species. The importance of bulb size is discussed and the remarkable similarities between bulbs and seeds in biological processes such as dormancy initiation and release. Last, the challenges and practical applications are discussed.

Chapter 2

Transfer of knowledge about flowering and vegetative propagation from model species to bulbous plants

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Abstract

The extensive characterization of plant genes and genome sequences summed to the continuous development of biotechnology tools, has played a major role in understanding biological processes in plant model species. The challenge for the near future is to generate methods and pipelines for an efficient transfer of this knowledge to economically important crops and other plant species. In the case of flower bulbs, which are economically very important for the ornamental industry, flowering time control and vegetative propagation constitute the most relevant processes for agronomical improvements. Those processes have been reasonably studied in reference species, making them excellent candidates for translational investigations in bulbous plant species. The approaches that can be taken for the transfer of biological knowledge from model to non-model species can be roughly categorized as 'bottom-up' or 'top-down'. The former approach usually goes from individual genes to systems, also known as a 'gene-by-gene' approach. It assumes conservation of molecular pathways and therefore makes use of sequence homology searches to identify candidate genes. 'Top-down' methodologies go from systems to genes, and are e.g. based on large scale transcriptome profiling via heterologous microarrays or RNA sequencing, followed by the identification of associations between phenotypes, genes, and gene expression patterns and levels. In this review, examples of the various knowledge-transfer approaches are provided and pros and cons are discussed. Due to the latest developments in transgenic research and next generation sequencing and the emerging of systems biology as a matured research field, transfer of knowledge concerning flowering time and vegetative propagation capacity in bulbous species come into sight.

Introduction

In the last decade the establishment of full genome-sequences and the development of new biotechnology tools have dramatically increased our knowledge of plant functioning. . For example, the genome sequence of Arabidopsis (~130 Mbp; dicot), rice (~380 Mbp; monocot) and maize (~2500 Mbp; monocot) were completed in 2000, 2002 and 2009,

respectively (AGI, 2000; Sequencing Project International Rice, 2005; Schnable et al., 2009). Molecular biology, genomic and transgenic research, such as loss-of-function mutagenesis and overexpression studies, have played a key role in exploiting and understanding biological and molecular functions of the thousands of genes present in the genome sequences. Nonetheless, the majority of these functional studies have been performed in plant model species, such as Arabidopsis, Medicago and rice. All together this provided a wealth of knowledge on the control of a large variety of biological processes and traits. Hence, the road has been paved for the implementation of this data and the transfer of knowledge from model species to relevant but less studied crop species, ultimately aiming to improve and optimize yield and quality for a sustainable agriculture.

Almost all bulbous plant species are monocots, including the economically important ornamentals tulip and lily. Bulbous plants are hardly studied at the molecular level and therefore this review will have a special focus on these species. Bulbous species were introduced in Western Europe in the 16th century and are nowadays primarily utilized for commercial bulb production, garden and forced fresh cut flower production and for landscape architecture. Cultivation occurs in temperate climate regions with the Netherlands being the leading producer world-wide. In total, seven species dominate the industry consisting of *Tulipa*, *Lilium*, *Narcissus*, *Gladiolus*, *Hyacinthus*, *Crocus* and *Iris* (Benschop et al., 2010). Flower bulbs propagate sexually through seeds and vegetative via initiation and outgrowth of axillary meristems, which are usually located in the underground storage organ (Kamenetsky and Okubo, 2013). Like other plants, bulbs propagated from seeds undergo three developmental phases: juvenile vegetative, adult vegetative and reproductive. The duration of the juvenile vegetative phase can take several years (e.g. *Tulipa* and *Narcissus*) and only upon the transition to the adult vegetative phase, the bulb becomes competent for flower initiating signals. The vegetative phase switch from juvenile to adult depends on the physiological age, weight and size of the bulb. Subsequently, taking tulip as an example, high temperatures can

induce the transition from adult vegetative to the reproductive phase, resulting in flower bud initiation. Simultaneously, dormancy is triggered and a prolonged period of cold is required for dormancy release and internal preparation for stem elongation and flower outgrowth in the next spring. This specific life cycle is not only seen in tulip, but is common for various bulbous species, including *Crocus* and *Hyacinthus* (Rees, 1966; Saniewski et al., 2000; Kamenetsky and Okubo, 2013). In order to improve bulb productivity and ornamental characteristics, it is necessary to increase genetic variation by breeding new cultivars and potentially this can highly benefit from the implementation of biotechnological and 'omics' tools. Currently, the development of a new tulip cultivar can take up to 20 years because of its long juvenile phase and low vegetative propagation rate (Podwyszyńska, 2005). Besides the long juvenile phase, which slows down the breeding process and the production of flowers, an agricultural problem is laid down in the precocious flower initiation by high temperatures in spring, resulting in early development of the flower bud. Consequently the flower bud is completely developed inside the bulb around harvest time, leading to either flower abortion or a decrease of flower quality in the next season because of dehydration during storage of the bulbs (Hartsema, 1961). In addition, natural vegetative propagation rates vary among flower bulbs, but on average are low due to the limited number of axillary meristems and a restriction in outgrowth of these meristems (Kamenetsky and Okubo, 2013). Together with the long juvenile phase, this makes the development of a new flower bulb cultivar a slow and time consuming process. Many efforts in understanding and improving the physiological nature of flowering and vegetative propagation in bulbous plants took place in the last decades (Beijer, 1952; Aung and Hertogh, 1979; Lambrechts et al., 1994; Balk and de Boer, 1999; Rietveld et al., 2000); however, the majority of these studies focused on physiological factors and limited molecular and genomic studies have been performed. Although various reasons can be brought forward for this, the large genome sizes for bulbous plants (Tulip ~25000 Mbp; Lily ~36000 Mbp) and technical difficulties in isolating e.g. RNA from bulb scales have been particularly

decisive in this (Shahin et al., 2012).

Here, we will briefly summarize the current knowledge on flowering time control and vegetative propagation gained from studies in model plant species, since these are the two most important biological processes for agronomical improvements of bulbous plant species cultivation. Subsequently, we will give an overview of approaches to transfer this type of knowledge from model plants to crop species and how transgenic and 'omics' technologies can be supportive. Various examples will be given from studies that used such a strategy, including an overview of the technologies that are relevant for bulbous plant species. In the final concluding section a prospect will be given how novel emerging technologies, bioinformatics, and systems biology can increase the efficiency and strength of this type of research and move the field from gene-by-gene approaches into a comprehensive genome-wide level.

What is known on flowering and vegetative propagation from model systems

Although the best studied model system, Arabidopsis, is a dicot, and the majority of bulbous plant species are monocots, the regulatory mechanisms underlying important agricultural traits appeared to be conserved in various cases. Hence, knowledge gained in Arabidopsis can be informative for studies in bulbous plants. Strong conservation between Arabidopsis and the monocot rice was observed e.g. for the genes involved in the photoperiod flowering time pathway (Izawa et al., 2003). The same holds for various hormonal signalling components and the key transcription factors involved in axillary meristem formation and outgrowth, which is directly related to vegetative propagation capacity in bulbous species (Finlayson, 2007; Kebrom et al., 2013). Nevertheless, various exceptions are known and in general best results are obtained when using a closely related model species as starting point. Therefore, we will discuss mainly knowledge gained from Arabidopsis, but when relevant, complemented with information from other dicot and monocot species.

Vegetative propagation

Shoot branching is a vegetative process determined by axillary meristems and it determines the architecture, biomass and reproductive success of a plant. Initiation of an axillary meristem results in the formation of a bud that will undergo a period of dormancy. Once the right environmental or endogenous plant factors release the bud from dormancy, it will grow and develop into a branch or a propagule in the case of flower bulbs (e.g. daughter bulb, bulblet, bulbil), a process known as bud outgrowth. Hence, the processes of axillary bud initiation and axillary bud outgrowth together determine the vegetative propagation rate in bulbous species (Fig. 1). Several genes promoting axillary bud initiation have been identified in different model species (Bennett and Leyser, 2006; Kebrom et al., 2013) and their supposed functions could be confirmed by transgenic approaches. For instance, a transcription factor of the GRAS family characterized in tomato, rice and Arabidopsis, - *Lateral suppressor* (*Ls*), *Monoculm1* (*MOC1*) and *LATERAL SUPPRESSOR* (*LAS*), respectively - is responsible for the establishment of an axil identity and maintenance of meristematic capacity via prevention of cell de-differentiation (Greb et al., 2003; Li et al., 2003; Ward and Leyser, 2004; Schmitz and Theres, 2005; Bennett and Leyser, 2006). A second key regulatory gene discovered in tomato, *BLIND* (*Bl*), encodes a MYB transcription factor that also promotes axillary bud initiation but its function is independent of *Ls*. Occurrence of bud outgrowth depends on the factors that release buds from dormancy. Apical dominance, which is the ability of the shoot apex of the plant to prevent outgrowth of axillary meristems, and therefore branching, is one of the most studied phenomena controlling dormancy in axillary buds.

The *Bl* ortholog in Arabidopsis is *REGULATOR OF AXILLARY MERISTEMS1* (*RAX1*) (Keller et al., 2006; Müller et al., 2006). A third regulator identified in Arabidopsis, *REGULATOR OF AXILLARY MERISTEM FORMATION* (*ROX*) has orthologs in rice *LAX PANICLE1* (*LAX1*) and maize *Barren stalk1* (*Ba1*), although the latter two also affect inflorescence branching (Yang et al., 2012).

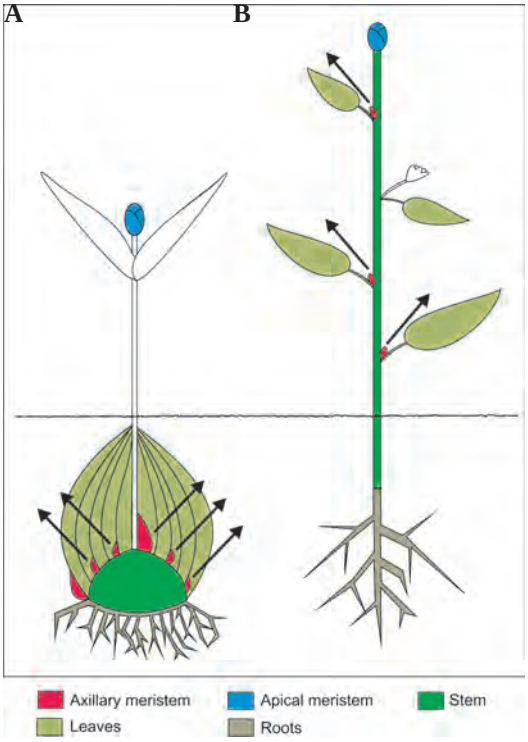

Figure 1. Architecture of a bulbous and a non-bulbous plant. (A) Tulip (B) Model dicot plant. Initiation of axillary meristems takes place in the axils of bulb-scales (A) or leaves (B). They form a bud like structure and undergo a period of dormancy. Once bud dormancy is broken, axillary buds grow out and develop into daughter bulbs in bulbous plants, or axillary branches in a typical dicot plant. In tulip, normally only two of the axillary buds will develop into daughter bulbs and once the apical bud blooms and dies, the closest axillary bud will become the apical bud for the next season. In bulbous plants the stem is called basal plate and it is a modified stem; Bulb-scales of bulbous plants are modified leaves. Arrows represent axillary bud outgrowth.

During vegetative development in Arabidopsis, *LAS* and *RAX1* influence the expression of *ROX* and axillary bud initiation occurs when *ROX* expression ceases (Yang et al., 2012). In contrast, *LAX1* transcripts in rice are detected only after the axillary bud has initiated (Oikawa and Kyozuka, 2009), suggesting that the molecular control of *ROX-like* genes may differ in timing between monocots and dicots. This control is mediated by a balanced hormonal signalling between auxin, cytokinin and the recently discovered strigolactones (Kebrom et al., 2013). Evidence for a role of strigolactones

in axillary bud outgrowth is given by *ramosus* (*rms*) mutants in pea, *decreased apical dominance* (*dad*) in petunia, *more axillary growth* (*max*) in Arabidopsis, and *dwarf* (*d*) or *high tillering dwarf* (*hdt*) in rice (Napoli, 1996; Morris et al., 2001; Booker et al., 2005; Ishikawa et al., 2005; Liu et al., 2009). In Arabidopsis *MAX1*, *MAX3* and *MAX4* are involved in strigolactone biosynthesis while *MAX2* plays a role in strigolactone signalling. Although the exact crosstalk between auxin, strigolactones and cytokinins in the control of shoot branching is not yet entirely understood, it is clear that auxin and strigolactones inhibit bud outgrowth while cytokinins promote it. In this system, a bud-specific gene that promotes bud arrest could be the key element to integrate the bud outgrowth pathway. Indeed, such a gene exist and is represented by T*eosinte branched1* (*TB1*) in maize and *BRANCHED* (*BRC1*) in Arabidopsis (dicot). *TB1* was first identified in maize and appears to encode for a transcription factor from the TCP family (Aguilar-Martínez et al., 2007). Evidence in Arabidopsis and pea show that the *TB1* ortholog *BRC1* is up-regulated by strigolactones and down-regulated by cytokinins (Aguilar-Martínez et al., 2007; Braun et al., 2012). A more recent study supports the idea of *BRC1* as a second messenger to induce and maintain bud arrest by negatively regulation of cell cycle, ribosome translation, and promotion of Abscisic Acid (ABA) signalling (González-Grandío et al., 2013). Because, outgrowth of axillary buds seems to be the major limiting factor in vegetative propagation of bulbs, the strigolactone signalling pathway and *TB1-like* genes are first targets of choice to study and optimize vegetative propagation in these plant species.

Flowering time control and flowering induction

Besides branching and axillary bud development, flowering time is an important trait influencing reproduction capacity in bulbous species. Plants are continuously sensing their environment, for being in the reproductive phase under optimal conditions and securing their reproductive success. Besides environmental cues, such as photoperiod and temperature, flowering time is also controlled by endogenous signals, including hormone levels and plant age (Lang, 1952). In the model plant Arabidopsis the vegetative phase

transition and floral induction, are well studied at the molecular level and the complex gene regulatory networks underlying these processes have recently been reviewed (Srikanth and Schmid, 2011; Andres and Coupland, 2012). We will discuss flowering time control here only briefly, with a focus on the pathways that are the most important for flowering in most of the bulbous species (Fig. 2), which are the aging and temperature pathways. The juvenile vegetative phase (aging pathway) can take up to seven years in bulbous species. Upon reaching the adult vegetative stage, the transition to reproductive development can be induced, which in tulip e.g. is triggered by relative warm temperatures in the spring or early summer (ambient temperature pathway). However, for development of the floral meristem into a complete flower and for elongation of the floral stem, a prolonged period of cold is essential (dormancy release), in analogy with bud dormancy release in trees (Cooke et al., 2012).

Plant age is one of the endogenous factors that can be linked with developmental phase transitions and competence of the shoot apical meristem for environmental signals triggering flowering. The age-dependent vegetative transition in Arabidopsis is regulated by *microRNA156* (*miR156*) and the *SQUAMOSA PROMOTOR BINDING PROTEIN-LIKE* (*SPL*) genes that are targeted by this microRNA. The repression of *miR156* results in up-regulation of several *SPL* genes which promotes vegetative transition (Fornara and Coupland, 2009). Two recently published studies showed that *miR156* levels are responding to sugars (Yang et al., 2013; Yu et al., 2013). Whereas a bulb is a storage organ and it is well known that sugars get re-located towards the shoot apical meristem and stem (sinks) upon flowering-inducing temperature changes (Lambrechts et al., 1994), it will be of interest to focus on this particular pathway in the hunt for signalling components involved in flowering time control of bulbous species.

After the switch from the juvenile to the adult vegetative phase, the plant becomes competent for flowering inducing external cues. Furthermore, reproductive development is triggered by the activation of

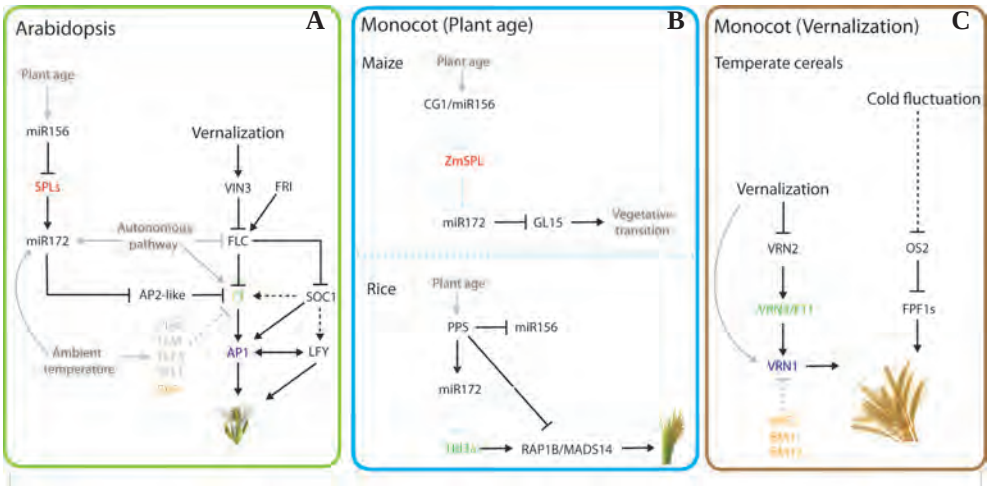

Figure 2. Comparison of the gene regulatory networks for flowering time control in dicots and monocots. In Arabidopsis (A) upon aging *miR156* is repressed leading to the up-regulation of selected SPL genes which promote the vegetative phase transition (aging pathway). External cues, e.g. ambient temperature, trigger the activation of *miR172* via the SPL genes leading to the repression of the floral repressors *AP2* and *AP2-like* genes. In order to be able to flower, winter annual Arabidopsis ecotypes first needs a prolonged period of cold leading to the activation of *VIN3* and repression of *FLC*. Ultimately, this results in the activation of the floral integrators *FT* and *SOC1*, followed by the activation of the floral meristem identity genes *AP1* and *LFY*. In the monocot maize (B, top), the vegetative phase transition is regulated by the suppression of the *AP2-like* gene *GLOSSY15* (*GL15*) through the activation of *miR172*. *CORNGRASS1* (*CG1*) encodes *miR156* and similar to Arabidopsis, might represses *ZmSPL* leading to the activation of *miR172*. In the monocot rice (B, bottom), *PETER PAN SYNDROME* (*PPS*) is involved in the repression of *miR156* and the activation of *miR172*. This might occur directly by *PPS* or indirectly (dotted blue arrow) through *miR156*. Upon unfavourable environmental conditions, *PPS* represses *RAP1B/MADS14*, independent of *Hd3a* (rice FT homolog). In monocot temperature cereals (C) the *FT* homolog *VRN3/FT* activates *VRN1* upon a prolonged period of cold, leading to flowering. Nevertheless, the *SVP* homolog *VRT2* represses *VRN1*. Shorter periods of cold repress *OS2* which inhibits stem elongation through *FPF1*s. Taking into account this knowledge from model species and assuming general conservation of the gene regulatory networks, a putative flowering controlling network can be designed for bulbous species. Genes with similar kind of functions in the different species are marked with the same colour.

microRNA172 (*miR172*) by the *SPL* genes, which results in the repression of a set of *APETALA2* (*AP2*)-like genes, acting as repressors of flowering (Zhu and Helliwell, 2011). Both microRNAs *miR156* and *miR172* are conserved in dicots and monocots (Axtell et al., 2007). Although, the age dependent phase transition is studied to a lesser extent in monocots (Fig. 2; Strable et al., 2008; Tanaka et al., 2011), performed experiments reveal a high level of conservation in the regulatory mechanisms controlling flowering time in between different species.

Vernalization is the requirement for a period of prolonged cold to overcome a block on flowering in winter annual plants. In Arabidopsis *FLOWERING LOCUS C* (*FLC*) is the key floral repressor in this process, and this transcription factor was shown to act as a direct transcriptional repressor of the so-called floral integrator genes *FT* (*FLOWERING LOCUS T*) and *SUPPRESSION OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (Fig. 2). *FLC* is activated by the positive regulator *FRIGIDA* (*FRI*) that acts in a large multi-protein complex. During winter, the transcriptional regulator *VERNALIZATION INSENSITIVE3* (*VIN3*) will respond to a prolonged period of cold, resulting in its gradual activation. As a consequence *FLC* will be repressed providing the shoot apical meristem competence for floral inducing cues, such as optimal temperatures and appropriate photoperiod conditions (Choi et al., 2011). In monocots however, *FLC-like* genes could not been identified. In wheat a different gene, *VERNALIZATION2* (*VRN2*), encoding for a Zinc finger-CCT domain containing transcription factor (Yan et al., 2004), is down-regulated by vernalization. This repression results in the activation of the *FT* homolog *VERNALIZATION3* (*VRN3*)/*FLOWERING LOCUS T1* (*FT1*) and the *APETALA1-like VERNALIZATION1* (*VRN1*) gene during a period of prolonged cold (Yan et al., 2006; Alonso-Peral et al., 2011). Three genes homologous to the Arabidopsis *SVP* gene; *VRT2*, *BM1* and *BM10* respectively, are able to repress *VRN1* but their role in vernalization or floral transition is not completely understood (Kane et al., 2005; Trevaskis et al., 2007). Besides a prolonged period of cold (vernalization response), short cold stresses repress the grass specific MADS box gene *ODDSOC2* (*OS2*).

A proposition was made that *OS2* is present in a pathway that delays the transition to reproductive development and that additionally inhibits stem elongation (Greenup et al., 2010). Altogether, this suggests that the vernalization response has evolved independently in monocot and dicot plants, although members from the MADS box transcription factor family play an important role in both. Bulbous plants, such as tulip, also require a prolonged period of cold. Though, in this case it is not essential for the meristematic switch from vegetative to reproductive development, but to release dormancy in the already existing floral bud and to induce stretching of the floral stem. Despite that this dormancy release is different from the vernalization response, more and more evidence is provided that the underlying regulatory mechanisms are comparable (Horvath, 2009). In addition to the vernalization response, flowering time also depends on relative small fluctuations in ambient temperatures. Genes involved in flowering time control and responding to changes in ambient temperature are e.g. *FLOWERING LOCUS M*(*FLM*)/*MADS AFFECTING FLOWERING1* (*MAF1*), *SHORT VEGETATIVE PHASE* (*SVP*), *EARLY FLOWERING3* (*ELF3*), *TERMINAL FLOWER1* (*TFL1*) and *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*) (Balasubramanian and Weigel, 2006; Kumar et al., 2012). Nevertheless, in contrast to the wealth of knowledge on the vernalization pathway, insight in the gene regulatory network underlying the ambient temperature pathway is just emerging.

Ways to transfer knowledge from model plants to economically important crop species

To transfer the wealth of knowledge gained from studies in model species towards crops and e.g. bulbous plant species, diverse roads can be taken. According to the methodology used to link the molecular basis of life (e.g. genes) with biological functions, such methods can be divided in bottom-up or top-down approaches (Fig. 3). The former one uses deductive reasoning, meaning that the knowledge is built from the constitutive parts (e.g. genes) to the systems, while top-down requires inductive reasoning: from systems to causal genes.

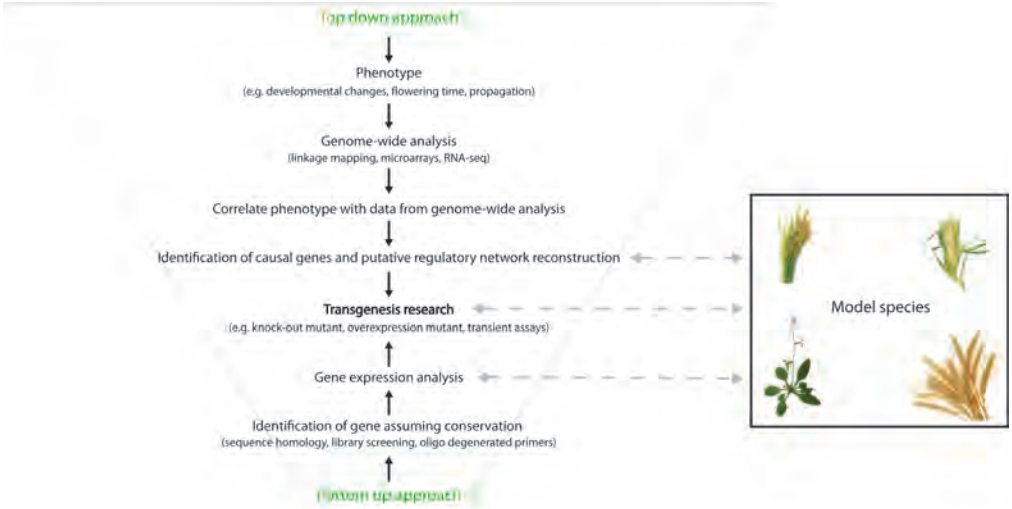

Figure 3. Flow chart how knowledge can be transferred from model species to economically important crops. Both bottom-up and top-down approaches are indicated and come together at the level of functional confirmation of candidate gene functions by transgenic research.

Bottom-up approach

The start point of this approach is the identification of putative orthologues genes in crops for genes of interest in model species (Salentijn et al., 2007). In general this is based on sequence homology and the assumption that the molecular pathways underlying the control of the biological processes, and hence the involved genes, are conserved. A widely used method in the past was the identification of highly homologous genes by genomic or cDNA library screenings (e.g. Xu et al., 1995; Sun et al., 1999). For this purpose hybridization can be applied or alternatively PCR-based methods, using degenerated oligonucleotides. Once the unknown target genes are identified, they can be sequenced and subsequently compared in silico with the gene sequences from the model species. An advantage of this method is that construction of such libraries does not require a priori genome sequence information. However, to date there are only limited comprehensive genomic libraries available for flower bulb species, likely due to the complex

genome sizes. When sequence information is available for the species of interest, identification of homologues is normally done *in silico* via BLAST-based sequence alignments (Altschul et al., 1990). However, there are limitations to the above discussed simplistic approaches, given by the fact that sequence similarity does not always imply functional similarity. This is nicely exemplified by differences in function for key genes in the vernalization pathway between monocots and dicots (e.g. *AP1-like* genes; Fig. 2). Furthermore, large-scale evolutionary events such as duplications can cause functional divergence for paralogues genes. When evolutionary events are taken into account, comparative studies, such as synteny mapping can provide information on orthology of the blasted sequences (McCouch, 2001). Whereas in the past, this was restricted to species for which the genome was sequenced or for which a detailed genetic map was available, integrating high-throughput Next Generation Sequencing (NGS) data makes it possible to apply this type of studies to crops that lack a reference genome sequence (Galvão et al., 2012) and hence, make it also possible to use synteny mapping for bulbous plant species in the near future. Regardless whether orthology will be taken into account, various experimental tools can be applied to guide the identification of genes or proteins with identical functions based on intrinsic characteristics of the molecules, such as protein-protein interaction capacity or their specific expression patterns.

Top-down approach

Top-down methodologies build vast amounts of high-throughput data in order to establish systems from which identifying causal genes would be feasible (Fig. 3). Large scale phenotyping platforms coupled to linkage mapping, and gene expression-based analyses, such as the generation of Expressed Sequenced Tags (EST) or genome-wide transcriptome profiling via microarray analyses or RNA-seq, are examples of sources for such large-scale data sets. EST datasets are a rich source for designing custom-made DNA microarrays (Lorenz et al., 2003), but for many species of interest there are no sufficient datasets available to create a proper

microarray platform. In that case, cross-species microarrays, in which probe sequences are derived from a model species and hybridization is performed with material from a crop of interest, is an attractive alternative to profile expression patterns (e.g. Moore et al., 2005; Wang et al., 2010). However results have to be interpreted carefully because of variance in efficiency of probe-transcript hybridization, caused by differences in sequence similarities or e.g. number of gene copies, due to species-specific duplication events (Lu et al., 2009). Unlike classical microarray experiments, RNA-seq does not require genome sequence information (Wang et al., 2009), neither *a priori* knowledge of gene functions. Furthermore, the method is highly sensitive and accurate providing detailed insight in gene transcription levels, as well as splicing variants across different physiological or morphological samples. Together, these characteristics make this technology an ideal tool to gain insight in the transcriptome of bulbous plants and to study differential gene expression for relevant biological process in these species. Nevertheless, assembling the enormous amount of short reads produced by RNA-seq is a bioinformatic challenge (Martin and Wang, 2011); especially for crops that lack a reference genome, which is the case for many economically important crops and in particular for bulbous plants. In absence of a reference genome, *de novo* transcriptome assembly is used as first approach (Garber et al., 2011). A successful example of the latter approach, was recently presented for grapes, that like bulbous species preferably sustain through vegetative propagation (Venturini et al., 2013). Besides transcriptomics data, information from other 'omics' types of approaches can be implemented. Currently, after transcriptomics the proteomics field is the most advanced and detailed quantitative information can be obtained at the protein level (Bindschedler and Cramer, 2011; Kaufmann et al., 2011). Also metabolomics is improving, but generated datasets are more fragmented and improvements of both throughput and reproducibility are needed (Saito and Matsuda, 2010).

The next step for all above mentioned top-down approaches, aiming to obtain information on gene activity and intrinsic gene product characteristics

at a genome-wide scale, is the identification of genes or sets of genes that behave in a manner associated to the biological process of interest. Subsequently, potential gene regulatory networks can be reconstructed based on this information, which can be compared to and fed back to knowledge from model species (Fig. 3). In this respect it is good to realize that for the usage of e.g. metabolomics data an additional hurdle needs to be taken in correlating metabolite concentrations to e.g. gene expression patterns and finally gene functions.

Verification of gene function

Both bottom-up and top-down approaches give a selection of genes that are potential key players in the biological process under study, and for which preferably the function should be validated. In Arabidopsis this is usually done through the selection of loss-of-function mutations in collections of T-DNA insertion plants (Slater et al., 2003). Alternatively, stable transformants can be generated or functions can be investigated based on transient expression assays by agro-infiltration or virus induced gene silencing (VIGS; Yang et al., 2000; Lu et al., 2003). The majority of methods that are available today for gene function verification depend on transgenic approaches. Despite that these technologies are already available for thirty years and have undergone various improvements over the last decades, it is still far from trivial to transform any desired plant species. Therefore, it is still common practise to perform gene function verifications by overexpression or complementation studies using a model species as target (cross-species analysis; Tsaftaris et al., 2012; Li et al., 2013).

Examples of successful knowledge transfer to bulbous plants

Bottom-up 'gene-by-gene' approach

Several of the above discussed methods to transfer knowledge from model species to crops have been used already in bulbous species. Probably one of the best known examples of the bottom-up approach is related to the specification of floral organ identities by MADS box transcription factor

genes according to the ABC-model (Ferrario et al., 2004; Litt and Kramer, 2010; Rijpkema et al., 2010). Floral organs in higher eudicots are organized in four concentric whorls, with sepals in the outer whorl, petals in whorl two, stamens in whorl three and carpels in the inner fourth whorl. The classical ABC model predicts the establishment of the four floral organ identities by the combinatorial action of MADS domain transcription factors and the accessory gene regulatory network appeared to be highly conserved. Based on the assumption that this network will also be conserved in bulbous flowers, hypotheses were generated to explain particular flower mutants in these species. Classical examples are the so called 'double flowers', in which stamens are converted into petals or petaloid organs, which in theory can be caused by alterations in B- or C-class MADS box genes. Expression studies in the double-flowered lily 'Elodie' provided evidence that this phenotype indeed was caused by the miss-expression of the putative Lily C-class gene *LelAG1* (Akita et al., 2008). Besides the C class gene, a putative A class (*AP1-like*) and other MADS box genes of the C/D class have been identified in *Lilium longiflorum* (Tzeng and Yang, 2001; Chen et al., 2008). Also in *Crocus sativus* a putative *AP1* gene was identified as well as a *SEPALLATA3* (*SEP3*)–like gene from the E-class (Tsaftaris et al., 2004; Tsaftaris et al., 2011). Despite strong conservations in flower organisation, plants belonging to the Liliaceae family have in general a slightly modified flower structure with two almost identical outer floral whorls, known as tepals. Based on this phenomenon a modified ABC model was proposed (van Tunen et al., 1993), suggesting that class B genes are also expressed in whorl one, leading to the same petaloid identity in the outer two whorls. The putative class B genes from *Tulipa gesneriana* were cloned and characterized (Kanno et al., 2003). In agreement with the hypothesized alternative model, the two *DEFICIENS* (*DEF*)–like genes *TGDEFA* and *TGDEFB* as well as one *GLOBOSA* (*GLO*)–like B-type gene *TGGLO*, were found to be all expressed in whorls one, two and three. The same model is also supported by the identification and analysis of B-class floral homeotic gene *PISTILLATA* (*PI*)/*GLO* in *Crocus sativus* (Kalivas et al., 2007).

All together, these examples show the power of a 'gene-by-gene' bottom-up approachin case of well-studied and strongly conserved biological processes.

Top-down 'transcriptome profiling' approach

Performing large-scale expression studies coupled to phenotyping is an advanced technology to identify key genes involved in a particular biological process. In lily e.g., a custom-made cDNA microarray was designed and generated, consisting of several cDNA's obtained from different pollen-related tissues (Huang et al., 2006). Following, a differentially expressed gene was identified encoding for a putative protein containing ankyrin repeats and a RING zinc-finger domain, named *LlANK*. Comparison of *LlANK* to functionally characterized genes in model plants suggested ubiquitin ligase activity for the gene product. Further experiments could confirm this molecular function and revealed an important role for this gene in polar pollen tube growth, showing the relevance of the followed approach. Despite the potential of this method and the large number of examples of success stories in a variety of food crops, the approach has been hardly explored in bulbous plant species.

Gene function verification using model species

Upon the identification of functional analogues genes, verification of the function is an important process. Monocots are known to be recalcitrant to *Agrobacterium*-mediated transformation and therefore most of the flower bulb transformations have been achieved through gene-gun techniques (e.g. Kamo et al., 1995; Watad et al., 1998; De Villiers et al., 2000). However, a major drawback of gene-gun transformation over *Agrobacterium*-mediated transformation is the lack of stable integrations on one hand and the unintended, but frequently observed integration of multiple gene copies in the case of a successful integration on the other hand. The latter can be a trigger for undesirable recombination events, genomic rearrangement, or silencing of the transgene (Hooykaas and Schilperoort, 1992). Conveniently,

evidence has been provided for the presence of certain *Agrobacterium* strains being able to infect flower bulb species such as *Ornithogalum* (Van Emmenes et al., 2008), *Gladiolus* (Kamo et al., 1995) and *Lilium* (Cohen and Meredith, 1992). More recently Li and collaborators proved that insertion and stable integration of *Zm401* gene in *Lilium* is possible via *Agrobacterium*-mediated transformation, which opens the door for more transgenic efforts in flower bulbs (Li et al., 2008). Nevertheless, in general transformation of bulbous plants is tedious and stable transformation frequencies are low (Lu et al., 2007; Wang et al., 2012). Therefore, heterologous complementation studies in Arabidopsis are widely used as an alternative to verify the function of a candidate gene found in bulbous species. For example, a homolog of *CENTRORADIALIS* (*CEN*)/*TERMINAL FLOWER1* (*TFL1*), *CsatCEN/TFL1* respectively, was cloned from *Crocus sativus* and functionally characterized in Arabidopsis. In Arabidopsis, *TFL1* controls axillary meristem identity, inflorescence development and flowering time (Alvarez et al., 1992). Overexpression of *CsatCEN/TFL1* in a *tfl1* Arabidopsis mutant background resulted in complementation of the mutant phenotype, indicating that the gene isolated from *C. sativus* is able to function as *TFL1* (Tsaftaris et al., 2012). A similar study revealed that a *FT-like* gene in *Narcissus tazetta* var. *chinensis*, known as *NFT1*, act as a flowering time regulator when ectopically and constitutively expressed in ft-3 mutant Arabidopsis plants. In these transgenic lines, *SOC1* a target of *FT* showed to be up-regulated as expected based on *FT* functioning in Arabidopsis (Li et al., 2013).

Besides stable transformation, transient technologies, such as VIGS, have been applied in bulbous species. A fragment of a putative *PDS* gene supposed to encoding phytoene desaturase, which is involved in carotenoid metabolism and photosynthesis, has e.g. been derived from lily and caused a bleaching phenotype in *N. benthamiana* after infiltration (VIGS). This phenotype was expected, because it is know that silencing of *PDS* results in photo bleaching symptoms caused by a decrease in leaf carotene.

This reveals that genes of monocot species can be used to silence their counterparts in the dicot *N. benthamiana* regardless of their distant evolutionary relationship (Benedito et al., 2004; Wang et al., 2009) and providing hints for possible functions of the used genes. Although the above mentioned examples show the success and power of heterologous functional analyses based on stable or transient transformation, it is good to realize that these type of experiments do in principle not indicate more than that a gene from a crop has sufficient sequence homology and overlap in functional domains to take over the activity of the endogenous gene in the model system. Consequently, this is no guarantee that a similar function can be assigned to the identified gene in the crop species. Difference in the spatial or temporal expression pattern might already withhold the gene from its supposed function based on the heterologous functional analysis.

Future directions and Challenges

So far most molecular-oriented research studies in recalcitrant crops and bulbous plants have focussed on the identification of a single candidate gene. Analyses of complete regulatory pathways, as is nowadays common in model species, are hardly done yet. However, with the speed NGS technologies are developing (Schneeberger and Weigel, 2011), molecular technologies become attractive tools to analyse important biological processes in non-model species. Shahin and collegues (2012) provided e.g. the first transcriptome dataset of lily and tulip by sequencing of ESTs with the 454 NGS technology (Roche; http://www.454.com/). Comparative genomics helped with the search for gene conservation between tulip and lily, and the contigs could be annotated on the basis of the rice genome annotation (Sequencing Project International Rice, 2005). Subsequently, molecular function, biological process and cell component were predicted for the identified genes that all together resemble about 40% of the lily and tulip transcriptome. Hence, this approach provides fast insight in the active part of bulbous plants genomes, with a limited investment and avoiding the need for deciphering the complete genome sequence, which in the case

of tulip is 200 times the size of the Arabidopsis genome. Although this is a great step forward, the authors realized and emphasized that deeper sequencing and analysis of time series for various tissues or cell types is essential to obtain sufficient information for extended comparative and functional gene studies. Furthermore, traditional sequencing techniques were producing long contiguous DNA sequence reads up to 1 kb in length; however, the majority of the latest introduced NGS platforms generate huge quantities of short sequence tags (50 to 100 bp), requiring sophisticated assembly algorithms and bioinformatics solutions (Nagarajan and Pop, 2013). Besides tackling this problem by a bioinformatic approach, technical improvements such as paired-end sequencing, helps to solve the assembly problem. Additionally, output from different platforms (e.g. PacBio; http://www.pacificbiosciences.com/) can be incorporated to overcome this problem to a certain extend. Nevertheless, the biggest barrier in this type of research will not be the generation of large scale data sets and the identification of complete gene sequences, but to extract the genes and alleles of importance for the process under study; or in other words, to find the needle in the haystack. In this respect it is good to take into account that the success rate of RNAseq experiments for gaining knowledge in a particular biological process strongly depends on a well-defined research question, followed by detailed temporal and spatial differential expression analyses (Van Verk et al., 2013). In addition to the correct input of biological material and the usage of optimal algorithms to extract genome-wide differential gene expression patterns, it is of utmost importance to improve the methods for the annotation of the identified genes. As discussed above, simple blast-based alignments are a good starting point, but in the case when no or only low homology exist with known gene sequences, other technologies are essential. Recently, bioinformatics and systems biology tools have been developed for this purpose, in which e.g. domain co-occurrence networks are generated (Wang et al., 2013) or information from various data sources or prediction programs is combined (Kourmpetis et al., 2011).

Despite the importance of bulbous plants for the ornamental industry, these species remained under investigated at the genetic and molecular level. However, thanks to the latest developments in transgenic research, the 'omics' area, and in the field of systems biology, the detailed study of flowering and vegetative propagation in bulbous plants, resembling the two most important biological processes for agronomical improvements, comes in sight. In a breeders perspective, shortening of the juvenile phase will help increasing the speed of selection processes for new varieties, with e.g. improved bulb productivity, ornamental characteristics and pathogen resistance. Hopefully, these developments will keep this sector flourishing in the coming century.

Acknowledgements

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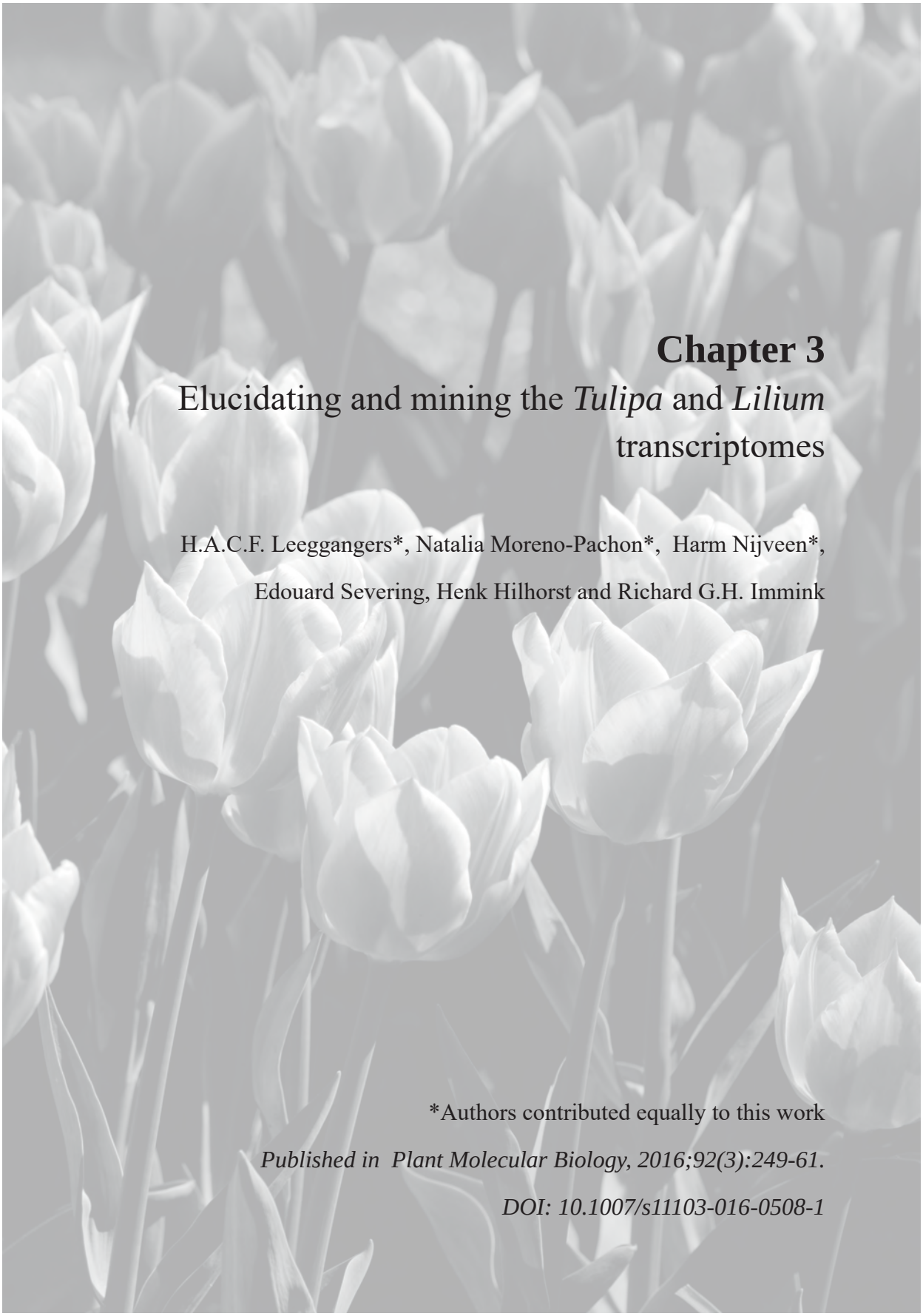

Chapter 3

Elucidating and mining the *Tulipa* and *Lilium* transcriptomes

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Abstract

Genome sequencing remains a challenge for species with large and complex genomes containing extensive repetitive sequences, of which the bulbous and monocotyledonous plants tulip and lily are examples. In such a case, sequencing of only the active part of the genome, represented by the transcriptome, is a good alternative to obtain information about gene content. In this study we aimed to generate a high quality transcriptome of tulip and lily and to make this data available as an open-access resource via a user-friendly web-based interface. The Illumina HiSeq 2000 platform was applied and transcribed RNA was sequenced from a collection of different lily and tulip tissues, respectively. In order to obtain good transcriptome coverage and to facilitate effective data mining, assembly was done using different filtering parameters for clearing out contamination and noise of the RNAseq datasets. This analysis revealed limitations of commonly applied methods and parameter settings used in de-novo transcriptome assembly. The final created transcriptomes are publicly available via a user friendly Transcriptome browser (http://www.bioinformatics.nl/bulbs/db/species/index). The usefulness of this resource has been exemplified by a search for all potential transcription factors in lily and tulip, with special focus on the *TCP* transcription factor family. This analysis and other quality parameters point out the quality of the transcriptomes, which can serve as a basis for further genomics studies in lily, tulip, and bulbous plants in general.

Introduction

Modern sequencing technology, also referred to as Next Generation Sequencing (NGS), quickly generates large amounts of sequence data at lower cost in comparison with traditional Sanger sequencing (Marguerat and Bähler, 2010; Schatz et al., 2010). While sequencing and assembly of large genomes still represent a technical challenge and a laborious procedure (Treangen and Salzberg, 2012), sequencing the expressed part of the genome, represented by the transcriptome, is nowadays achievable and can level down the complexity and provide useful information (Riesgo et al., 2012).

Therefore, transcriptome sequencing may represent an alternative to whole genome sequencing for species with large complex genomes when the aim is to generate a comprehensive database of genomic resources, suitable for gene identification, allele mining, or genome wide expression studies (Hou et al., 2011; Liu et al., 2012; Duangjit et al., 2013).
Bulbous plants, also classified as geophytes, represent species with economic relevance, large genomes and relatively scarce genomic resources. In short, geophytes are plants with storage organs and renewal buds resting in underground structures (Fig. 1, Kamenetsky and Okubo, 2013). Tulip and lily (*Tulipa sp* and *Lilium sp*) are ornamental geophytes with an estimated genome size of 25 and 36 GB, respectively (Shahin et al., 2012). One of the first studies of a transcriptome characterization for both species was done by Shahin et al. in 2012 using 454 pyro-sequencing technology of messenger RNA (mRNA) from leaves (Shahin et al., 2012). They obtained 81,791 unigenes for tulip with an average length of 514 bp and 52,172 unigenes for lily with an average length of 555 bp. Later studies have e.g. focused on sequencing the transcriptome of leaves (Wang et al., 2014), bulblets (Li et al., 2014) and meristem-enriched tissue (Villacorta-Martin et al., 2015) of different *Lilium* cultivars, using the Illumina HiSeq sequencing platform. These studies resulted in the identification of 37,843 unigenes for leaves (Wang et al., 2014), 52,901 unigenes in bulblets (Li et al., 2014) and 42,430 genes for the meristem-enriched lily tissues (Villacorta-Martin et al., 2015).

Despite continuous efforts to broaden the genetic resources of the bulbous species tulip and lily, characterization of their entire transcriptome is far from being completed. The information generated to date only covered leaf and meristem-enriched tissues and, furthermore, the data is difficult to access and mine for non-bioinformaticians. Our study aimed to generate a high quality and extensive transcriptome of these two bulbous species and making this valuable resource publicly available through a user-friendly and freely accessible web-based interphase, allowing easy data mining. The Illumina HiSeq platform was used to sequence a pooled sample for lily

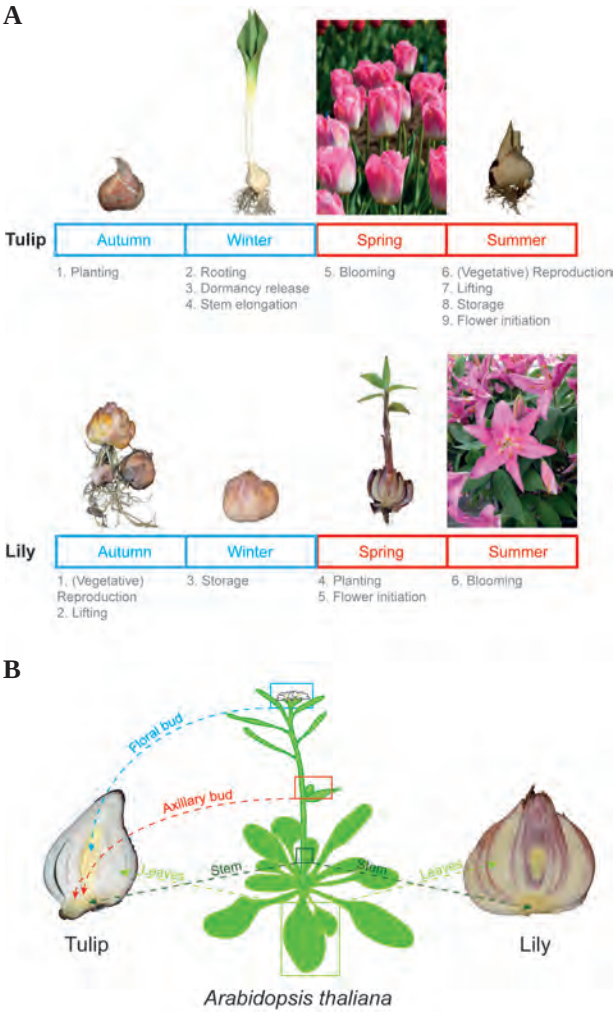


Figure 1. Life cycle and architecture of tulip and lily bulbs. (A) Tulip and lily yearly growth cycle. Note that their growth cycle is very similar. Both require a period of cold, but for different purposes and blooming occurs in different seasons. (B) Bulbs can be regarded as modified plants where the stem has shorten into a basal plate, the leaves have been modified into bulb-scales. In the tulip bulb the axillary buds are located in the axils of the bulb-scales and the floral bud is located in the center on top of the basal plate.

and for tulip, each made up of a mixture of equal amounts of poly adenylated mRNA obtained from flowers, stem, leaves, bulb and bulblets. Even though short reads are generated with the Illumina HiSeq platform, a tremendous throughput can be reached, resulting in an improved coverage of rare transcripts in comparison to the other platforms used in some of the previous transcriptome studies of bulbous species (Shahin et al., 2012; Kamenetsky et al., 2015).

The generated data was used to assemble reference transcriptomes for tulip and lily. For this purpose, different assembly settings were explored, aiming to generate an optimal transcriptome for gene mining. To proof the quality of the generated data sets, a comparison was made between

the transcripts found in the bulbous species tulip and lily and the genes of the model species *Arabidopsis thaliana* and *Oryza sativa* (rice). In addition, we searched for potential transcription factors present in both transcriptomes and compared their distribution with the distribution of transcription factors in the model species Arabidopsis and *O. sativa*. Subsequently, a web-based interface (Kamei et al., 2016), which we call '*Transcriptome Browser*', was implemented for data presentation and mining. The various possibilities of this browser are exemplified by zooming-in on a particular plant-specific gene family and the identification of all potential members of this transcription factor family. This activity enlightens the usefulness of the tulip and lily transcriptome browser in mining high-throughput sequencing data and identifying sequence information from lowly expressed, but important regulatory genes. Furthermore, these analyses revealed the quality of our data set and show how this resource can be explored in the future to study biological processes in bulbous plants at the molecular level.

Methods

Plant material

Tulip and lily tissues of several developmental stages were collected throughout the year of 2013 in The Netherlands. Adult tulip bulbs of the cultivar 'Dynasty' (*Tulipa gesneriana*) were planted in October 2012 in the field at Wageningen University (51.9667° N, 5.6667° E). Tulip bulb-scales, axillary buds, stem, leaves and floral bud were collected in January when all organs were entirely below ground; in March when the stem and leaves emerged above ground; and in May during blooming at full anthesis of the flowers. Roots and just initiated and dormant flower buds inside the buds during summer have not been sampled. Tissues of lily cultivar "McAleese" (*Lilium*, oriental hybrid group) were collected from regenerated bulblets and from fully grown plants. Regenerated bulblets were obtained by incubating detached bulb-scales in moist chambers without exogenous hormonal application at 23°C for six weeks,

followed by 12 weeks at 4°C. Newly regenerated bulblets were dissected under a stereo microscope and collected at the developmental stages S0 (proximal side of the explant at the start of the culture); S1 (proximal side of the explant at one day after culture); S2 (thickened structures of proximal side of the explant); D (dome formation); P (bulb-scale primordium formation); B (bulblet formation; Marinangeli et al., 2003). Fully formed regenerated bulblets were also collected at six weeks after culture under 23°C (bulblets are thought to enter a resting phase at this moment); and at 18 weeks after culture, from which the first six weeks were at 23°C followed by 12 weeks at 4°C (bulblets are out of the resting phase and ready to sprout into leaflets or a true stem). In addition to the regenerated bulblets, fully grown leaves, closed and open flowers, stem, and stem axils containing axillary buds were collected at the moment of blooming from greenhouse-grown plants (In the Netherlands; Long day (~16 hrs of light) conditions and 20-25°C). After collection of both tulip and lily plant material, the tissues were ground in liquid nitrogen and stored in -80°C until use.

RNA isolation

Total RNA was extracted from tulip bulb-scale tissue with the Tripure protocol (Roche, The Netherlands) according to the manufacturer's manual, with the addition of 2% Polyvinylpyrrolidone (PVP, w/v) and 2% β-mercaptoethanol (β-ME, v/v) to the extraction buffer. Isolated RNA was DNase treated with RQ1 (Promega, The Netherlands) followed by a phenol/chloroform (1:1) extraction and ethanol precipitation. RNA from the other tulip tissues was extracted with the Invitrap spin plant RNA mini kit (Invitek, ISOGEN Life Science, The Netherlands) and DNase treated with DNaseI (Qiagen, The Netherlands). Total RNA from all tissues collected from lily plants was isolated following the Tripure protocol (Roche, The Netherlands) with modifications. The modifications consisted of an initial removal of starch using an SDS-containing buffer (buffer I, (Li, 2005)) followed by phenol/chloroform extraction; and a final RNA purification of the eluted pellet using the Invitrap spin column (Invitrap spin plant RNA mini kit, Invitek, ISOGEN Life Science,

The Netherlands). DNA was removed from the samples by DNAse treatment with RQ1 (Promega, The Netherlands) according to the manufacturer's specification.

Quantity and quality of isolated RNA was assessed by agarose gel electrophoresis and NanoDrop spectrophotometer ND1000. Samples with a 260 to 280 ratio ranging from 1.7 to 2.1 were selected and mixed into equal RNA quantities into a separated lily and tulip pool. These two pooled RNA samples were sent to Wageningen UR Greenomics (Wageningen, The Netherlands) for cDNA library preparation and subsequent sequencing.

cDNA library preparation and sequencing

A cDNA library for each pooled sample was prepared following the TruSeq Stranded Total RNA Sample Preparation kit with Ribo-Zero Plant (Illumina, The Netherlands). The Ribo-Zero Plant kit removes ribosomal RNA (rRNA) from total RNA using biotinylated probes and the obtained rRNA-depleted RNA is first and second cDNA transcribed keeping strand specificity. Quality and quantity of each library was checked using a Bioanalyzer 2100 DNA1000 chip (Agilent technologies) and Qubit quantitation platform using Quant-iT PicoGreen (Invitrogen, Life Technologies). Library sequencing was done on a HiSeq2000 platform. The tulip and lily transcriptomes raw data were submitted to The National Center for Biotechnology Information (NCBI) under the numbers SRR3105600 (tulip) and SRR3105700 (lily).

Sequencing analysis

Paired-end reads were sequenced using Illumina Hiseq 2000. The quality of the reads was examined by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters were removed and paired-end reads were trimmed using Trimmomatic (Bolger et al., 2014) with settings: "ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:20 MINLEN:70 HEADCROP:5". The transcriptomes were assembled de novo using Trinity version 2.0.6 (Haas et al., 2013) with default settings, except max_memory 150G and SS_lib_type RF.

Transcriptome statistics were determined using the TrinityStats.pl script, which is part of the Trinity package. Transcripts abundances were quantified using RSEM version 1.2.22 (Li and Dewey, 2011) with default settings. To assess the level of contamination contained in both assemblies, NCBI's non-redundant protein database was searched using Diamond (Buchfink et al., 2015) with default settings and the results were analysed using MEGAN (Huson et al., 2007). CEGMA analysis (Parra et al., 2007) was used as a rough measure of the completeness and quality of the assemblies. Coding sequences on the transcripts were predicted using TransDecoder version 2.0.1 (Haas et al., 2013) as follows: first the longest open reading frames (ORF) were determined and translated using a cut off of 60 amino acids as the minimal protein length. The resulting protein sequences were used as queries to search the SwissProt section of the UniProt protein database (Consortium, 2015) with blastp (E-value cut-off 1e-5), and they were also scanned for conserved protein domains from the Pfam (Finn et al., 2014) database using Pfamscan. The Blast hits and Pfam results were used as input for the TransDecoder.Predict tool. Subsequently, the longest peptides per transcript on the (+) strand were selected using a custom Python script.

Translated sequences were clustered with orthologous proteins from the monocots rice, maize, Brachypodium, sorghum, switchgrass, barley and the dicots soybean, Arabidopsis, grape, poplar and tomato using OrthoFinder (Emms and Kelly, 2015).

Search Transcription Factor Families

For the identification of transcription factor families a PFAM analysis was performed on all the proteins present in the transcriptome from both lily and tulip. The families were divided according to the family assignment rules used in the Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/help_famschema.php). Transcription factor families without a Pfam domain were identified with BLAST by using the known *Arabidopsis thaliana* transcription factors in a particular family.

Tulip and lily Transcriptome mining

Tulip and lily putative TCP transcripts were retrieved using the Transcriptome Browser in three successive steps. The first screen was achieved making use of the sequence search tool, option Pfam (PF03634). In the second step, new TCP transcripts were identified by selecting all tulip and lily transcripts from the first screen and using the 'Seed BLAST' tool without default parameters. In the last step every oc cluster containing tulip, lily, Arabidopsis and rice transcripts with a PF03634 hit were screened manually. The TCP domain sequence of each transcript was retrieved manually from the '*Transcriptome Browser*' and aligned using Geneious software (Drummond et al., 2010). All Arabidopsis, rice, lily and tulip transcripts resulting from the Pfam (PF03634) search were clustered using the Neighbour-joining tree option of the '*Transcriptome Browser*'. Primer design was achieved using the cDNA alignment tool followed by the 'Specific' primer design option. The primers used can be found in Table S3.

Data availability

The 'Transcriptome Browser' containing the Tulipa and Lilium transcriptomes is publicly available via the website http://www.bioinformatics.nl/bulbs/db/species/index. The sequence data are available in the Transcriptome Shotgun Assembly Sequence Database of NCBI (http://www.ncbi.nlm.nih.gov/genbank/tsa/) under numbers SRR3105600 (tulip) and SRR3105700 (lily).

Results

Transcriptome sequencing and assembly

The Illumina HiSeq 2000 platform was used to sequence the tulip and lily transcriptome of a wide range of tissues varying from bulb scales to flowers. After trimming and removal of low quality reads, a similar number of paired end reads were obtained for both libraries: 169,920,574 reads for tulip and 165,031,389 for lily. Subsequently, Trinity software (Grabherr et al., 2011) was used to assemble both transcriptomes de novo and this assembly yielded to 499,780 transcripts for tulip and 569,305 for lily with an average

length of 561 bp and 487 bp, respectively. When not taking the isoforms into account and without applying additional data filtering, Trinity predicted 380,091 genes for tulip and 467,241 for lily (Table 1). Transcript over-estimation is common in de novo sequencing studies because the lack of a reference transcriptome or genome limits the assembly of sequences that represent non-overlapping pieces of the same gene. Transcripts expressed at extremely low levels can also cause noise because they may not be reliably assembled (http://cole-trapnell-lab.github.io/cufflinks/cufflinks/). Furthermore, it is difficult to distinguish between isoforms of one gene versus the existence of more gene copies as a consequence of duplications (Chang et al., 2015). Therefore, filtering out lowly expressed transcripts is a routine procedure applied during transcriptome assembly to get rid of noise and contamination, and it yields, in general, significantly reduced numbers of predicted transcripts and genes. To compare and find the optimal parameters for our two datasets, but retaining the full complexity of the tulip and lily transcriptomes, we generated three additional assemblies based on different abundance filtering settings. The three new assemblies consisted of transcripts with equal or more than 10 or 20 counts; and transcripts occurring at least more than once per million (TPM), respectively. As summarized in Table 1, increasing the cut-off value to filter out transcripts with low abundance leads to a dramatic decrease in the number of predicted transcripts and genes, but improves the N50 and average transcript length.

The number of obtained transcripts and predicted genes, in combination with the average transcript length, is generally used as a quality indicator of de novo transcriptome assemblies. In an ideal situation, the number of predicted genes should be close to the number of genes expected for the species. Based on this criterion, using counts per transcript upward of 20, seemed to be the best parameter since it reached a reasonable number of genes taking into account the number of genes found in sequenced plant genomes (e.g. rice (Sequencing Project International Rice, 2005); Arabidopsis (Initiative., 2000); poplar (Tuskan et al., 2006);

Table 1. Summary statistics of the tulip and lily transcriptomes generated by non-filtered data and upon applying three different filtering parameter settings.

	Non-filtered		Counts per transcript ≥10		Counts per transcript ≥20		TPM ≥1	
	Tulip	Lily	Tulip	Lily	Tulip	Lily	Tulip	Lily
Contigs	499,780	569,305	174,442	252,040	112,256	131,912	39,171	38,688
Genes	380,091	467,241	115,167	198,613	70,634	94,283	29,523	29,188
GC %	42,74	41,79	43,62	42,1	43,98	42,64	45,4	45
N50	695	514	1226	913	1478	1322	1573	1717
Average length	561	487	933	703	1,139	989	1,017	1,035

TPM: transcripts per million

loblolly pine (Neale et al., 2014)). Furthermore, this filtering resulted in a high average transcript length, suggesting a high percentage of complete and fully covered mRNA sequences in this assembly.

Nonetheless, it is important to realize that the high number of transcripts and predicted genes in the non-filtered transcriptome may not only be the result of miss-assemblies and non-plant contamination, but also because of the presence of incomplete or truncated rare, but valuable transcripts. Such incomplete transcripts may be the result of incomplete cDNA amplification, or mRNA degradation and breakage, and in general lowly expressed transcripts are more prone to be assembled as fragments due to limited sequencing coverage. To investigate this option in more detail, we studied − using the lily transcriptome as an example − how filtering out lowly expressed transcripts affects the number of transcripts encoding plant orthologues as well as the transcripts considered to be contamination (Fig. 2). As expected, the three filtering options improved the raw transcriptome in terms of contamination, but surprisingly decreased also dramatically the number of plant orthologues retained. For example, TPM larger or equal to one reduced the contamination with almost 100% efficiency, but only retained a bit more than 20% of the plant orthologues from the non-filtered

transcriptome database.

This observation prompted us to gain more insight in the nature of the transcripts with low abundance. For this purpose, all removed transcripts per filtering method were compared with the Arabidopsis Information Resource (TAIR) database using the Basic Local Alignment Search Tool (BLAST). Within the removed transcript sequences many important gene products where present, e.g. encoding putative meristem signalling peptides (CLAVATA3/ESR) (Wang and Fiers, 2010), which are known to be short in sequence and lowly expressed. Furthermore, transcript fragments of genes expected to be very locally and lowly expressed, such as some basic helix-loop-helix (bHLH) transcription factors, wound-responsive protein-related and flowering promoting factors, were identified in these filtered-out transcript sets (Supplemental Table 1). Hence, the use of a filtering method may lead to a transcriptome with improved quality based on average transcript length, but, it results on the other hand in the removal of a substantial number of transcript fragments corresponding to important plant genes. Based on these observations, we decided to continue with a non-filtered transcriptome, including short, truncated, and incomplete transcripts, since this increases the chances of identifying sequence information of rarely expressed genes. In order to evaluate the completeness of these final assembled and selected tulip and lily non-filtered transcriptomes, Core Eukaryotic Genes Mapping Approach (CEGMA) analysis was used (Parra et al., 2007), showing that the generated transcriptomes of tulip and lily contain nearly 100% of the 248 core eukaryotic proteins (98.79% for both species).

Functional annotation

TransDecoder 2.0.1 (Haas et al., 2013) has been used to predict coding sequences in the tulip and lily transcriptomes. Subsequently, the UniProt protein database (Consortium, 2015) and the Pfam conserved domain database (Finn et al., 2014) were used to predict protein coding genes.

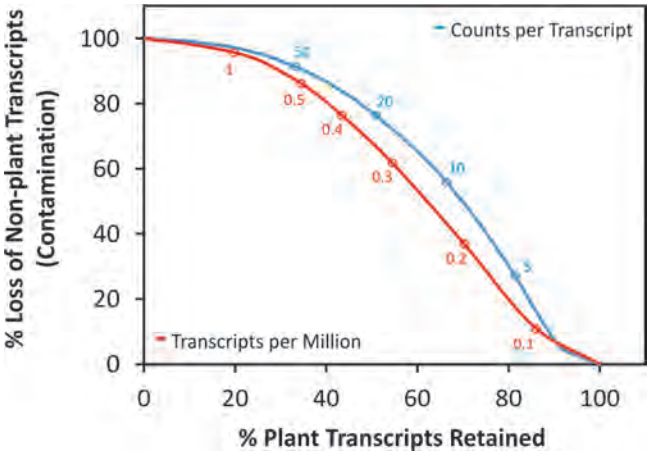


Figure 2. Effect of transcriptome filtering on the percentage of "non-plant" and plant transcripts retained. Filtering done based on counts per transcript.

In total 147,101 transcripts of tulip were identified, resulting into 89,530 predicted protein coding genes and 144,801 transcripts of lily, giving rise to 101,312 predicted genes. Those predicted genes represent nearly 50% of the transcripts in the non-filtered transcriptomes. In a follow-up step, the predicted proteins of tulip and lily were grouped in so-called Orthology clusters using OrthoFinder (Emms and Kelly, 2015). The clusters also contained the monocots rice, maize, Brachypodium, sorghum, switchgrass, barley and garlic; and the dicots soybean, Arabidopsis, grape, poplar and tomato. A total of 15,296 orthology groups were found to contain lily and tulip proteins, 10,014 of these also included one or more Arabidopsis proteins. A search for orthology groups that only contained proteins from the bulbous species tulip, lily, and garlic (Kamenetsky et al., 2015), revealed a set of 281 unique groups that might represent bulbous plant specific genes.

To get a better impression of the quality and completeness of the functional annotated datasets, we compared our transcriptomes and annotation with previously published transcriptomes of tulip and lily (Shahin et al., 2012). Initially, we performed a BLAST search at the nucleotide level to determine how well we covered the transcripts present in these publicly available datasets. Depending on the cultivar we used for this comparison, we found a BLAST hit for 87-95% of the published tulip contigs and for 80-85% of the lily contigs. These numbers reveal that we found evidence for the

presence of the majority of potential genes in the published datasets in our transcriptomes. Subsequently, we determined how many potential tulip and lily genes with a putative Arabidopsis ortholog were unique in either our transcriptomes, or the published datasets of Shahin and co-workers (2012). For this purpose a BLAST screening (blastx, e-value cut-off of 1e-5) on the Arabidopsis proteome was performed for the individual datasets. In this analysis we found 1345 and 95 unique tulip hits, for the transcriptomes described in this study and the published tulip datasets, respectively. For lily these numbers were 647 and 164. So on average almost eight times more additional and unique sequences with a BLAST hit to the Arabidopsis proteome were identified in this study in comparison to the previous study. In Supplemental Table 2, an overview is presented of the unique hits in the individual lily datasets as an example. As expected, a large part of the unique sequences in our transcriptomes in comparison to the published transcriptomes resemble genes that are expressed in tissues other than leaves, which was the only tissue sampled by Shahin and co-workers (2012). In addition, sequences were uniquely identified in this study that are potentially encoding for rare and low expressed genes. Examples are three out of 22 known members of the novel seed plant-specific family of small peptides encoding genes, *ROT-FOUR LIKE1-22* (*RTFL1-22*) (Narita et al., 2004).

Transcriptome coverage assessed by the identification of Transcription Factor families

In the plant kingdom a large number of transcription factor families can be found and they are involved in several processes, ranging from plant development to abiotic and biotic stress responses (Riechmann et al., 2000; Zhang et al., 2011). Transcription factors orchestrate several networks by controlling when and where certain genes will be expressed (Lee et al., 2006) and, therefore, have been well studied and characterized in plants. However, even though they function as master regulators, transcription factors are often expressed at relatively low abundance (Jones et al., 2015).

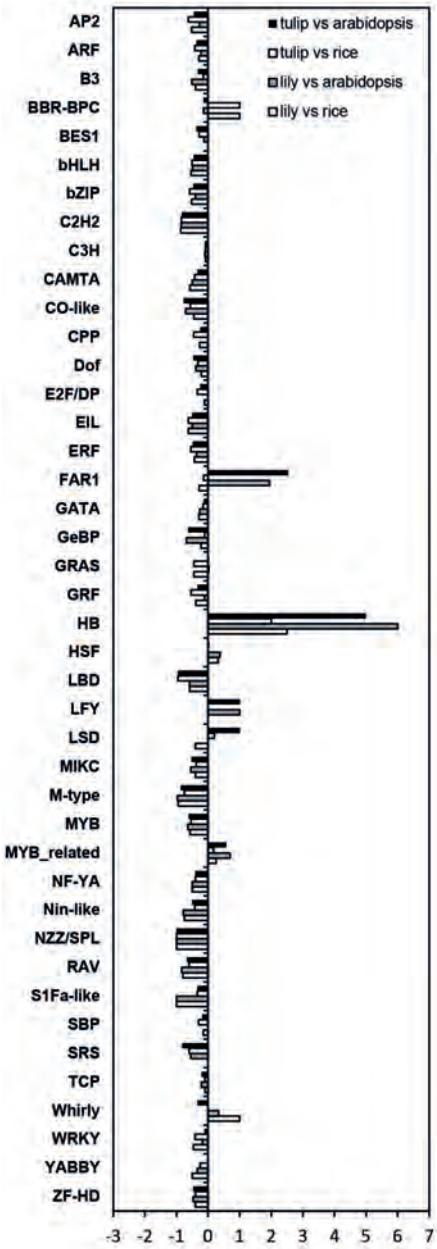


Figure 3. Overview of 42 transcription factor families identified in lily and tulip in comparison to rice and Arabidopsis. The bar represents the relative number of transcription factors present in each family in comparison to the number of transcription factors present in the model species Arabidopsis and rice, respectively. A value below one indicates under-representation in lily or tulip in comparison to rice or Arabidopsis and a value above one shows over-representation.

This low level of expression makes transcription factors suitable markers to further assess the sequencing depth and coverage of our two generated transcriptomes. Therefore, a comparison was made between the 42 known transcription factor families in the model species Arabidopsis and rice, and our generated transcriptomes of tulip and lily. For this purpose, the putative transcription factors of each family were identified based on Pfam domains (Finn et al., 2014). The outcome of this analysis is summarized in Table S2. A large number of transcription factors were identified in the transcriptome data of both lily and tulip with an expected distribution over families, but some families in both tulip and lily seemed to contain more putative members than expected based on their abundance in model species (Fig. 3). Examples are the homeodomain (HB) family and the MYB related transcription factor family. For the FAR1 family, over-representation is observed in comparison to Arabidopsis but the numbers found in lily and tulip, are almost equal in comparison to rice. This might point to a monocot specific expansion of this specific transcription factor family. In general, the number of transcription factor members in a particular family is rather similar in the two bulbous plant species. However, exceptions can be found for the zinc finger LSD and the Whirly family. The LSD family is over-represented in tulip while the Whirly family is over-represented in lily, based on our datasets. These examples might point to species-specific family expansions, though additional analyses are needed before firm conclusions can be drawn.

Examples are the M-type and MIKC MADS domain transcription factor family clades, AP2 and RAV, B3 and ARF, and HB-other and HB-PHD (Riechmann et al., 2000). In Fig. 4 an overview is given of the distribution of TF protein domains within each species. As expected, the overall distribution is similar between the model species and the bulbous plants tulip and lily. One of the largest groups of transcription factors, which covers ~13-15% of all transcription factors of the 42 families, contains a zinc finger domain. The second largest group is represented by the MYB

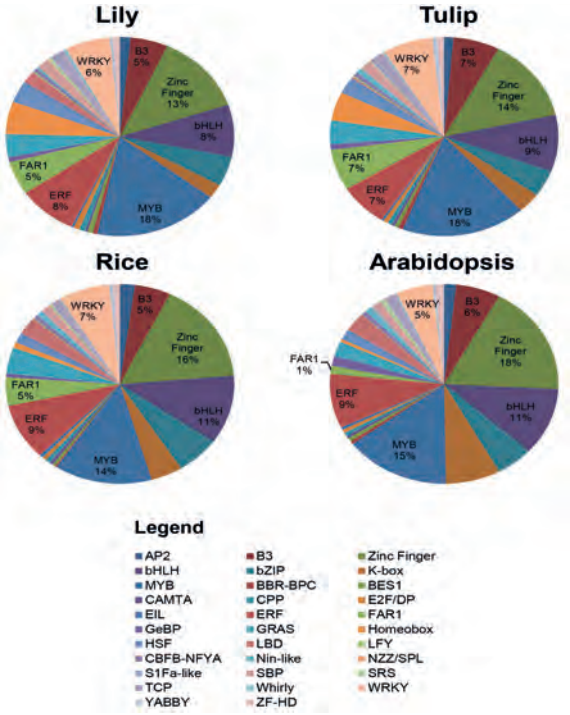


Figure 4. Distribution of transcription factors based on conserved protein domains in lily, tulip, Arabidopsis and rice. The transcription factor family distribution in tulip and lily is similar to the distribution in rice and Arabidopsis. However, in comparison with Arabidopsis, the FAR1 transcription factor family is larger inthe monocots tulip, lily, and rice.

transcription factors (~12-15%), followed by the bHLH domain containing transcription factors (~7-10%). A major and remarkable difference is observed between monocotyledonous and dicotyledonous species for the FAR1 domain containing transcription factors, as was already mentioned above. Approximately 5-6% of the total transcription factors used in this analysis has the FAR1 domain in lily, tulip and rice. Nevertheless, in Arabidopsis only ~1% of the transcription factors contain this domain. The biological relevance of the expansion of this particular transcription factor family in tulip and lily is currently not known, but it seems not to be an assembly artefact, since the overrepresentation is also found in the completely sequenced rice genome (Sequencing Project International Rice, 2005).

Mining high throughput data with the *Transcriptome Browser*: Identification of the TCP gene family

Once a transcriptome is assembled, one of the biggest challenges for researchers is to explore the large dataset in search for sequences with biological relevance. To support in mining data using open sources, we decided to deposit our generated transcriptomes in a web-browser (http://www.bioinformatics.nl/bulbs/db/species/index) based on recently developed open software (Kamei et al., 2016). This web-based interface offers basic bioinformatics search tools, identification of candidate transcripts based on phylogenetic relationships between orthologous sequence data and design of specific and degenerate primers for expression studies of transcripts of interest (Fig. 5).

To explore the usefulness of this data resource, we mined the datasets aiming to identify members of the *TCP* gene family in lily and tulip. The TCP transcription factor family, named after its founder members *TEOSINTE BRANCHED1*, *CYCLOIDEA*, and *PROLIFERATING CELL FACTOR*, has in general around 25-30 members in eudicots (Nicolas et al., 2015). *TCP* genes are expressed in a wide range of tissues and they control flower, leaf, and lateral shoot growth by activating or inhibiting cell proliferation (Martín-Trillo and Cubas, 2010; Mondragón-Palomino and Trontin, 2011; Nicolas et al., 2015). Furthermore, evidence from Arabidopsis expression studies indicates that several *TCP* members are lowly expressed in the above ground tissues (Danisman et al., 2013).

The expected wide-range in tissue and level of expression of *TCP* genes was our reason to choose this gene family to assess the power of the Transcriptome Browser in mining high throughput sequencing data. All putative lily and tulip *TCP* sequences were identified by using the sequence search tool (setting Pfam PF03634), followed by seed BLAST analyses with different parameter settings, and an additional manual search scrolling through the orthology (oc) clusters. The Pfam search resulted in 38 tulip and 33 lily transcripts, the seed BLAST search into two additional tulip transcripts and the oc search identified two extra transcripts for each species.

This total of 42 tulip and 35 lily transcripts, represented 24 and 22 potential *TCP* genes respectively.

The following step was to corroborate the TCP identity of the resulting tulip and lily transcripts based on the characteristic features of the TCP domain described by Martín-Trillo and Cubas. As shown in figure 6, the two putative *TCP* transcripts identified by seed BLAST search, as well as the remaining lily transcript found by oc search contained only a partial fragment of the TCP domain and this was the reason why they failed to pop-up within the PFAM search. However they can be considered true TCPs based on their characteristic features. This example shows the power of using the *Transcriptome Browser* in data mining and highlights the importance of our choice to maintain truncated transcripts into the final assembly.

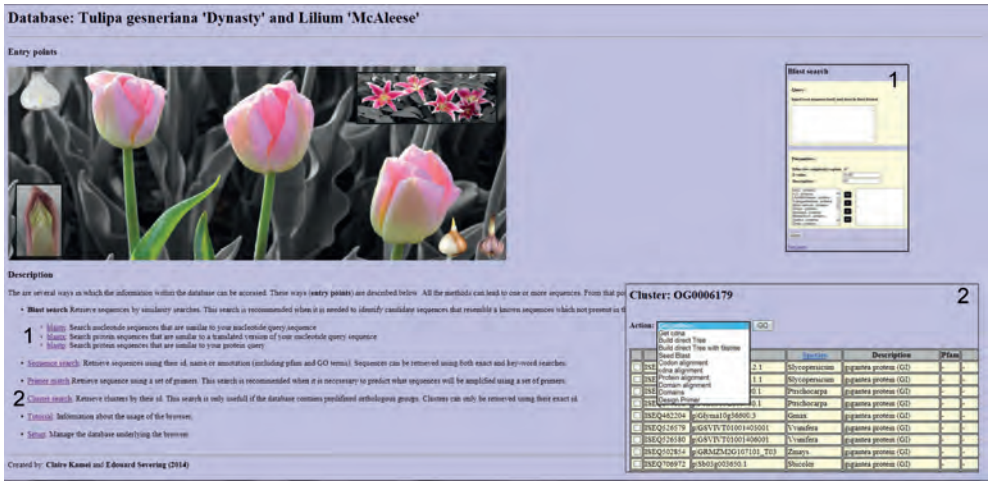


Figure 5. Screenshots of the Transcriptome browser. In panel 1 the interphase of the BLAST search is shown. The input sequence can be blasted against the tulip and lily transcriptomes as well as other plants species such as *Arabidopsis thaliana*, *Oryza sativa* and *Vitis vinifera*. In panel 2 an example is given of the output of the cluster search. Here different actions can be chosen such as protein alignment, primer design and build a direct tree (phylogenetic tree). Note that the browser has a tutorial option, in which the exact procedure how to perform the different tasks and actions is explained.

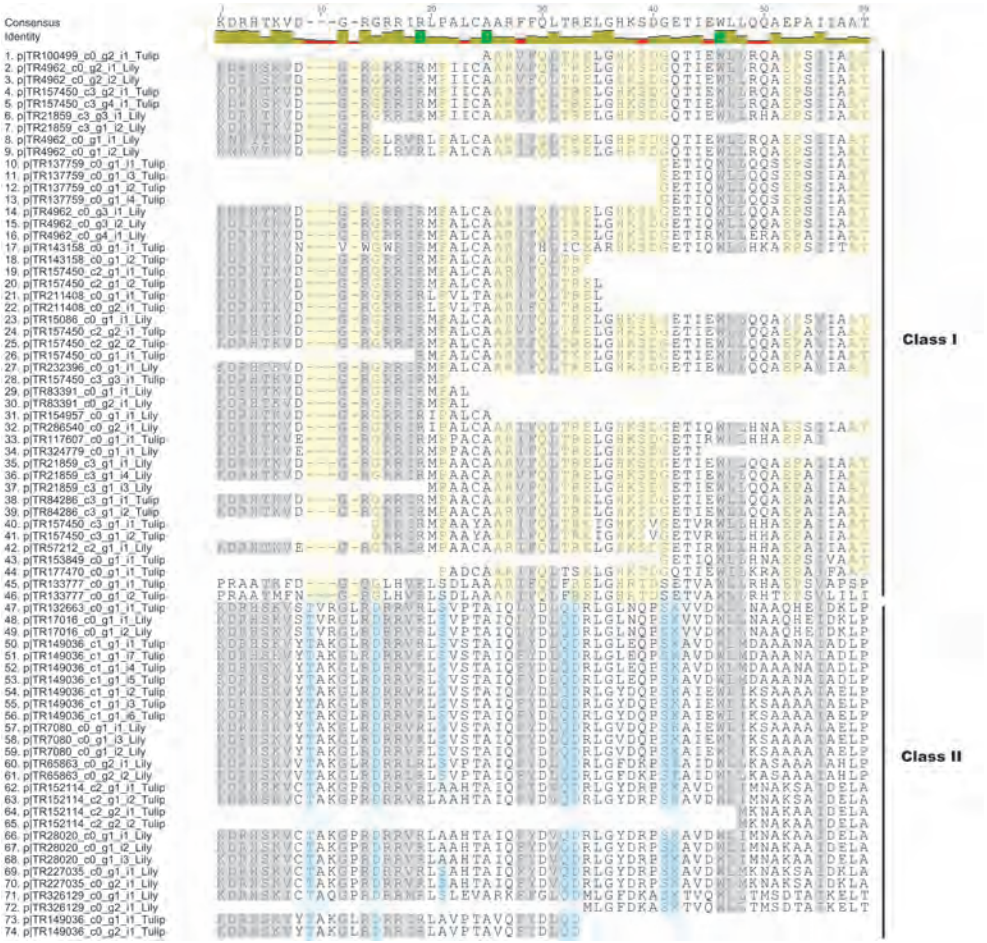


Figure 6. Sequence alignment of the domain of 74 TCP transcripts found in tulip and lily. Sequences are clustered in class I and class II based on the classification by Martín-Trillo and Cubas (Martín-Trillo and Cubas, 2010). Sequences 64 (p|TR152114_c2_g2_i1_Tulip) and 65 (p|TR152114_c2_g2_i2_Tulip) were found by seed BLAST search only, and sequence 7 (p|TR21859_c3_g1_i2_Lily) was identified by the orthology cluster (oc) search option. Yellow shaded regions indicate characteristic features of class I, blue characteristic features for class II and grey for all other amino acids.

Although the aim of this study was not to characterize the identity of each *TCP* transcript found in tulip and lily, we wanted to test the capacity of the Transcriptome Browser in clustering the tulip, lily, rice and Arabidopsis TCP sequences, based on sequence similarity. All lily, tulip, Arabidopsis

and rice protein sequences which contained the TCP domain (from the initial Pfam search) were selected to build an unrooted tree using the Neighbour-Joining algorithm (Fig. S1). Once again, the browser was able to distinguish between transcripts from class I and II. Also, most of the clades contained transcripts of all four species, which might help in further approaches to characterize the TCP identity of the tulip and lily transcripts.

Last, we tested the capacity of the 'specific primer design tool' offered in the *Transcriptome Browser* (Kamei et al., 2016). This tool designs primers in unique regions, given a set of similar sequences. PCR amplification of unspecific fragments or fragments without the expected size might indicate assembly errors. Therefore, five *TCP* genes were selected randomly for each bulbous species. The browser was able to design unique primers in all chosen sequences and PCR amplification with the expected band size was observed in nine out of the ten selected genes (Fig. S2). Overall, this result highlights the power of the *Transcriptome Browser* in designing specific and unique primers given from e.g the members of a gene family.

Discussion

Despite various large-scale sequencing efforts, we still lack a comprehensive transcriptome for many species. In this study a large-scale lily and tulip transcriptome was generated and this resource has been made available in a web-browser for easy mining.

Filtering out transcripts with low abundance reduced the number of retained plant orthologue hits

The number of transcripts and predicted genes in our non-filtered transcriptomes may be highly over-estimated taking into account that there are only 27,024 protein coding gene models in the recently sequenced monocot genome of pineapple (Ming et al., 2015), 39,045 genes reported for rice — the monocot model species — (Sequencing Project International Rice, 2005) and 81,791 for tulip, based on a previous transcriptome

sequencing effort (Shahin et al., 2012). Nevertheless, we expect that the large number is certainly not all because of noise, whereas the methodology we selected for sequencing assures high depth coverage and strand specificity. These aspects make the identification of rare and lowly expressed transcripts for both coding and non-coding RNAs possible. Additionally, both tulip and lily are in general vegetative propagated and therefore heterozygosity is maintained, being a source for a higher number of different transcripts. Although in other bulbous studies filtering out low abundant sequences reduced significantly the number of predicted genes to a level that gets close to what is reported for model species (Villacorta-Martin et al., 2015), we proved that in our data this filtering reduced dramatically the percentage of transcripts with substantial homology to a known plant gene. Therefore, our non-filtered transcriptomes may not reflect the true number of genes but they rather represent extensive transcriptome coverage for both tulip and lily. Despite the fact that there is some contamination (non-plant hits) retained in the non-filtered databases, both transcriptomes contained nearly 100% of a core set of eukaryotic proteins, which is an indication of the completeness of the assemblies. Furthermore, we showed the power of these transcriptomes in finding rarely expressed genes, such as genes belonging to the CLV/ESR family encoding for small size ligands that act as important developmental signalling molecules (Wang and Fiers, 2010).

Transcriptome coverage assessment

In addition to the core eukaryotic proteins, the transcription factor family distribution analysis in tulip and lily has also confirmed the quality of the transcriptome assembly. A large number of transcription factors could be identified even though not all tissues, developmental stages, and common biological process -such as stress responses and floral primordium formation- of the bulbs were collected for RNA-sequencing. To mention an example, tulip tissues were collected from January until May, leaving out the months June to December. During this latter period of time, the floral

primordium inside the tulip bulbs is formed (Khodorova and Boitel-Conti, 2013) and therefore transcription factors specifically involved in this process might be absent. When zooming in on the members of each transcription factor family found in tulip and lily, some families contain more members in comparison to the model species or vice versa. Although, we cannot rule out miss-assembly as a reason for over-representation in particular transcription factor families, a few nice examples of expanded families have been found that based on comparison with other monocots seem to be present and probably unique to monocots or bulbous species. Having more members in a family can be due to the large genome that both tulip and lily have which might be partially due to additional gene duplication events. It will be of interest to study in the future whether there are bulbous-plant-specific functions for these additional genes, proving their biological relevance. Though, before going into laborious in-depth functional studies it is essential to confirm a correct assembly of these potential novel genes by wet-lab experiments, other sequencing methods such as PacBio, or using software such as Recognition of Errors in Assemblies using Paired Reads (REAPR; Hunt et al., 2013).

Functionality of the Transcriptome Browser in mining the extensive tulip and lily transcriptomes

Mining high through-put data often requires advanced programming skills or access to user friendly commercial software. Most of the publicly available software tools offer limited options, forcing researchers to use a combination of open software packages, requiring in general different formats and operational systems (Deng, 2011). Based on the identification of the putative *TCP* transcripts for both bulbous species, we confirmed that the Transcriptome Browser (Kamei et al., 2016) represents a reliable and user-friendly web based interface, able to identify gene families and build phylogenetic relationships with other species.

Conclusion

The methodology implemented in this study to assemble de novo transcriptomes demonstrates that there is a trade-off between transcriptome quality and the amount of information retained. Filtering out data that are considered 'noise' improves the values of the parameters that are commonly used to assess the quality of a transcriptome. However, such filtering methods may limit the power of data mining by e.g. reducing dramatically the chances of finding rare or lowly expressed genes. This study resulted in extensive transcriptome resources for both tulip and lily that can be easily mined. The limited number of molecular studies performed in these two bulbous species to date, states the need for such a user-friendly resource. Although, genome sequencing has undergone an enormous revolution over the last decade, it will most likely take some time before a high-quality and well-assembled genome sequence of lily and tulip will become available. Until that moment, the transcriptome browser presented here will be of pivotal importance for gene identification in these two bulbous plant species.

Acknowledgements

We thank Van der Gulik Tulpen B.V. for providing Dynasty tulip bulbs, and Hans van der Heijden and Enthius for providing the lily material. We are grateful to Paul Bijman for technical advice and like to thank TTI-Green Genetics and the Dutch Ministry of Economic Affairs for financial support.

Supplemental material

Table S1. Blast result for the transcripts filtered out of the RNAseq dataset using commonly applied filter settings. (http://link.springer.com/article/10.1007%2Fs11103-016-0508-1)

Table S2. Overview of lily transcripts with a blastx hit to the Arabidopsis TAIR database and unique in the transcriptome presented in this study or in a recently published lily transcriptome. (http://link.springer.com/article/10.1007%2Fs11103-016-0508-1)

Table S3. Overview of the identified putative transcription factors in lily and tulip, classified over 42 families.

(http://link.springer.com/article/10.1007%2Fs11103-016-0508-1)

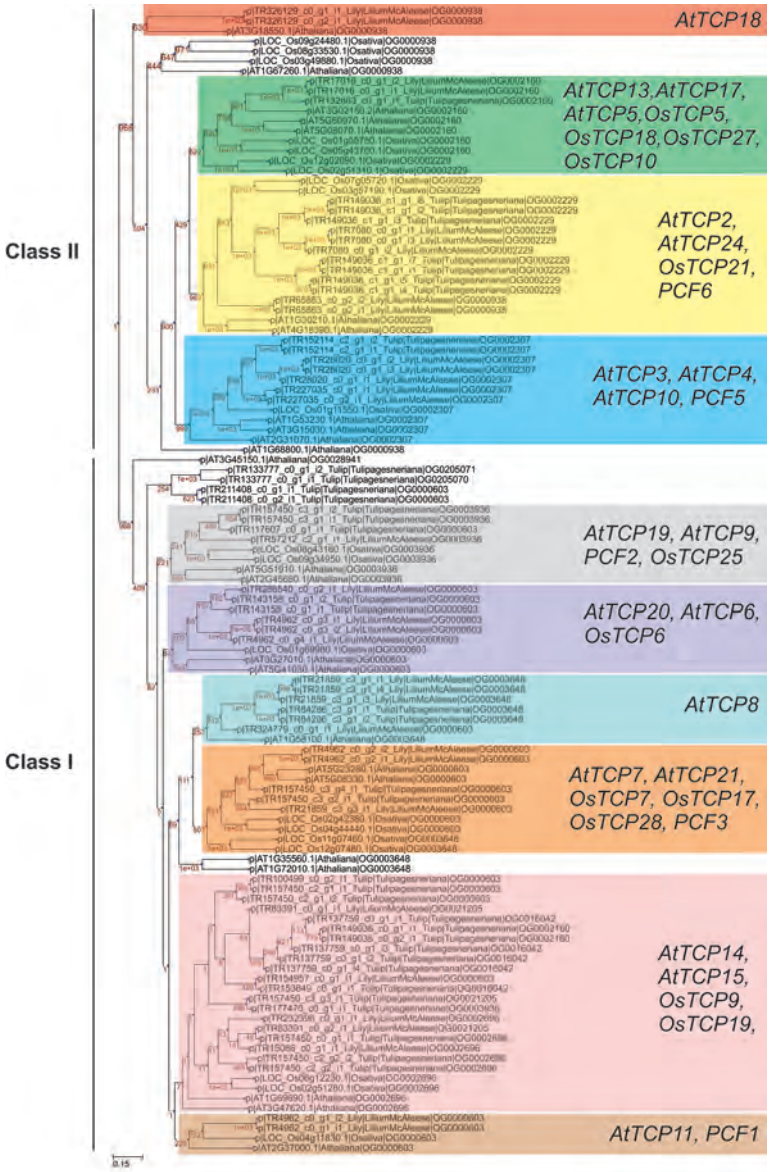


Figure S1. Neighbour-joining tree of the TCP sequences of Arabidopsis, rice, lily and tulip. The sequences from rice and Arabidopsis follow the nomenclature used in the review of Martín-Trillo and Cubas 2010.

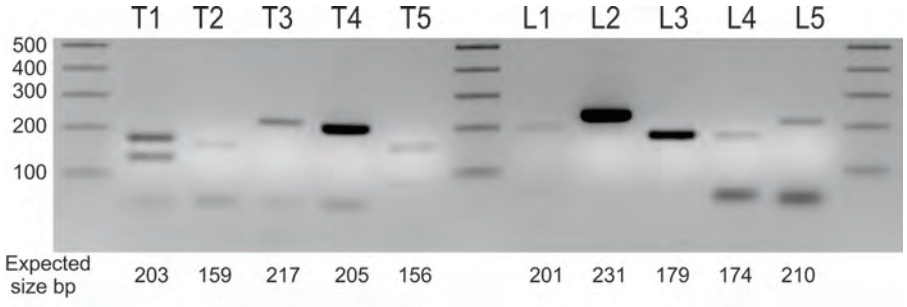
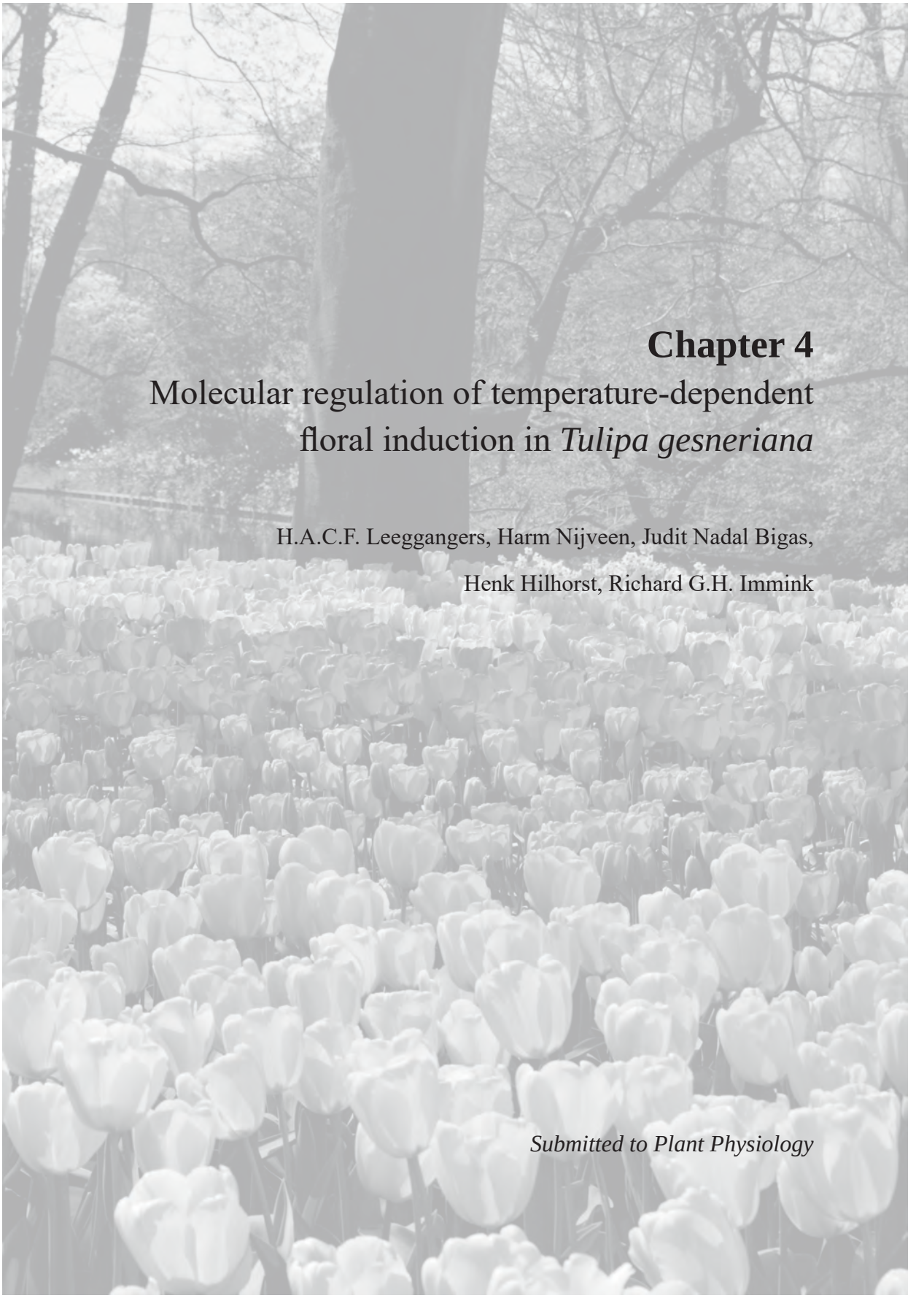


Figure S2. PCR amplification of fragments of 10 putative *TCP* genes. Five tulip (T1 to T5) and lily (L1 to L5) genes were chosen at random to proof the capacity of the Transcriptome Browser in designing unique primers. PCR amplification was successful in nine out of tten cases. T1 failed to produce the expected fragment size. The primers were designed on the following transcripts: T1= p|TR157450_c3_g2_i1_Tulip; T2= P|TR157450_c2_g2_i2_Tulip; T3= p|TR157450_c0_g1_i1_Tulip; T4= p|TR149036_c1_g1_i5_Tulip; T5= p|TR84286_c3_g1_i2_Tulip; L1= p|TR28020_c0_g1_i1_Lily; L2= p|TR4962_c0_g2_i2_Lily; L3= p|TR4962_c0_g3_i1_Lily; L4= p|TR4962_c0_g1_i1_Lily; L5= p|TR232396_c0_g1_i1_Lily.



Chapter 4

Molecular regulation of temperature-dependent floral induction in *Tulipa gesneriana*

H.A.C.F. Leeggangers, Harm Nijveen, Judit Nadal Bigas,

Henk Hilhorst, Richard G.H. Immink

Submitted to Plant Physiology

Abstract

The vegetative to reproductive phase change in adult tulip bulbs is promoted by increasing temperatures during spring after the cold winter. The warm winters of recent years interfere with this process, affecting especially Dutch tulip breeders, and calling for new cultivars that are adapted to this climate change. A better understanding of the underlying molecular mechanisms would be of great help, but unlike the model plant Arabidopsis, very little is known about the molecular control of floral induction in tulip. To shed light on the gene regulatory network controlling floral induction in tulip RNA-sequencing was performed on meristem-enriched tissue collected under two contrasting temperature conditions, low and high. The start of flower development correlated with rounding of the shoot apical meristem and induction of *TGSQA* expression, an *AP1-like* gene. Gene Ontology (GO)-enrichment analysis of differentially expressed genes showed over-representation of genes potentially involved in floral induction, bulb maturation, and dormancy establishment. Subsequently, the knowledge of the flowering time controlling gene regulatory network in plant model species was used to identify homologous and potential flowering time regulators in tulip. Expression analysis revealed that *TgTFL1* and *TgSOC1-like1* might be repressors, whereas *TgSOC1-like2* likely is an activator of flowering. Subsequently, flowering time associated expression of eight potential flowering time genes was confirmed in three tulip cultivars grown in the field. Additionally, heterologous functional analyses in Arabidopsis resulted in flowering time phenotypes in line with *TgTFL1* being a floral repressor and *TgSOC1-like2* a floral activator in tulip. Taken together, we have shown that long before morphological changes occur in the shoot apical meristem, the expression of floral repressors in tulip is suppressed by increased ambient temperatures, preparing the bulb for the vegetative to reproductive phase change. This leads, either directly or indirectly, to the activation of potential flowering activators shortly before commencement of the phase change.

Introduction

The monocotyledonous species *Tulipa* originates from Central Asia and grows in mountain rich areas with a temperate climate (Christenhusz et al., 2013; Kamenetsky and Okubo, 2013). Most cultivated tulips are produced in The Netherlands, which has a temperate maritime climate, fairly resembling the climate of the tulip's region of origin (Compton et al., 2007). The growth cycle of cultivated tulips starts in autumn, when the bulbs are planted in the field. At that moment all organs such as the stem, leaves and flower are already present inside the bulb. A subsequent period of prolonged cold is required for fast stem elongation, as well as for internal preparation of the flower to bloom in spring (Lambrechts et al., 1994; Rietveld et al., 2000). After this cold winter period the stem elongates, the leaves stretch and unfold, and blooming occurs around April or May, depending on the cultivar. The mother bulb is completely consumed after blooming and the main daughter bulb, also known as axillary bud A, replaces the mother bulb (Botschantzeva and Varekamp, 1982). Increasing ambient temperatures in spring are assumed to induce the vegetative to reproductive phase change (floral induction) at the shoot apical meristem (SAM) in the daughter bulb, leading to the development of the floral organs and the induction of dormancy (Steward et al., 1971; Gilford and Rees, 1973; De Hertogh and Le Nard, 1993). Once the flower is completely developed inside the bulb, the growth cycle starts again.

The morphology of the SAM during floral induction was well characterized by Beijer (1952); however, until now, its molecular regulation has not been thoroughly investigated. In contrast, this process has been studied extensively in the model dicotyledonous species Arabidopsis thaliana. In Arabidopsis floral induction can be triggered by long days (LD) after a period of prolonged cold (vernalization response), which leads to the down-regulation of the flowering repressor *FLOWERING LOCUS C* (*FLC*) gene. This repression of *FLC* facilitates flowering by making the SAM sensitive to flower-inducing cues such as ambient temperature and long days (Choi et al., 2011). When the days are getting longer, the photoperiod pathway is

induced, leading to the activation of *FLOWERING LOCUS T* (*FT*) by the zinc finger transcriptional regulator *CONSTANS* (*CO*). The perception of changes in the photoperiod is located in the leaves, but floral induction occurs at the SAM. In this respect, FT acts as a 'florigen'. The FT protein is transported via the phloem to the SAM where it interacts with the bZIP transcription factor FLOWERING LOCUS D (FD). The interaction between FT and FD results in the activation of the floral integrator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *SQUAMOSA BINDING PROTEIN-LIKE* (*SPL*) genes and finally of the flower meristem identity genes *APETALA1* (*AP1*), *LEAFY* (*LFY*) and *FRUITFULL* (*FUL*) (Huijser and Schmid, 2011; Andres and Coupland, 2012).

In the absence of the photoperiod pathway in Arabidopsis, the phytohormone gibberellin (GA) plays a major role in the regulation of flowering. GA is known to promote the expression of *SOC1* and *LFY*, dependent or independent of the DELLA-mediated pathway, leading to the activation of the so called A, B and C class genes (Lee and Lee, 2010). Together with GA, it is believed that other endogenous (e.g. other hormones and carbohydrates) and external signals (e.g. nutrients and ambient temperature) also play a role in the floral induction (Mutasa-Göttgens and Hedden, 2009; Galvão et al., 2015). The molecular regulation of the phase change by ambient temperature has been studied to a lesser extent in comparison with the vernalization and photoperiod pathway. Several genes involved in flowering time control have been shown to be responsive to changes in ambient temperature. Examples of genes playing a role in temperature mediated flowering are *FLOWERING LOCUS M* (*FLM*), *SHORT VEGETATIVE PHASE* (*SVP*), *EARLY FLOWERING3* (*ELF3*), *TERMINAL FLOWER1* (*TFL1*) and *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*) (Balasubramanian and Weigel, 2006; Strasser et al., 2009; Kumar et al., 2012; Box et al., 2015).

Even though it has been shown that flowering time genes in Arabidopsis are regulated by changes in temperature, the change in day length (photoperiod)

is the key seasonal cue to trigger the reproduction process (Park et al., 1999; Jeong and Clark, 2005; Osnato et al., 2012). However, for tulip it is assumed that a high ambient temperature is the most important seasonal signal to trigger the floral induction (Khodorova and Boitel-Conti, 2013). In addition, Arabidopsis is a dicot while tulip is a monocot, and this large evolutionary distance may raise the question of how much of the flowering time network has been conserved, i.e. to which extend knowledge can be transferred from Arabidopsis to tulip?

In the monocotyledonous model species *Oryza sativa* (rice) the homologous gene of *FT* (*Heading Date 3a*, *Hd3a*) also acts as an activator of flowering, but under short day (SD) conditions (Komiya et al., 2008). This, and many other examples, reveals that there is at least some similarity between dicots and monocots in the molecular mechanisms underlying flowering time control (Blümel et al., 2015). To date, only a few genes with high levels of similarity to key Arabidopsis flowering time genes have been identified and characterized in ornamental geophytes, such as tulip. A study by Noy-Porat and colleagues (2013), focusing on *Narcissus tazetta* (daffodil), identified two homologs of genes involved in the floral induction known from Arabidopsis, namely *FT* and *LFY*. In *N. tazetta*, *NtFT* was shown to be induced by high temperatures at the end of the growth period (Noy-Porat et al., 2013). Next to these single gene approaches, Villacorta-Martin and colleagues (2015) published for example a genome-wide study focusing on the vernalization response and flowering in *Lilium longiflorum* by transcriptome profiling. Although these are valuable examples, the total number of studies in ornamental geophytes is limited.

In the present study a genome-wide approach was undertaken to elucidate the molecular mechanism underlying the floral induction and the integration of temperature responses in tulip. In the Netherlands, the warm winters and high temperatures during spring in recent years interfered with the floral induction process, resulting in dehydration of the flower (floral bud blasting) or low quality tulip flowers (van Dam and van Haaster, 2011). This problem calls for development of new cultivars that are adapted to this climate change and hence

detailed molecular knowledge of the process is required. An experimental set-up was designed with contrary environments, low and high temperature, to identify genes induced by high temperature and their possible role in floral induction. RNA-sequencing (RNA-seq) was performed to identify differentially expressed genes in vegetative and reproductive meristem enriched tissues collected at the different temperatures. Subsequently, both a bottom-up and a top-down approach were followed to identify potential flowering time genes in tulip. For the bottom-up approach a clustering analysis was performed to obtain an overall picture of the transcriptional changes, followed by a Gene Ontology (GO)-enrichment analysis. For the top-down approach, a direct search based on high similarity with known flowering time genes was performed. Eight genes were further characterized and their correlation with the flowering time response was validated in different tulip cultivars. Additionally, heterologous functional analysis of a small number of potential tulip key flowering time regulators was performed in Arabidopsis to confirm their proposed role in the control of this important phase transition.

Material and Methods

Plant material and growth conditions

Tulipa gesneriana cv. "Dynasty" (size 10/11) bulbs were planted in crates in the field in early November 2012 and transferred to two different controlled temperature conditions, 8-9°C and 19°C, respectively, at the beginning of June 2013 after decapitation of the flower. The bulbs were planted in crates to prevent damage to the roots by transfer to the temperature-controlled climate cells. The bulbs were maintained in the climate cells at the two indicated temperature regimes with a 16 h photoperiod, with 100 μmol s-1 m-2 of light, for nine weeks. A mix of meristem-enriched tissues (square cutting of 0.5 by 0.5 cm including the meristem and leaf primordia, Fig. 1A), derived from five individual tulip bulbs, were dissected with a scalpel and pooled together to form one biological replicate and this was repeated three times, once every week in the afternoon (Central European time 1 pm – 3 pm).

Each mix of meristem-enriched tissue was homogenized by the use of liquid nitrogen, mortar and pestle and stored at -80°C until use. In addition to artificially stimulating or preventing the floral induction by controlled temperature conditions, six cultivars (Northgo, Purple Prince, Dynasty, Ile de France, Strong Gold and Yellow Flight) were planted directly in the field at the end of October 2014 and harvested in June 2015. After harvest, the bulbs were placed at 25°C for 10 days to dry. After these 10 days the bulbs were stored at 17-20°C in the dark. Samples of meristem-enriched tissue were collected during the cycle as described above and stored at -80°C until use. *Arabidopsis thaliana* seeds were stratified for 2-3 days at 4°C, germinated, and a segregating plant population (30-50 plants) was grown under LD conditions (16/8 hours light/dark) at 20°C on Rockwool blocks. Flowering time was scored by counting the number of rosette leaves at the moment the inflorescence reached a length of one centimeter.

Microscopic imaging

Morphological changes of the shoot apical meristem region inside the bulb during the vegetative and reproductive phase were monitored with a Carl Zeiss Stereomicroscope SV11 (Zeiss, The Netherlands) and pictures were taken with a Nikon digital sight DS-Fi1 camera (Nikon, Germany).

Total RNA extraction and cDNA synthesis

To extract the total RNA of the meristem-enriched tissue, the Tripure protocol (Roche, The Netherlands) was used according to the manufacturer's instructions with the addition of 2% Polyvinylpyrrolidone (PVP, w/v) and 2% β-mercaptoethanol (β-ME, v/v) to the extraction buffer. Subsequently, a DNase treatment with RQ1 (Promega, The Netherlands) was performed to remove DNA, followed by a phenol/chloroform (1:1) extraction and ethanol precipitation. A total amount of 500 ng was used for first-strand cDNA synthesis using M-MuLV Reverse Transcriptase (Thermo Scientific, The Netherlands) following the protocol from the manufacturer and oligo-dT primers. All reactions were performed in a Bio-rad MyCycler (Bio-rad, The Netherlands).

Strand-specific RNA-sequencing

Total RNA of meristem enriched tissues, collected between June and late July 2013, was used for RNA-seq. For the preparation of the RNA-seq cDNA library, the TruSeq stranded mRNA sample preparation kit (Illumina, The Netherlands) was used according to the manufacturer's instructions. The quality of the libraries was examined with the Bioanalyzer 2100 DNA 1000 chip (Agilent Technologies, United States). The Illumina Hiseq2000 platform was used for obtaining 100 bp paired-end reads.

RNA-seq data analysis

The quality of the reads obtained from RNA-sequencing was examined by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimming of the reads by Trimmomatic version 0.32 (Bolger et al., 2014) was used to improve their quality. After trimming, a de novo assembly was performed using Trinity version 2.0.6 (Haas et al., 2013). Trinity assembles short read RNA-seq data into contigs, which are likely (parts of) transcripts. Low abundant transcripts can be assembled in two or more contigs if regions of the transcript are not covered by any read. Based on sequence similarity contigs are grouped together with the assumption they represent isoforms derived from the same genetic locus (Trinity 'genes'). Kallisto version 0.42.1 (Bray et al., 2016) was used to quantify gene expression. Differential gene expression analysis was donw with EdgeR package version 3.10/5 (Robinson et al., 2010), using the estimated counts produced by Kallisto as input. Each transcript was annotated with the best Arabidopsis hit. For this, Arabidopsis was chosen because of its well annotated genome and an extensive amount of functional analysis has been performed in comparison to the monocot *Oryza sativa* (rice).

Gene ontology (GO)-enrichment analysis

Based on the differentially expressed genes, identified in the high temperature condition, the Plant GeneSet Enrichment Analysis Toolkit (PlantGSEA) was used for gene ontology enrichment. From these sets of differentially expressed genes, the genes also showing

differential expression in the cold environment, were removed for each weekly interval. The hypergeometric statistical test method and the Yekutieli (FDR under dependency) multi-test adjustment method settings were used for the analysis. The significance level and the False Discovery Rate (FDR) were set at 0.05.

Clustering analysis

Clustering of the transcripts with a similar expression pattern was done with the R-package hclust, using Pearson correlation as the distance measure (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/hclust.html). To include as many as possible transcripts, all transcripts were annotated with the name of the best Arabidopsis hit (cut-off value 1e-05). The expression values were normalized per gene and then the z-scores per time point were plotted. If the z-score for a gene at a time point is 1, this means that the expression value differs by one standard deviation from the mean of the expression of this gene over all time points.

Identification of potential flowering time regulators

Protein sequences of AtSOC1, AtFT, AtSEP1, AtTFL1 and all SPLs were used for BLASTx (cut-off 1e-05) to identify the transcript with the highest similarity. All matching sequences were aligned, including the Arabidopsis gene, and the hit with the highest sequence similarity (>50%) was chosen for further characterization.

Phylogenetic analysis

For the AP1-like proteins TGSQA and TGSQB a maximum likelihood tree was reconstructed with MEGA 5.0 (Tamura et al., 2011). The alignment was made with the default ClustalW settings in MEGA 5.0. For the construction of the maximum likelihood tree the WAG model was used as the substitution model and 500 bootstraps were generated to test the reliability of the tree. In addition, the setting “gaps/missing data treatment” was changed to partial deletion with 95% as the site coverage cut off. These same settings were

used to generate the maximum likelihood tree of the FT/TFL1 protein sequences. Here, a cut-off of 70% for the bootstrap value was used to adjust the branches. The neighbor joining (NJ) tree of the MADS-box proteins was constructed in a similar way, but using the Dayhoff model.

Real-time PCR for expression analysis

Real-time PCR reactions were performed in a total volume of 20 µl containing 10 µl of iQ SYBR Green Supermix (Bio-rad, The Netherlands), 5 µl of each forward and reverse primer (0.05 µM; Primer details are listed in table S1) and 5 µl of a 1:15 dilution of the cDNA reaction mixture as template. Reactions were performed on a CFX Connect real-time PCR detection system (Bio-rad, The Netherlands) with an initial 3 min denaturation at 95°C followed by 40 cycles of 95°C for 10s and 60°C for 30s for the amplification. Final steps used for elongation were 95°C for 1 min, 55°C for 10s and 95°C for 30s with afterwards a melt curve determination. Normalized expression levels were calculated by the ΔΔCt method (Livak and Schmittgen, 2001) with TgACT as the reference gene. Calculations were based on three technical replicates and two to three biological replicates.

Construction of overexpression lines in *Arabidopsis thaliana*

Two selected genes were amplified from cDNA by PCR with the primers *TgTFL1* (forward 5'-ATGGCAAGAGTGCTGGAGC-3' and reverse 5'-TCACTGCTCCCACTTAACAT-3') and *TgSOC1L2* (forward 5'-ATGAAGAGGGGGAAGACACA-3' and reverse 5'-CCATCCAATATGCAAGTCCG-3'). The PCR fragments of the flowering time genes were cloned in the Gateway overexpression vector pGD625 (Immink et al., 2002), driving ectopic expression of the transgene from the CaMV35S promoter. All generated constructs were introduced into *Agrobacterium tumefaciens* AGL0 and transformed into Arabidopsis Colombia-0 (Col-0) plants using the floral dip method (Clough and Bent, 1998).

Yeast two hybrid assays

Yeast two hybrid screens were performed according to (de Folter and Immink, 2011). All baits have been tested for auto-activation capacity prior to the screening for potential protein-protein interactions. None of the tested baits showed auto-activation capacity.

Results

Morphological characterization of floral induction and early flower development under high temperature conditions

Tulips bloom in spring and during development towards blooming the resources in the mother bulb are completely consumed. The mother bulb is replaced by a small number of daughter bulbs including one main daughter bulb (known as 'axillary A'), which is competent to flower (Botschantzeva and Varekamp, 1982). The SAM within this daughter bulb is present in the middle of the bulb on top of the basal plate and is surrounded by fleshy scales that function as storage organs and provide energy for growth (Fig. 1A; Van der Toorn et al., 2000). The vegetative to reproductive phase transition occurs in the main daughter bulb shortly after blooming of the mother bulb and is supposed to be induced by high temperatures during spring (Khodorova and Boitel-Conti, 2013). To prove whether temperature is indeed the primary trigger for the floral induction and to investigate the process of floral induction at the morphological level, tulip bulbs of the cultivar Dynasty were lifted from the field at the end of spring. The bulbs were transferred to controlled climate cells with LD conditions to match with field conditions; they were separated into two groups and exposed to low (8-9 ºC) or high (18 ºC) ambient temperature conditions. The temperature courses during the growth season in the field and in the climate cells were monitored (Fig. S1A-B). Figure 1B shows the morphological changes of the SAM that were observed in the main daughter bulb at 8-9 ºC in comparison to 18 ºC. Based on the morphological changes of the SAM, Beijer (1952) divided flower induction and development into seven stages (Fig. S2). In order to confirm that FM identity is indeed

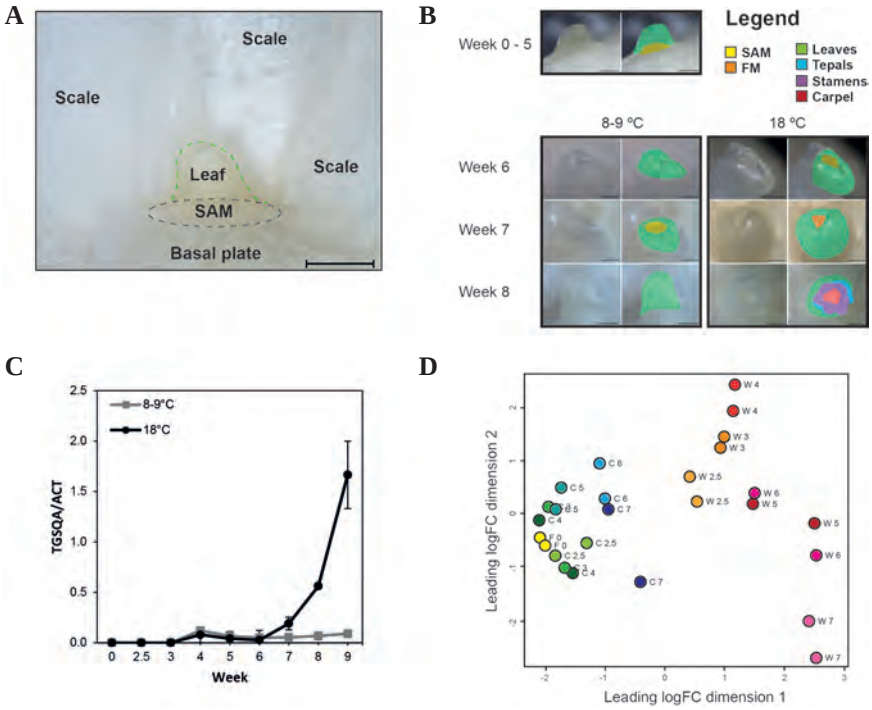


Figure 1. Morphology of the vegetative to reproductive phase change at the shoot apical meristem (SAM) and transcriptional changes over time. (A) Morphology of the SAM inside the bulb and its surrounding tissues in spring prior to the temperature experiment. Note that the SAM is still vegetative and that one leaf primordium has developed. (B) Morphological changes at the shoot apical meristem inside the main daughter bulbs of T.gesneriana cv. "Dynasty", during low and high temperature conditions. In the first five weeks of both temperature conditions only one leaf primordium developed (green) and the SAM remained flat (yellow). After six weeks at 18 ºC the SAM got a dome-like appearance, which is the first morphological change marking the switch from vegetative to reproductive development. Shortly after, the floral meristem (FM; orange) gives rise to the development of the different floral organs (tepals: cyan, stamens: violet, carpel: red). Note that the SAM of bulbs at the low temperature (8-9 ºC) condition remains vegetative and flat for the complete period of eight weeks. The bar in the bottom right corner indicates one millimeter. The different tissues have been artificially colored in the right panel of each picture. (C) Expression pattern of *TGSQA* at low (8-9 ºC) and high (18 ºC) temperature conditions. (D) MDS plot revealing the global transcriptional changes over time. The bulbs from the low (8-9 ºC) temperature condition remain in a relative stable transcriptional state, whereas the bulbs from the high (18 ºC) temperature condition show significant transcriptional changes over time associated with the switch from the vegetative to a reproductive phase.

established around stage II, the expression of a putative FM identity gene was investigated. In Arabidopsis *APETALA1* (*AP1*) has been identified as an FM identity gene, which is not expressed during the vegetative stage of development, but specifies FMs from the earliest moment onwards (Irish and Sussex, 1990; Sundström et al., 2006). In tulip two genes belonging to the SQUAMOSA (SQUA) subfamily were identified, showing high similarity with the *AP1-like* MADS-box gene *OsMADS28* of *Oryza sativa* (rice) (Fig. S3; Yamaguchi and Hirano, 2006). These two *AP1-like* genes, known as *TGSQA* and *TGSQB*, were previously identified in viridifloral tulips (Hirai et al., 2010). Therefore, we adapted the previously given names *TGSQA* and *TGSQB*. The expression of *TGSQA* was investigated in both low and high temperature conditions (Fig. 1C). This analysis shows that FM identity is indeed established just after the moment that the SAM enlarges and transforms into a dome-like structure, which is approximately six weeks after the start of the high temperature treatment. This confirms that the staging, as proposed by Beijer (1952), is correct. Based on this observation the same classification is used in this study.

During the first five weeks, under both temperature conditions, the SAM of the main daughter bulbs were morphologically still in stage I and displayed a similar appearance of one leaf primordium developed and the SAM remaining flat (Fig. 1B). The first morphological differences between the 8-9 ºC and 18 ºC treatments were observed from six weeks onwards. The main daughter bulbs at 8-9 ºC continued to develop the first leaf primordium and the SAM remained flat, while at 18 ºC the SAM started rounding and forming a dome-like structure (stage II). At seven weeks the first floral organ primordium appeared (stage P1), and two additional leaf primordia began to develop. More defined tepal, stamen and carpel structures were observed after eight weeks at 18 ºC (stage A2+). In contrast, bulbs at 8-9 ºC developed one leaf primordium only and the SAM remained vegetative, even after eight weeks of low temperature treatment. Above the soil the mother plants remained green at the low temperature condition (Fig. S1C)

whereas the mother plants at the high temperature condition senesced completely, quite similar to normal field conditions (Fig. S1D).

Transcriptome analysis during the floral induction: a top-down approach

To obtain a better understanding of floral induction in tulip, transcriptional changes were investigated. RNA-seq was performed on RNA collected from SAM enriched daughter bulb material collected from week zero (one day before transfer) up to seven weeks after the transfer to the low and or high temperature environment. Transcripts were reconstructed de novo using Trinity (Haas et al., 2013). A total number of 346,016 transcripts were reconstructed, representing 244,383 Trinity 'genes' (Table S1). This large number of putative genes is not unusual when using de novo assembly in the absence of a reference genome. In addition, no filtering was used after the transcriptome assembly to prevent the loss of lowly expressed transcripts (Moreno-Pachon et al., 2016). The MDS plot in figure 1D shows global transcriptional changes over time in the SAM at the two different temperature regimes. In the low temperature condition, very few morphological changes are occurring in the SAM inside the bulb (Fig. 1B), which is accompanied by only few transcriptional changes. In contrast, gene activity in bulbs at the high temperature condition is changing substantially over time. The samples taken from week two and a half until week six cluster together, while samples of seven weeks after the transfer form a separate cluster. This clustering reveals that high temperatures have an immediate effect and that floral induction, and likely other high temperature induced processes, are affected directly from the start of the temperature treatment (week two and a half). Subsequently, based on global transcriptional changes, the bulbs remain in this stage for several weeks, followed by a second change in global expression at week seven. This later burst of differential expression coincides perfectly with the morphological changes of the SAM (Fig. 1B) and induction of flowering, as confirmed by the increase of *TGSQA* transcript abundance (Fig 1C).

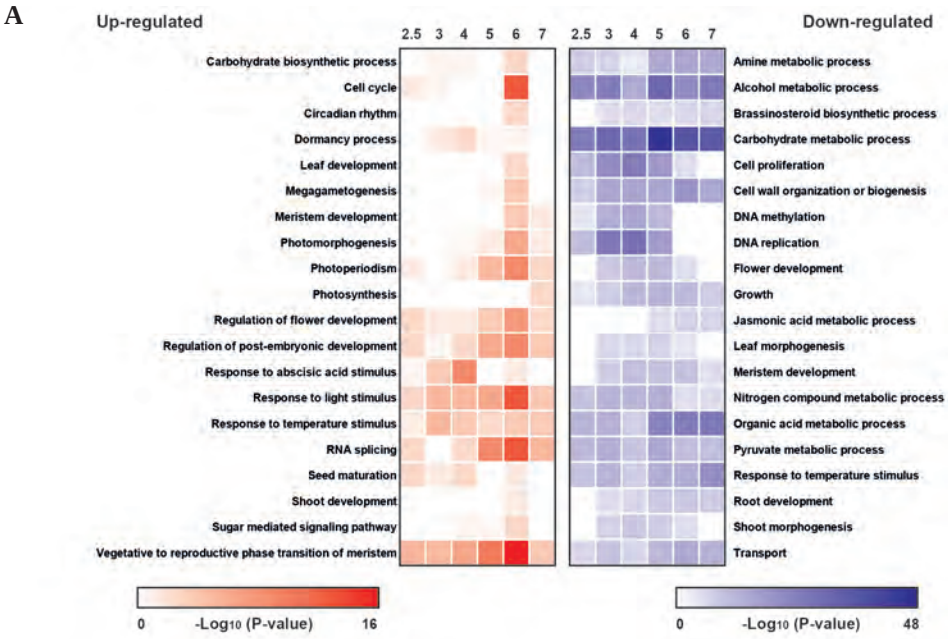
For further identification of putative flowering time controlling genes and to gain insight in the global transcriptional changes, an initial top-down approach was followed (Leeggangers et al., 2013). The top-down approach consisted of an untargeted analysis using GO-enrichment and a clustering analysis. In the GO-enrichment analysis genes differentially expressed upon high temperature treatment were selected to get an indication of the biological processes affected by this treatment. For this purpose, transcript abundance at each interval was compared to the situation at the moment just before transfer to controlled environmental conditions (week zero). Figure 2A displays a selection of GO-terms which were found to be over-represented in the significantly up- and down-regulated genes. A more complete overview of over-represented GO-terms can be found in Supplementary figure S4. As expected, the panel of up-regulated genes contains GO-terms related to the flowering process such as *circadian rhythm*, *regulation of flower development* and *vegetative to reproductive phase transition of meristem*. These GO-terms corroborate the morphological changes (Fig. 1B). Besides these directly flowering-related GO-terms, several others such as *sugar mediated signaling pathway*, *cell cycle*, *response to temperature stimulus* and *RNA splicing* are connected with the vegetative to reproductive phase transition. One example of sugar-mediated signaling involved in the flowering process is trehalose-6-phosphate (T6P) signaling in Arabidopsis, for which the gene *TREHALOSE-6-PHOSPHATE SYNTHASE 1* (*TPS1*) is required for the timing of the initiation of flowering (Wahl et al., 2013). Also the process of RNA splicing has been shown to play a role in ambient temperature-mediated flowering time control in Arabidopsis (Verhage et al., 2014; Capovilla et al., 2015).

In addition to direct flowering-related GO-terms, other GO-terms related to plant metabolism are over-represented in both groups of up- and down-regulated genes. One example is *carbohydrate biosynthetic process* that is found to be over-represented in the up-regulated genes, while carbohydrate metabolic process is over-represented in the down-regulated genes. When comparing the genes connected to these GO-terms, different parts of the

metabolic pathways show up (Fig. 2B). Up-regulated genes are mostly present in secondary metabolite biosynthesis and glycan biosynthesis/ metabolism, while the down-regulated genes are mostly present in lipid metabolism (fatty acid biosynthesis) and metabolism of other amino acids. In this respect it is good to realize that at the same moment that a 'decision' is made to flower or not to flower, parts of the mother bulb (e.g. leaves, stem and scales) are senescing and the daughter bulbs mature and become dormant (De Hertogh and Le Nard, 1993). Therefore, it is very well possible that the over-representation of these metabolism specific terms is not only related to floral induction, but also to these physiological changes.

The GO-terms over-represented in the down-regulated genes in the high temperature condition are mostly related to metabolic processes such as *amine metabolism* and *alcohol metabolism*, but also hormone related, such as brassinosteroid (BR) biosynthesis and jasmonic acid (JA) metabolism. Thus, the GO-enrichment analysis revealed that among the up-regulated differentially expressed genes flowering-related GO-terms are present together with GO-terms related to bulb maturation and the induction of dormancy.

As a second top-down approach, a co-expression clustering analysis of all transcription factors was performed to focus specifically on regulatory genes for which the expression correlates with high-temperature induced morphological changes of the floral induction in tulip. Of the clusters with an expression pattern that can be related to floral induction, three selected clusters contain at least one gene with high sequence similarity with an Arabidopsis flowering time regulator (Fig. S5; Fig. 3). In cluster 17 a transcript showing high similarity with the floral repressor *APETALA2* (*AP2*) (Jofuku et al., 1994) is present that shows a steep drop in expression after week four (Fig. 3A). This is approximately two weeks before the SAM obtains its characteristic dome-like structure. In addition to *AP2*, also a transcript showing high similarity with the floral repressor *ALBINO3* (*ALB3*) (Wang and Wang, 2009) is present in this cluster. Other putative transcription factor genes in this cluster are


A
Up-regulated
2.5 3 4 5 6 7
Carbohydrate biosynthetic process
Cell cycle
Circadian rhythm
Dormancy process
Leaf development
Megagametogenesis
Meristem development
Photomorphogenesis
Photoperiodism
Photosynthesis
Regulation of flower development
Regulation of post-embryonic development
Response to abscisic acid stimulus
Response to light stimulus
Response to temperature stimulus
RNA splicing
Seed maturation
Shoot development
Sugar mediated signaling pathway
Vegetative to reproductive phase transition of meristem
0 -Log10 (P-value) 16
Down-regulated
2.5 3 4 5 6 7
Amine metabolic process
Alcohol metabolic process
Brassinosteroid biosynthetic process
Carbohydrate metabolic process
Cell proliferation
Cell wall organization or biogenesis
DNA methylation
DNA replication
Flower development
Growth
Jasmonic acid metabolic process
Leaf morphogenesis
Meristem development
Nitrogen compound metabolic process
Organic acid metabolic process
Pyruvate metabolic process
Response to temperature stimulus
Root development
Shoot morphogenesis
Transport
0 -Log10 (P-value) 48

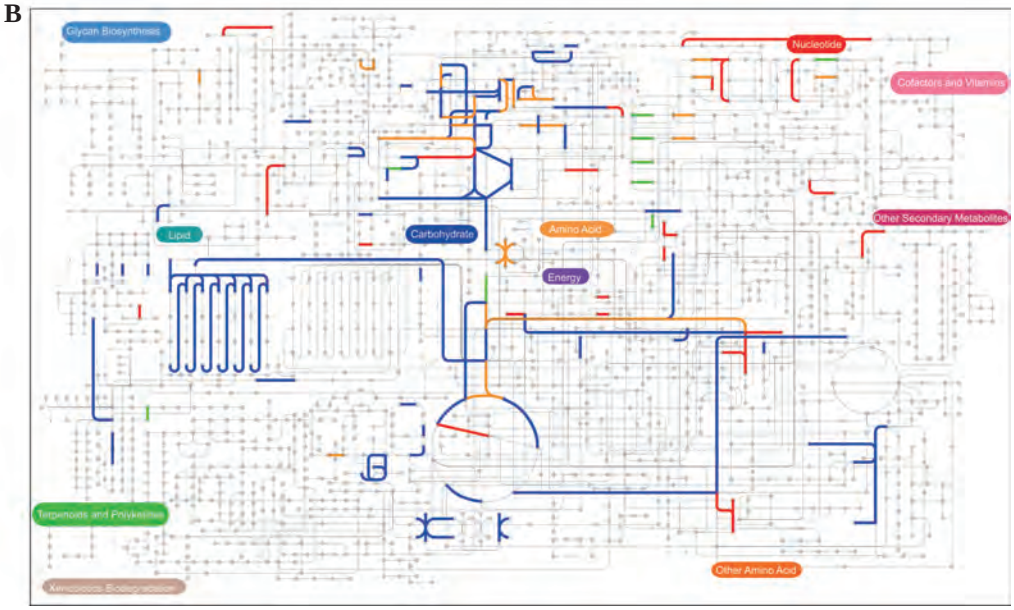

B
Glycan Biosynthesis
Nucleotide
Cofactors and Vitamins
Other Secondary Metabolites
Lipid
Carbohydrate
Amino Acid
Energy
Terpenoids and Polyketides
Other Amino Acid

Figure 2. Overview of the GO-enrichment analysis in the transcriptome data of the floral induction in tulip. (A) Output GO-enrichment analysis by comparing each week to week zero. On the left side in red, the GO-terms listed are over-represented in the up-regulated genes upon high temperatures. On the right side in blue, the GO-terms over-represented in the down-regulated genes are shown. (B) Comparison between the GO-term 'Carbohydrate biosynthetic process' from the up-regulated (red lines) and 'Carbohydrate metabolic process' from the down-regulated (blue lines) GO-enrichment analysis. Overlap between the up- and down-regulated genes, but different metabolic genes, is represented by orange lines and similar genes are represented by green lines.

INOSITOL 3-PHOSPHATE SYNTHASE 2 (*ATIPS2*), *AUXIN RESPONSIVE FACTOR 22* (*ARF22*), and *JASMONATE-ZIM-DOMAIN PROTEIN 1* (*JAZ1*). Although these genes are not directly associated with flowering in Arabidopsis, their expression pattern suggests a relation with repression of flowering. Further detailed analyses are needed to explore possible roles for these regulatory genes in the flowering time response. Cluster 37 contains a transcript showing high similarity with the Arabidopsis *CENTRORADIALIS* (*ATC*) gene, another repressor of flowering (Huang et al., 2012). Its expression decreased gradually until week five (Fig. 3B). The fact that both clusters 17 and 37 contain putative flowering repressors suggests that the block on flowering is removed around week four after high-temperature induction. Other transcripts present in cluster 37 have been related in Arabidopsis to trichrome branching and seed coat development (*MYB5*) (Li et al., 2009), cell-wall biosynthesis (*GALACTURONOSYLTRANSFERASE 15*, *GAUT15*) (Persson et al., 2007) and lignin biosynthesis (*PINORESINOL REDUCTASE 2*, *PRR2*) (Nakatsubo et al., 2008). Finally, in cluster 238 two transcripts showing high similarity with known flowering time functions in Arabidopsis were present. The first is the flowering time gene *FLOWERING LOCUS K* (*FLK*) which acts as a repressor of *FLC* in Arabidopsis (Mockler et al., 2004). The second gene is *TWIN SISTER OF FT* (*TSF*), which is in Arabidopsis the closest homolog of *FT* and, similar to *FT*, a floral integrator (Yamaguchi et al., 2005). The expression of these putative flowering

time genes steadily increased from week zero onwards until they reached a plateau of maximum expression around week four. This interesting cluster contains also the genes *TATA-BOX-BINDING PROTEIN 2* (*TBP2*) and *EMBRYO SAC DEVELOPMENT ARREST 35* (*EDA35*). It is attractive to address a potential function as flowering inducer to these genes, but it is good to realize that at the same moment during development other biological processes are active to which these genes might be related. Hence, we cannot exclude that their correlation with the morphological flowering and a few selected putative flowering time inducers is coincidental.

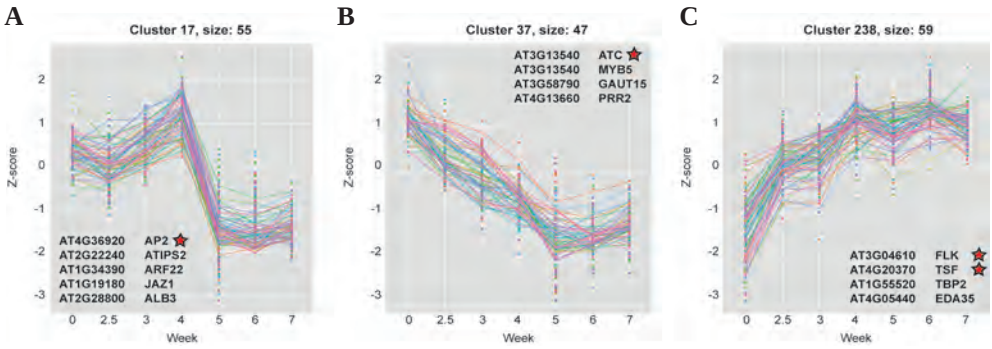


Figure 3. Three selected clusters from the cluster analysis of all tulip transcripts that have high similarity with known transcription factors in Arabidopsis. The clusters are representing transcripts of the high temperature condition. On the x-axis the different time points are plotted and on the y-axis the z-score (normalized Counts Per Million (CPM)). (A) Expression of the genes in cluster 17 remains stable until week four, where after their expression decreases. The cluster includes *APETALA2* (*AP2*), *INOSITOL 3-PHOSPHATE SYNTHASE 2* (*ATIPS2*), *AUXIN RESPONSE FACTOR22* (*ARF22*), *JASMONATE-ZIM-DOMAIN PROTEIN 1* (*JAZ1*) and *ALBINO3* (*ALB3*). (B) Expression of the genes in cluster 37 slowly decreasing over time. This cluster includes *ARABIDOPSIS CENTRORADIALIS* (*ATC*), *MYB5*, *GALACTURONOSYLTRANSFERASE 15* (*GAUT15*) and *PINORESINOL REDUCTASE 2* (*PRR2*). (C) Expression of the genes in cluster 238 increasing from week zero onwards and reaching a plateau around week four. This cluster includes *ENHANCER OF AG-4 1* (*HUA1*), *FLOWERING LOCUS K* (*FLK*), *TWIN SISTER OF FT* (*TSF*), T*ATA-BOX-BINDING PROTEIN 2* (*TBP2*) and *EMBRYO SAC DEVELOPMENT ARREST 35* (*EDA35*). Transcripts with a high similarity toa known flowering time gene in Arabidopsis are marked with a red star.

Identification and characterization of putative flowering time genes: a bottom-up approach.

The top-down approach provided first insights into the flowering time gene regulatory network and pointed towards genes potentially involved in a variety of high temperature-induced biological processes, including floral induction. However, it also revealed limitations of the identification of key regulatory genes of a single defined process solely based on correlation. Therefore a bottom-up approach was followed as well, guided by the wealth of knowledge on the molecular network of flowering time control in Arabidopsis. In this model species over 170 genes have been identified and described that are known to play a role in flowering time control (Fornara et al., 2010).

In total eight genes, with a high sequence similarity with *AtTFL1*, *AtSOC1*, *AtFT*, *AtSEP1* and *SPL* genes, respectively, were selected to further study their potential role in the control of the floral induction in tulip. To confirm their expression pattern (Fig. S6), as well as the overall quality of our RNA-seq assembly and differential gene expression analysis, the expression patterns of these selected genes were confirmed by qPCR (Fig. 4). Among these eight genes is a gene with high sequence similarity to the floral repressor *TFL1*, designated *TgTFL1* (Fig. S7A). In the high-temperature condition the expression of this gene decreased instantly after the start of the treatment, while under the low-temperature condition transcript abundance decreased gradually but slowly over the whole period of eight weeks (Fig. 4A). A similar pattern was observed for the gene belonging to the TM3 subfamily *TgSOC1-like1* (*TgSOC1L1*) (Fig. 4B; Fig. S7B). Based on these expression patterns both genes seem to act as repressors of flowering. For *TgTFL1* this is expected, based on Arabidopsis data (Hanano and Goto, 2011), but for *TgSOC1-like1* this is a surprising observation taking into account the function of the floral integrator *AtSOC1* (Lee and Lee, 2010). However, besides this *TgSOC1L1* gene, another member of the TM3 subfamily clade was identified and named *TgSOC1-like2* (*TgSOC1L2*; Fig. S7B). Expression of *TgSOC1L2* increased between week four to week six and decreased again between week

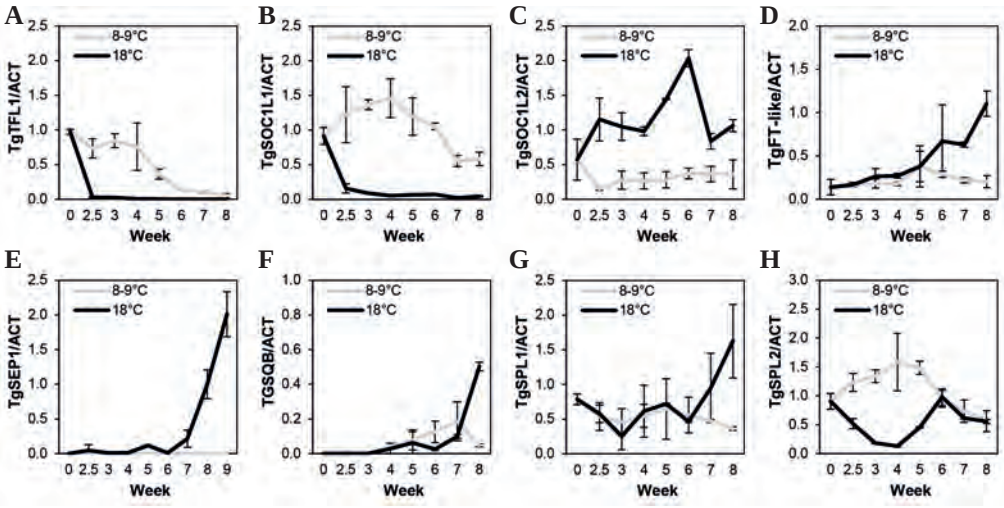


Figure 4. Expression analysis by qRT-PCR of eight putative tulip flowering time genes in the shoot apical meristem region of the main daughter bulb during eight weeks of high or low temperature treatment. (A) Expression of *TgTFL1*. (B) Expression of *TgSOC1L1*. (C) Expression of *TgSOC1L2*. (D) Expression of *TgFT-like*. (E) Expression of *TgSEP1*. (F) Expression of *TGSQB*. (G) Expression of *TgSPL1*. (H) Expression of *TgSPL2*.

six and seven in the high temperature condition (Fig 4C). *AtSOC1* showed a similar increase in expression towards the vegetative to reproductive phase change, after which its expression diminished during further flower and floral organ development (Lee and Lee, 2010). Another potential floral integrator that could be identified is showing similarity with Arabidopsis *FT*, designated *TgFT-like* (Fig. S7B). Abundance of this *TgFT-like* transcript also increased from week four onwards, but instead of a decrease in expression, as *TgSOC1L2*, throughout the whole measured period (Fig 4D). In the low temperature condition, both genes were not expressed, and, hence, for both genes a positive correlation with floral induction was observed, providing evidence that these genes might act as activators of flowering in tulip.

Morphological data and the expression of the *AP1-like* gene *TGSQA* in tulip (Fig. 1C) suggested that flower development starts in week six and the expression of the putative floral organ identity gene *SEPALLATA1* (*SEP1*)

and *TGSQB* correlates with this (Fig. 4E and F, Fig. S6). Furthermore, these genes were not expressed in the low temperature condition in which the SAM remains vegetative. *TGSQA* is homologous to *AP1* from Arabidopsis and the Antirrhinum majus *SQUAMOSA* (*SQUA*) gene. *SQUA* genes are regulated by *SQUAMOSA-PROMOTOR BINDING PROTEIN* (*SBP*) – box genes (Preston and Hileman, 2010) of which two were identified in the tulip transcriptome, designated *TgSPL1* and *TgSPL2*. Both genes have a different expression pattern (Fig. 4G and H). *TgSPL2* might act as a floral repressor, because its expression decreased from week zero to four under high temperature conditions but increased under the low temperature condition. In contrast, *TgSPL1* was specifically induced by high temperature and this increase coincided with the up-regulation of *TGSQA*, making it a putative candidate as upstream regulator of *TGSQA* and suggesting conservation of this link between Arabidopsis and tulip.

Genetic diversity as a tool to confirm the role of putative tulip flowering time genes

To date, no efficient tools are available to transform Tulipa species (Kanno et al., 2007). Therefore genetic diversity was used as a tool to obtain additional confirmation on the proposed role of a selection of genes in the flowering time response. Furthermore, the potential tulip flowering time regulators were identified under controlled temperature conditions and therefore the experiment was repeated with more cultivars under their natural conditions in the field. Unfortunately, no detailed information is available about the moment of floral induction in different tulip genetic backgrounds. However, the moment of blooming in spring has been reported for a large number of tulip cultivars and we hypothesized that there is a direct correlation between the timing of blooming and the moment of the vegetative to reproductive phase change inside the main daughter bulb. Initially, six tulip cultivars were selected with variable blooming times in spring (Fig. S8A). After blooming of the mother bulb, bulbs of all cultivars were lifted at the same time. From one month before lifting until

eight weeks after lifting the morphological changes related to the floral induction were monitored (Fig. S8B). Surprisingly, earliness in the floral induction appeared not to be correlated with early blooming in spring. One of the latest-blooming cultivars (Strong Gold) of our selection showed to be one of the first making the developmental switch from the vegetative to the reproductive phase. However, it is important to note that Strong Gold is known to be a temperature sensitive cultivar. The differences in the timing of the phase change appeared to be limited to approximately one to two weeks only and all cultivars reached stage P1 (first whorl of tepals formed) almost at the same time (Fig. S8B). After reaching this stage, the floral buds developed at a similar speed. Based on these observations, the most diversified cultivars in the moment of the floral induction were selected for molecular analysis (Strong Gold, early; Purple Prince, mid; Dynasty, late; Fig. 5A). From these three cultivars, Purple Prince and Strong Gold have one parent in common (Yokohama).

Eight putative flowering time genes were selected and their expression was monitored in the three cultivars (Fig. 5B-I). The expression of *TgTFL1* decreased first in Strong Gold, starting from four weeks before lifting and followed one to two weeks later in Purple Prince and Dynasty (Fig. 5B). In the case of *TgSOC1L1*, the expression in all three cultivars decreased in a similar manner (Fig. 5C). The same was observed for the putative floral inducer *TgSOC1L2*, the expression of which increased from one week before lifting (week -1) and, after reaching a high steady-state level, started to decrease slowly after the transition to reproductive development (Fig. 5D). Also TgFT-like expression increased one week before lifting (week -1) in all cultivars, but remained high over time (Fig. 5E). The expression of *TgSEP1* and *TgSPL2* was similar for all cultivars and increased from three weeks after lifting onwards (Fig. 5G and I). For the *TGSQA* and *TgSPL1* genes a slightly earlier induction was observed in Strong Gold, which is in line with the earlier floral induction in this cultivar (Fig. 5F and H).

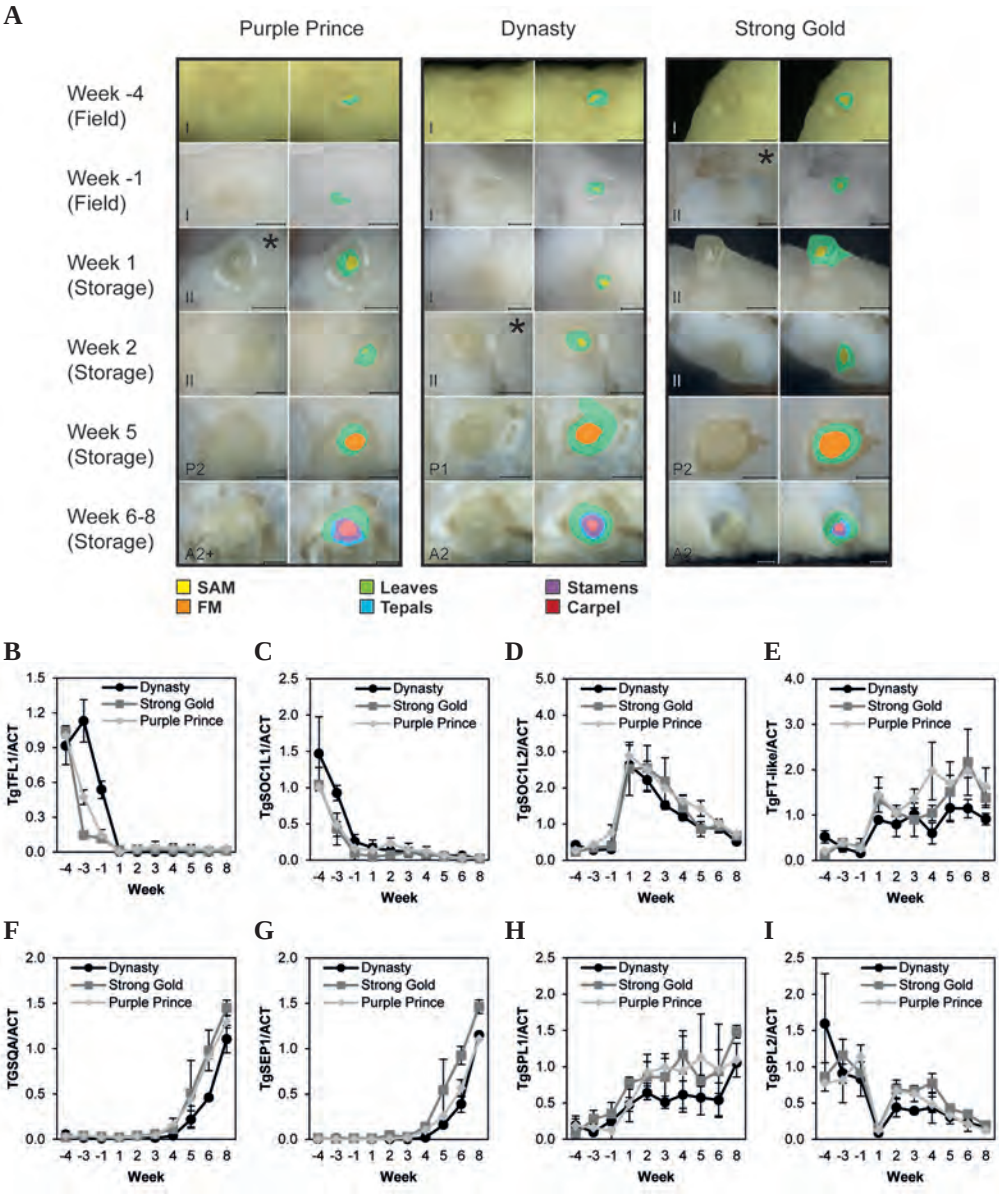


Figure 5. Morphological and molecular analysis of the vegetative to reproductive phase change in three tulip cultivars. (A) Morphological analysis of the changes at the SAM in the tulip cultivars Purple Prince, Dynasty and Strong Gold. I: vegetative, II: reproductive, P1: first whorl of tepals, P2: second whorl of tepals, A1: first whirl of stamens, A2: second whorl of stamens, A2+: beginning of carpel development. (B) Expression of *TgTFL1*. (C) Expression of *TgSOC1L1*. (D) Expression of *TgSOC1L2*. (E) Expression of *TgFT-like*. (F) Expression of *TGSQA*. (G) Expression of *TgSEP1*. (H) Expression of *TgSPL1*. (I) Expression of *TgSPL2*.

In conclusion, all selected genes showed a similar behavior in expression pattern in this field experiment performed in 2015 as in the previous controlled climate chamber experiment in 2013. The observed expression patterns and levels were in line with the supposed function of the analyzed genes in flowering time control, and as such provided additional evidence for their proposed roles in this biological process. Whereas for some of the genes no differences in expression could be observed at the exact moment of repression or induction in the three cultivars, others showed expression changes tightly linked to the small differences in the phase switch from vegetative to reproductive development.

Heterologous expression of tulip flowering time genes in Arabidopsis

To further investigate the potential function of a small selection of potential tulip flowering time regulators, heterologous over-expression studies were performed in Arabidopsis. Transgenic Arabidopsis lines in which the tulip genes *TgSOC1L2* and *TgTFL1* were placed under control of the constitutive CAMV35S promoter (Odell et al., 1985) were generated and phenotyped for flowering time. Over-expression of *TgSOC1L2* resulted in a weak early flowering phenotype (Fig. 6A, E-G) while over-expression of *TgTFL1* resulted in a severe late flowering phenotype (Fig. 6B, H-J). In addition to the late flowering phenotype upon overexpressing *TgTFL1*, floral organ morphological changes were observed which are similar to those observed when ectopically expressing *AtTFL1* (Fig. 6C and D; Shannon and Meeks-Wagner, 1991), confirming that *TgTFL1* is similar in function and behavior to *AtTFL1*.

Protein interaction partners of potential tulip flowering time regulators

For both AtSOC1L2 and AtTFL1 protein-protein interaction studies have been reported (de Folter et al., 2005; van Dijk et al., 2010; Hanano and Goto, 2011) which provide information about their biological and molecular functions. In total 25 protein-protein interactions between AtSOC1 and other MADS domain transcription factor proteins have been shown

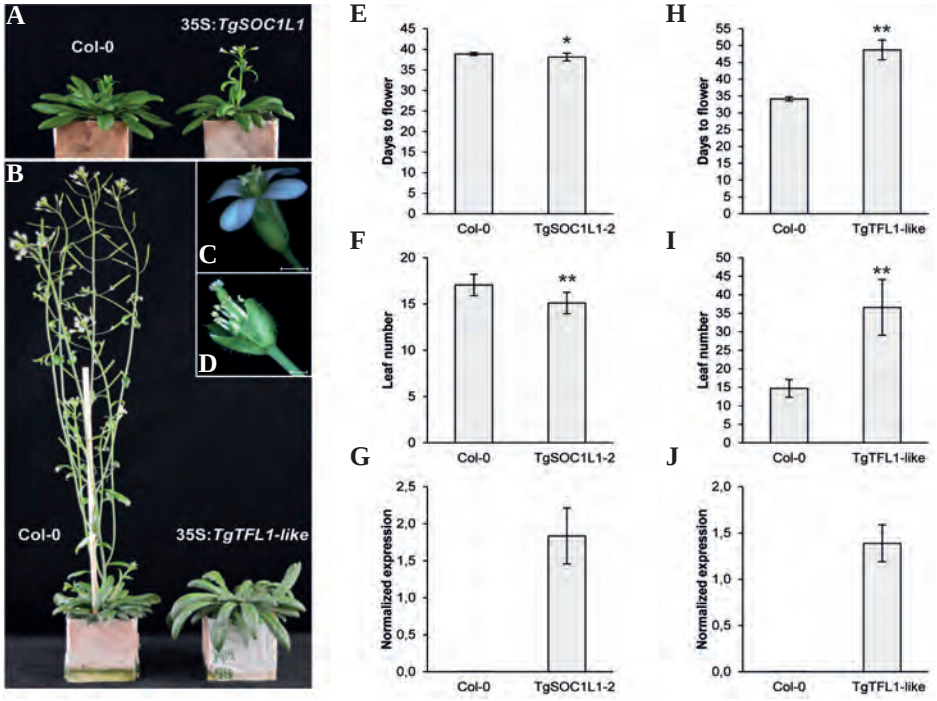


Figure 6. Phenotypic data of Arabidopsis overexpressing different potential tulip flowering time genes. (A) *35S:TgSOC1L2*. (B) *35S:TgTFL1*. (C) Wild type flower. (D) *35S:TgTFL1* flower. (E) Number of days to flowering for *35S:TgSOC1L2*. (F) Leaf number of *35S:TgSOC1L2* when the inflorescence is reaching a length of one cm. (G) Expression of *TgSOC1L2* in the overexpression line *TgSOC1L2-2* in comparison to Col-0. (H) Number of days to flowering for *35S:TgTFL1*. (I) Leaf number of *35S:TgTFL1* when the inflorescence reaches a length of one cm. (J) Expression of *TgTFL1* in the overexpression line *TgTFL1-1* in comparison to Col-0.

in the study of de Folter and colleagues (2005). To test whether the tulip homolog TgSOC1L2 is able to interact with the same set of MADS domain proteins as AtSOC1, protein-protein interaction between TgSOC1L2 and the collection of Arabidopsis MADS domain proteins was studied. Yeast two hybrid analyses revealed that TgSOC1L2 is able to interact with 18 Arabidopsis MADS domain proteins (Fig. 7A), of which, remarkably only four are in common with AtSOC1 (AGAMOUS-LIKE (AGL) 12/ XAANTAL1 (XAL1), AGL17, AGL19 and AGL44/ARABIDOPSIS NITRATE REGULATED 1 (ANR1)). This difference in protein-protein,

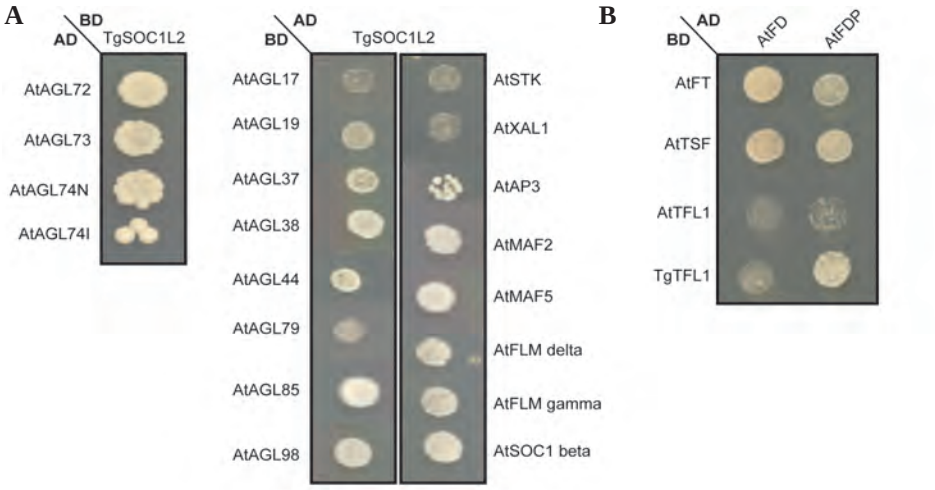


Figure 7. Yeast two hybrid assay of tulip flowering time regulators. (A) Protein-protein interactions between TgSOC1L2 and Arabidopsis MADS domain proteins (SD glu medium –LWH and 1 mM 3-AT). (B) Protein-protein interaction of TgTFL1 and Arabidopsis FD and FDP proteins (SD glu medium –LWH and 1 mM 3-AT). AtFT and AtTSF were added to the assay as positive control.

interaction pattern between AtSOC1 and TgSOC1L2 might explain the weak early flowering phenotype upon overexpressing TgSOC1L2 in Arabidopsis (Fig. 6). The lack of interaction with the classical ABC-class proteins also could explain why no flower phenotypes appeared upon ectopic expression of *TgSOC1L2*, in contrast to what has been found when ectopically expressing *AtSOC1* in flowers (Borner et al., 2000). In AtSOC1 certain interaction motifs are characterized and required for protein-protein interactions (van Dijk et al., 2010). When aligning the TgSOC1L2 and AtSOC1 protein sequences, mutations are present at almost all important motifs for protein-protein interactions, except for motif 2 (Fig. S9). Thissupports the difference observed in the protein-protein interactions of TgSOC1L2. Overexpression of *TgTFL1* in Arabidopsis gave a similar phenotype as overexpression of *AtTFL1* (Ratcliffe et al., 1998). It is known that in Arabidopsis both AtFT and AtTFL1 can interact with the bZIP transcription factor FD (Hanano and Goto, 2011). To test whether the tulip homolog of AtTFL1 is able to interact with AtFD, a yeast two hybrid assay

was performed. TgTFL1 showed interaction with AtFD and AtFDP (Fig 7B) suggesting that, similar to AtTFL1, TgTFL1 can interfere with the FT/FD-dependent transcriptional activation of flowering (Hanano and Goto, 2011).

Discussion

In this study a deep-sequencing RNA-seq approach was followed to shed light on the transcriptional changes occurring prior to and during the switch from vegetative to reproductive development in the bulbous plant species Tulipa gesneriana. A broad range of in silico analyses and confirmation of observed expression patterns by qRT-PCR provided strong evidence for a set of tulip genes to represent regulators of flowering. For two of the identified genes, their supposed roles as repressor and activator of flowering could be confirmed by heterologous functional analyses in Arabidopsis. We showed that high ambient temperatures are inducing flowering in tulip and that transcriptional changes associated with the flowering time response occur already four to five weeks before the first flowering-related morphological changes of the SAM become visible.

Flowering induction co-occurs with bulb maturation and initiation of dormancy

Simultaneously with flower initiation in the SAM, the daughter bulbs mature and are prepared for a period of dormancy (De Hertogh and Le Nard, 1993). In line with these developmental and physiological conditions, we identified over-representation of GO-terms such as dormancy process, seed maturation and response to abscisic acid in the GO analysis of the differentially expressed genes. The phytohormone abscisic acid (ABA) is often associated with the establishment and maintenance of seed dormancy (McCarty, 1995). Seeds are prevented from precocious germination by the presence of ABA, the osmotic environment and, possibly, by limiting the availability of energy and nutrients (Garciarrubio et al., 1997; Bewley et al., 2013). In the transcriptome data of tulip something similar is observed as many genes annotated with metabolite-associated GO-terms, such as *amine*

metabolic process and *carbohydrate metabolism*, are down-regulated in the meristem-enriched tissue collected from tulip. Down-regulation of metabolism likely is associated here with the preparation or establishment of dormancy, very similar to what is observed in seeds. Thus, based on the transcriptome, maturation and preparation for dormancy in tulips resembles the process of maturation and dormancy induction in seeds. In addition, several studies have shown that ABA can inhibit or promote flowering, depending on the species (Wang et al., 2002; Frankowski et al., 2014). In Arabidopsis the bZIP transcription factor *ABSCISIC ACID-INSENSITIVE MUTANT5* (*ABI5*) is involved in the repression of the floral transition by up regulation of the vernalization responsive gene *FLC* (Wang et al., 2013). Also in *Pharbitis nil* (Japanese morning glory) ABA has been shown to have an inhibitory effect on flowering, likely through the modulation of ethylene biosynthesis (Frankowski et al., 2014). In contrast to Arabidopsis and *P. nil*, in which ABA inhibits flowering, ABA promotes flowering in *Litchi chinensis* (Lychee Nut). In this species, application of exogenous ABA promoted flowering and this was impaired by expression of *LcAP1*, the homologue of Arabidopsis *AP1* (Cui et al., 2013). Thus, ABA has been associated to several biological processes, ranging from metabolic arrest, dormancy initiation and tissue maturation, to control of flowering time. Obviously, more research is required to pin-point the exact function of ABA during floral bud initiation in tulip.

Functioning of a tulip TFL1 homolog as potential flowering repressor

We have identified *TgTFL1* as a potential inhibitor of flowering in tulip. Down regulation of its expression appears to be temperature-dependent and is initiated four to five weeks prior to the switch to reproductive development. In the dicot Arabidopsis, *TFL1* also acts as a flowering repressor in the ambient temperature pathway and was identified as hub between the photoperiodic and ambient temperature pathways (Strasser et al., 2009). Also in *Fragaria vesca* (strawberry) the *AtTFL1* homologue *FvTFL1* integrates photoperiod and temperature signals in order to repress

flowering (Rantanen et al., 2015). Different from Arabidopsis and strawberry, tulip is a day neutral plant and therefore, photoperiod is not supposed to play a role in the regulation of flowering time (Kamenetsky and Okubo, 2013). However, surprisingly, GO terms related to photoperiod, such as photoperiodism and response to light stimulus, were found to be overrepresented in the genes upregulated by high flowering-inducing ambient temperatures and overall their expression patterns perfectly correlated with the genes belonging to the GO category vegetative to reproductive phase transition of meristem. A bulb is an underground plant structure, but when these specific genes are induced, the plants still have green leaves above ground that may translate a light- or photoperiodic signal to the SAM in the daughter bulbs. It will be of great interest to investigate whether the observed expression differences of photoperiodic genes plays a role in the induction of flowering in tulip and whether the function of *TFL1* as integrator of the photoperiod- and ambient temperature pathways is conserved in the monocots and in bulbous plant species such as tulip. Furthermore, the life cycles of tulip and the SD plant *F.vesca* have a lot in common. Under SD and low temperature conditions in autumn, *F.vesca* makes the transition from the vegetative to reproductive phase change. Then after the winter period, the flowers emerge and blooming happens in spring (Koskela et al., 2012). *FvTFL1* is a strong regulator controlling the seasonal flowering of *F. vesca* (Rantanen et al., 2014). Not only in *F. vesca*, but likely also in other perennials, homologs of *TFL1* play an important role in the timing of flowering and the duration of blooming.

TgTFL1 is not only of interest in relation to flowering time control, but also to the function of *AtTFL1* in maintaining inflorescence meristem identity, and, as such, being essential for indeterminate inflorescence development and the production of multiple flowers. A mutation in the Arabidopsis *TFL1* gene transforms the indeterminate inflorescence into a terminal floral meristem (Shannon and Meeks-Wagner, 1991), which is similar to tulip reproductive stage morphology. The difference between these two species can most likely be explained by the fact that, in contrast with Arabidopsis,

TgTFL1 expression remains low throughout flower development following its reduction towards the phase switch. Nonetheless, when overexpressing *TgTFL1* in Arabidopsis, not only flowering is delayed but also petals of most flowers are absent, suggesting that *TgTFL1* in Arabidopsis is able to repress *AtAP1*. This shows that the sequences of *TgTFL1* and *AtTFL1* are sufficiently similar and conserved to maintain this function in the repression of *AtAP1* and that terminal flower formation in tulip is most likely not due to a mutation in the *TgTFL1* protein, but to a mutation of the *TgTFL1* expression pattern.

Existence of *TgSOC1-like* genes with possible antagonistic and novel functions in flowering

Two *SOC1-like* genes were identified in tulip that surprisingly appeared to respond in an opposite manner to the temperature treatments. The gene that we designated *TgSOC1L1* has an expression pattern typical for a flowering repressor, whereas *TgSOC1L2* resembles the expression of *AtSOC1* and that of a flowering inducer. In line with this observation, overexpression of *TgSOC1L2* in Arabidopsis caused a weak but significant early flowering phenotype. In this respect, it should be realized that the tulip's life cycle is different from the life cycle of Arabidopsis (Anderson, 2006; Sofo, 2016). This difference is not only in duration, but also when the transition to the reproductive phase is made and blooming occurs. In Arabidopsis flowering directly commences upon the switch from vegetative to reproductive development, whereas tulip flower buds become dormant after their initiation and still require a period of prolonged cold in order to bloom in spring (Lambrechts et al., 1994).

Conclusions

This study has confirmed that high temperature is an important trigger of the vegetative to reproductive phase transition in tulip. A large number of potential flowering time regulators have been identified which partially appear to be conserved when compared to the dicot Arabidopsis.

Our results are summarized in a proposed model of the molecular regulation of the floral induction in tulip (Fig. 8). We have identified a large number of potential novel flowering time regulators which might be bulbous plants or tulip specific. The initiation of flower development, maturation of the bulb and establishment of dormancy all take place at the same moment. Therefore it is of great interest to study the interactions between these processes in more detail and to resolve the complexity of events occurring in daughter bulbs when from the outside nothing seems to be happening and the bulbs are establishing summer dormancy.

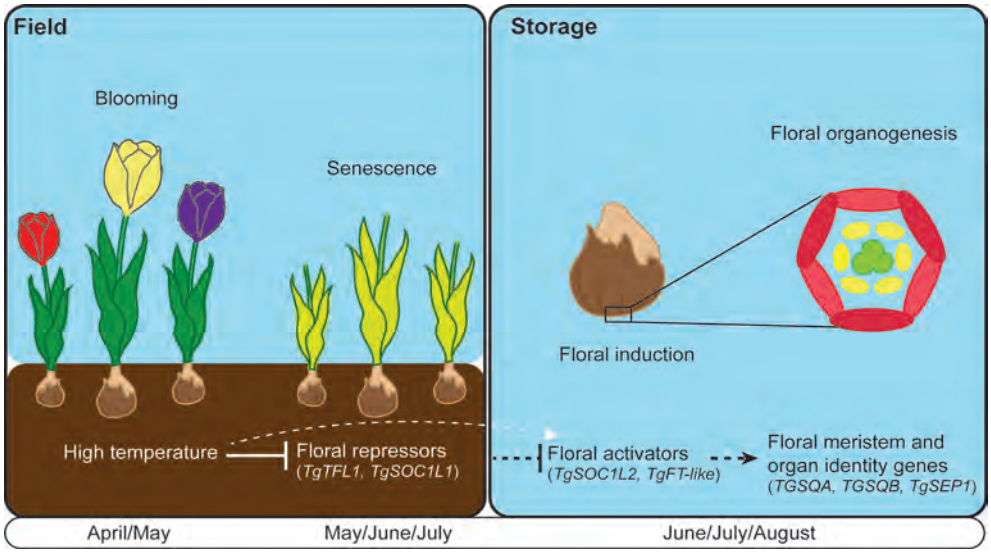


Figure 8. Proposed model of the vegetative to reproductive phase change in tulip. During spring high temperature induces the floral induction in tulip by first repressing *TgTFL1* and *TgSOC1L2*. After this suppression, the floral activators *TgSOC1L2* and TgFT-like are induced, leading to direct or indirect activation of floral meristem and organ identity genes (*TGSQA*, *TGSQB* and *TgSEP1*).

Acknowledgements

We thank Gebroeders Klaver, Maliepaard bloembollen and Van der Gulik tulpen B.V. for the tulip bulbs. We also thank Maarten Holdinga and Alex Silfhout for planting of the bulbs in the field and Juliette Silven for her help with cloning of some of the flowering time genes. We also thank the group

Bioscience of Plant Research International for making use of the Carl Zeiss Stereomicroscope SV11 and Mariana Silva Artur for suggestions on the construction of phylogenetic trees. Funding of the project was kindly provided by TTI-Green Genetics and the Dutch Ministry of Economic Affairs.

Supplemental material

Table S1. Statistical overview of the de novo assembly by Trinity.

Statistics	No.
Trinity transcripts	346,016
Trinity 'genes'	244,383
GC (%)	43.17
N50	1175
Average contig length (bp)	741.35

Table S2. Quantitative PCR primers for expression analysis of tulip flowering time genes.

Gene	Forward	Reverse
TGSQA	5'GGCAGACTGAAGGCTAAGGT 3'	5'GCTCGTTGAAGATCGGCAAT 3'
TGSQB	5'GGAGCAGAAGTCCAAGGCTT 3'	5'GCTCCATCCTCTTCGTTGCT 3'
TgSEP1	5'TGCCCTTCGACTATCATGGG 3'	5' CAGGAGCATAGCCATCACCA 3'
TgTFL1	5'GGCAGAGGAGAGGACAGGTA 3'	5' AAGAGAGTGTCAGTCAGCGG 3'
TgFT-like	5'GTGGATCCTGATGCTCCGAG 3'	5' AAAACAAACACGAGGCGGTG 3'
TgSOC1L1	5'AGAGGGTTTGGTAGAGCAAAGT 3'	5' GGTCTGATGCGGGGATTCTT 3'
TgSOC1L2	5'TCACAGCTCAGAGGAAAGGA 3'	5' GCAAGTCCGTCTCCACATCT 3'
TgSPL1	5'CCGCGTGAGTCACCTTCTAA 3'	5' GTACAGCGCTCCACCTGG 3'
TgSPL2	5'CCAGAAATGTCGGAGCCCAG 3'	5' AGATGGAACCTGCTGCACTG 3'

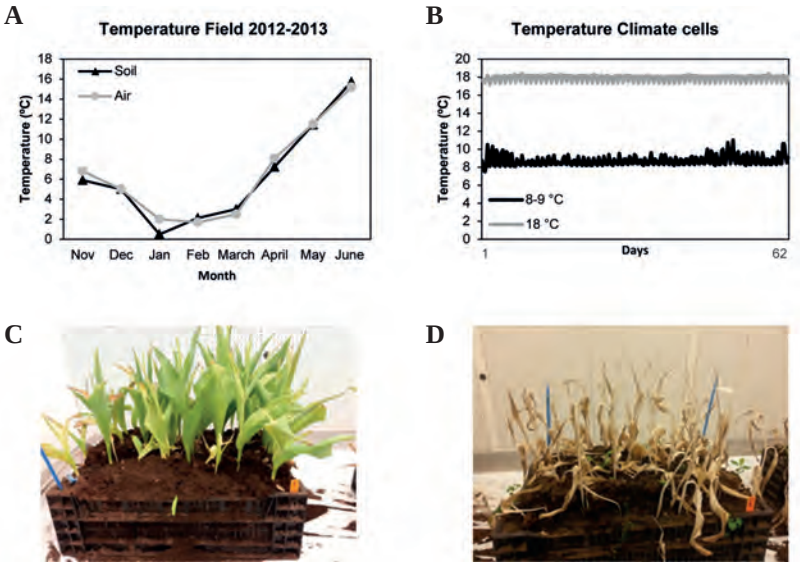


Figure S1. Outside temperature overview of 2012-2013 and tulip morphology after transfer to the climate cells. (A) Average field temperature of the soil (30 cm deep) and air in 2012-2013. January was the coldest month during winter and after this month the temperature kept increasing. (B) Temperature in the climate cells of both low (8-9°C) and high (18ºC) temperature conditions. (C) Morphology of the tulips above ground several weeks after transfer to 8-9 ºC (D) Morphology of the tulips above ground several weeks after transfer to 18 ºC.

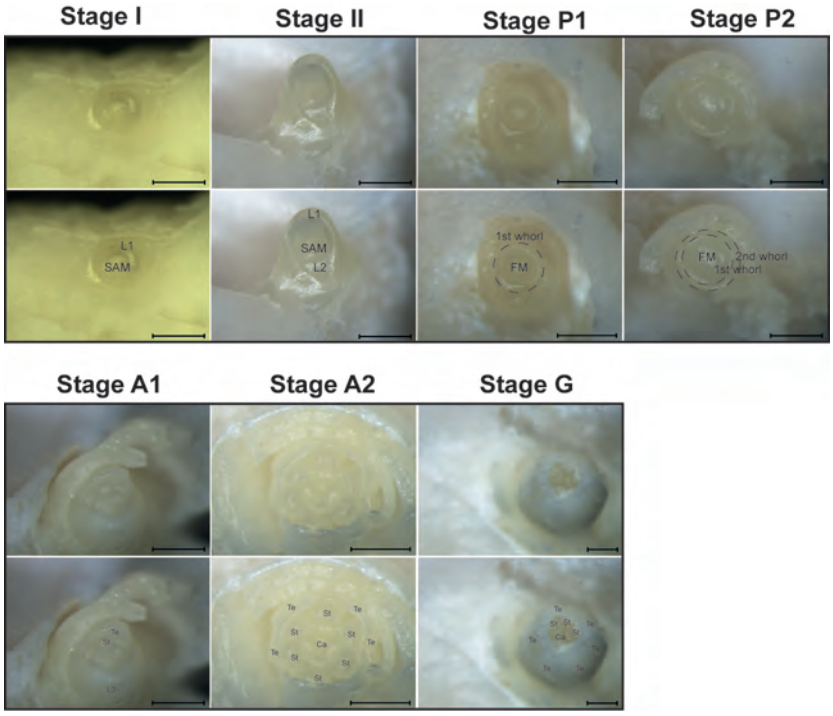


Figure S2. Morphological changes in relation to the vegetative to reproductive phase in tulip. Staging oth the different morphological changes as suggested by Beijer (1952). In stage I the SAM is vegetative and flat. In stage II the vegetative to reproductive phase transition is proposed to commence and the SAM transforms into a dome-like structure with floral meristem (FM) identity. Subsequently, the first whorl of tepals is formed from the FM, which is named stage P1. Stage P2 represents the formation of the second whorl of tepals. In stage A1 and A2 the first and second whorl of stamens are formed and finally in stage G the carpel is fully developed, leading to the completion of the flower structure.SAM: shoot apical meristem, L:leaf, 1st whorl: tepals, 2nd whorl: tepals, Te: tepals, St: stamen, Ca: carpel.

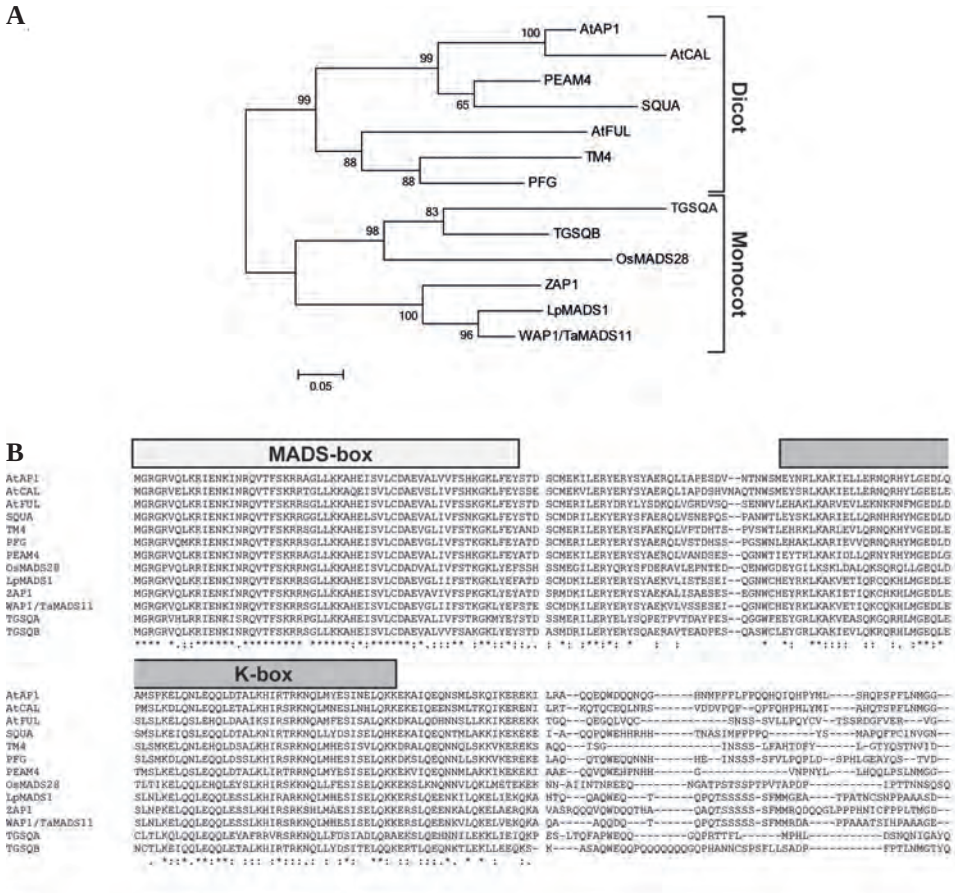


Figure S3. Phylogenetic analysis of TGSQA and TGSQB. (A) Maximum likelihood tree including AtAP1 (AT1G69120), AtCAL (AT1G26310) and AtFUL (AT5G60910) from Arabidopsis, SQUA of Antirrhinum (CAA45228.1), TM4 of *Solanum lycopersicum* (Q40170.1), PEAM4 of *Pisum sativum* (AJ279089), OsMADS28 of *Oryza sativa* (Q0D4T4.1), LpMADS1 of *Lolium perenne* (AAO45873.1), ZAP1 of *Zea mays* (NP_001105333.2), WAP1 of *Hordeum vulgare* (AB007504.1), TGSQA (BAJ09453.1) and TGSQB (BAJ09452.1) of *Tulipa gesneriana*. (B) Alignment of AP1-like proteins, AtCAL and AtFUL.

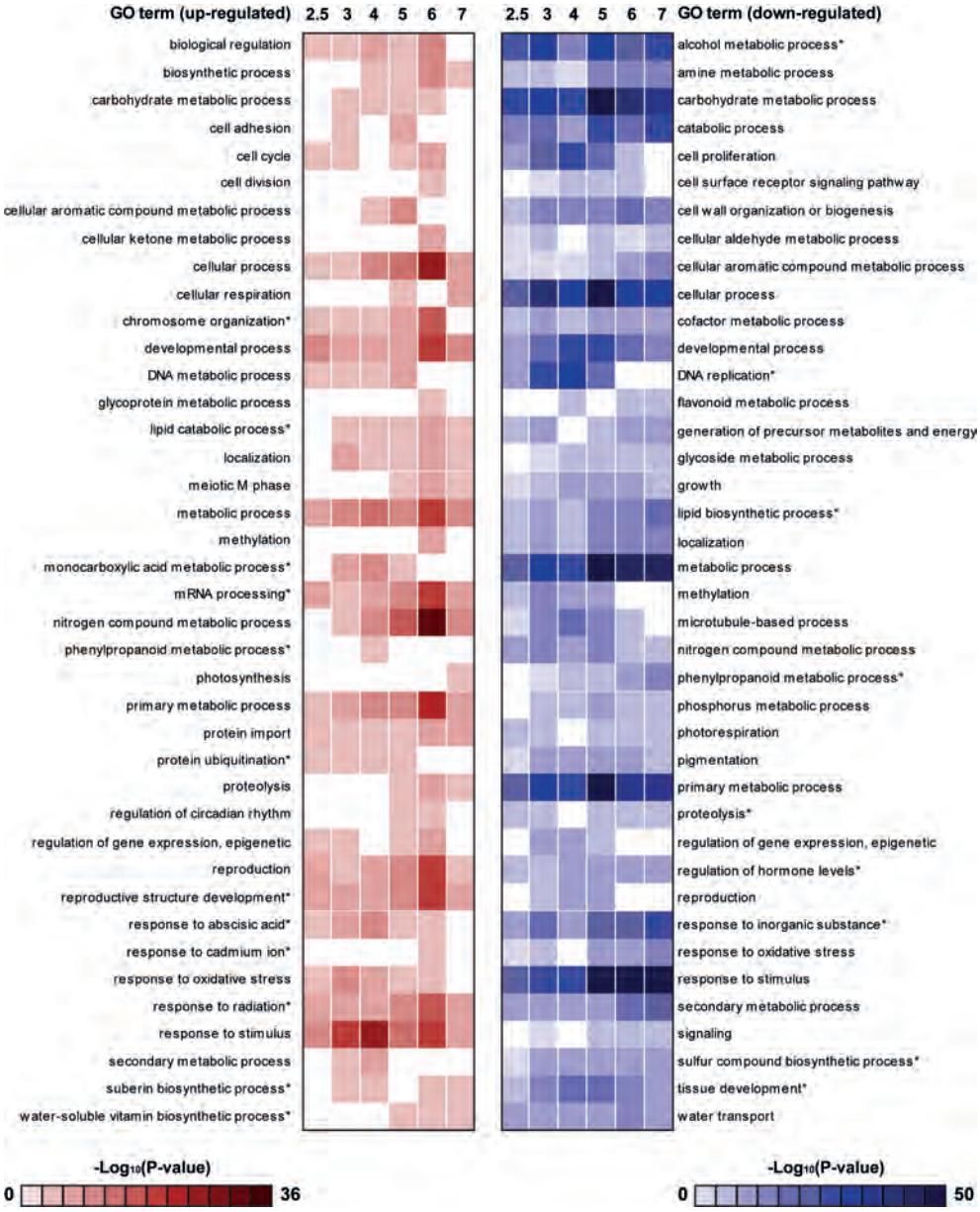


Figure S4. General overview of the GO-enrichment analysis. GO-terms with an asterisk are a clustering of several GO-terms.

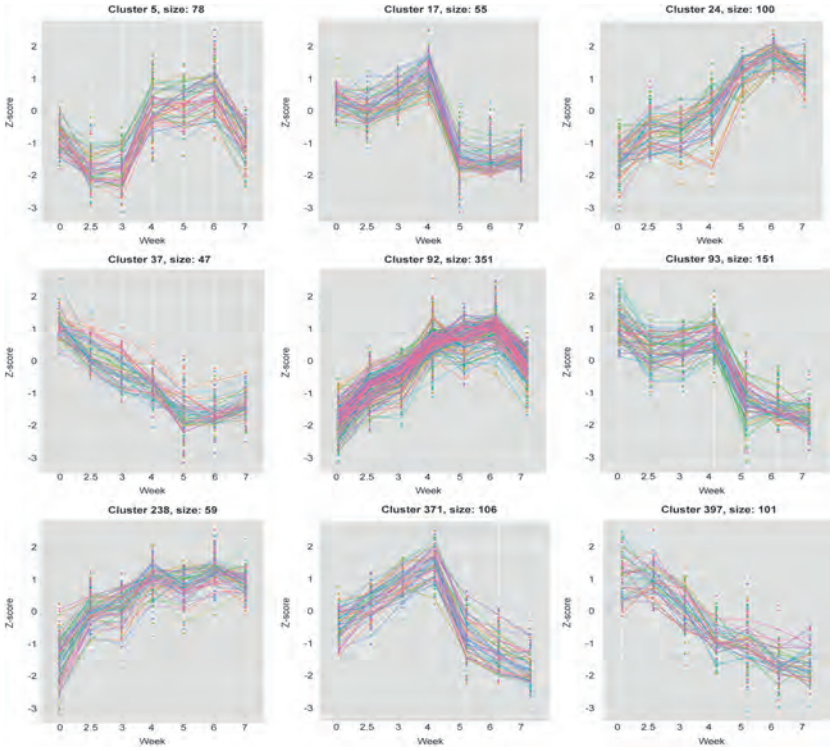


Figure S5. Selection of clusters (high temperature condition) with potential flowering time regulators. All clusters contain transcripts named after the best hit with Arabidopsis transcription factors identified in the RNA-seq data of the floral induction of tulip.

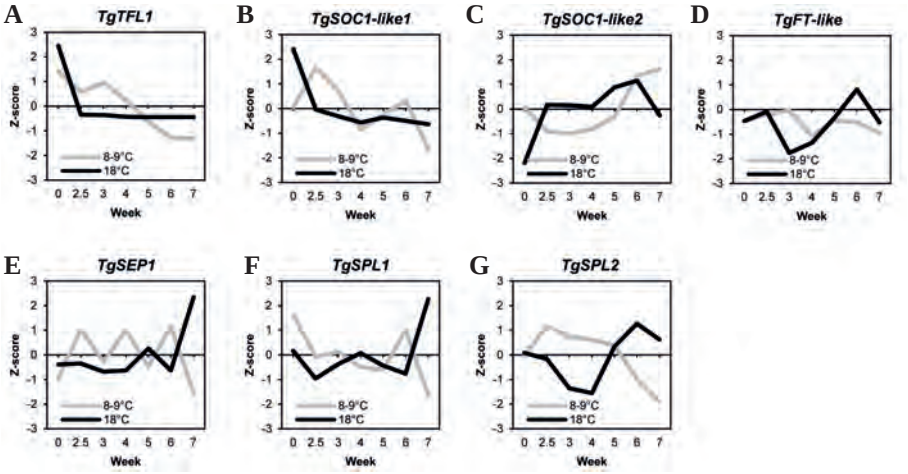


Figure S6. Expression patterns of potential tulip flowering time genes identified in the RNA-sequencing data. (A) TgTFL1, (B) TgSOC1L1, (C) TgSOC1L2, (D) TgFT-like, (E) TgSEP1, (F) TgSPL1 and (G) TgSPL2.

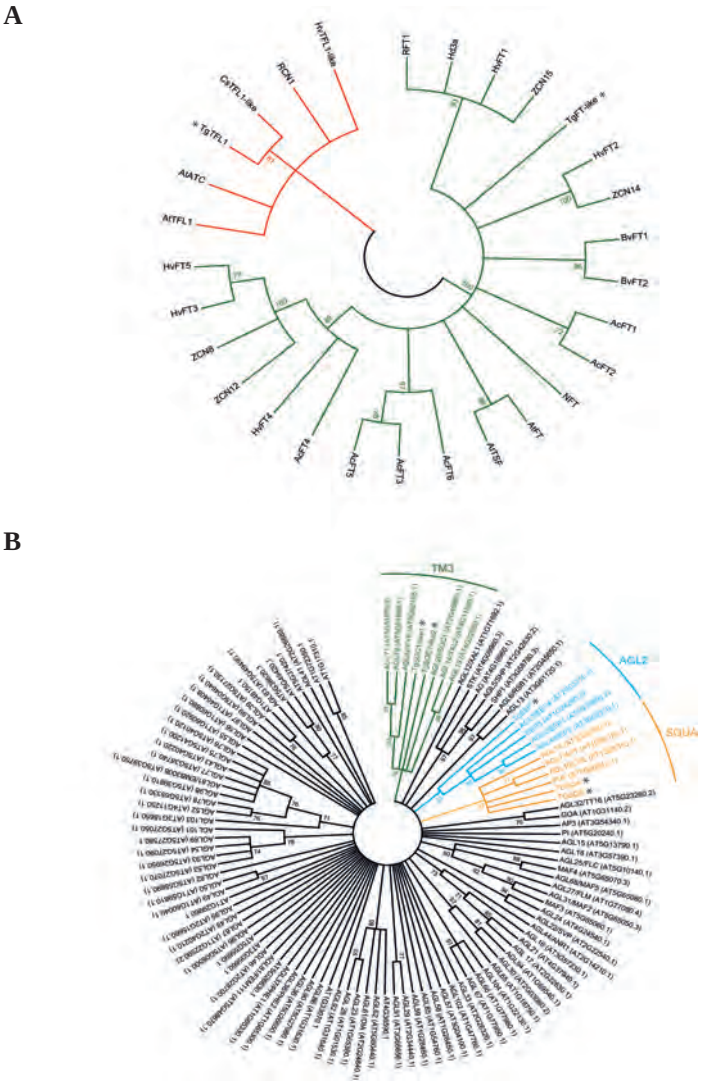


Figure S7. Phylogenetic analysis. (A) Maximum likelihood tree of FT-like and TFL1-like proteins identified in dicot and monocot species including: Hd3a and RFT1 of *Oryza sativa* (BAB61030.1 and BAO03081.1), ZCN8, ZCN12, ZCN14, ZCN15 and RCN1 of *Zea mays* (NP_001106247.1, ABX11014.1, ABX11016.1, ABX11017.1 and ACG45277.1), BvFT1 and BvFT2 of *Beta vulgaris* (ADM92608.1 and ADM92609.1), HvFT1-5 of *Hordeum vulage* (ABD75336.2, ABD75337.1, ABB99414.1, ABV59396.1 and ABF85670.1), AcFT1-6 of *Allium cepa* (AGZ20207, AGZ20208, AGZ20209,AGZ20210, AGZ20211 and AGZ20212), CsTFL1-like of *Crocus sativus* (ACX53295.1) and NFT of *Narcissus tazetta* (AFS50164.1). (B) Neighbor joining tree of all Arabidopsis MADS-box proteins and the tulip proteins TgSEP1, TGSQA, TGSQB, TgSOC1L1 and TgSOC1L2.

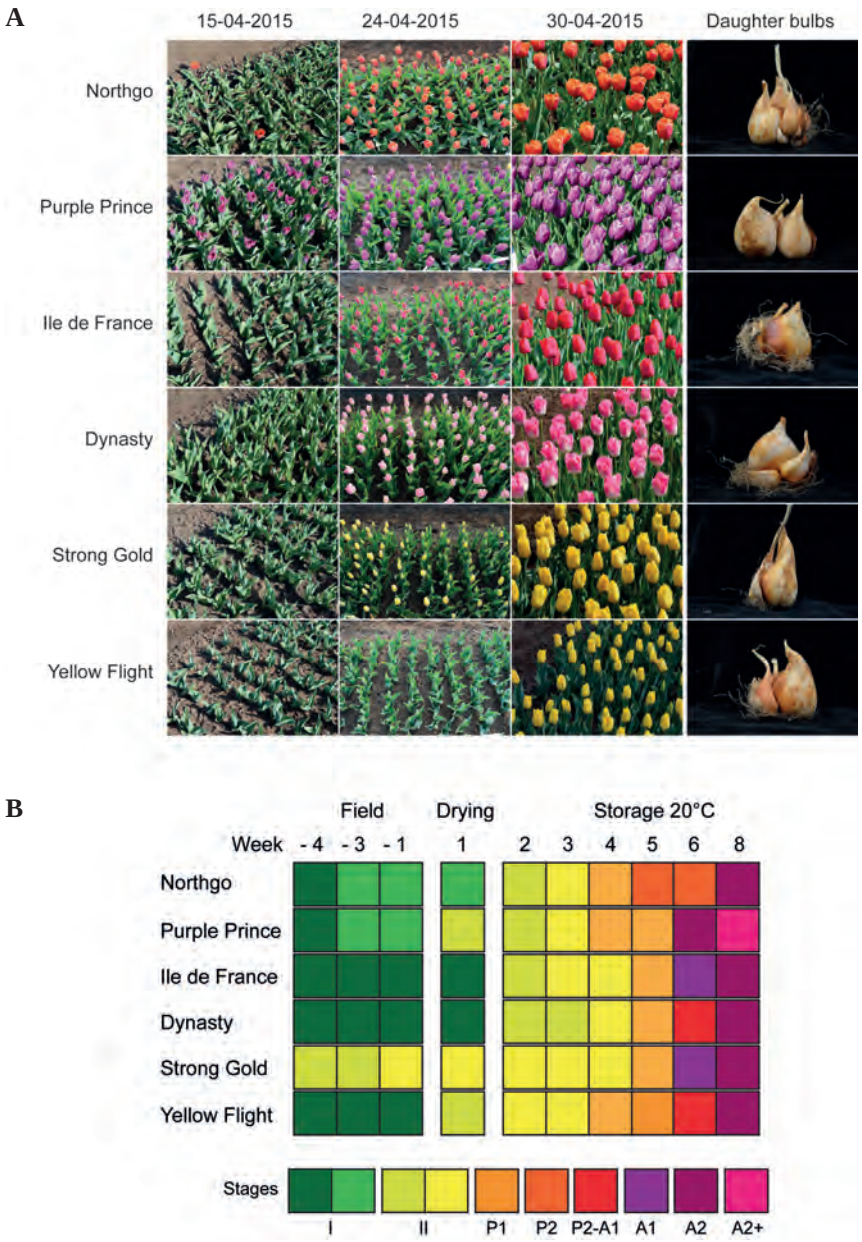


Figure S8. Morphological analysis of the six different tulip cultivars. (A) Blooming of the six different cultivars in the field and the daughter bulbs after drying. Northgo and Purple Prince are early cultivars, Ile de France and Dynasty are mid cultivars, and Strong Gold and Yellow Flight are late cultivars. (B) Stage determination of the six different cultivars in relation to the floral induction. I: vegetative, II: reproductive, P1: first whorl of tepals, P2: second whorl of tepals, P2-A1: start of stamen development of the first whorl, A1: first whorl of stamens, A2: second whorl of stamens, A2+: start of carpel development.

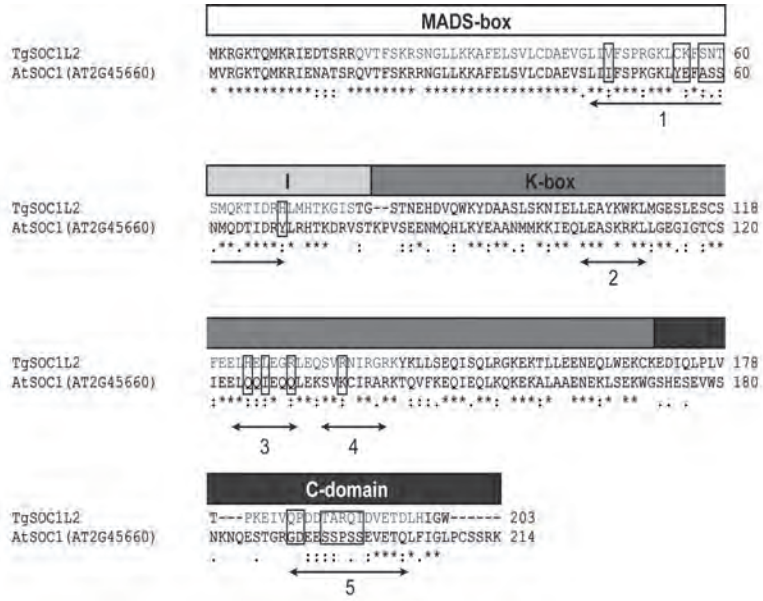


Figure S9. Alignment of tulip TgSOC1L2 and Arabidopsis AtSOC1 protein sequences. The numbers indicate five different positions that are important for the loss or gain of protein-protein interactions.

Chapter 5

Characterization of *Tulipa gesneriana* and *Lilium longiflorum* genes from the *PEBP* family and their putative roles in flowering time control

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*Both authors contributed equally to this work

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Abstract

Floral induction in *Tulipa gesneriana* and *Lilium longiflorum* is triggered by contrasting temperature conditions, high and low temperature respectively. In Arabidopsis, the floral integrator *FLOWERING LOCUS T* (*FT*), member of the PEBP gene family, is a key player in flowering time control. In this study, four PEBP genes were identified from lily and tulip of which one in lily (*LlFT*) and three in tulip (*TgFT1*, *TgFT2* and *TgFT3*). Over-expression of these genes in Arabidopsis resulted in an early flowering phenotype for *LlFT* and *TgFT2*, but a late flowering phenotype for *TgFT1* and *TgFT3*. In addition, over-expression of *LlFT* in *L. longiflorum* also resulted in an early flowering phenotype, confirming its proposed role as a flowering time controlling gene. The tulip PEBP genes *TgFT2* and *TgFT3* have a similar expression pattern in tulip, but show a different behaviour in Arabidopsis. Therefore, the difference between these two proteins was further investigated by interchanging amino acids known to be important for the FT function. This resulted in the conversion of phenotypes in Arabidopsis upon overexpressing the substituted *TgFT2* and *TgFT3* genes, showing the importance of these interchanged amino acid residues. Based on all obtained results we hypothesize that *LlFT* is involved in creating meristem competency to flowering related cues and *TgFT2* is considered to act as a florigen involved in the floral induction. The function of *TgFT3* remains unclear, but phylogenetic analysis suggests a bulb specific function for this gene.

Introduction

The ornamental geophytes *Tulipa gesneriana* and *Lilium longiflorum* are both members of the Liliaceae family (Patterson and Givnish, 2002). Despite this relationship, floral induction in tulip and lily is triggered by contrasting temperature conditions (Fig 1A; Khodorova and Boitel-Conti, 2013; Okubo and Sochacki, 2013). During spring, high ambient temperature induces the transition from the vegetative to the reproductive phase in the daughter bulbs of tulip. This switch is accompanied by the development of the floral bud inside the bulb. Around August-September, the bulb is already

dormant and a fully developed floral bud (stage G) is present within the bulb. A period of prolonged low temperature (< 10°C) is required to re-activate the development of this floral bud inside the bulb. At the end of winter (February-March) the bulb becomes fully active, resulting in blooming in April-May, depending on the cultivar (Gilford and Rees, 1973). In contrast, the vegetative to reproductive phase change in lily does not occur within the bulb, but rather after shoot elongation and production of several leaves (Roh and Willkins, 1977a). However, a prolonged period of cold at the bulb stage or during vegetative development is required to fulfil the vernalization requirement of the plant and induce floral transition (Miller, 1993). In some cases, long day (LD) photoperiod hastens floral transition (Roh and Willkins, 1977a; Miller, 1993; Dole and Wilkins, 1994) and may replace cold exposure as a trigger for floral induction (Lazare and Zaccai, 2016). The typical life cycle of lily starts with bulb planting in the fall (October-November), followed by shoot elongation and leaf development. The plant is then exposed to the low temperatures of the winter, which enables the switch of the shoot apical meristem (SAM) to an inflorescence meristem (IM) and subsequent floral meristems (FM), and flowering occurs in late spring. Flowering time is inversely correlated to the length of cold exposure (2-10°C), hence off-season blooming can be reached by bulb storage at low temperatures before planting (Roh and Willkins, 1977a; Dole and Wilkins, 1994; Holcomb and Berghage, 2001; Lugassi-Ben Hamo et al., 2015; Lazare and Zaccai, 2016).

In many plant species, such as the model species Arabidopsis thaliana and *Oryza sativa*, the floral integrator *FLOWERING LOCUS T* (*FT*) has been shown to be a key regulator of flowering time (Komiya et al., 2008; Turck et al., 2008). The activity of *FT* is regulated by environmental and endogenous signals. In winter accessions of Arabidopsis, the vernalization responsive gene *FLOWERING LOCUS C* (*FLC*) represses *FT*. After a cold period during which *FLC* is down-regulated (Sheldon et al., 2006), and increase of day length, *FT* is activated in the leaves. The FT protein is transported via the phloem to the SAM, where it interacts with the bZIP transcription

factor FD (Jaeger and Wigge, 2007). This protein complex is assumed to contain two FT monomers and two FD bZIP transcription factors, as well as a dimeric 14-3-3 protein acting to bridge the FT-FD interaction (Li et al., 2015). The resulting florigen activation complex (FAC) leads to the direct activation of the floral meristem identity genes *APETALA1* (*AP1*) and *FRUITFULL* (*FUL*) (Lee and Lee, 2010). Besides the vernalization response, high ambient temperature is also able to influence the activity of *FT* in Arabidopsis. The regulation of this process is not yet fully understood, but several genes, such as *PHYTOCHROME INTERACTING FACTOR3* (*PIF3*), *PIF4*, *FLOWERING LOCUS M* (*FLM*) and *TERMINAL FLOWER1* (*TFL1*), have been identified as temperature responsive genes and acting on *FT* (Hanano and Goto, 2011; Thines et al., 2014; Verhage et al., 2014).

FT is a member of the phosphatidylethanolamine-binding protein (PEBP) gene family and besides *FT*, five additional genes have been identified as members of this family in Arabidopsis. These members are *TWIN SISTER OF FT* (*TSF*), *BROTHER OF FT* (*BFT*), *MOTHER OF FT* (*MFT*), *ARABIDOPSIS THALIANA CENTRORADIALIS* (*ATC*) and *TERMINAL FLOWER 1* (*TFL1*). The three PEBP genes *FT*, *TSF* and *MFT* are floral activators, while *TFL1*, *ATC* and *BFT* can act as floral repressors (Yoo et al., 2004; Yamaguchi et al., 2005; Huang et al., 2012). All members of this family contain a PEBP domain, which is a characteristic and common feature. Detailed molecular and biochemical studies revealed that the function of being a repressor or activator of flowering is at least partially determined by several unique amino acid (AA) residues in the protein sequence (Ho and Weigel, 2014). Floral activators such as FT contain a Tyr (Y) at position 85, while floral repressors contain a His (H) at the analogous position 88 (Hanzawa et al., 2005). Also AA residues at position 140 can affect the function of the protein. For the activator FT a Glu (Q) is present at position 140 and for the repressors an Asp (D) is positioned at the same analogous position (Ahn et al., 2006).
Besides functioning as activators or repressors of flowering, members of the

PEBP family are also involved in other processes. In perennial species, *FT-like* or *TFL1-like* genes have been linked to the regulation of growth cessation, better known as dormancy. For example in *Picea abies* (Norway spruce) *PaFTL2*, an *FT/TFL1-like* gene, determines bud set as well as growth cessation (Karlgren et al., 2013). Additionally, in *Actinidia* spp. (kiwifruit) *FT* and *CEN* have been shown to be involved in the regulation of growth cessation, through the integration of developmental and environmental signals (Varkonyi-Gasic et al., 2013). Another organ in which *FT-like* genes are expressed is the fruit. In *Ficus carica* L., *FcFT1* is regulated by light and likely plays a role in fruit set (Ikegami et al., 2013). Even within Arabidopsis FT is not only involved in flowering time control at the SAM, but is, for example, also involved in the outgrowth of axillary meristems (branching). Related to this specific function the FT protein interacts with the key regulator of axillary meristem outgrowth *BRANCHED1/TEOSINTE BRANCHED1-LIKE 1* (*BRC1*) (Hofmann, 2013). All together this shows the importance of the PEBP gene family in plant development processes and the great diversity in functions of the encoded FT-like proteins.

In addition to these studies of individual PEBP family genes, for a few sequenced plant species the complete PEBP family has been investigated. For example, in *Glycine max* (soybean) 23 PEBP genes were identified (Wang et al., 2015), four times the number of PEBP genes identified in Arabidopsis. Within this family *GmFT2a* and *GmFT5a* control flowering time in a photoperiod-regulated manner, similar to Arabidopsis *FT* (Nan et al., 2014). In the model monocotyledonous species *Oryza sativa* (rice) 19 PEBP genes were identified of which *Heading date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*) are the most studied members (Chardon and Damerval, 2005). *Hd3a* is the key player in the activation of flowering in rice under short-day (SD) conditions. Besides *Hd3a*, *RFT1* also plays an essential role in the control of flowering time. *RFT1* is the closest homologue of *Hd3a* and it is believed that *RFT1* may function as an auxiliary to *Hd3a* in controlling flowering time, when *Hd3a* is repressed

(Komiya et al., 2008). In *Zea mays* (maize) a total of 24 PEBP genes were identified (Danilevskaya et al., 2008). Among those, only ZCN8 seems to possess the characteristics for protein movement from the leaves to the SAM and might therefore be the FT-like protein similar to Arabidopsis FT (Meng et al., 2011). Overall the amount of genes in the PEBP family can differ, but often there is only a single gene that encodes for a protein acting as a flowering time activator.

Also in non-model monocotyledonous species several *FT* and *TFL1-like* genes have been identified. In *Narcissus tazetta* the *FT* homolog *NFT* was shown to be responsive to heat and to correlate with floral induction independently of photoperiod and vernalization (Li et al., 2013; Noy-Porat et al., 2013). In addition to *NFT*, seven *FT-like* genes were identified in the bulbous species *Allium cepa*. *AcFT2* appears to promote flowering time in response to temperature as well as photoperiod (Lee et al., 2013). Surprisingly, *AcFT1* and *AcFT4* have been shown to be involved in bulbing, where *AcFT1* acts as a promoter of bulbing and *AcFT4* as inhibitor (Lee et al., 2013; Manoharan et al., 2016).

In this study we aimed to identify and characterize PEBP family genes from the ornamental geophytes *T. gesneriana* and *L. longiflorum*, and to study their potential biological functions, with a focus on flowering time control. Three tulip PEBP family genes were identified (*TgFT1*, *TgFT2* and *TgFT3*) and one lily PEBP family gene (*LlFT*, Villacorta-Martin et al., 2015). The expression patterns of the four identified genes were determined and functional characterization was performed to reveal the potential role of the isolated PEBP family genes in flowering time control of tulip and lily.

Material and methods

Plant material and growth conditions

Tulipa gesneriana cv. “Dynasty” bulbs (size 10/11) were planted early November 2012 in the field at Wageningen University (51.9667° N, 5.6667° E). Material of the stem, leaves, scales and floral buds were collected

every three weeks from January 2013 until May 2013 in three biological replicates, each containing tissues collected from five bulbs. Samples were always collected in the morning. Each sample was grinded and homogenized by the use of liquid nitrogen, mortar and pestle and subsequently stored at -70°C until use. L. longiflorum cv. 'White Heaven' bulbs (size 12-16) were purchased from a commercial nursery in August 2014. After sanitation, bulbs were stored in moist medium mixture of peat and vermiculite (1:1, v/v) at 25 °C or at 4 °C (cold treatment) in the dark until planting on November 2014 in a temperature-controlled glasshouse, with a constant average temperature of 24.4 ± 2.2 °C. Long day (LD, 16/8 hours light/dark) or short day (SD, 8/16 hours light/dark) conditions during growth were achieved using an automatic system of curtains and day extension (for LD) with incandescent light bulbs, in different chambers. Meristems and leaves were collected at different time points under the different conditions during growth. The meristem developmental stage (vegetative or floral transition) was determined by observation under a Stereo Microscope Leica M125 (Germany).

Arabidopsis thaliana seeds were stratified for 2-3 days at 4°C and plants were grown under LD conditions (16/8 hours light/dark) at 20-23°C in 5x5 cm pots with soil or on Rockwool. For tulip genes, in total two independent segregating transgenic lines of each construct were selected and of each line 30-50 plants were used for phenotyping and genotyping. Flowering time was scored by counting the number of rosette leaves at the moment the inflorescence reached a length of one centimeter. For the statistical analysis flowering time scores of wild type plants were removed from the segregating populations using PCR-based genotyping. For lily genes, two homozygous lines of LlFT were selected and 30-50 plants were used for phenotyping and genotyping.

Total RNA extraction and cDNA synthesis

To extract the total RNA from the scale and stem tissue of tulip, the Tripure protocol (Roche, The Netherlands) was used according to the

manufacturer's instructions with the addition of 2% Polyvinylpyrrolidone (PVP, w/v) and 2% β-mercaptoethanol (β-ME, v/v) to the extraction buffer. Subsequently, a DNase treatment with RQ1 (Promega, The Netherlands) was performed to remove DNA, followed by a phenol/chloroform (1:1) extraction and ethanol precipitation. RNA from the leaves and floral bud of tulip were extracted with the InviTrap Spin Plant RNA Mini Kit (STRATEC Molecular, the Netherlands), including a DNase treatment on the column. A total amount of 500 ng was used for first-strand cDNA synthesis using M-MuLV Reverse Transcriptase (Thermo Scientific, The Netherlands). All reactions were performed in a Bio-rad MyCycler (Bio-rad, The Netherlands).

For lily, total RNA was extracted using the Aurum Total RNA Mini kit (Bio-Rad Laboratories), which included a DNase treatment. cDNA was produced with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Reactions were performed in a DNA Engine® (PTC-200) Peltier Thermal cycle (MJ Research, Inc., USA).

Identification of PEBP family genes

The tulip PEBP family gene sequences were identified from the transcriptome data published by Moreno and colleagues (2016) using protein BLAST in the *Tulipa* and *Lilium* Transcriptome browser (Moreno-Pachon et al., 2016). Complete sequences, as well as the PEBP domain only, of all six PEBP family genes of A. thaliana (AT1G65480, AT1G18100, AT4G20370, AT5G62040, AT5G03840, and AT2G27550) were used as query for this BLAST search. The lily PEBP family gene sequence, *LlFT* was obtained from a lily transcriptome and blasted against UniProtKB database, as described in Villacorta-Martin et al., 2015.

Construction and generation of over-expression lines in *Arabidopsis thaliana*

The three PEBP family genes of tulip were amplified from cDNA by PCR with the primers *TgFT1* (forward 5'-ATGAGTAGAGAAAGGGATCC-3'

and reverse 5'-TCACGGGTACAGGCGCC-3'), *TgFT2* (forward 5'-ATGGAGAACAGCAGTGATCC-3' and reverse 5'-TCAAGGGTACATCCTCCGG-3') and *TgFT3* (forward 5'-ATGTCCTCGATCCGTTCG-3' and reverse 5'-TCAGCTTGTCTTAGAACCTT-3'). The *L. longiflorum LlFT* gene was amplified from cDNA, with following primers, 5'-ACTAGTATGAATATGCGAAGGAGCTC-3', forward and 5'-GAATTCTCAGGTTGTTGGTAGCCTTC-3', reverse, including restriction sites for SpeI and EcoRI, respectively. The PCR fragments of the PEBP family genes from tulip were cloned in the Gateway overexpression vector pGD625 described by Folter and colleagues (2006), while the LlFT amplicon was cloned into the pCAMBIA23R plasmid (http://www.cambia.org/daisy/cambia/585.html). *LlFT* gene was amplified using the primers 5'- TTGTGGTACCATGAATATGCGAAGGAGCTCCGG-3' (forward) and 5'- TTGTTCTAGATCAGGTTGTTGGTAGCCTTCTT-3' (reverse). Both plasmids carry the nptII gene for kanamycin resistance in plants for future selection of positive transformants. The pGD625 constructs containing the tulip PEBP family genes were transformed into *Agrobacteria tumefaciens* AGL0. The *LlFT* overexpression construct was introduced into *A. tumefaciens* strain EHA105. Both constructs were transformed into Arabidopsis using the floral dip method (Clough and Bent, 1998).

Construction of the substitution lines

The gene sequence of *TgFT2* and *TgFT3*, including two substitutions and Gateway compatible att-cloning sites, were synthesized by Genscript (United States). In the substitution line of TgFT2 the codon of leucine at position 128 was substituted for the glutamic acid codon of TgFT3 and the codon of glutamine at position 140 was substituted for the proline codon of TgFT3. The substitutions made in *TgFT3* were opposite of the ones made in *TgFT2*. The synthesized genes were delivered in the pUC57 plasmid. Before the BP reaction with pDONR207, the plasmid was linearized using HindIII. Also here the vector pGD625 was used for overexpression of

the *TgFT2* and *TgFT3* substitution lines (*35S:TgFT2(L128E/Q140P*) and *35S:TgFT3(E125L/P137Q*)) in Arabidopsis as described in construction of overexpression lines in Arabidopsis.

Prediction of protein structure

The structure of the tulip PEBP proteins was modeled using Modeller version 9.16. The protein structure available in the Protein Data Bank with the highest similarity to the tulip PEBP proteins is the rice FT protein Hd3a with identifier 3axy (Taoka et al., 2011). It has sequence identity of 73% for TgFT2 and 58% for TgFT3, which indicates it is a suitable template for homology modeling. 1,000 structure models were generated both for TgFT2 and TgFT3 using the standard auto-model approach in Modeller. The best one based on objective score was selected.

Generation and growth of *L. longiflorum* transgenic plants

Lily plants were transformed with the same plasmid and *A. tumefasciens* strain as Arabidopsis (see previous section), using the method described in (Núñez de Cáceres et al., 2011). Transformation was validated in resistant bulblets using the specific primers (5'-GAGGCTATTCGGCTATGACT-3', forward and 5'-AATCTCGTGATGGCAGGTTG-3', reverse, within the nptII gene for RT-PCR amplification from lily cDNA. PCR conditions were 95◦C, 5 min followed by 40 cycles of (1) 95◦C, 1 min, (2) 54◦C, 1 min, (3) 75◦C, 3 min; 75◦C, 10 min.

Transgenic and non-transgenic lily bulbs from tissue culture were potted into soil mixture and transferred to the controlled greenhouse described in the "Plant materials and growth conditions" section, under SD conditions. In order to avoid unwanted effects derived from in vitro regeneration, scales were detached from the bulbs and transferred into new pots for production of plants used in flowering time experiments.

Real-time PCR for expression analysis

Real-time PCR reactions for tulip were performed in a total volume of 20 µl

containing 10 µl of iQ SYBR Green Supermix (Bio-rad, The Netherlands), 5 µl of each forward and reverse primer (0.05 µM) and 5 µl of a 1:15 dilution of the cDNA reaction mixture as template. Reactions were performed on a CFX Connect real-time PCR detection system (Bio-rad, The Netherlands) with an initial 3 min denaturation at 95°C followed by 40 cycles of 95°C for 10s and 60°C for 30s for the amplification. Final steps used for elongation were 95°C for 1 min, 55°C for 10s and 95°C for 30s with afterwards a melt curve determination. Relative expression levels were calculated by the ΔΔCt method (Livak and Schmittgen, 2001) with *TgEF1α* and *TgACT* as reference genes.

In the case of lily, gene expression was determined using the 7300 Real-Time PCR System (Applied Biosystems) and primers were designed by Primer-Express (Version 2.0, Applied Biosystems). Amplicon lengths were between 80 and 85 bp. Each reaction involved 100 ng cDNA, 10 µl Power SYBR Green PCR MasterMix (Applied Biosystems), 500 nM of each primer for the tested gene and 700nM for the reference gene UBQ. Real-time program was as follows: 50oC for 2 min, 95oC for 10 min, then 40 cycles of 95oC for 15 s and 60oC for 1min each, a dissociation stage of 95oC for 15 s, 60oC for 1min, and 95oC for 15 s. RNA relative quantification was performed using the 7300 System SDS software (Applied Biosystems). Alternatively, gene expression was determined by high throughput qPCR using the BioMarkTM 96.96 dynamic arrays (Fluidigm, USA) and run by the Department of Biological Services, Weizmann Institute of Science (Israel). As for tulip, relative gene expression was calculated using the ΔΔCt method. The lily polyubiquitin gene (UBQ from *L. longiflorum*, Accession AAF21992) was used as reference.

For all expression analyses calculations were based on three technical replicates and two-three biological replicates.

Yeast two hybrid assay

The protein-protein interaction capacity of the various PEBP family proteins from tulip and lily was determined by a yeast two hybrid assay as

described by (Folter and Immink, 2011). Auto-activation was determined for all bait vectors used in this study and only TgFT1 showed auto-activation.

Phylogenetic analysis of the FT-like proteins in bulbous species

A maximum likelihood tree was constructed with MEGA 5.1 (Tamura et al., 2011) to investigate the phylogenetic relationship of the PEBP family proteins identified in tulip and lily. The alignment for the phylogenetic tree was performed with CLUSTALW default settings. The amino acid sequences of the bulbous species *Allium cepa* (AGZ20207, AGZ20208, AGZ20209, AGZ20210, AGZ20211 and AGZ20212), *Allium sativum*, *Crocus sativus* (ACX53295.1), *Narcissus tazetta* (AFS50164.1), *Tulipa gesneriana*, *Lillium longiflorum* and the model species Arabidopsis (AT1G65480, AT4G20370, AT5G03840 and AT2G27550) *Oryza sativa* (BAO0348.1 and BAO03215.1), *Zea mays* (NP_0011062471.1, ABX11014.1, ABX11016.1, ABX11017.1 and ACG45277.1), *Beta vulgaris* (ADM92608.1 and ADM92609.1), *Hordeum vulgare* (ABD75336.2, ABD75337.1, ABB99414.1, ABV59396.1 and ABF85670.1), *Malus domestica* (AB16112.1, FJ555224.1, NM_001293958.1), *Solanum tuberosum* (NP_001307981.1, AY186735, AY186737) and *Populus trichocarpa* (POPTR_008s07730.1, POPTR_0010s18680.1) were included in this analysis. Putative PEBP protein sequences (AsFT1-AsFT7, AsTFL1-1 and AsTFL1-2) of Allium sativum were identified in the data of Kamenetsky et al., (2015), by an assembly of the raw data with Trinity (Haas et al., 2013). The other *Lilium* FT-like sequences LiFTL1, LiFTL2 and LiFTL3 were identified in a dataset produced by P. Arens internally available at Wageningen University. For the construction of the maximum likelihood tree the WAG model was used as the substitution model and 500 bootstraps were generated to validate the relationship between sequences. In addition, the setting “gaps/missing data treatment” was changed into partial deletion with 95% as the site coverage cut off. For the substitution analysis, an alignment was built in Geneious version 8.1.8. using the ClustalW settings. All known bulbous PEBP family protein sequences and

the Arabidopsis and *O. sativa* PEBP domain containing protein sequences were used in this alignment.

Results

Identification of PEBP family gene sequences in tulip and lily

In a previous study by Villacorta-Martin and colleagues (2015) one PEBP family gene was described in relation to the vernalization response in *L. longiflorum*. This gene, known as *LlFT* is further characterized in this study. The PEBP family genes of tulip were discovered in the RNA sequencing data described by Moreno-Pachon et al. (2016). In total three putative PEBP family genes were identified in tulip using BLAST. The translated cDNA sequence of these genes was compared to FT, TSF and TFL1 of Arabidopsis as well as Hd3A, RFT1 and RCN1 of *O. sativa*, and TgTFL1 of tulip (Leeggangers et al., submitted; Fig. 1B). The lily LlFT sequence was ~65% similar to AtFT and AtTSF and 70% similar to Hd3a and RFT1 (Fig. 1B). In contrast, similarity of LlFT was only ~50% with AtTFL1, RCN1 and TgTFL1. Similar low percentages of similarity to TFL1 and TFL1-like sequences were also observed for the three tulip PEBP family proteins; showing that all four identified sequences are more similar to FT than TFL1. Therefore, we now refer to the lily PEBP family gene as *LlFT* and the three tulip PEBP family genes as *TgFT1*, *TgFT2* and *TgFT3*. Based on sequence identity, the TgFT1 protein was most similar to AtFT (75%), whereas TgFT2 (67%) and TgFT3 (55%) showed lower percentages of similarity to AtFT. However, in comparison to Hd3a and RFT1 from monocot species, TgFT2 revealed to be more similar to these sequences (~74%), than TgFT1 (~73%) and TgFT3 (~50%). Remarkably, TgFT3 has a low percentage of similarity with both FT-like and TFL1-like proteins. Several amino acids are known to be important for the specific and unique FT and TFL1 functions (Ahn et al., 2006; Ho and Weigel, 2014). In the conserved ligand binding pocket motif (Asp-Pro-Asp-X-Pro) of PEBP proteins, the first Pro (P) at position 68 is substituted by Ala (A) in TgFT3 (Fig. 1B). Segment B is located at

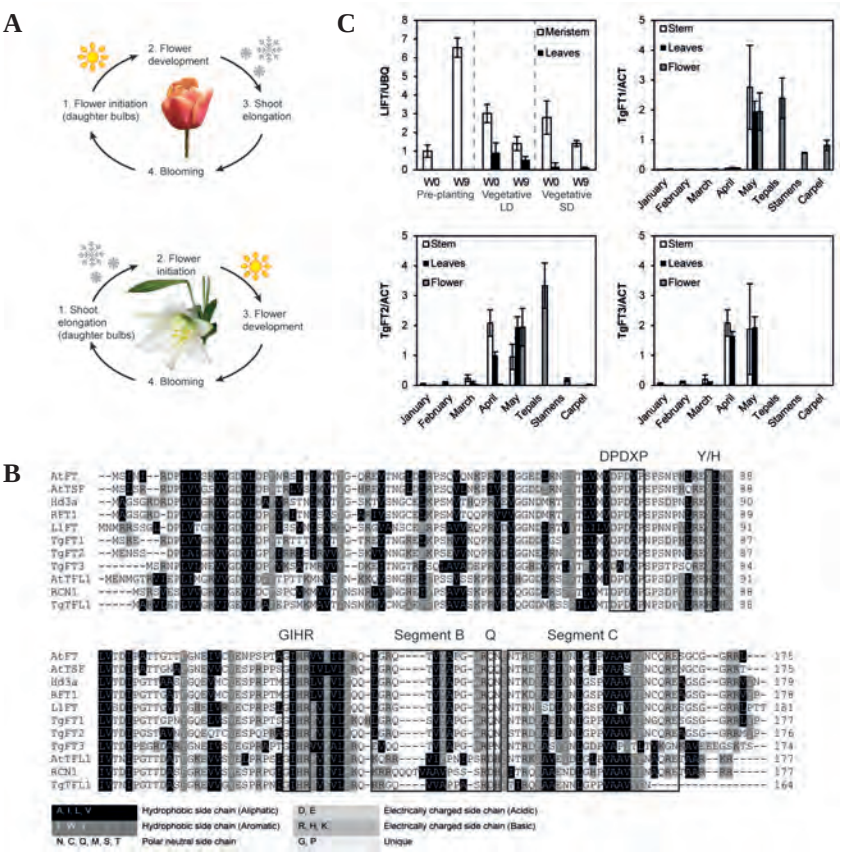


Figure 1. Identification and characterization of the lily and tulip PEBP family genes and proteins. (A) Schematic overview of the flowering process in the yearly growth cycle of tulip and lily. Flower initiation (1) inside the tulip daughter bulbs is triggered by high temperatures in spring and upon completing floral organ development the flower bud becomes dormant (2). After a period of low temperatures, the shoot elongates (3) and outgrowth of the flower occurs, ending in blooming (4). In L. longiflorum the shoot first elongates (1) followed by flower initiation triggered by low temperatures (2) and finally outgrowth of the flower resulting in blooming (3-4). (B) Multiple sequence alignment of AtFT, AtTSF and AtTFL1 proteins of Arabidopsis and O. sativa and the newly identified PEBP family protein sequences of lily and tulip. The black and white and grey shading of the amino acids represent the classification of amino acid conservation by Taylor (1986). (C) Gene expression analysis of LlFT (top panel, left) in lily meristems and leaves before planting (Pre-planting) and in plants at the vegetative stage (VEG), under different vernalization and photoperiod conditions. 0W, 9W: weeks of bulb exposure to 4oC before planting. SD, LD: short day and long day conditions, respectively, in the period after planting. Gene expression analysis of TgFT1 (top panel, right), TgFT2 (bottom panel, left) and TgFT3 (bottom panel, right) in different organs during the annual growth cycle.

the N-terminus of the protein, which is easily accessible and exposes an external loop in the 3D structure available for potential interactions (Ahn et al., 2006). In previous studies this segment is described as invariable; however, in TgFT3 several substitutions are present in this part of the protein. This includes the important amino acid Gln (Q), which is substituted by Pro (P) at position 137. Also in segment C differences between TgFT3 and the other FT/TFL1-like sequences are present, especially the end of the N-terminus. Taken together, LlFT and both TgFT1 and TgFT2 are very similar to FT-like proteins, whereas TgFT3 differs at certain levels of the protein sequence in comparison to canonical FT proteins such as AtFT (Fig. 1B). Nevertheless, TgFT3 still contains some important amino acids essential for the FT function, such as Tyr (Y) at position 81.

Expression analysis of lily and tulip PEBP family genes

Lily and tulip respond to different temperature conditions, allowing the transition from the vegetative to the reproductive phase (Fig. 1A). To further investigate the role of the identified lily and tulip PEBP family genes and their potential function in flowering control, the expression pattern of these genes was determined during the growth cycle (Fig. 1C). In lily, *LlFT* expression was up-regulated in the bulb meristem by cold exposure (9 weeks at 4oC) before planting (Fig. 1C; Fig. S1B). However, after planting this effect was diminished, in the vegetative meristem (Fig. 1C; Fig. S1B). Rather, *LlFT* expression was higher in meristem from non-cooled bulbs. *LlFT* was upregulated by LD in the leaves but not in the meristem. Generally, the level of gene expression was much lower in the leaves than in the meristems. The three tulip PEBP family genes were expressed more or less from the start of shoot elongation onwards in early spring (Fig. 1C; Fig. S1A). *TgFT1* is expressed in the stem, leaves and flower at the moment of blooming in May, when these organs are already completely developed (Fig. 1C). Dissecting the flower at blooming time revealed that *TgFT1* was expressed in all parts of the flower. *TgFT2* and *TgFT3* expression was initiated earlier

and increased in stem and leaves during rapid elongation of the shoot (Fig. 1C). *TgFT2* was also expressed in the flower at blooming time in May, which is comparable to *TgFT1*. Within the flower, the expression of *TgFT2* was low in the stamens but high in the tepals.

Functional characterization of *LlFT* in *A. thaliana* and *L. longiflorum*

The potential role of *LlFT* in flowering time was further investigated by heterologous expression of *LlFT* in Arabidopsis and overexpression of *LlFT* in *L. longiflorum*. When overexpressing *LlFT* in Arabidopsis (Fig. 2A) a mild early flowering phenotype was observed. Nevertheless, a significant reduction in the time to flowering and in the number of leaves was observed in both transgenic lines compared to wild type Col-0 (Fig. 2A-C). In addition, lily plants (N=2) transformed with *35S:LlFT* grown from scales under non-inductive conditions of temperature and photoperiod, flowered 8.9 and 9.8 months after planting and developed 45 and 50 leaves, respectively (Fig. 2D, Left). Under these conditions, non-transgenic control *L. longiflorum* plants did not switch to the reproductive phase but remained vegetative (Fig. 2D, Right). Thus, *LlFT* has the potential to be an inducer of the floral transition in *L. longiflorum*.

Functional characterization of *TgFT1, TgFT2* and *TgFT3* in *A. thaliana*

Unfortunately, stable transformation in tulip is laborious and very inefficient (Wilmink et al., 1992). Therefore functional analysis of the three identified tulip PEBP family genes was performed in the model dicotyledonous species Arabidopsis. Constitutive overexpression lines were created and analyzed for flowering time. For each gene two independent transgenic lines were selected for the screening. Ectopic expression of the *FT-like* genes in these lines was confirmed by qRT-PCR (Fig. S2B-D). Upon overexpression of *TgFT1* and *TgFT3* a slight, but significant delay in flowering time is observed (Figure 3A-C and G-I). Besides this mild flowering time phenotype, no obvious phenotypic changes were observed. In contrast, overexpression of *TgFT2* resulted in very early flowering with a significant

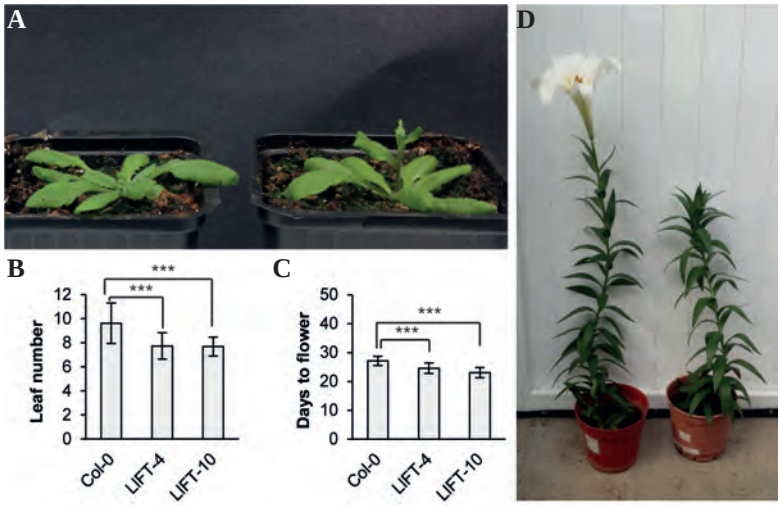


Figure 2. Functional characterization of *LlFT* in Arabidopsis and *L. longiflorum*. (A-C) Overexpression of *LlFT* in Arabidopsis. Wild type Col-0 is depicted left and *35S:LlFT* on the right. The triple asterisk indicates a significant difference with a p-value of ≤0.001. (D) Overexpression of *LlFT* in *L. longiflorum*. Transgenic (left) and wild type (right) plants.

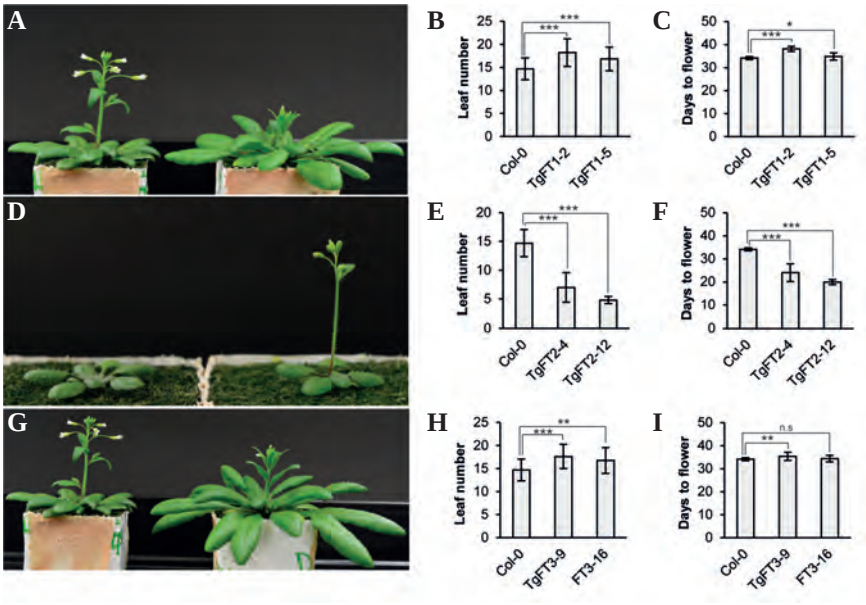


Figure 3. Flowering time phenotypes of the tulip *FT-like* genes overexpression lines in Arabidopsis. (A-C) Overexpression of *TgFT1*. (D-F) Overexpression of *TgFT2*. (G-I) Overexpression of *TgFT3*. Wild type Col-0 is depicted on the left and the overexpression lines on the right. The single asterisk indicates a significant difference with a p-value of ≤0.05, the double asterisk a p-value of ≤0.01 and the triple asterisk a p-value of ≤0.001.

reduction of leaf number and days to flowering in comparison to wild type Col-0 (Figure 3D-F). Thus, *TgFT2* seems to be a positive regulator of flowering, while *TgFT1* and *TgFT3* may act as negative regulators of flowering in tulip.

Protein-protein interaction capacity of the lily and tulip PEBP proteins

Overexpression of lily *LlFT* and tulip *TgFT2* resulted in an early flowering phenotype, suggesting that both bulbous plant FT proteins can interact with the bZIP transcription factor AtFD from Arabidopsis. This hypothesis was tested by yeast two hybrid analyses (Fig. 4A). Both LlFT and TgFT2 were able to interact with AtFD and AtFD PARALOG (AtFDP), confirming our hypothesis and providing an explanation for the early flowering phenotype observed in Arabidopsis. Remarkably, TgFT1 was also able to interact with both AtFD and AtFDP, but its ectopic expression resulted in late flowering. In contrast, TgFT3 did not interact with AtFD or AtFDP in yeast. Besides bZIP transcription factors, AtFT is able to interact with specific members of the TCP family (Taoka et al., 2013) and this unique interaction pattern can be used to distinguish FT from TFL. All twenty four members of the Arabidopsis TCP family were tested for interaction with the lily and tulip PEBP proteins (Fig. 4B). The lily FT-like protein LlFT appeared to interact with AtTCP3, AtTCP8, AtTCP14, AtTCP15, AtTCP16, AtTCP18, AtTCP20 and AtTCP23. Remarkably, TgFT1 is able to interact with almost all members of the TCP family, except for AtTCP2, AtTCP6 and AtTCP8. When comparing the interaction pattern of TgFT1 with AtTSF, there is a large overlap. For example, AtTSF is able to interact with AtTCP5, TCP9 and TCP10, just like TgFT1, while AtFT is not interacting with these specific TCP proteins. TgFT2 appeared to be the most similar protein to AtFT in terms of interaction pattern. In the case of TgFT3 a limited number of interactions was observed (AtTCP14, AtTCP16, AtTCP18, AtTCP20, AtTCP22 and AtTCP23). Thus, the yeast two hybrid assays revealed differentiation in protein-protein interaction capacity within the tulip PEBP protein family.

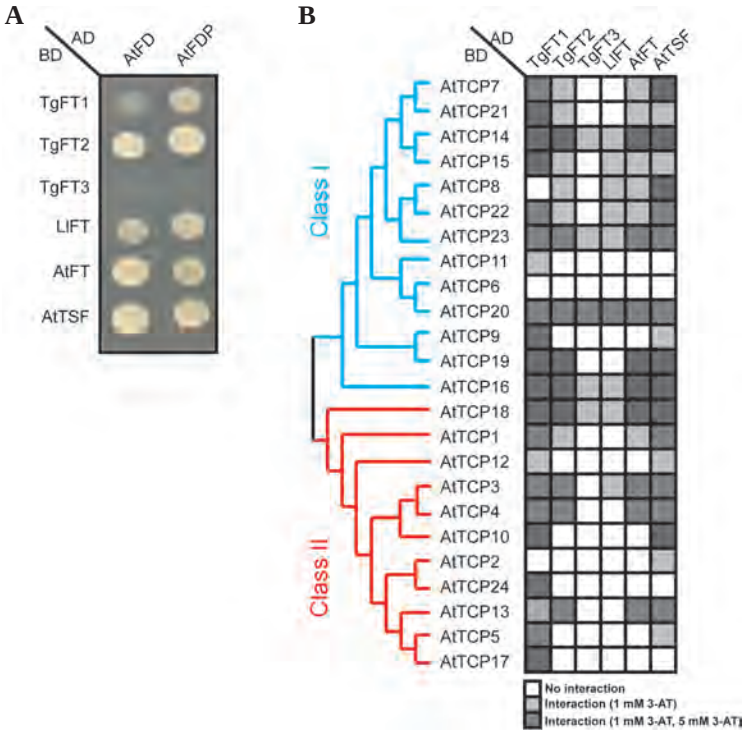


Figure 4. Overview of protein-protein interaction patterns of the lily and tulip PEBP proteins. (A) Protein-protein interactions of lily and tulip PEBP proteins with AtFD/AtFDP. (B) Protein-protein interaction screening of lily and tulip PEBP proteins with all Arabidopsis TCP transcription factors (red: class I; blue: class II).

Conversion of *TgFT2* and *TgFT3* function

TgFT2 induced early flowering in Arabidopsis, while *TgFT3* weakly repressed the floral transition (Fig. 3). In addition, the protein-protein interaction profile of TgFT2 and TgFT3 varied to a large extent (Fig. 4). Nevertheless, their expression patterns overlap during development in tulip, with the exception of *TgFT2* which is expressed in the flower until May (Fig. 1C). To investigate which amino acid difference might be responsible for the differences in functioning between TgFT2 and TgFT3, substitution lines were created based on native differences in conserved domains between these two PEBP proteins. L128E and Q140P substitutions were generated in TgFT2 and the complementary E125L and P137Q were made in TgFT3. These particular positions in the protein are known to be critical

for FT function in Arabidopsis (Ho and Weigel, 2014). For both substitution overexpression lines were created and flowering time was determined in the obtained *35S:TgFT2(L128E/Q140P)* and *35S:TgFT3(E125L/P137Q)* transgenic lines (Fig. 5). When overexpressing *TgFT2* with L128E and Q140P substituted, the plants flowered later than the wild type (Fig. 5A-C), in contrast to the early flowering observed upon ectopic expression of the native TgFT2 (Fig. 3). Overexpression of TgFT3 with E125L and P137Q substituted resulted in earlier flowering than wild-type for one of the lines (Fig. 6D-F), whereas the native TgFT3 protein represses flowering in Arabidopsis. Nevertheless, both lines were flowering significantly earlier in comparison to the native TgFT3 overexpression lines. To get more insight in the molecular reason for this observed functional switch, yeast two hybrid assays were performed. Despite a different functioning of the mutated proteins in Arabidopsis, their heterologous interaction with selected Arabidopsis proteins did not change (Fig. S3).

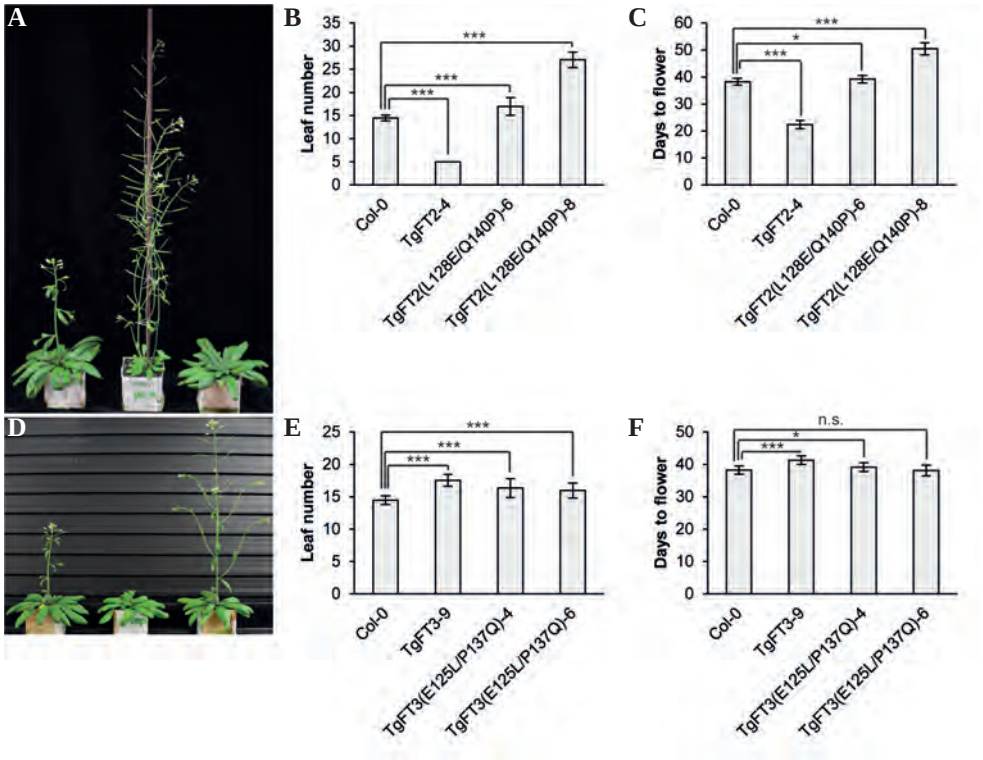


Figure 5. Phenotype of the substitution lines of *TgFT2 (35S:L128E/Q140P)* and *TgFT3 (35S:E125L/P137Q).* (A-C) Overexpression of *TgFT2* with substitutions L128E and Q140P. (D-F) Overexpression of *TgFT3* with substitutions E125L and P137Q. Wild type Col-0 is depicted on the left, overexpression of the original PEBP genes in the middle and the substitution lines on the right. The double asterisk indicates a significant difference with a p-value of ≤0.01 and the triple asterisk indicates a p-value of ≤0.001.

Predicted protein structure of TgFT2 and TgFT3

The lack of changes in protein-protein interactions for the substituted TgFT2 and TgFT3 proteins raised the question if TgFT2 and TgFT3 may differ in 3D structure and properties of motifs not involved in the analyzed protein-protein interactions. Therefore, the structures of both TgFT2 and TgFT3 proteins were predicted using homology modelling (Fig. 6). The resulting structure models indicate that the two substituted residues (L128, Q140) do not map close to the interface on FT important for the interaction with the bZIP proteins FD/FDP that is bridged by 14-3-3 proteins (Taoka et al., 2013). This suggests that the FAC complex still can be formed to activate the floral meristem identity genes (Li et al., 2015). Both residues are however located at the surface of the FT-like protein and therefore, could potentially be involved in interaction with an unknown ligand or co-factor.

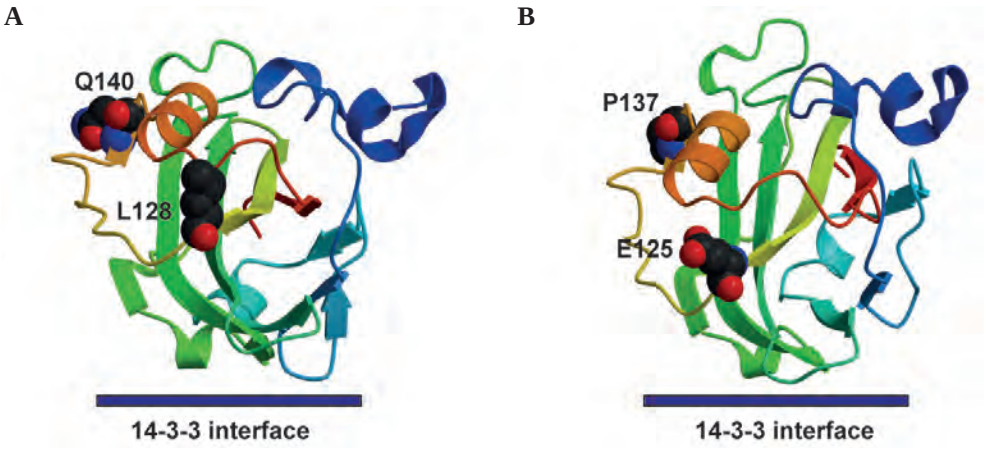


Figure 6. Predicted protein structure of TgFT2 (A) and TgFT3 (B). Mutated residues are indicated, as is the interface for interaction with 14-3-3 proteins.

Phylogenetic analysis of PEBP protein sequences identified in various bulbous species

In this study we have identified four new PEBP family genes in the bulbous species lily and tulip. In several previous studies PEBP family genes of bulbous plants were identified, in *Allium cepa* (onion), *Allium sativum* (garlic), *Narcissus tazetta* (daffodil), *Crocus sativus* (Saffron crocus) and Tulipa gesneriana (Tsaftaris et al., 2012; Lee et al., 2013; Noy-Porat et al., 2013; Kamenetsky et al., 2015; Leeggangers et al., submitted). In order to estimate the relationship between PEBP sequences of these bulbous plants, a phylogenetic analysis was performed (Fig. 7).

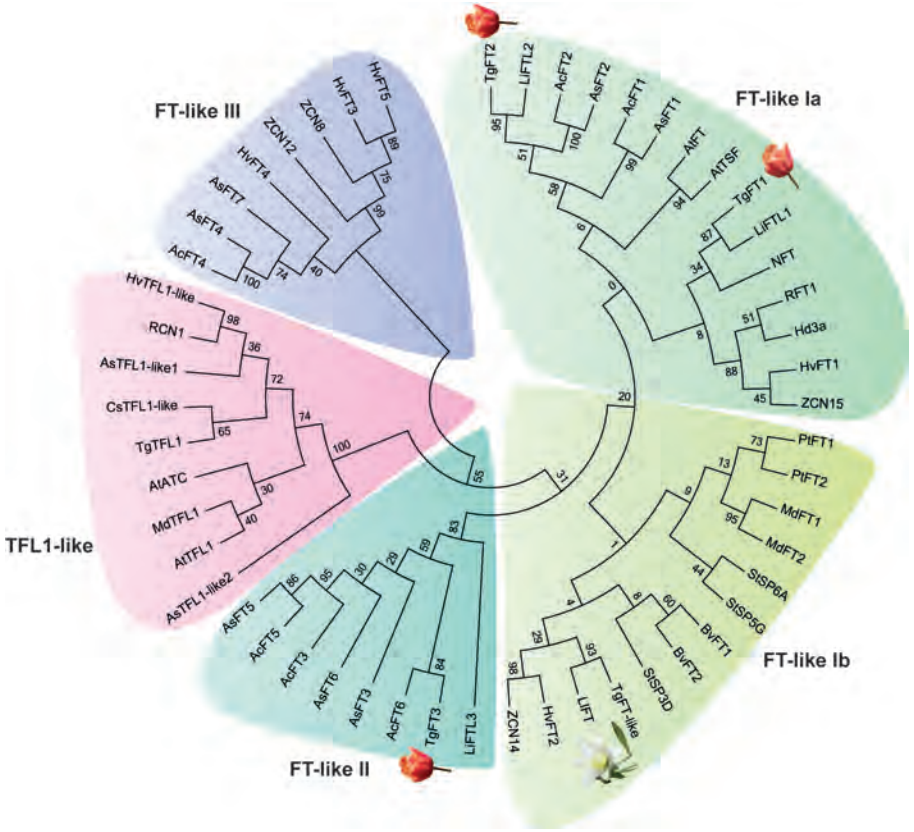


Figure 7. Maximum likelihood tree with PEBP family protein. Included are FT(-like) and TFL1(-like) sequences from *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Hordeum vulgare*, *Beta vulgaris*, *Populus trichocarpa*, *Solanum tuberosum*, *Malus domestica*, *Allium cepa*, *Allium sativum*, *Narcissus tazetta*, *Crocus sativus*, *Lilium longiflorum*, *Lilium* spp. and *Tulipa gesneriana*.

In the constructed maximum likelihood tree, four FT-like clades and one TFL1-like clade could be distinguished. The FT-like Ia clade contains TgFT1 and TgFT2 from tulip as well as AtFT and AtTSF from Arabidopsis. Furthermore, additional bulbous FT-like sequences are present in this clade which, based on this phylogenetic classification, are hypothesized to act as FT. Numerous other monocot FT-like proteins, including LlFT and a few eudicot FT-like proteins are present in FT-like clade II. Other functionally characterized FT-like proteins present in this clade are the antagonistic FT proteins of *Beta vulgaris* (sugar beet; BvFT1 and BvFT2; Pin and Nilsson, 2012), the flowering time regulators Hd3a and RFT1 from *O. sativa* (Komiya et al., 2008) and the tuberization controlling protein StSP6A of *S. tuberosum* (potato; Navarro et al., 2015). Remarkably, the FT-like II clade represents FT-like proteins of bulbous species only, including TgFT3 of tulip, which has been analyzed in this study. In the TFL1-like clade all TFL1 and CENTRORADIALIS (CEN) proteins are present. In FT-like clade III the bulbing controlling AcFT4 of *A. cepa* is present and it is highly homologous to an FT sequence identified in the RNA-sequencing data described in the study of Kamenetsky et al. (2015) on garlic, designated as AsFT4. Other closely-related sequences within this clade are from *H. vulgare* and *Zea mays* (maize). In the TFL1-like clade, it appears that A. sativum likely has two TFL1-like sequences, here designated as AsTFL1-like1 and AsTFL1-like2. Nevertheless, functional analysis is required to confirm their function as flowering repressors in *A. sativum*. Furthermore, the two bulbous proteins TgTFL1 and CsTFL1-like are closely related; together with HvTFL1-like from *H. vulgare* and RCN from *O. sativa*. This analysis shows that until now identified bulbous PEBP family sequences are scattered through the maximum likelihood tree and therefore likely have diversified their function, e.g. AcFT4 is involved in bulbing and StSP6A in tuberization (Lee and Lee, 2010; Navarro et al., 2011).

Discussion

The floral integrator *FT*, a member of the PEBP gene family, is one of the most studied genes in the control of flowering time. In many plant species,

varying from the biofuel plant Jatropha curcas to the evergreen conifer *Pinus sylvestris* (Scots pine), *FT-like* genes have been identified (Avia et al., 2014; Li et al., 2014). In this study, four PEBP family genes of *L. longiflorum* and *T. gesneriana* are described and functionally characterized in Arabidopsis, and *LlFT* as well in *L. longiflorum*.

Based on phylogeny, LlFT of lily and TgFT1 and TgFT2 of tulip can be classified as FT-like proteins, while TgFT3 shared a weaker similarity (~50%) with AtFT, Hd3a and RFT1. Consequently, it grouped in another FT-like sub-clade that consisted of bulbous plant PEBP proteins only. This classification and the importance of specific conserved amino acid residues is further discussed in view of the potential role of these proteins in bulbous species, with a special focus on flowering time control.

The power of amino acid substitutions in TgFT2 and TgFT3 and their change in function

Several studies have focused on exchanging amino acids between FT and TFL1 to convert their flowering activating and repressing functions, respectively. Additionally, random mutagenesis of conserved amino acids shed light on important amino acid residues and domains in these PEBP proteins (Hanzawa et al., 2005; Ho and Weigel, 2014). In this study we exchanged conserved amino acids that are assumed to be important for the flowering activating FT function and that are naturally occurring in tulip TgFT2 but only partially in TgFT3. In the study of Ho and Weigel (2014), it was shown that changing Leu-128 into a charged Lys in the AtFT protein resulted in a late flowering phenotype upon overexpression, mimicking weak TFL1 activity. TgFT2 with L128E substituted also undergoes a change from a hydrophobic to a charged residue, which in combination with the Gln-140 to Lys substitution led to a late flowering phenotype upon ectopic expression in Arabidopsis. The two substituted amino acid residues are close to a binding pocket, which, based on findings in non-plant species, is supposed to be important for the binding of anions, phosphate groups, and phospholipids. Both TgFT2 and TgFT3 have Y85 in this pocket,

but only TgFT2 contains Q140, while TgFT3 has a Pro at the analogous position. This change might affect the binding pocket and therefore also the binding of the co-factor. Previously, it has been suggested that the TCP family of transcription factors are candidates for mediating differential activity of FT and TFL1, because of specific differential interactions of FT and TFL1 with TCP family proteins (Ho and Weigel, 2014). However, for the tulip TgFT2 and TgFT3 proteins, interactions with Arabidopsis TCPs were not changed upon the amino acid substitutions. Similar observations with mutations at analogous positions have been shown in the study of Ho and Weigel (2014). Hence, the complex formation with TCPs seems not to make the functional difference in this particular case. This still leaves open the option of differential interactions with other co-factors. Nakamura and colleagues (2014) revealed that FT can interact with the phospholipid phosphatidylcholine (PC). The increase in PC levels accelerates flowering, while a decrease in PC levels suppresses flowering (Nakamura et al., 2014). Based on the position of the mutations in the two tulip proteins it is tempting to speculate that interactions with phospholipids are impaired in TgFT3 and TgFT2(L128E/Q140P), causing the differential flowering time response.

Possible functions of FT-like proteins in lily and tulip

LlFT

In lily, *LlFT* is noticeably up-regulated by cold exposure. Overexpression in transgenic lily led to flowering under non-inductive conditions – no cold and SD. Under these conditions, wild type plants produce a large number of leaves but do not flower (Lugassi-Ben Hamo et al., 2015; Lazare and Zaccai, 2016). These results clearly hint at the involvement of LlFT in the vernalization response of lily, and are able to substitute for cold exposure. *LlFT* may even act as a major regulator of flowering within the vernalization pathway as reported for BvFT2 in *B. vulgaris* (Pin and Nilsson, 2012), PtFT1 in poplar (Hsu et al., 2011) and *AcFT2* in A. cepa (Lee et al., 2013).

On the other hand, *LlFT* expression was lower in meristems of plants developing from cooled bulbs than in meristems of plants developing from

non-cooled bulbs. As plants from non-cooled bulbs will remain vegetative and plants from cooled bulbs will flower, the difference between these two groups is the competence of their meristem to flower. Moreover, *LlFT* expression decreases even further in the meristem at the floral transition stage and in small flower buds (Mazor, 2015). It is therefore tempting to assume that *LlFT* is involved in generating meristem competence for flowering inducing signals during vernalization (and its over-expression was enough to confer flowering competence without vernalization), but that it does not act as flowering inducer afterwards. In line with this, its expression declines when the meristem approaches the reproductive stage. In this sense, LlFT may be compared to PtFT2 from poplar, found to promote vegetative growth (Hsu et al., 2011) and proposed to be involved in creating meristem competence for flowering signals as well (Pin and Nilsson, 2012). Furthermore, PtFT2 controls dormancy in poplar (Hsu et al., 2011). This makes LlFT and PtFT2 interesting candidates to investigate the somewhat overlapping mechanism of vernalization and dormancy break (Brunner et al., 2014).

LlFT was able to interact with AtFD and AtFDP, and, consequently, transgenic Arabidopsis lines over-expressing *LlFT* showed an early flowering phenotype. However, this phenotype was rather mild, which is in line with the assumed role of LlFT in the vernalization response rather than acting as a true 'Florigen' protein. It is reasonable to assume that additional *FT-like* genes in lily will have other functions, including flowering and induction.

TgFT1 and TgFT2

In the FT-like Ia sub-clade, which contains the Arabidopsis 'florigen' proteins AtFT and AtTSF, two tulip proteins were found, TgFT1 and TgFT2. In this sub-clade also the *A. cepa* (onion) AcFT2 protein is present, which was shown to act as an inducer of flowering (Lee et al., 2013). Furthermore, the *A. sativum* (garlic) AsFT2 protein is present here and expression analysis for the gene encoding this protein revealed a perfect correlation with flowering induction (Shalom et al., 2015). Altogether, these findings

strongly support a function of TgFT1 and TgFT2 as flowering inducers in tulip; however, as discussed later this may not be the true function of TgFT1. For both these two tulip FT-like genes, an increase in the expression was detected in the vegetative organs during plant development, culminating in May, when floral induction is initiated in the daughter bulbs (De Hertogh and Le Nard, 1993).

For TgFT2 the hypothesized role in flowering induction is further supported by the strong early flowering phenotype observed in the transgenic Arabidopsis plants over-expressing *TgFT2* and the interaction of the protein with both AtFD and AtFDP. Hence, we propose that *TgFT2* acts as a flowering enhancer. Surprisingly, ectopic expression of *TgFT1* in Arabidopsis resulted in delayed flowering. Based on this it may be hypothesized that *TgFT1* acts in an antagonistic way with *TgFT2* to regulate floral transition in tulip. A similar antagonistic functioning of FT-like proteins has been reported for other biennial and perennial species, such as BvFT1 and BvFT2 in *B. vulgaris* (sugar beet) (Pin and Nilsson, 2012), FT1 and FT2 in poplar (Hsu et al., 2011), and DlFT1-3 in *Dimocarpus longan* (longan) (Heller et al., 2014). Based on these observations, it seems that this balancing activity between two counteracting FT-like proteins is a specific characteristic of biennial and perennial species and in a recent review the term 'antiflorigen' was introduced for the counteracting FT proteins (Putterill and Varkonyi-Gasic, 2016) Nevertheless, further functional studies and detailed expression studies are essential to proof that *TgFT1* indeed acts as a repressor of the floral transition in tulip in competition with *TgFT2*.

The tulip *TgFT1* and *TgFT2* genes are also expressed in the flower organs, suggesting a possible role in flower maturation even at later stages. *FT-like* gene expression has also been observed during florogenesis in the bulbous species *Narcissus* (Noy-Porat et al., 2013), and in flowers and fruits in apple (Kotoda et al., 2010). Furthermore, in Arabidopsis it was suggested that high *FT* expression after the initial induction of the reproductive phase and during inflorescence development prevents a reversion to the vegetative stage (Liu et al., 2014). In order to identify a possible function associated

associated to the 'late' flower expression of these two tulip *FT-like* genes, additional functional studies are needed.

TgFT3

The tulip TgFT3 protein is part of a PEBP family sub-clade FT-like II that contains solely proteins from geophytes. As observed in this study for *TgFT3*, overexpression of the onion *AcFT3* and *AcFT5* from this specific sub-clade resulted in late flowering in Arabidopsis. How TgFT3 can act as repressor of flowering in Arabidopsis is not clear. In contrast to LlFT, TgFT1 and TgFT2, TgFT3 was not able to interact with either AtFD or AtFDP. However, AtBRC1 (TCP18) is interacting with TgFT3 in the two-hybrid assay and this protein was shown to interact with Arabidopsis FT in order to delay the floral transition in the axillary meristem (Poza-Carrión et al., 2007). Commonly cultivated tulip cultivars only have one flower, but some cultivars are multi-flowered (e.g. cultivar *Tricolette*). In the multi-flowered cultivars, with only one stem, the flower appears from the floral stem when the apical bud is blooming (Fig. S4). *TgFT3* is expressed in the stem at the moment before and during blooming and therefore might prevent the formation or growth of "axillary" floral buds. However, it is too speculative to draw any conclusions in this direction based on the current knowledge about *TgFT3*.

Diversification of PEBP functions in bulbous and other plant species

As FT-like genes are characterized in an increasing number of plant species, it becomes obvious that these genes are involved in an array of functions, additional to the important role of being a florigen. For example, StSP6A from *Solanum tuberosum* (potato) controls tuberization (Navarro et al., 2011) and this activity is counteracted by StSP5G (Navarro et al., 2015; Abelenda et al., 2016). Furthermore, AcFT1 and AcFT4 from *A. cepa* control bulbing in a similar counteracting manner (Lee et al., 2013). In geophytes, the storage structure is actively involved in reproduction. It contains the vegetative reproduction organs and it serves as an energy

source for flowering and sexual reproduction. Therefore, this group of plants is particularly interesting as they can reveal the diverse functions of *FT-like* genes within the same structure, the bulb. Even though only four PEBP genes of lily and tulip have been analyzed in this study, their distribution over the phylogenetic tree in the different clades with divergent PEBP proteins of other species can be interpreted as vast diversification of the *FT-like* genes. Essentially, tulip *TgFT2* might be considered as florigen, acting on the switch from vegetative to reproductive stages. On the other hand, LlFT was clearly associated with the vernalization response of the plant. This discrepancy might stem from the basic difference in the flowering physiology of both species. In tulip, floral transition occurs within the bulb and is induced by high temperatures. This is the case of many other geophytes (Kamenetsky and Okubo, 2013), including *Narcissus* (Noy-Porat et al., 2013) and saffron (Tsaftaris et al., 2012). An overview of PEBP family proteins identified in tulip and lily and their potential role during plant development is given in figure 8.

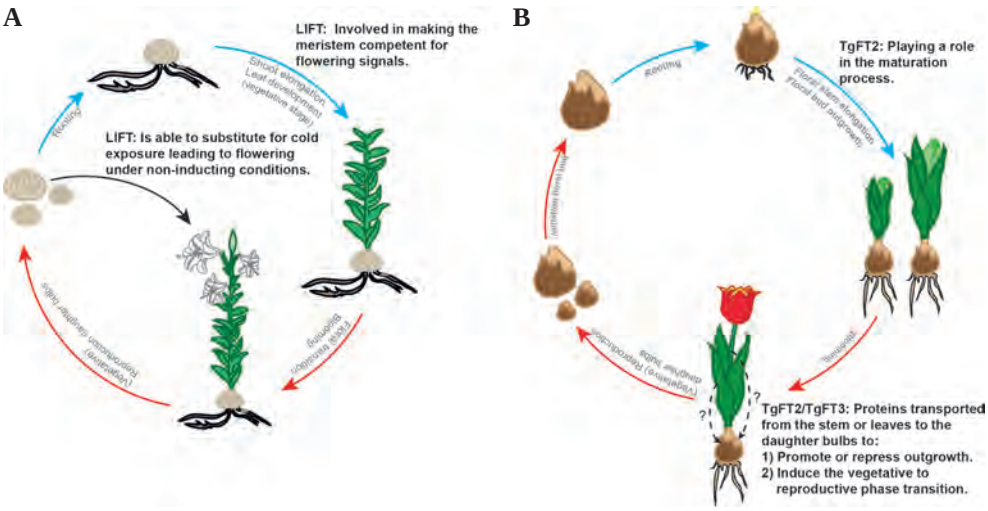


Figure 8. Schematic overview of the hypothesized roles of LlFT in lily (A) and TgFT1, TgFT2 and TgFT3 in tulip (B). An overview of the annual growth cycle is given with an indication of the different *FT-like* genes and their possible function during development. The blue arrows indicate the cold period and the red arrows indicate the warm period.

Acknowledgements

We thank Froukje van der Wal and Martijn Fiers of Wageningen University and Research, Bioscience for growing wild-type Col-0 plants for transformation and Maarten Holdinga for planting and taking care of the tulips in the field. We thank Twan de Groot who was involved in the cloning of the tulip FT-like genes and Dr. P. Arens from Wageningen University and Research, Plant Breeding for delivering additional lily sequences for the phylogenetic study. We thank Silit Lazare for lily pictures and plant growth and Rachel Shami for technical help. Part of the lily research was funded by the Marie Curie Actions—Industry-Academia Partnerships and Pathways (IAPP) (Project number 286175). Funding of the tulip project was kindly provided by TTI-Green Genetics and the Dutch Ministry of Economic Affairs.

Supplemental material

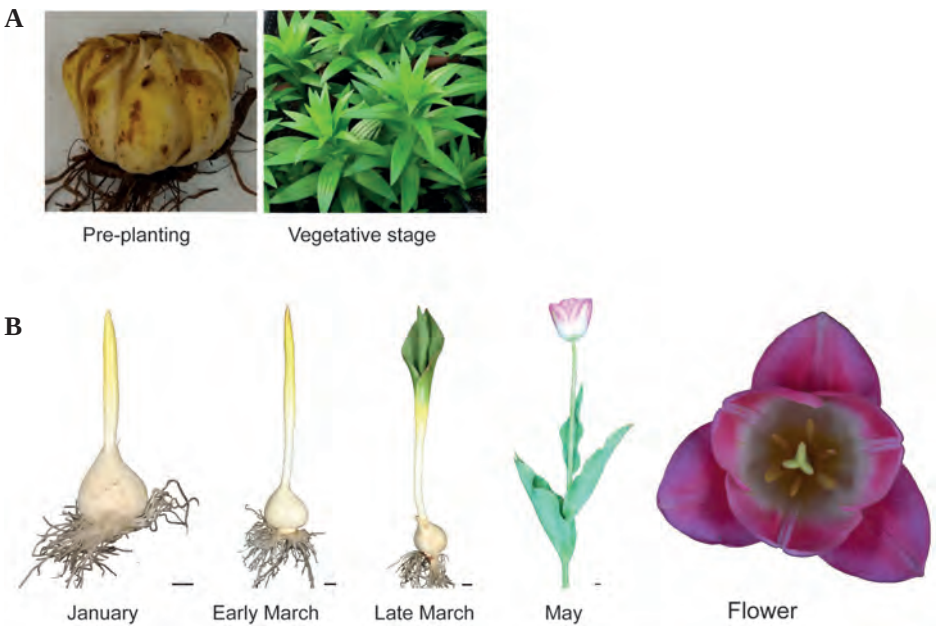


Figure S1. Morphology of lily and tulip tissues at different developmental stages. (A) Morphology of the lily tissues used for determination of the expression of the *FT-like genes*. (B) Morphological changes during development in tulip.

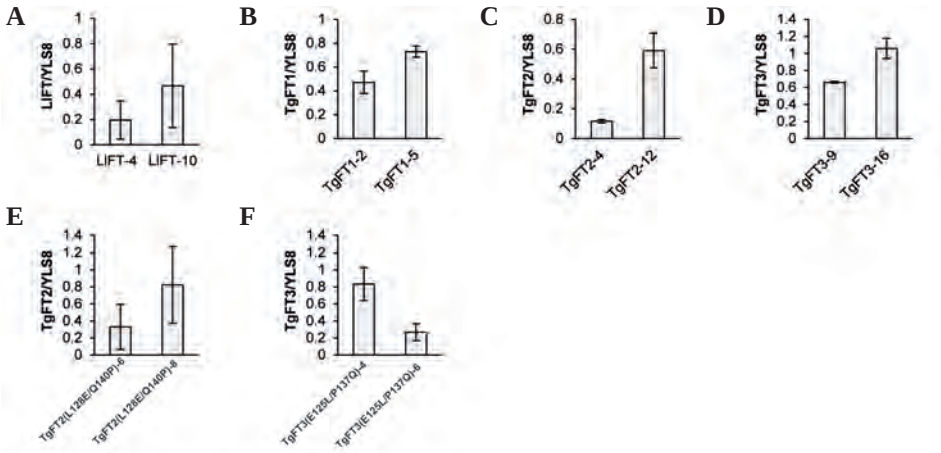


Figure S2. Expression analysis of the introduced lily and tulip PEBP transgenes in *Arabidopsis thaliana*. (A) Expression of *LlFT* in the lines LlFT-4 and LlFT-10. (B) Expression of *TgFT1* in the transgenic lines TgFT1-2 and TgFT1-5. (C) Expression of *TgFT2* in the lines TgFT2-4 and TgFT2-12. (D) Expression of *TgFT3* in the lines TgFT3-9 and TgFT3-16. (E) Expression of *TgFT2* in the substitution lines. (F) Expression of *TgFT3* in the substitution lines.

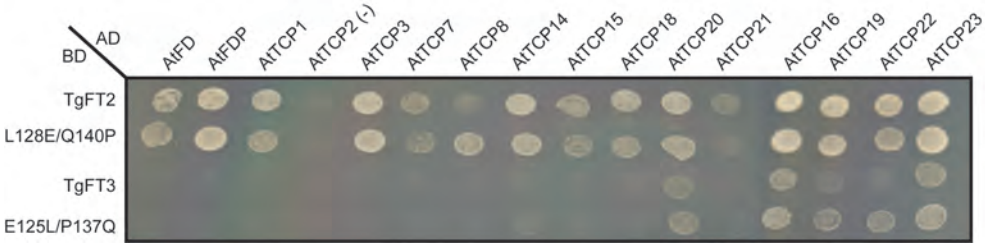
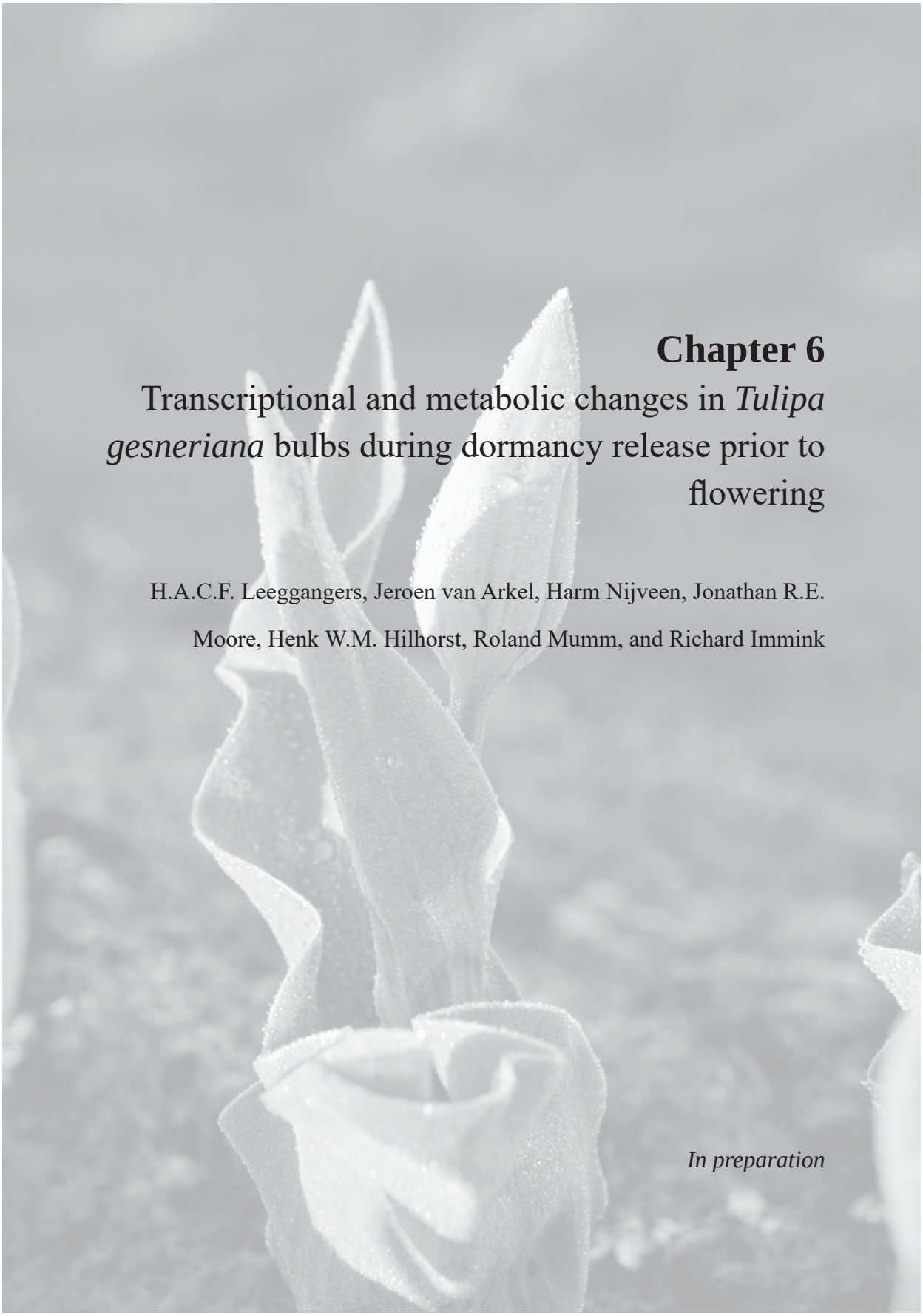


Figure S3. Yeast two hybrid assay of TgFT2 (L128E/Q140P) and TgFT3 (E125L/P137Q). After mating yeast was spotted on SD glucose -LWH and 5 mM 3-AT selective medium and picture was taken after five days incubation at 20°C.

Fig S4. Example of a multi-flowered tulip.



Chapter 6

Transcriptional and metabolic changes in *Tulipa gesneriana* bulbs during dormancy release prior to flowering

H.A.C.F. Leeggangers, Jeroen van Arkel, Harm Nijveen, Jonathan R.E. Moore, Henk W.M. Hilhorst, Roland Mumm, and Richard Immink

In preparation

Abstract

In the ornamental geophyte *Tulipa gesneriana* dormancy is established during the summer period. After development of the floral bud inside the bulb, tulips require a period of prolonged cold to release dormancy in order to flower the following spring. Molecular changes during this cold period are important for floral stem elongation and preparation of the floral bud to flower. However, little is known about these changes and therefore the morphological, transcriptional, and metabolic changes were studied in the floral stem, floral bud and developing leaves, starting during the storage period and continued in the field during the cold winter period. For all measured tissues, increase in length was correlated with an increase of glucose content. Surprisingly, during the storage period, the floral bud appeared to be very active at the transcriptional level; however, shortly upon planting in the field, the bud becomes the least active tissue in transcription and growth. This suggests a type of floral bud dormancy in tulip bulbs, which seems to be epigenetically regulated and resembling bud dormancy in trees. A combined view on the transcriptome and primary metabolite changes during the winter in the floral bud suggests that during this dormancy period cell division occurs, which is accompanied by cell wall formation. After approximately ten weeks in the field (end of January), all tissues displayed acceleration of growth and dormancy is released in all tissues. At this moment, photosynthesis related genes are starting to be expressed in the leaves, suggesting preparation for photosynthetic activity while still beneath the soil surface. Altogether, these observations shed light on the processes ongoing inside a tulip bulb during the cold winter period and our preliminary analyses set the stage for additional investigations in the future to obtain a full understanding of how the bulb prepares for full flowering in spring.

Introduction

Plants have evolved a survival mechanism which allows them to anticipate environmental changes during the growth season. This survival mechanism, often referred to as dormancy, secures the plants reproduction success by the temporary arrest of growth and development under harsh or less desirable conditions. Dormancy is widely studied in seeds, apical buds and vegetative axillary buds of annual and perennial plants (Finch-Savage and Leubner-Metzger, 2006; Rohde and Bhalerao, 2007). Nonetheless, also in geophytes the temporarily arrest of growth is embedded in their annual growth cycle (Borochov et al., 1997; Kamenetsky et al., 2003). In the ornamental geophyte *Tulipa gesneriana*, dormancy is induced in the daughter bulbs by high temperatures in spring after flowering of the mother bulb. Subsequently a period of so-called summer dormancy takes place in which the daughter bulbs are surrounded by a tunic (dried scale) and seem to be completely inactive. Nevertheless during the first weeks of this period the further development of the floral bud occurs inside the bulb (De Hertogh and Le Nard, 1993). After completion of floral bud development (stage G), the tulip bulbs require a period of prolonged cold to break the dormancy, in order to be able to flower the following year in spring (Khodorova and Boitel-Conti, 2013). Without a sufficient period of low temperature during the tulip's annual cycle, the growth of the sprout is slow, the floral stem elongates poorly and the flowers are small or will even abort (De Hertogh and Le Nard, 1993).

The dormancy process in ornamental geophytes is studied to a lesser extent than e.g. in seeds or axillary buds of trees. In seeds the dormancy process is controlled by a balance of the phytohormones abscisic acid (ABA) and gibberellin (GA). During the maturation stage of seed development, endogenous ABA is present and induces seed dormancy to prevent precocious germination of seeds on the mother plant (vivipary). Antagonistically, GA breaks dormancy and stimulates germination by suppressing ABA-induced seed dormancy (Finch-Savage and Leubner-Metzger, 2006; Rodríguez-Gacio et al., 2009). Important genes controlling

seed dormancy are *ABA INSENSITIVE 3* (*ABI3*), *ABI4*, *DELAY OF GERMINATION* (*DOG1*) and *MOTHER OF FLOWERING LOCUS T* (*MFT*) (Finkelstein, 1994; Bentsink et al., 2006; Xi et al., 2010; Shu et al., 2013).

In trees several other genes have been associated with the dormancy process. These include the *DORMANCY ASSOCIATED MADS BOX* (*DAM*) genes, which share a high percentage of homology with *SHORT VEGETATIVE PHASE* (*SVP*) and *AGAMOUS-LIKE24* (*AGL24*) of *Arabidopsis thaliana*. *DAM* genes were identified in *Euphorbia esula* (leafy spurge), *Malus domestica* (apple), *Pyrus pyrifolia* (Asian pear), P*runus persica* (peach) and *Actinidia chinensis* (kiwifruit; Li et al., 2009; Horvath et al., 2010; Wu et al., 2011; Mimida et al., 2015; Niu et al., 2016). Detailed expression studies revealed that their activity correlates perfectly with the dormancy state of the vegetative buds, with high expression during the low temperature period which decreases prior to dormancy release or bud burst (Leida et al., 2012). However, whether these transcripts are regulators of dormancy or are changed in abundance as a consequence of a changed dormancy status remains to be seen. A more established regulatory network is known for the control of growth cessation in trees and this network involves the *CONSTANS* (*CO*) and *FLOWERING LOCSUS T* (*FT*) genes. In Arabidopsis, both CO and FT play a role in the regulation of flowering time in response to the photoperiod (Imaizumi and Kay, 2006). The tree ortholog of FT, identified in *Populus trichocarpa* (*PtFT*), is involved in the control of growth cessation and bud set in fall (Böhlenius et al., 2006). Both growth cessation and bud set are photoperiodically controlled. Besides CO and FT, this photoperiodic induction also involves photoreceptors and the circadian clock (Cooke et al., 2012). Furthermore, in some species growth cessation and dormancy can be induced by low temperature, which, for example, is the case for *M. domestica* and *Pyrus communis* (pear). These species develop so-called winter buds after growth cessation. Once the winter buds are established by temperatures below 12°C, dormancy is induced, which can then be released by a prolonged period of winter cold (Olsen, 2010).

The required period of winter cold, often referred to as chilling requirement, represents a natural process of breaking dormancy. In seeds this mechanism ensures that germination occurs in spring, and in trees it ensures that the buds burst after winter (Probert, 2000). Similar to tree buds, low temperatures break dormancy in tulip, stimulating floral stem elongation and full flowering in spring (Moe and WickstrØm, 1973; Lambrechts et al., 1994; Kamenetsky et al., 2003). The effect of low temperature on floral stem elongation and flowering in tulip has been extensively studied at the physiological level over the last decade (Lambrechts et al., 1994; Saniewski and Okubo, 1997; Rietveld et al., 2000). The hormones auxin and GA were identified to play a role in the elongation of the floral stem and sensing of low temperature (Aung et al., 1969; Saniewski and Okubo, 1997; Rietveld et al., 2000). Furthermore, the carbohydrate status of the tulip bulbs during winter has often been investigated in relation to floral stem elongation. One of the main events during low temperature perception is the degradation of starch in the tulip bulb scales, resulting in the accumulation of monosaccharides, such as glucose and sucrose (Lambrechts et al., 1994; Smith et al., 2005). This degradation of starch correlates with the increase of alpha-amylase activity in the scales. According to Moe and Wickstrom (1973), besides alpha-amylase, various other metabolic enzymes, e.g. invertases, are induced by low temperature (chilling) in tulips. Chilling also enhances the mobilization of fructans and sucrose in the scales (Moe and WickstrØm, 1973; Ho and Rees, 1975; Lambrechts et al., 1994).

Surprisingly little is known about the influence of low temperature on molecular changes in tulip bulbs and whether chilling has distinct effects on the various bulb organs and tissues. Additionally, most studies in the past investigated changes at low temperature during the storage period prior to planting. This treatment is commonly used to control the timing of flowering and aiming to produce tulips early during winter (‘forcing’; Kamenetsky and Okubo, 2013). In this study, we decided to follow transcriptional and metabolic changes in tulip bulbs that had been stored at ambient

temperature (18-20°C) conditions followed by planting in the field and development during winter. Initially, a detailed morphological investigation of the dormancy period was performed, starting in storage from one month before planting until four months after planting of the bulbs in the field. During this time frame, samples were collected from various bulb tissues for expression analysis and metabolic status investigations. These analyses revealed that the floral bud is the least active tissue in the field during winter, in comparison to the floral stem and leaves. However, based on the transcriptome and primary metabolite data, processes in relation to growth, such as cell division and -extension, are occurring in this phase. All tissues displayed acceleration of growth around week ten, which suggests dormancy release of the complete bulb. For all activities until this moment the bulb scales are the energy source. After dormancy is released, photosynthesis-related genes are expressed in the leaves, signifying the preparation for photosynthesis. Taken together this suggests a transition in the leaves from sink to source to provide energy for the flowering process.

Material & Methods

Plant material and growth conditions

Tulipa gesneriana cv. 'Strong Gold' bulbs (size 9/10) were stored from August 2013 to mid-November 2013 at 18-20°C. Two weeks prior to planting the storage temperature was dropped to 18°C and the bulbs were planted mid-November 2013 in the field at Wageningen University (The Netherlands). During this whole period, the temperature was measured with an EasyLog EL-USB-1-PRO (Lascar Electronics, United Kingdom). Samples of the second scale (counted from outer to inner), floral stem, floral bud and leaves were collected almost every two weeks from October 2013 until the end of February 2014 (week -4, 0, 2, 4, 6, 8, 10, 12, 14 and 16). All samples were collected in the afternoon between 1 pm – 3 pm Central European Time. Three times ten bulbs, each a biological replicate, were used for dissecting the scale, floral stem, floral bud and leaf tissue.

During dissecting the length of the floral stem, floral bud and leaves were measured by a ruler. All tissue was homogenized by the use of liquid nitrogen, mortar and pestle and stored at -80°C until further usage.

Total RNA extraction and cDNA synthesis

To extract RNA of the scale tissue, the Tripure protocol (Roche, The Netherlands) was used according to manufacturer's manual with the addition of 2% polyvinylpyrrolidone (PVP, w/v) and 2% β-mercaptoethanol (β-ME, v/v) to the extraction buffer. Subsequently, DNase treatment was performed with RQ1 (Promega, The Netherlands) followed by a phenol/chloroform (1:1) extraction and ethanol precipitation. RNA extraction of the leaves, stem and floral bud was performed with the Invitrap spin plant RNA mini kit (Invitek, ISOGEN Life Science, The Netherlands), which was used according to the manufacturers manual. DNase of Qiagen was used for the DNase treatment at the column. 500 ng of total RNA of each sample was used for cDNA synthesis, using M-MuLV Reverse Transcriptase (Thermo Scientific, The Netherlands), and Oligo dT primers. All reactions were performed in a Bio-rad MyCycler.

Strand-specific RNA-sequencing

Total RNA from scale, leaf and floral bud tissue collected between October 2013 and February 2014, was used for RNA-seq. For the preparation of the RNA-seq cDNA library, the TruSeq stranded mRNA sample preparation kit (Illumina, The Netherlands) was implemented according to the manufacturer's instructions. The quality of the libraries was examined with the Bioanalyzer 2100 DNA 1000 chip (Agilent Technologies, United States). The Illumina Hiseq 2500 platform was used for obtaining 250 bp paired-end reads.

RNA-seq expression analysis

The quality of the reads obtained from RNA-sequencing was examined by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Trimming of the reads by Trimmomatic (Bolger et al. 2014) was performed to improve the quality. After trimming, a denovo assembly by Trinity (Haas et al., 2013) was made with the RNA-seq data from scale, floral bud and leaf tissue. Kallisto version 0.42.1 (Bray et al., 2016) was used to quantify gene expression. Differential gene expression analysis was done with EdgeR package version 3.10/5 (Robinson et al., 2010), using the estimated counts produced by Kallisto as input.

Gene ontology (GO)-enrichment analysis

Based on the differentially expressed genes, the Plant GeneSet Enrichment Analysis Toolkit (PlantGSEA) was used for gene ontology (GO)-enrichment analysis. The hypergeometric statistical test method and the Yekutieli (FDR under dependency) multi-test adjustment method settings were applied for the analysis. The significance level and the False Discovery Rate (FDR) were set at 0.05.

Real-time PCR

Real-time PCR reactions were performed in a total volume of 20 µl containing 10 µl of iQ SYBR Green Supermix (Bio-rad, The Netherlands), 5 µl of each forward and reverse primer (0.05 µM) and 5 µl of a 1:15 dilution of the cDNA reaction mixture as template. Reactions were performed on a CFX Connect real-time PCR detection system (Bio-rad, The Netherlands) with an initial 3 min denaturation at 95°C followed by 40 cycles of 95°C for 10s and 60°C for 30s. Final steps used for elongation were 95°C for 1 min, 55°C for 10s and 95°C for 30s with afterwards a melt curve determination. Relative expression levels were calculated by the ΔΔCt method with TgACT as reference gene. Calculations were based on three technical replicates and two to three biological replicates.

Metabolic profiling

Profiling of the polar metabolites was done by coupled gas chromatography-time of flight mass spectrometry (GC-ToF-MS) similarly as described by

Lisec et al. (2006) and Carvalho et al. (2015). For this purpose, the collected material as described in the 'plant material and growth conditions' section was freeze dried followed by metabolic profiling. Before freeze drying the fresh weight was determined and after freeze drying the dry weight. For the bulb scales 100 mg dry weight was used for the extraction, while for the floral stem, floral bud and leaves 50 mg was used. In total 1.4 ml of a cold methanol solution (16.8 ug/ml Ribitol (Fluka), -20°C) was added to 2 ml safe-lock Eppendorf tube with the freeze dried material. Samples were shaken for 10 min at 70°C in a thermomixer (Vortemp, Labnet) at 950 rpm. After mixing, the samples were centrifuged for 10 min at 14000 rpm in an Eppendorf centrifuge and 500 ul of the supernatant was transferred to a 2.0 ml safe-lock Eppendorf tube. Successively, 375 µl chloroform (-20°C) and 750 µl cold dH2O (4°C) was added to the supernatant followed by mixing the samples for 10s and centrifuging for 15 min at 14000 rpm. Aliquots (100 µl) of the upper phase were dried in glass vials with 100 µl glass inserts by vacuum centrifugation for 16h. The dried samples were derivatised online as described by Lisec et al. (2006) using a Combi PAL autosampler (CTC Analytics AG).

The metabolic profile of the derivatized samples was analyzed by a GC-TOF-MS system consisting of an Optic 3 high-performance injector (ATAS GL Int., Eindhoven, the Netherlands), an Agilent 6890 gas chromatograph (Agilent Technologies), equipped with a VF-5 ms capillary column (Varian), and a Pegasus III time-of-flight mass spectrometer (Leco Instruments) detector. The instruments settings were used as described by Carvalho et al. (2015), with the adaption that the detector voltage was set to 1800v. GC-TOF-MS data processing methods were used as described by (Carvalho et al., 2015).

The analysis was done separately for the four different tissues. Principal component analysis (PCA) was done in Galaxy Biostar using the settings Log transformation and pareto scaling for transformation and scaling. Non pre-processed data was used for generating the PCA plots.

HPAEC analysis

Total carbohydrate content was extracted from freeze-dried bulb scale, stem, leaf and floral bud tissue according to van Arkel et al. (2012). 10mg dry weight of each tissue was mixed with 600 µl 20 mM phosphate buffer (pH 7) and incubated for 30 min at 85°C in thermomixer (Vortemp, Labnet) with shaking every 5 min . After incubation samples were centrifuged for 15 min at 4000 rpm. The supernatants were collected and extracted as described above with 600 µl 20 mM phosphate buffer (pH 7). After two extractions, the supernatants of both extractions were mixed and de-ionized with an ion exchange buffer (1:1 Q-Sepharose and S-Sepharose suspended in 20 mM phosphate buffer pH 7). The extraction and ion exchange buffer were mixed 1:2 and mixed for 5 min on a shaker at 500 rpm followed by centrifuging for 5 min at maximum speed. A total volume of 120 µl was transferred to polycarbonate vials. The content of glucose, fructose and sucrose was determined with the Dionex ICS-5000+ HPIC equipped with a CarbopacTM PA1 (Thermo Scientific, The Netherlands). The gradient and column specifications and quantification methods are described in (van Arkel et al., 2012).

Results & Discussion

Morphological and physiological changes during storage and the winter period in the field

Growth

After flowering in spring, the vegetative tulip daughter bulbs finalize their growth period. At this moment, the floral bud is initiated inside daughter bulbs of sufficient size and the floral organs are all initiated (Khodorova and Boitel-Conti, 2013). Around this period growth of the bulb organs ceases due to the initiation of dormancy. A widely adapted definition of dormancy, in relation to seeds, is ‘an endogenously controlled but environmentally imposed temporary suspension of growth, accompanied by reduced metabolic activity and relatively independent of ambient environmental

conditions' (Amen, 1968). In contrast to seeds, the definition of dormancy suggested for bulbs is 'a complex and dynamic physiological state during which there are no apparent external morphological changes or growth. Internally, however, many physiological and/or morphological events are occurring' (De Hertogh and Le Nard, 1993). Furthermore, it has been proposed for tulip that the inactivity of meristem can be considered as true dormancy (Rees, 1981). To investigate these definitions, the growth of the internal tissues (floral stem, leaves and floral bud), dry weight (DW), and carbohydrate content were measured in bulbs of the tulip cultivar Strong Gold. Measurements started one month before planting, when the bulbs were still in storage, until February when sprouting was accelerated (Fig. 1A). During this period the soil temperature was also measured, enabling to correlate observed temperature effects (Fig. 1B). Overall growth of the internal tissues (sprout) was slow from one month before planting until ten weeks after planting. After ten weeks of low temperature perception in the field, a more rapid growth was observed (Fig. 1C), despite that no large differences in temperature were observed at this moment (Fig. 1B). When separating the sprout into the floral stem, floral bud, and leaves, different growth patterns could be observed for floral bud and leaves. In comparison to the other tissues, the floral bud had an overall slow growth rate, making this tissue the least active within the bulb. This suggests that tulip have a kind of floral bud dormancy, as has been suggested by Rees (1981) when considering the apical bud as a meristem. In contrast, the leaves seemed to be the most actively growing tissue. Accelerated leaf growth was observed after ten weeks in the field, which was also the case for the floral stem. At this moment the leaves are still below the surface and are not yet photosynthesizing. This suggests that around week ten, after two and a half months in the field, dormancy is released, and preparations have been made for optimal flowering in April/May.

Dry weight and water content

Besides the growth measurements, relative dry weight (DW) was

determined in the different samples of the various tissues (Fig. 1D). In the second bulb scale, the relative DW increased around week six. Since the scale is not growing, it is likely that the water loss is more rapid than the re-mobilization of nutrients, proteins and carbohydrates. To confirm this, absolute values are essential, but these were not measured in this study. The relative DW of the floral stem, floral bud and leaves was comparable to the increase of length of the different tissues (Fig. 1C). Increase in relative DW can also be explained by the presence of a large number of cells being ready to elongate or drying of the tissue. In contrast to the floral stem and leaves, the relative DW in the floral bud was decreasing until approximately week six and increased again after week 12. This suggests that at the beginning of these measurements the floral bud was in a kind of 'desiccated' dormant state, which requires water uptake in order to expand (swelling) and/or to prepare for an increase in metabolic activity. Further research is required to pin-point the importance of the water content in the floral bud in relation to growth.

Carbohydrates

Ten weeks after planting, growth appeared to be re-activated in all tissues inside the bulb. In the study of Kamenetsky and colleagues (2003) it was shown that cold induced dormancy release is accompanied by an increase in soluble carbohydrate content. Therefore, the carbohydrates glucose, fructose and sucrose were measured in the collected material (Fig. 2). From week ten onwards glucose levels in the floral stem and leaves increased strongly whereas in the scale and floral bud the increase was more gradual (Fig. 2A). The increase of glucose levels in the tissues correlated with the advancement of growth, which implies that approximately ten weeks of low temperature is sufficient to remobilize the sugars (Fig. 2B). The correlation of glucose content with growth can be explained by the fact that glucose is known to contribute to both cell division and elongation (Khodorova and Boitel-Conti, 2013).

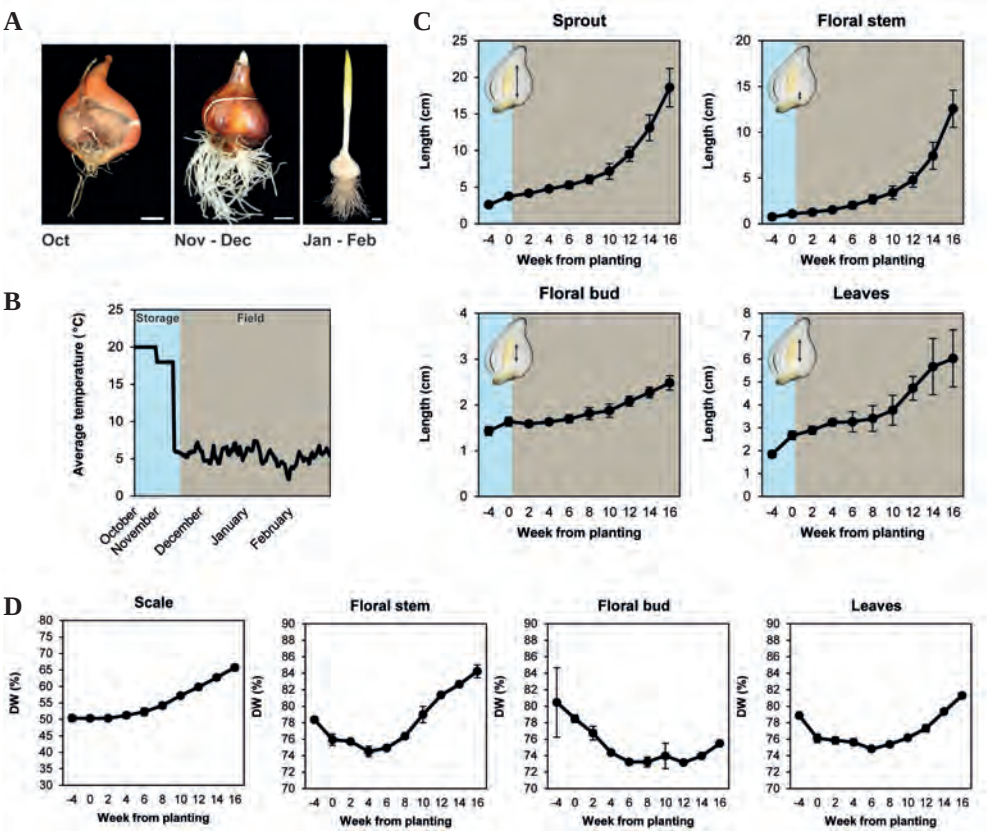


Figure 1. Morphological changes outside and inside tulip bulbs during the winter period in the field. (A) Overview of bulb morphology during storage, first month after planting when roots are being formed, and three months after planting when sprouting accelerates. (B) Average temperature during storage and soil temperature in the field in the winter period. (C) Growth analysis of the total sprout, floral stem, floral bud, and developing leaves. The arrow in the bulb (left corner) indicates the area of the measurement and position of the tissue inside the bulb. The period in storage is marked in blue and the period in the field in grey. The error bars represent the standard deviation of 30 measurements. (D) Dry weight (DW) percentage of the scale, floral stem, floral bud and leaves.

However, scales do not grow anymore and the sugars remobilized from these organs due to starch degradation, are assumed to be transported to growing sink tissues (De Hertogh and Le Nard, 1993). For fructose, a

similar pattern was observed. Although levels of this monosaccharide increased strongly in both floral stem and scales, the levels in the floral bud remained more or less constant over the period of investigation (Fig. 2C). Similar to glucose, the increase of fructose in the floral stem and leaves correlated with the growth of these tissues (Fig. 2D). Sucrose contents in the scales increased from week two onwards but showed only limited fluctuations in all other analysed tissues (Fig. 2E). In conclusion, observed carbohydrate patterns are in accordance with current consensus on the

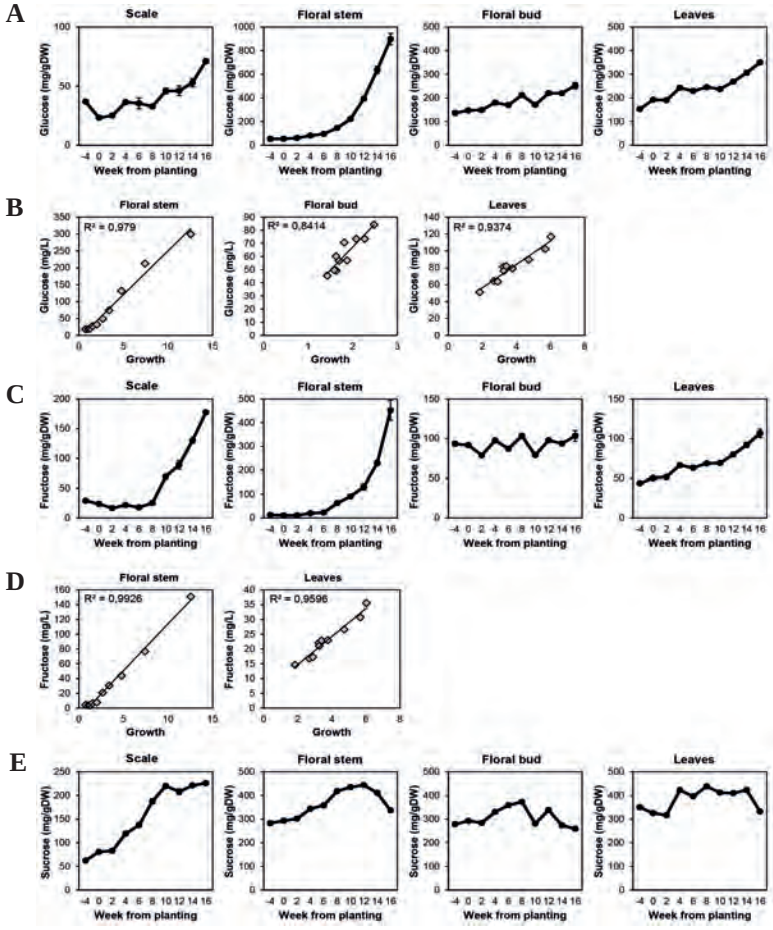


Figure 2. Carbohydrate status of Strong Gold tulip bulbs during storage and in the field in winter. (A) Glucose content in the scale, floral stem, floral bud and leaves. (B) Correlation between growth and the glucose content in floral stem, floral bud and leaf tissue. (C) Fructose content. (D) Correlation between growth and the fructose content in the floral stem and leaves. (E) Sucrose content.

role of carbohydrates in growth. Around week ten, dormancy appeared to be released resulting in a boost of growth. This suggests that prior to this moment important physiological and perhaps also transcriptional changes are occurring, which are required for re-activation of growth leading to flowering in spring.

Transcriptome analysis of bulb tissues

To investigate the changes occurring inside the tulip bulb in relation to dormancy release, RNA-sequencing (RNA-seq) was performed on scale, floral bud, and leaf tissues. Scale tissue collected at week minus four (storage), zero (just prior to planting), and two, eight, and twelve weeks after planting in the field, were used for the transcriptome analysis by RNA-seq. In total 698,828 transcripts from tissue of the second bulb scale were assembled by using Trinity (Haas et al. 2013), of which 413,284 were Trinity 'genes' (Table 1). RNA-seq analysis of floral bud tissue collected in week minus four, time point zero, two, six, eight, twelve and sixteen weeks after planting resulted in 1,017,621 assembled transcripts encoding for 757,761 Trinity 'genes' (Table 1). The RNA-seq data of the leaves consisted of samples collected in week zero (planting), two, eight and twelve weeks after planting. In total 967,556 transcripts were assembled encoding for 667,425 Trinity 'genes' (Table 1). No filtering was applied on this data in order to minimize the loss of information of rarely expressed genes, and consequently, a large part of the Trinity 'genes' is probably resembling partial fragments of the same transcript or gene (Moreno-Pachon et al., 2016). Nevertheless, quality characteristics, such as GC content and average contig length were similar for the different tissues (Table 1) and showed values that are not far off from expectations.

Initially, the overall transcriptional changes over time in the different tissues were studied, as shown in multidimensional scaling (MDS) plots (Fig. 3). For each investigated tissue, strongest separation was found for samples taken during storage (weeks minus four and zero) and the samples taken from the field. Only for the floral bud a more gradual change over time was

observed. Planting of the bulbs (comparison of time point zero with time point two) had considerable impact on the transcriptional changes in each tissue. This variation is likely caused by the change in temperature (Fig. 1B) and the initiation of root development. Nevertheless, only for leaves this resulted in a considerably greater effect on gene expression in comparison to the other time points. For both scales and floral bud no increased variation in overall gene expression could be observed at this particular moment. However, planting of the bulbs may have resulted in a transient stress response and altered gene expression patterns, which is missed in our analysis, because the first sampling after planting was done at two weeks.

Table 1. Statistical overview of the assembled RNA-sequencing data of the different tulip bulb tissues.

Tissue	Trinity 'genes'	Trinity transcripts	GC (%)	N50*	Average contig length (bp)
Scale	413,284	698,828	43,33	717	548,08
Floral bud	757,761	1,017,621	42,91	641	521,70
Leaves	667,425	967,556	42,90	861	609,02

**: Statistical measure of average length of the assembled contigs.*

Subsequently, the number of significant differentially expressed transcripts was determined for each tissue (Fig. 3B). A comparison was made between each consecutive sample in time (e.g. week minus four was compared to week zero). For this analysis, we separated transcripts, that based on sequence similarity, could be annotated with an Arabidopsis gene from those for which no annotation could be obtained (Fig. 3B). The number of differentially expressed transcripts in the scale tissue increased until week ten and declined thereafter (Fig. 3B, left). The number of differentially expressed genes was rather low in the scale tissue as compared to the floral bud. Surprisingly, in the floral bud almost 3000 transcripts were differentially up-regulated and over 4000 transcripts were down-regulated when comparing between one month before planting (week minus four) and the day before planting (week zero; Fig. 3B, middle). Despite the fact that

the bulb appears to be in rest in this period, various genes are under transcriptional control in the floral bud in this period. Once the bulbs were planted in the field, the amount of differentially expressed genes declined until three months after planting (week 16) when the number increased again. This suggests that the floral bud is almost inactive for the first three months in the field and is thereafter re-activated in order to develop into a fully developed and open flower in spring. For leaves only three time points were included for RNA-seq and comparison showed a large number of differentially expressed genes between the sampling points (Fig. 3B, right).

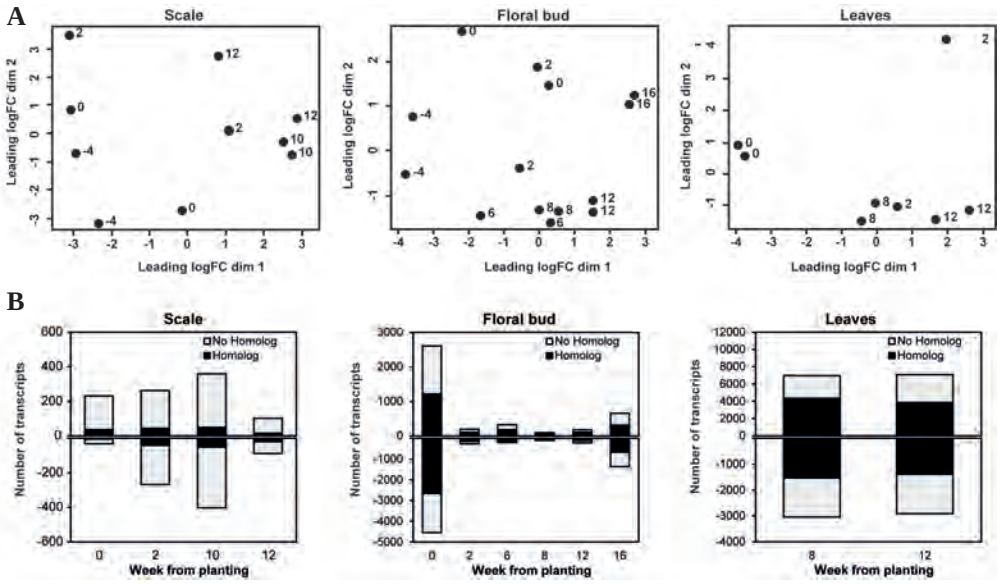
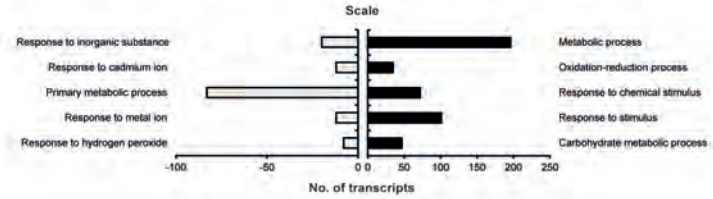


Figure 3. Transcriptional changes in the scale, floral bud and leaf tissues as determined by RNA-seq. (A) Multidimensional scaling (MDS) plot of transcriptome data generated from scale (left), floral bud (middle) and leaves (right). The numbering of the samples is according to the sampling moments of the bulbs (-4: in storage, four weeks before planting, 0: one day before planting; 2,6,8,10,12, 16: two, six, eight, 10, 12 and 16 weeks after planting in the field, respectively). (B) Number of differentially expressed transcripts in the scale (left), floral bud (middle) and leaves (right). No homolog: transcripts lacking a BLAST hit (cut-off 1e-05) with an Arabidopsis gene, Homolog: transcripts showing a BLAST hit with an Arabidopsis gene.

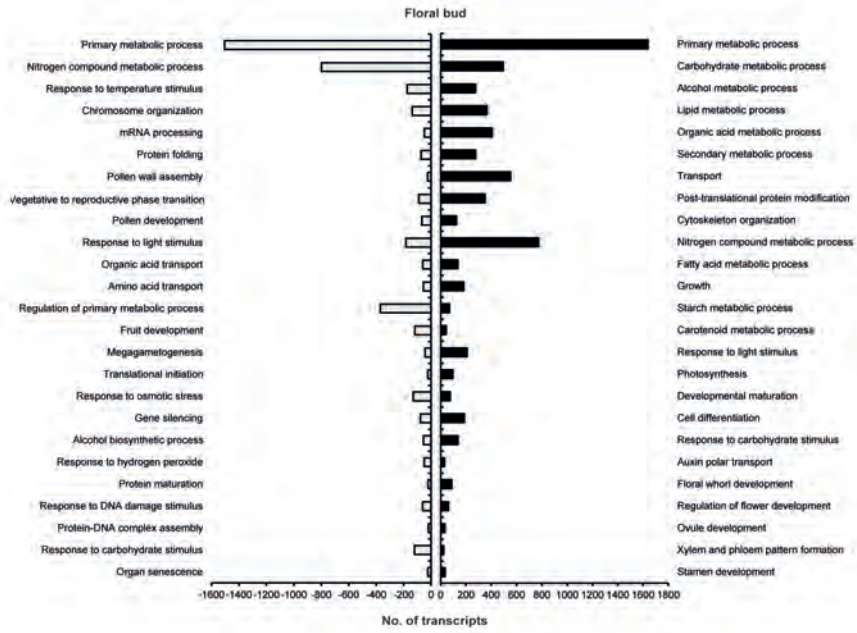
Gene Ontology enrichment analysis – storage versus field

Both the MDS plots and the number of differentially expressed transcripts imply that numerous transcriptional changes are occurring over time. To obtain insight in the specific biological processes that are associated with these transcriptional changes, a Gene Ontology (GO)-enrichment analysis was performed, comparing samples from bulbs collected during storage conditions and after a long period (twelve or sixteen weeks) in the field. Due to the relatively low number of differentially expressed transcripts in the scale tissue, a small number of over-represented GO-terms was found (Fig. 4A). In line with expectations and the proposed function of the scales, GO terms such as *Metabolic process* and *Oxidation-reduction process* were found for the up-regulated transcripts (Fig. 4A). For the floral bud several GO-terms were found to be overrepresented for the differentially up- and down-regulated transcripts (Fig. 4B). Mainly metabolic-related GO-terms were over-represented in the set of up-regulated transcripts, such as *Primary metabolic process*, *Carbohydrate metabolic process* and *Lipid metabolic process*. Additionally, over-represented GO-terms associated with the later stages of flower development included *Ovule development*, *Stamen development* and *Developmental maturation*. Apparently, the floral bud that is already present inside the bulb undergoes further differentiation of the floral organs. Remarkably, *Pollen wall assembly* and *Megagametogenesis* was overrepresented in the down-regulated transcripts. In tulip, pollen development is arrested at the uninucleate stage for a long period after planting and approximately one week before flowering the pollen continues to develop into the binuclear stage followed by maturation (Xu et al., 2005).
Also for leaves a large number of overrepresented GO-terms was found in the differentially expressed transcripts (Fig. 4C). Even though the leaves are still pale yellow and in soil below the surface, numerous GO-terms for the up-regulated transcripts were related to photosynthesis and plastid formation. Specific examples are *Photosynthesis (light reaction)*, *Plastid organization*, *Chlorophyll metabolic process*, *Photosystem II assembly* and *Photorespiration*. It is surprising that transcripts related to these processes

A


Scale
Response to inorganic substance
Response to cadmium ion
Primary metabolic process
Response to metal ion
Response to hydrogen peroxide
Metabolic process
Oxidation-reduction process
Response to chemical stimulus
Response to stimulus
Carbohydrate metabolic process
-100 -50 0 0 50 100 150 200 250
No. of transcripts

B


Floral bud
Primary metabolic process
Nitrogen compound metabolic process
Response to temperature stimulus
Chromosome organization
mRNA processing
Protein folding
Pollen wall assembly
Vegetative to reproductive phase transition
Pollen development
Response to light stimulus
Organic acid transport
Amino acid transport
Regulation of primary metabolic process
Fruit development
Megagametogenesis
Translational initiation
Response to osmotic stress
Gene silencing
Alcohol biosynthetic process
Response to hydrogen peroxide
Protein maturation
Response to DNA damage stimulus
Protein-DNA complex assembly
Response to carbohydrate stimulus
Organ senescence
Primary metabolic process
Carbohydrate metabolic process
Alcohol metabolic process
Lipid metabolic process
Organic acid metabolic process
Secondary metabolic process
Transport
Post-translational protein modification
Cytoskeleton organization
Nitrogen compound metabolic process
Fatty acid metabolic process
Growth
Starch metabolic process
Carotenoid metabolic process
Response to light stimulus
Photosynthesis
Developmental maturation
Cell differentiation
Response to carbohydrate stimulus
Auxin polar transport
Floral whorl development
Regulation of flower development
Ovule development
Xylem and phloem pattern formation
Stamen development
-1600 -1400 -1200 -1000 -800 -600 -400 -200 0 0 200 400 600 800 1000 1200 1400 1600 1800
No. of transcripts

C

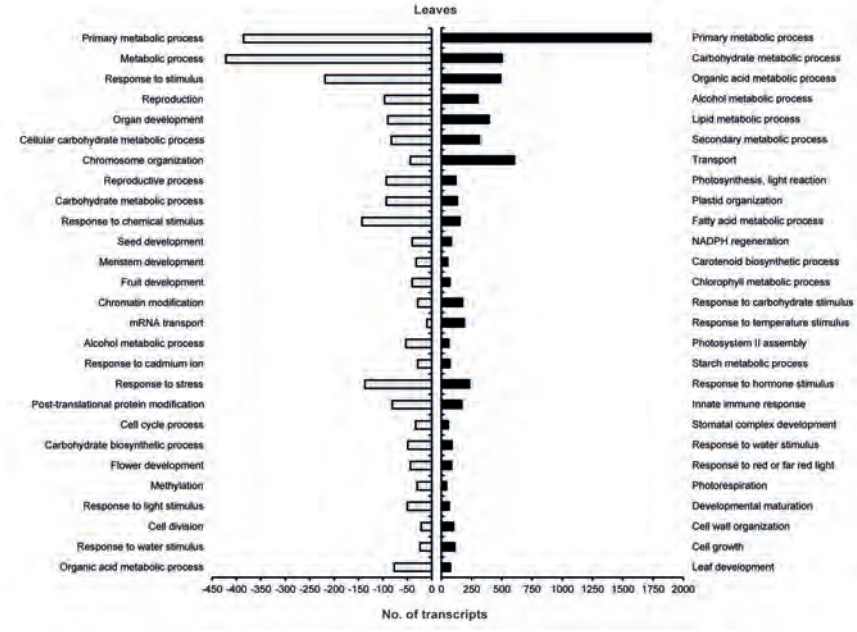

Leaves
Primary metabolic process
Metabolic process
Response to stimulus
Reproduction
Organ development
Cellular carbohydrate metabolic process
Chromosome organization
Reproductive process
Carbohydrate metabolic process
Response to chemical stimulus
Seed development
Meristem development
Fruit development
Chromatin modification
mRNA transport
Alcohol metabolic process
Response to cadmium ion
Response to stress
Post-translational protein modification
Cell cycle process
Carbohydrate biosynthetic process
Flower development
Methylation
Response to light stimulus
Cell division
Response to water stimulus
Organic acid metabolic process
Primary metabolic process
Carbohydrate metabolic process
Organic acid metabolic process
Alcohol metabolic process
Lipid metabolic process
Secondary metabolic process
Transport
Photosynthesis, light reaction
Plastid organization
Fatty acid metabolic process
NADPH regeneration
Carotenoid biosynthetic process
Chlorophyll metabolic process
Response to carbohydrate stimulus
Response to temperature stimulus
Photosystem II assembly
Starch metabolic process
Response to hormone stimulus
Innate immune response
Stomatal complex development
Response to water stimulus
Response to red or far red light
Photorespiration
Developmental maturation
Cell wall organization
Cell growth
Leaf development
-450 -400 -350 -300 -250 -200 -150 -100 -50 0 0 250 500 750 1000 1250 1500 1750 2000
No. of transcripts

Figure 4. Gene Ontology (GO)-enrichment analysis of the differentially expressed genes in each bulb tissue. (A) Over-represented GO-terms among the up- and down-regulated transcripts in the scale, when comparing week minus four and week 12. (B) Over-represented GO-terms of the up- and down-regulated transcripts in the floral bud tissue, when comparing week minus four and week 16. (C) Over-represented GO-terms of the up- and down-regulated genes, when comparing week zero and week 12. GO-terms from top to bottom are ordered according to significance score (High to low p-value).

are already accumulating, although the leaves were not exposed to light yet. Nevertheless, this does not seem to be a unique characteristic of tulip. In spring, the shoots of ephemeral plants, which have a short life cycle, develop underground and are photosynthetically competent when emerging above the surface (Mamushina et al., 2002). Another example is in relation to seeds, where at the testa rupture stage of seed germination already gene expression of photosynthesis-related genes is observed (Silva et al., 2016). In the down-regulated transcripts, GO-terms related to cell proliferation can be found suggesting that the leaves are already in their differentiation phase and that the observed growth is mainly due to cell elongation (Fig. 4C).

Gene Ontology enrichment analysis in floral buds – storage

A large number of differentially expressed genes was found in the floral bud tissues during the last four weeks of storage. To obtain more insight in these changes and their biological meaning, another GO-enrichment analysis was performed (Fig. 5). Over-represented GO-terms found in the up-regulated transcripts included Primary metabolic process, Secondary metabolic process and Sucrose metabolic process. This suggests that even before commencement of the low temperature period, metabolic changes may occur. Other GO-terms that are indicative of activity in the floral bud are Response to hormone stimulus, Transport, Signalling and Response to water stimulus. Hormones have shown to be important in plant development, especially the cross-talk among the various hormones. In plants, several

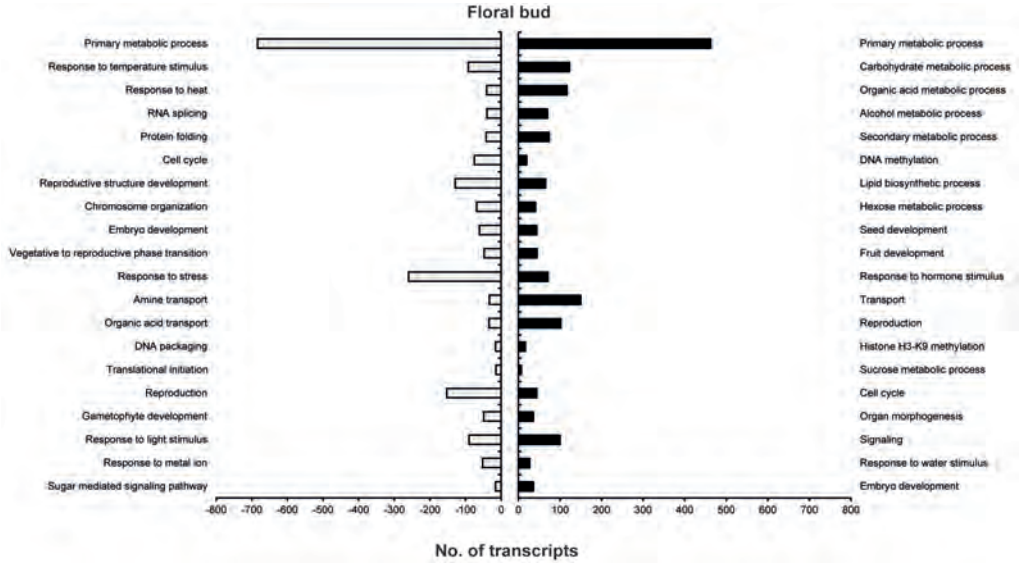


Figure 5. Gene ontology (GO)-enrichment analysis of the differentially expressed transcripts in the floral bud during the last four weeks of storage. Comparison of week minus four and week zero. GO-terms from top to bottom are ordered according to significance score (High to low p-value).

major classes of hormones have been characterized, which can be connected to growth regulation, even in a tissue-specific way (Vert and Chory, 2011). The floral bud and the leaves are connected to the floral stem, which is connected to the basal plate. In later stages of development it is known in tulip that the floral bud and the leaves are the major sources of auxin. The elongation of the floral stem is believed to be auxin-induced and GA-biosynthesis is another requirement for proper elongation (Rietveld et al., 2000). This suggests interaction of different tissues in a developmental context. A possibility is that already during storage these tissues are interconnected to each other via signalling and/or transport of hormones to regulate each other's growth. Nevertheless, further research is essential to unlock this complex crosstalk in regulation.

After the storage period, the number of differentially expressed transcripts dropped dramatically, indicating the start of an inactive period (Fig. 3B). The GO-terms that can be linked with this observation are *DNA methylation*

and *Histone H3-K9 methylation*. Histone H3K9 methylation is associated with gene silencing and predominately exists as H3K9 dimethylation1 (H3K9me1) and H3K9me2 in Arabidopsis (Liu et al., 2010). One of the transcripts belonging to the over-represented GO-term *Histone H3-K9 methylation* is *CHROMATIN REMODELLING17* (*CHR17*). This gene is one of the *Arabidopsis thaliana IMITATION SWITCH* (*AtSWI*) genes which is involved in maintenance of the vegetative phase. CHR17 interacts with both RINGLET1 (RLT1) and RLT2 to regulate their common downstream genes. These downstream targets include several key flowering and floral genes, such as *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) and *SEPELLATA1* (*SEP1*; Li et al., 2012). At this stage tulip is already in its reproductive phase, but regulation of this type of genes can explain the delay in further flower development, which is counteracted by a period of low temperature. Nevertheless, which particular groups of genes are targeted by this chromatin modification at this time point during tulip development remains to be elucidated, but the increased DNA methylation and H3K9 methylation provide a perfect explanation for the massive inactivation of gene expression shortly after.

In the down-regulated set of transcripts, GO-terms such as *Response to temperature*, *RNA splicing*, *Gametophyte development* and *Reproductive development* could be found. The temperature at one month before planting (week minus four) is 20°C, which is reduced to 18°C two weeks prior to planting. This drop in temperature may have triggered down-regulation of particular temperature-responsive genes. Transcripts involved in reproductive development are also down-regulated, which is in line with decelerated reproductive development. Overall, this GO-enrichment analysis shows the complexity of processes occurring in the floral bud just prior to planting when the bulb seems to be in a quiescent state.

Gene expression analysis in leaf tissue – from the dark to the light

The leaves are the first tissues, originating from inside the bulb, to emerge above the soil. Before this happens the scale tissue is the most important

source for carbohydrates and nutrients for internal bulb sink tissues such as the floral bud. Once above the soil, the leaves become the new energy source of photoassimilates (Ho and Rees, 1975). Plant growth is regulated by the availability of sucrose, generated by photosynthesis (Osorio et al., 2014). Photosynthesis can be influenced by light and temperature, but also water availability and CO2. In *Rhododendron simsii hybrids* (azalea) suboptimal light conditions, resulting in a decrease of carbohydrates in the leaves, could negatively influence the flowering process (Christiaens et al., 2016). Therefore, continuation of growth of the floral stem and floral bud, as well as flowering in spring, is most likely dependent on the accumulation of carbohydrates generated by photosynthesis. In the GO-enrichment analysis of leaves, the GO-term *Photosynthesis* was over-represented in the set of up-regulated transcripts. Photosynthesis is a complex process and depends on two main complexes, photosystem I (PSI) and photosystem II (PSII) (Zhang et al., 2016; Fig. S1A). A selection of 14 different photosynthesis-related genes had similar expression patterns (Fig. S1). Three genes were selected to be confirmed by qPCR. *LHCB2.2* was one of the selected genes, which encodes a protein that is part of the light harvesting antenna complex of PSI and PSII. The light harvesting complex II is an important component of the photosystem, because it plays a role in light capturing and acclimation to changing light conditions (Longoni et al., 2015). The expression of this gene remained low in the leaves until week 10 when its expression rapidly increased (Fig. 6A). Another selected gene was *PSBW*, which encodes for the low molecular mass subunit of PSII (Plöchinger et al., 2016). Also the expression of this gene increased from week 10 onwards (Fig. 6B). In addition, the *PORA* gene was selected to be confirmed by qRT-PCR. This gene is important for skotomorphogenesis and normal photomorphogenic development (Paddock et al., 2012). Differently from *LHCB2.2* and *PSBW*, the expression of *PORA* increased from week 12 onwards (Fig. 6C). It is possible that the leaves are switching from skotomorphogenic (etiolated) to photomorphogenic (de-etiolated) development. In seeds a similar developmental switch of the hypocotyls is observed, when a buried seed

tries to emerge through the soil to reach light. This developmental switch will prepare the leaves for optimal photosynthesis when reaching above the soil surface (Sullivan and Deng, 2003). It is not known in tulip when the leaves are able to sense light, and whether this is possible when still being under the soil surface. Therefore it can be that the early activation of photosynthesis genes is developmentally programmed or alternatively, that light is able to penetrate deep enough into the soil.

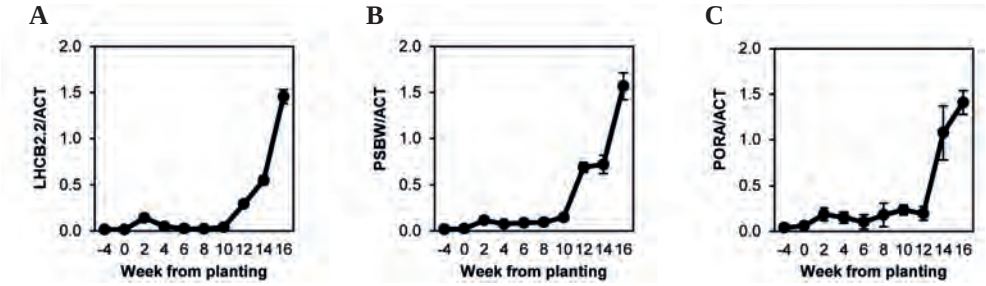


Figure 6. Expression analysis of a selection of photosynthesis related genes determined by qPCR in the leaves. (A) Expression of *LHCB2.2*, encoding for a protein that is part of the antenna complex of the two photosystems (B) *PSBW*, a gene which codes for a core protein of PSII (C) *PORA*, a gene coding for photo protectant of the dark to light transition.

Primary metabolites

Previous studies have indicated that the metabolic changes within the tulip bulbs are important for the process towards flowering and vegetative propagation of the daughter bulbs (Lambrechts et al., 1994; Rietveld et al., 2000). Here, a large number of differentially expressed transcripts were related to metabolic processes. To gain deeper insights in the metabolic changes apart from sucrose, glucose and fructose, a profiling experiment of the major primary metabolites was performed using GC-ToF-MS of derivatised extracts. Data were processed using an untargeted metabolomics approach. Relative amounts of amino acids, organic acids, and carbohydrates present in the scale, floral stem, floral bud and leaves were analysed. Principal component analysis (PCA), which was done per tissue, showed a global change of the metabolome over time (Fig. 7). For thescale tissue, a separation can be seen between storage weeks and two weeks

after planting, and the remaining weeks in the field (Fig. 7A). This reveals that the metabolic profiles are clearly different between these phases. In the PCA of the floral stem a similar pattern can be observed, but the separation of samples is different. The storage weeks until six weeks after planting are clustered together, followed by the remaining weeks in the field, with week eight in between the two clusters (Fig. 7B). This suggests a metabolic transition at approximately week eight, which is also the moment after which active growth is observed (Fig. 1C). Also the primary metabolism of the floral bud is changing over time, but gradually (Fig. 7C). Similar to the floral bud, metabolic changes are occurring more gradually over time in the leaves with the exception of week 16 clearly separated from the other later weeks in the field (Fig. 7D).

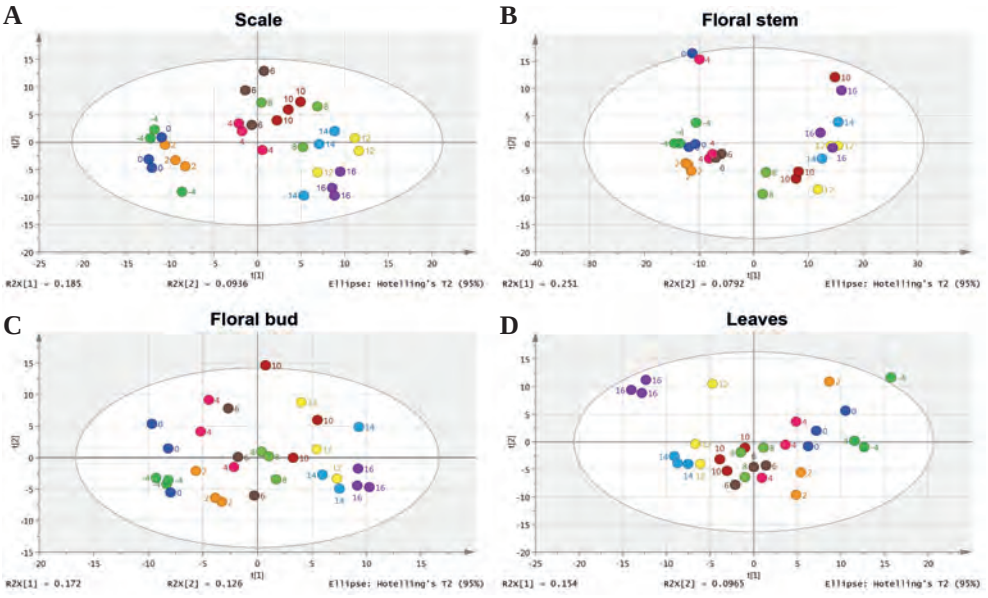


Figure 7. Principal component analysis (PCA) of the primary metabolite data. For each PCA plot the first two principal components are shown, first on the x-axis (t[1]) and second on the y-axis (t[2]). The numbers indicate the week from planting and the different colours indicate a particular week with its three replicates. Colouring: -4: green, 0: blue, 2: orange, 4: pink, 6: brown, 8: light green, 10: dark red, 12: yellow, 14: light blue and 16: purple. (A) Scale, t[1]: 18.5%, t[2]: 9.36%. (B) Floral stem, t[1]: 25.1%, t[2]: 7.92%. (C) Floral bud, t[1]: 17.2%, t[2]: 12.6%. (D) Leaves, t[1]: 15.4%, t[2]: 9.65%.

A first analysis of the floral bud metabolites revealed that all measured amino acids decreased over time, with the exception of proline and phenylalanine (Fig. 8A; Fig. S2). In contrast with the decrease in amino acid content, the xylose and mannose contents increased (Fig. 8B). Amino acids are important building blocks for the biosynthesis of proteins, but they can also play a role in signalling and plant stress (Hildebrandt et al., 2015). The accumulation of proline can be related to environmental stress, it acts as a protectant, but it can also be involved in plant development signalling (Szabados and Savouré, 2010). Proline levels increased upon the decrease in temperature and remained stable until week 12 (Fig. 1B; Fig. 8A). The decrease in proline content was not correlated with an increase in temperature, which raises the question if proline is acting here as a protectant in the context of freezing tolerance. A more likely function is the inhibition of growth, because the timing of its increase matches with the inhibition/reduction of growth (Fig. 1C). This has also been reported for Arabidopsis seeds, in which proline treatment inhibited germination and for Petunia plants in which proline restricts growth (Szabados and Savouré, 2010). The decrease of amino acid contents and the increase expression of the amino acid biosynthetic genes *GLUTAMINE SYNTHASE* and *ASPARAGINE SYNTHASE*, are suggesting that at least these amino acids are used (Fig. 8C). However, no obvious growth at organ level could be measured in the floral bud during this period (Fig. 1C). Growth is a result of various cellular processes, in particular cytoplasmic growth, turgor-driven cell wall extension, cell division and endoreduplication (Sablowski and Carnier Dornelas, 2013). The carbohydrates xylose and mannose are required for the formation of hemicelluloses (Scheller and Ulvskov, 2010). Besides the physiological data, a transcript homologous to the Arabidopsis *KORRIGAN1* (*KOR1*) gene showed to be differentially up-regulated (Fig. 8C). *KOR1* is involved in cellulose biosynthesis (Mansoori et al., 2014). The gene expression pattern showed an increase in expression from week zero onwards, revealing that also at the transcriptional level changes related to cell wall expansion/formation may occur. Together with an over-representation of cell-cycle related genes in the transcriptome

(Fig. 4B) this makes cell division activity during this period a likely scenario. Three transcripts homologous to the Arabidopsis *GROWTH REGULATING FACTOR5* (*GRF5*), *KEULE* (*KEU*) and *CYCLIN A1-1* (*CYCA1-A*), which all are involved in the cell division process (Heese et al., 1998; Bulankova et al., 2013; Vercruyssen et al., 2015), were differentially up-regulated between week zero and two. Histological data is required to determine what may occur at the cellular level.

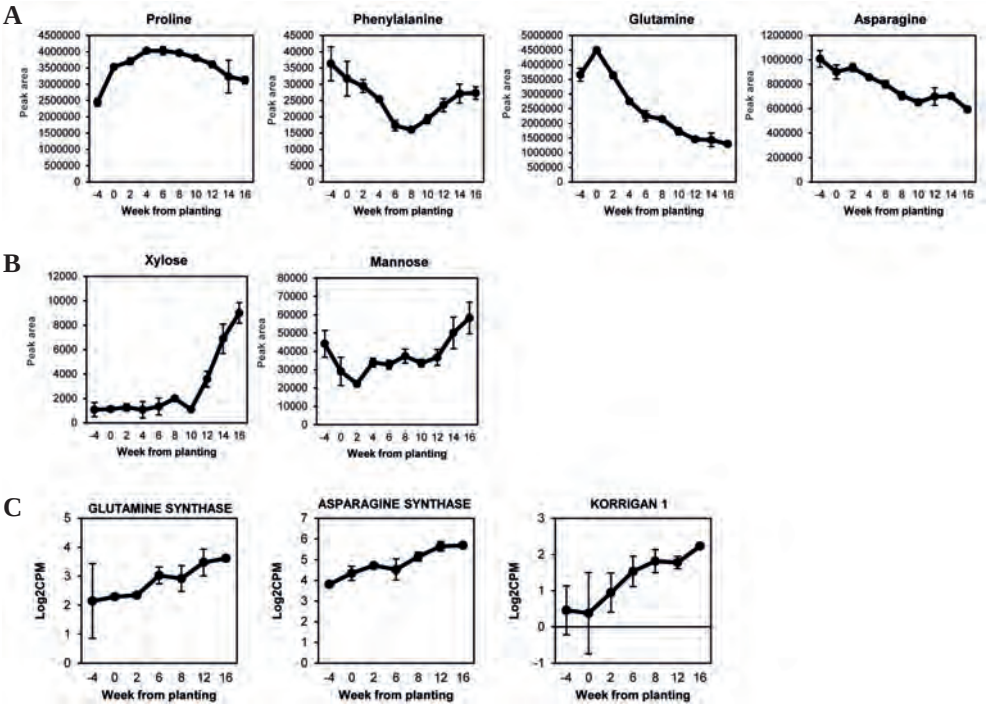


Figure 8. Primairy metabolites and differentially expressed genes in the floral bud. (A) Amino acid content of proline, phenylalanine, glutamine and asparagine. (B) Levels of xylose and mannose. (C) Two transcripts related to amino acid biosynthesis, *GLUTAMINE SYNTHASE* and *ASPARAGINE SYNTHASE*, and cellulose biosynthesis, *KORRIGAN1*.

Conclusion

In tulip, a low temperature period is required for proper outgrowth and development of the internal tissues that are already formed during the storage period. Glucose content could be correlated with the length of the floral stem, floral bud and leaves and is a perfect marker to determine

whether bulbs have obtained the required quantity of low temperature hours. However, further investigations of different cultivars, varying in flowering time, over different growth years are required to show the broader applicability of this correlation. The floral bud is the most inactive tissue during the winter period, which is apparent at both morphological and transcriptional levels. The leaves are the most actively changing tissue, including preparation for photosynthesis in relation to becoming the new source of carbohydrates. This new energy source is required for the last stretches of the life cycle of the bulb and flowering, but also in particular for the formation of new daughter bulbs. Further comprehensive and combinatorial analyses of the transcriptomic and metabolic data are essential to obtain a complete overview of the changes during the last month of storage and afterwards in the field.

Acknowledgements

We thank Gebroeders Klaver and Van der Gulik tulpen B.V. for the tulip bulbs and Maarten Holdinga for planting them. We also thank Wilco Ligterink for carefully reading of this chapter. Funding of the project was kindly provided by TTI-Green Genetics and the Dutch Ministry of Economic Affairs. The metabolomics work was funded by a grant obtained from ZonMW (2013).

Supplemental data

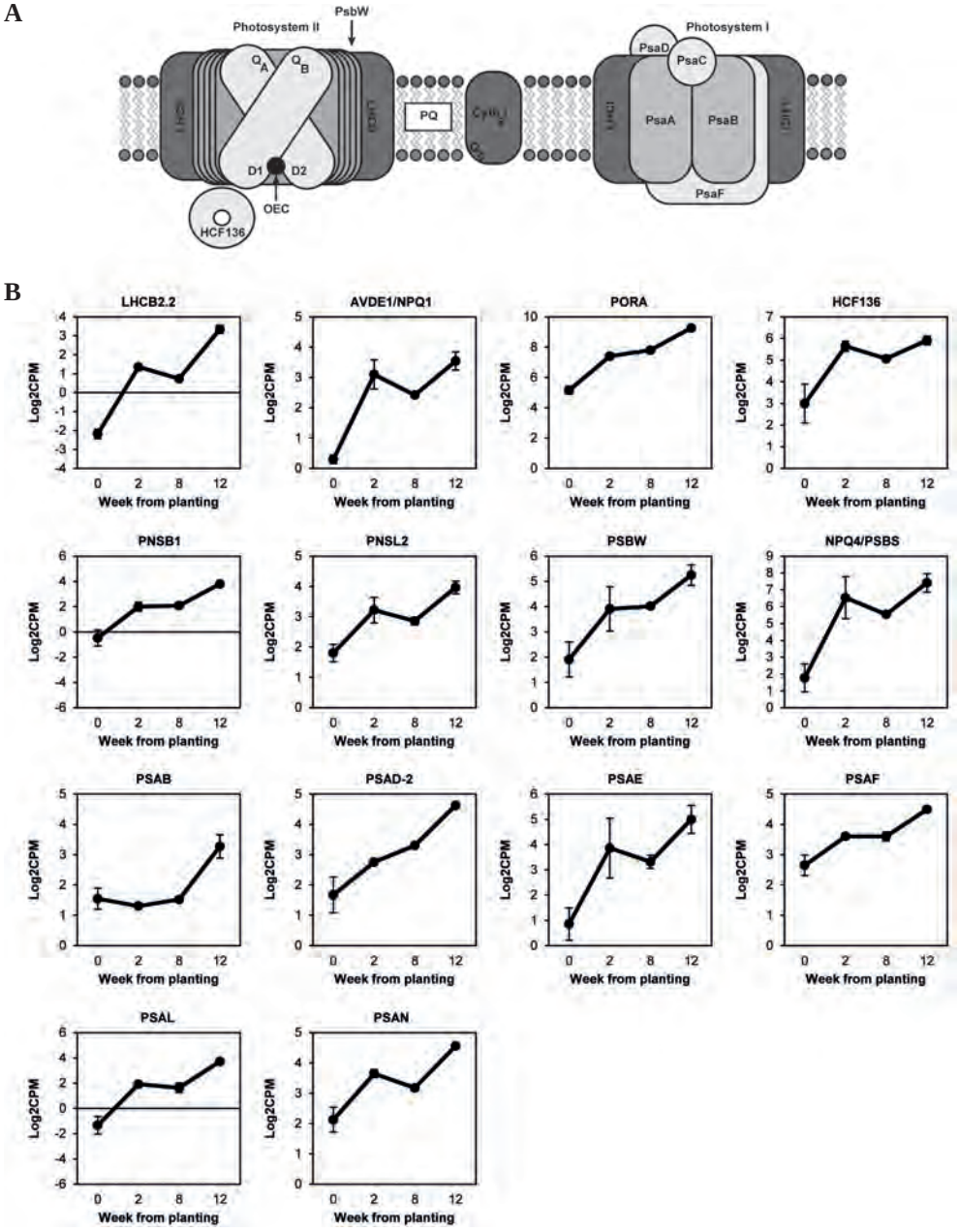


Figure S1. Selection of differentially expressed genes in the transcriptome data of tulip leaves. (A) Schematic overview of the photosynthetic machinery in plants. OEC stands for Oxygen Evolving Complex. (B) Selected photosynthesis related genes, which are differentially expressed according to the RNA-seq data when comparing week zero with week twelve.

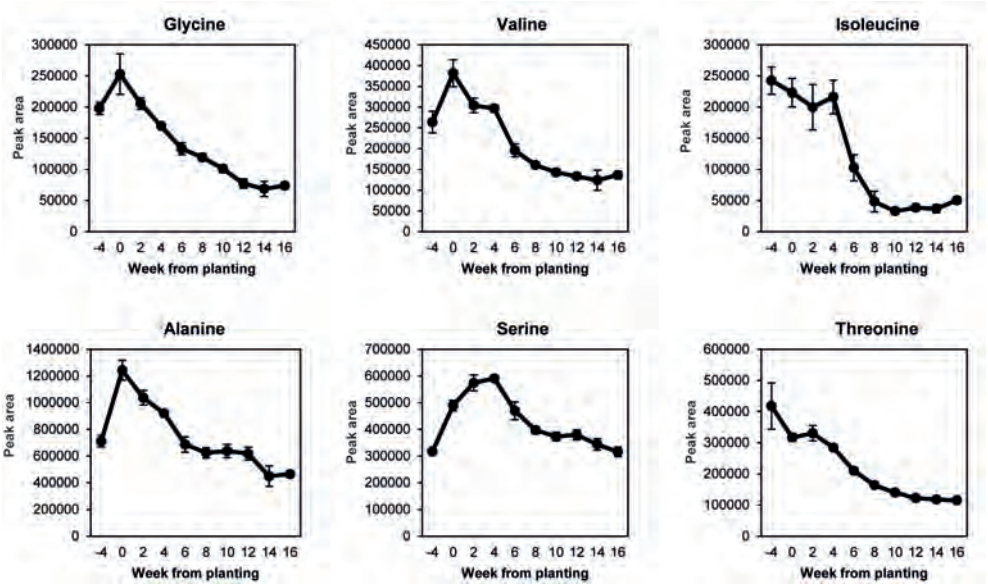
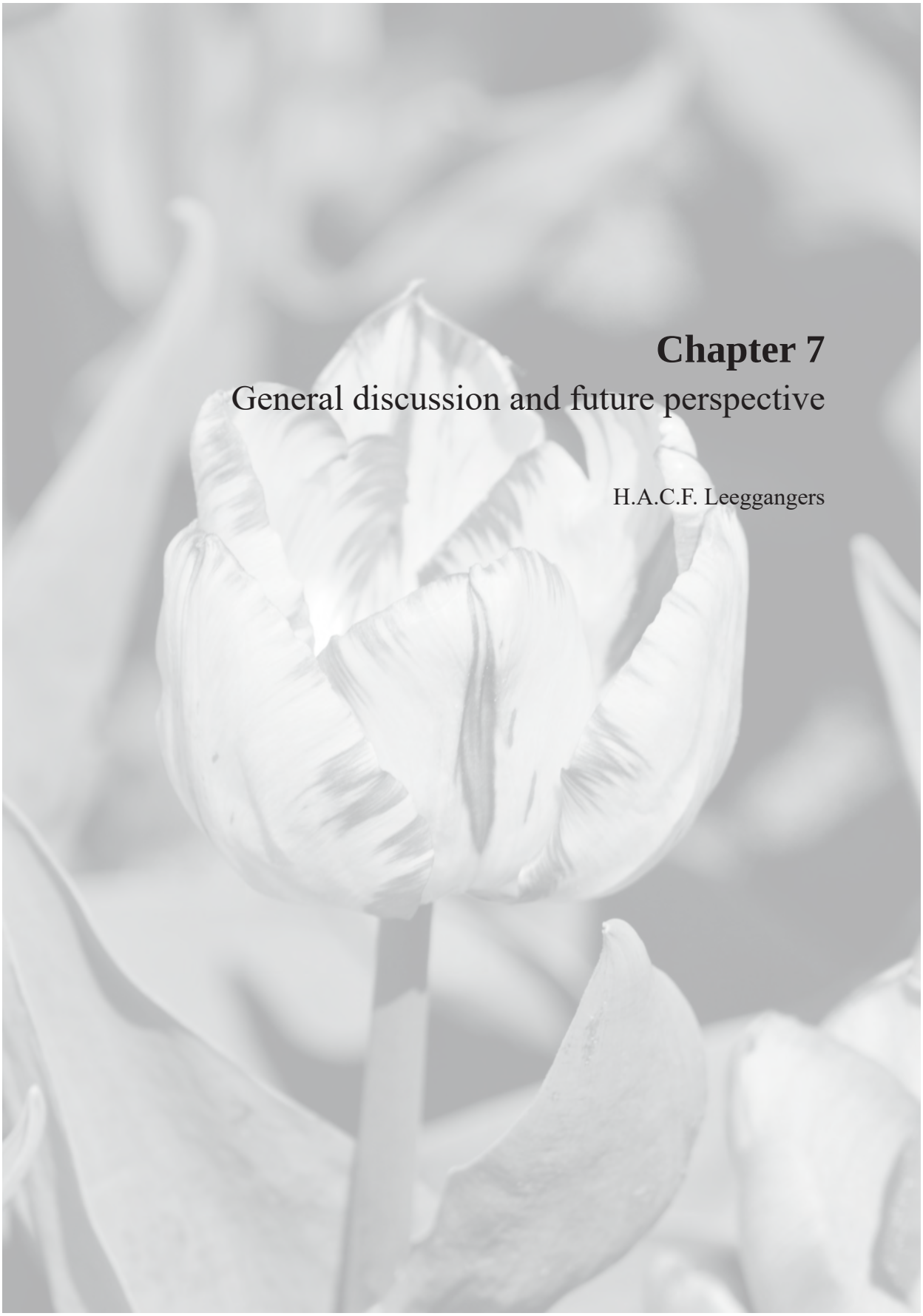


Figure S2. Amino acids measured in the floral bud.



Chapter 7
General discussion and future perspective

H.A.C.F. Leeggangers

Introduction

The ornamental geophyte tulip is the most cultivated bulbous species in the Netherlands and is widely grown in the field for vegetative propagation purposes and in greenhouses for the production of high quality cut flowers (De Hertogh and Le Nard, 1993). In this thesis, the effect of temperature on the floral induction and the transcriptional and metabolic changes that are occurring during winter in relation to dormancy release, have been studied in tulip. It is important to investigate these two temperature-dependent processes as the world is facing rapid climate change, which has negative effects on plant growth and development (Morison and Morecroft, 2006). The main focus for tulip breeders is on developing new cultivars with different colours, shape and/or size of the flower, but also plant height and pathogen resistance. Breeding and establishment of these new cultivars can take up to twenty five years, mostly due to the long juvenile phase (three to five years) and the slow propagation rate (van Eijk, 1971; Botschantzeva and Varekamp, 1982). In addition to these developmental constrains, environmental cues are interfering regularly with cut flower production. For example, floral bud blasting caused by high temperature during development in spring and summer has been occurring more frequently over the last decade (Fig.1A-B) (Hanks and Rees, 1977). These high temperatures coincide with climate change and can lead to reoccurring events of floral bud blasting in the future, especially when these sensitive cultivars are used for breeding purposes. The newly bred cultivars can inherit this temperature sensitivity. Furthermore, not only the temperatures are increasing during spring and summer, but also winters are becoming milder. In order to be able to flower, a period of prolonged cold is required for internal preparation of floral stem elongation and outgrowth of the flower (Fig. 1C-D) (Lambrechts et al., 1994; Rietveld et al., 2000). This might not have a direct effect on cut flower production in greenhouses (controlled growing conditions), but will have impact on the vegetative propagation process in the field.

Figure 1. Negative effect of unfavorable temperatures on development in tulip. (A) Example of floral bud blasting in the cultivar Strong Gold. The top leaves and floral bud are crinkled and do not fully develop. High ambient temperatures sensed by the daughter bulbs in late spring are supposed to be the cause of this phenomenon. (B) Normal floral bud development of Strong Gold. (C) Flowering of the cultivar Strong Gold without a period of winter cold. Bulbs were kept continuously at 20 °C after planting. Note that the stem is not elongating and that the flower remains closed. (D) Flowering of Strong Gold after a period of winter cold in the field. The stem is fully stretched and the leaves are unfolded, giving enough space for the flower to stretch and open.

The tulip three-ways life style: perennial, biennial and annual.

Tulips are mostly vegetatively propagated, because of the long juvenile phase, unless new cultivars need to be bred. Depending on which reproduction path is followed tulips can be described as perennial, biennial, or annual plant species.

Perennial – a botanical perspective

The time from a seed to grow into a bulb that is capable to produce a floral bud can vary between three to five years. In these years tulips grow monopodially from the displaced growing point until the adult vegetative phase is reached (Botschantzeva and Varekamp, 1982). This means that only after five to seven years, bulbs are able to reproduce. The long breeding cycle is not only observed for tulip, but also for woody perennial plant species, such as Populus trichocarpa (black cottonwood) (van Nocker and Gardiner, 2014). In this respect, tulip can be considered a perennial when grown from seeds.

The long duration of the juvenile phase is likely due to the number of growth cycles that are required to gain sufficient weight to switch from the juvenile vegetative to the adult vegetative phase (vegetative phase change). In the first years after germination from seed, the shoot apical meristem (SAM) is not competent to respond to the floral induction signals (Huijser and Schmid, 2011). Not much is known about this time consuming process in tulip, urging the need to study the juvenile phase and vegetative phase switch in the future in order to speed up the process. In contrast to tulip, this phase change has been studied at the molecular level in plant species such as *Arabidopsis thaliana* and *Zea mays* (maize) (Evans and Poethig, 1995; Wu and Poethig, 2006). An important regulator of maintaining the juvenile vegetative phase, by repression of *SQUAMOSA PROMOTOR BINDING PROTEIN LIKE* (*SPL*) genes, is *micro RNA 156* (*miR156*). Only repression of this *miRNA* in the shoot, initiated by a leaf derived signal, will result in the vegetative phase change by activation of its targets *SPL9*, *SPL13* and *SPL15* (Wu et al., 2009; Yang et al., 2010; Xu et al., 2016). The miR156/SPL module is conserved and this has been functionally confirmed in e.g. *Oryza sativa* (rice), *Brassica rapa* (Chinese cabbage) and *Populus* spp. (Wang and Wang, 2015)(Wang & Wang 2015). Detailed analyses in Arabidopsis revealed that a gradual increase in sugar content is serving as one of the triggers to make the switch from juvenile to adult vegetative phase by repressing *miRNA156* (Yang et al., 2013; Yu et al., 2013). Therefore, high expression of *miR156* and the repression of *SPL* genes due to the lack of sufficient free sugar could be one of the explanations of the long juvenile phase in tulip.

Besides the *miR156* and *SPL* genes, an *FT-like* gene in poplar was shown to have potential to shorten the juvenile phase (Hsu et al., 2006). Increasing *FT2* expression in poplar resulted in a faster change to the adult vegetative phase and, consequently, earlier first flowering. Furthermore, expression studies showed a gradual increase in *FT2* expression from one to two to eleven years old poplar trees. Together, these results suggest a role for this specific *FT* gene in the juvenile to adult phase transition. However, *FT*

is known to be a floral integrator and therefore might not be directly involved in the transition (Lee et al., 2006). In Arabidopsis, *Antirrhinum majus L.* (snapdragon) and *Olea europaea L.* (olive), the increase of *FT* expression towards the end of the juvenile phase is regulated by *TEMPRANILLO* (*TEM*). *TEM* is required to establish and control the length of the juvenile phase in these species and this function might be conserved in more (Sgamma et al., 2014). In this thesis several *FT-like* genes have been identified in tulip, with *TgFT2* as the best candidate to be the key regulator of seasonal flowering (Chapter 5). In case a similar regulatory mechanism for the juvenile to adult vegetative phase transition is present in tulip, as described above for poplar, *TgFT2* will be a good candidate gene to focus on. However, I identified two other *FT-like* genes in the tulip transcriptome and it might be that the regulation is more complex with a role for these other family members as well.

Biennial – from vegetative bud to reproductive bud

After the vegetative phase change, the SAM inside the bulb is responsive to floral inducing signals such as high temperature (Chapter 4). In addition, the bulb has no longer monopodial growth but sympodial as the apical bud in the mother bulb will terminate and growth is continued by several axillary meristems (daughter bulbs) (Botschantzeva and Varekamp, 1982). At planting time around October/November, the mother bulb contains an axillary bud (daughter bulb) in each axil of the scales. The innermost axillary bud (A) grows the largest and will replace the mother bulb after flowering in the coming spring. These daughter bulbs initiate their own daughter bulbs around February, which is at the same moment that bulb enlargement (bulbing) starts (De Hertogh and Le Nard, 1993). From this moment until termination of the flower, three generations are present inside the bulb. This means that two growth seasons are needed for an axillary meristem to develop into a mother bulb and flower in spring. In this respect tulip can be regarded as a biennial plant species. This implies that during the formation of these axillary buds, the temperature of that particular year can

have an effect on later stages of development. It has not been investigated if the development of the daughter bulbs is affected by past temperature experience of the (grand) mother plant. However, in seeds it has been shown that the growth environment of the mother plant has an effect on germination control of seed progeny. In Arabidopsis the *FT* gene also plays an important role but in the fruit tissues rather than the SAM. The temperature experienced during the vegetative phase of development is remembered by the parental plant and later used to control dormancy of the ripe seed (Chen et al., 2014). In relation to climate change it would be important to determine if tulip bulbs have a similar maternal effect on development of the (grand) daughter bulbs and how this is controlled.

Annual – Horticultural perspective

More commonly, tulip is referred to as an annual plant species, especially from a horticultural perspective. Growers plant the mother bulbs, containing a floral bud, in autumn and they will be replaced by daughter bulbs in the following spring (De Hertogh et al., 2013). These daughter bulbs are lifted, when the mother plant has completed senescence, and the bulbs are dried and afterwards stored at 17-20°C. Before lifting, or shortly thereafter, the vegetative to reproductive phase change (floral induction) is made in these daughter bulbs, which completes the annual growth cycle. High temperatures are required for the floral induction, while low temperatures are required for the preparation towards flowering (Chapter 4 and 6). This response to winter cold is different to, for example, Arabidopsis and Lilium longiflorum, where a period of prolonged cold (vernalization response) is required to trigger the floral induction (Dean et al., 2000; Villacorta-Martin et al., 2015). In Arabidopsis *FLOWERING LOCUS C* (*FLC*) is the key regulator in the vernalization response and only after a sufficient period of cold, *FLC* will be epigenetically silenced by the Polycomb Repressive Complex 2 (PRC2)(Kim and Sung, 2014). However, *FLC* dependent regulation of the vernalization response seems mostly restricted to the Brassicaceae, with the exceptions of *Beta vulgaris* (sugar beet),

Brachypodium distachyon (Purple false brome) and *Malus domestica* (apple) (Reeves et al., 2007; Alexandre and Hennig, 2008; Ruelens et al., 2013; Porto et al., 2015). In cereals, such as *Hordeum vulgare* (barley), the vernalization response is regulated through the activation of *VERNALIZATION1* (*VRN1*). The base sequence of this gene is highly similar to the MADS-box gene *APETALA1* (*AP1*) of Arabidopsis (Alonso-Peral et al., 2011). *VEGETATIVE TO REPRODUCTIVE TRANSITION 2* (*VRT2*), an *AGAMOUS LIKE 24* (*AGL24*) and *SHORT VEGETATIVE PHASE* (*SVP*) homologue, represses *VRN1* until a sufficient amount of cold is perceived and *VRT2* is down-regulated (Distelfeld et al., 2009). In oriental lily two vernalization related genes, *LoSVP* and *LoVRN1*, have been identified as candidates for playing a predominant role in the development and response to flowering (Villacorta-Martin et al., 2015; Li et al., 2016). Even though tulip lacks the vernalization response, low temperatures are still required in order to flower in spring. Whether this cold-induced outgrowth and flower maturation are regulated by the same type of genes is unknown. However, a preliminary targeted analysis did not provide any indications in this direction (Chapter 6).

Floral induction – size matters!

The regulation of (grand) daughter bulb initiation and bulbing in the annual cycle of an adult tulip remains a mystery, but they are important processes for the vegetative daughter bulb in order to reproduce and to flower the following spring. The vegetative daughter bulb must reach a certain size before it can flower. This is an important factor that determines whether the SAM produces only one leaf primordium or whether more leaves and in addition a floral bud is being developed (Khodorova and Boitel-Conti, 2013). The main vegetative daughter bulb (axillary A) is always of sufficient size to produce a floral bud (Chapter 4), but the remaining daughter bulbs do not always meet the size requirement for the establishment of a floral bud in summer. There are two distinctive scenarios which can possibly explain this: 1) the resources available for these daughter bulbs are not sufficient for

proper development of the flower or/and 2) the meristem of these particular daughter bulbs is incompetent for floral inducing signals. Smaller sized daughter bulbs often have a lower number of scales in comparison to a larger size daughter bulb (Rees, 1972). The scales are storage organs and provide energy for developing tissues in the appearance of carbohydrates (Botschantzeva and Varekamp, 1982). Fewer scales will result in a lower quantity of resources and therefore the lack of sufficient energy to maintain the development of a floral bud or to produce one. It is not known if the development is terminated earlier or if the SAM is incompetent for floral inducing signals. However, Rees (1972) describes that a small bulb connected to a large bulb is still able to produce a small flower, but of lower quality. An example of this is axillary H, which is attached to the outer scale of the mother bulb. This shows that the resources available in the scale tissue are at least determining the quality and size of the flower.

Temperature is the most important environmental signal to trigger floral induction at the SAM of the daughter bulb (Chapter 4). Small sized bulbs might be incompetent to this environmental signal, leading to the development of just one leaf primordium. In Tulipa fosteriana it has been shown that after replacement of the mother bulb, the daughter bulbs continue to multiply vegetatively for several years. Apical and axillary buds form so called ‘sinkers’ and even though several large fleshy scales are formed, they do not flower. A cultivar known as ‘Keizerskroon’ has been maintained for over 200 years through vegetative propagation and did not show any sign of degeneration. It is believed that the daughter bulbs are partly rejuvenated, meaning that the bulbs switch temporally back to the juvenile vegetative phase. This has also been observed in the tulip species *Tulipa kaufmanniana* and *Tulipa greigii* (Botschantzeva and Varekamp, 1982). How, or if the vegetative phase transition can be reversed is not known, but perhaps *miR156* is again up-regulated in these bulbs. A more recent view on the regulation of the vegetative phase transition is related to the carbohydrate metabolism. Arabidopsis plants with defective sugar signaling and floral repressor genes (e.g. *GLUCOSE INSENSITIVE* (*GIN1*)

and *TERMINAL FLOWER 1* (*TFL1*)) have a shorter juvenile phase, showing the importance of these genes in the vegetative phase transition (Matsoukas et al., 2013). This reveals that the turnover of starch into available carbohydrates is another factor influencing the vegetative phase transition and may enable the reversion of the transition. This means that the miR156/SPL module in tulip may be important at different moments of development, both in bulbs descended from seeds and vegetatively propagated bulbs. In addition to this, sometimes in perennials a minority of meristems is kept in the vegetative state to produce flowers and fruit at a more favourable moment (Polycarpic growth) (Bergonzi and Albani, 2011). Examples of perennial species in which this has been studied are *Arabis alpina* (Alpine rock-cress) and *Fragaria vesca* (strawberry). In both species, the homologue of AtTFL1 plays an important role in meristem competency for floral inducing signals in axillary meristems (Wang et al., 2011; Koskela et al., 2012). The same might acquire for the vegetative daughter bulbs in tulip. In axillary A the expression of *TgTFL1*, which has a high sequence similarity with *AtTFL1*, decreases in expression prior to the floral initiation. The timing of this decrease is different in the three cultivars Purple Prince, Dynasty and Strong Gold (Chapter 4). It would be of great interest to investigate the expression of *TgTFL1* in the other axillary buds that might not develop a floral bud after experiencing high temperatures. In case of a correlation, tulip rather resembles a perennial plant species throughout its whole development cycle than an annual. Therefore, it is conceivable that tulip and other perennial plant species share a common mechanism in the regulation of reproduction through axillary meristems.

Dormancy

After completion of the floral bud inside the main daughter bulb and the establishment of the dry tunic on the outside, the bulb is dormant (Kamenetsky and Okubo, 2013). The term ‘dormancy’ can be a misleading term, because inside the tulip bulb there is still continuous growth of the various tissues. Examination of the different internal tissues has led to the

conclusion that only the floral bud is not showing any growth over a period of several weeks (Chapter 6).

Sink to source

Growth during the dormancy period is still significant in the cultivar Strong Gold that was investigated, but at the transcriptome level a small number of changes are occurring in the floral bud (Chapter 6). Possibly, the floral bud is not a strong sink in comparison to the other tissues, which are developing faster. In most plants, the leaves are green and above the soil surface before any signs of flowering are observed. In those species, the leaves are the most important source for the distribution of sucrose (Ho and Rees, 1975). For the majority of its growth cycle tulip has leaves inside the bulb or under the soil surface; these leaves will only appear above the surface in February/March. Scales are modified leaves and are an important energy source for the developing tissue, while still under the soil surface. Ho and Rees (1976) have shown that the two major sinks during the first half of the low temperature period are the roots and the leaves. In chapter 6 of this thesis, a similar observation was made but also the floral stem seems to be an important sink organ at this time. Once the leaves come above the soil surface, it is likely that the leaves make the switch from sink to source. More carbohydrates are then transported from the leaves to the floral stem, floral bud and the vegetative daughter bulbs in the axils of the scales.

Carbohydrates, especially sucrose, are often transported from the source to the sink via the vasculature or, more specific, the phloem (Lemoine et al., 2013). The GO-analysis of the floral bud in chapter six suggests that the xylem and phloem are formed after planting and during the winter period in the field. This can be an explanation of why the floral bud is not directly a strong sink. The floral bud may have to mature first to become a strong sink in order to develop a proper flower. This phenomenon is not uncommon in plants when comparing for example bulbs with seeds. The formation of vasculature tissue is paused during embryogenesis (seed maturation) and will be completely formed before germination (Heo et al., 2014). In this

comparison the floral bud substitutes the embryo and germination is replaced by sprouting.

Bulbs versus seeds

In the past scientists have tried to compare the dormancy process of seeds with tulip bulbs, but their conclusion was that they do not share a common genetic basis for this process (Fortanier and van Brenk, 1975). The regulation of dormancy might not directly share a common mechanism, but in general the overall development of a seed or tulip bulb shows a remarkable overlap. Both contain a protective skin on the outside of the tissue (seed coat and tunic). Furthermore, both have specialized storage tissues (endosperm and scales) to provide enough energy for the embryo versus shoot apical meristem, until another energy source becomes available. Finally, both structures continue development to produce offspring (germination and sprouting). The Gene Ontology (GO)-enrichment analysis of the differentially up-regulated genes in relation to the floral induction, showed seed related GO-terms. In addition, GO-terms over-represented in the down-regulated genes were related to metabolism (Chapter 4). In seeds, dormancy is induced during the seed maturation phase resulting in a quiescence of metabolic activity (Fait et al., 2006; Graeber et al., 2012). This is also suggested by the GO-enrichment analysis of tulip. The two key players for the induction of dormancy in Arabidopsis seeds are the phytohormone abscisic acid (ABA) and the *DELAY OF GERMINATION1* (*DOG1*) gene (Bentsink and Koornneef, 2008). Similar to the floral repressor *AtFLC*, *AtDOG1* is mostly identified in Brassicaceae relatives, such as *Brassica rapa* and *Lepidium sativum* (garden cress), but also in cereals (Shu et al., 2015). A more conserved dormancy-inducing gene is *ABA INSENSITIVE3/VIVIPAROUS1* (*ABI3/VP1*). Arabidopsis *ABI3*, *VP1* of maize, and their orthologous genes in other plants species are components of a network that controls the expression of ABA responsive genes during seed maturation (Graeber et al., 2010). It is possible that dormancy induction in tulip bulbs is regulated by a similar genetic network. First evidence for this

hypothesis is provided by the GO-enrichment analysis of differentially up-regulated genes described in Chapter 4, showing the over-representation of the GO-term *Response to abscisic acid*. Genes marked with this GO-term and present in the RNA-seq data are a homolog of *AtABCG40* and *ABA INSENSITIVE 5* (*ABI5*). *ABCG40* is involved in the transport of ABA from the endosperm to the embryo, which will be the floral bud in the case of tulip (Kang et al., 2015). *ABI5* is important for ABA signaling during seed maturation and germination (Finkelstein and Lynch, 2000). Further research is required in tulip to show the importance of ABA related genes in dormancy establishment.

Dormancy release in seeds, resulting in germination, is accompanied by the change in balance between ABA and gibberellin (GA) (Bewley, 1997). Environmental stimuli, such as light and temperature, are able to alter the germination potential by changing the levels or sensitivity for ABA and GA. GA is required for seed germination, but also for stem elongation and fertility (Hauvermale et al., 2015). *Repressor of GA1-3 like 2* (*RGL2*) plays a major role in the repression of seed germination in Arabidopsis. RGL2 is one of the five DELLA proteins that acts immediately downstream of the GA receptor. The other four DELLA proteins (RGA, GA INSENSITIVE (GAI), RGL1 and RGL2) are involved in modulation of floral development (Tyler et al., 2004). During dormancy release, the expression of *GIBBERELLIN-INSENSITIVE DWARF1* (*GID1*) increases. Due to this increase, GA can bind on the GID1 receptor, which stimulates GA responses by targeting the degradation of the repressing DELLA proteins. The GID1-GA-DELLA composition is recognized by SLEEPY1 (SLY1), resulting in the ubiquitination of DELLA and dormancy release (Hauvermale et al., 2015). In bulbs the alteration in ABA/GA balance is also linked to dormancy release (Kamenetsky et al., 2003). It is possible that also in tulip bulbs GA-receptors and DELLA genes play a role in dormancy release. Among the differentially expressed transcripts in the floral bud, when comparing week minus four with week 16, a homolog of AtGAI was 3.2 fold down-regulated and a homolog of AtSLY1 was 2.4 fold up-regulated (Chapter 6). This

shows that there is a possibility that seeds and bulbs share a common mechanism to release dormancy. However, further investigations and exploration of the RNA-seq data is needed.

Challenges of fundamental research on a non-model species

Studying certain processes, such as the floral induction and dormancy release, in tulip comes with challenges. The growth cycle of tulip is relatively long and no genome is available to map RNA sequencing data and/or to improve the assembly of this data (Chapter 3). Furthermore, there are limited tools to perform functional analyses. In 1992 Wilmink and colleagues (Wilmink et al., 1992) managed to transform tulip and to obtain bulbs transiently expressing GUS. However, it was a laborious procedure and very inefficient. Identification of genes can be considered as one of the easiest steps nowadays in the era of Next Generation Sequencing (NGS). However, collecting knowledge on the molecular functions of these genes in a certain developmental process is challenging.

It would be of great help to have an efficient protocol to make transgenic tulip bulbs. In other bulbous species, such as *Narcissus*, *Lilium* and *Allium*, stable transformation protocols are available. The common factors in these protocols are transformation mediated by Agrobacterium and the use of embryonic cultures as starting material (Eady et al., 2000; Lu et al., 2007; Núñez de Cáceres et al., 2011). Generating bulbs from in vitro propagation protocols is at the moment one of the limiting steps in developing a transformation protocol for tulip (Kamenetsky and Okubo, 2013). However, other techniques, such as virus-induced gene silencing (VIGS) can be explored as alternative methods to provide insight in the exact function of identified candidate genes. In Gladiolus VIGS was successfully used for comparative functional studies (Zhong et al., 2014; Wu et al., 2015). VIGS is often applied to non-model species of which stable genetic transformation protocols are inefficient or are lacking a transformation protocol. The VIGS system uses the RNA defense system of the plant in order to silence a gene.

In most studies a virus, such as the Tobacco Mosaic Virus (TMV) or Tobacco Rattle Virus (TRV), carries the plant *PHYTOENE DESATURASE* (*PDS*) gene. This gene is highly conserved and is involved in the carotenoid biosynthesis pathway. Carotenoids involved in chlorophyll protection can be found downstream of phytoene (Siefermann-Harms, 1987). Therefore, when the viral vector containing the *PDS* gene is introduced in the plant via Agrobacterium, leaves with a bleaching phenotype can be observed (Lu et al., 2003; Senthil-Kumar and Mysore, 2014). This system makes it easy to test whether VIGS can be used for creating (transient) transgenic plants. For tulip the same approach was taken by us and the *PDS* gene was amplified from the cultivar Strong Gold and cloned into the TRV2 vector. The *PDS* gene of tulip was able to induce bleaching of *Nicotiana benthamiana* leaves by agro-infiltration, showing that the generated vectors were correct and revealing strong conservation of the coding sequence of this gene. Unfortunately, in tulip no bleaching was observed in the leaves. Therefore further research is required to create a good and efficient transformation protocol for tulip.

Future prospective and practical applications

Le Nard and De Hertogh (2002) identified research areas of which they thought would be needed to understand tulips and their development. One of these research areas is the performance of basic research to understand fundamental processes such as bulbing, floral induction, and flower differentiation. In this thesis, one of the first integrated and multidisciplinary approaches was undertaken to investigate the floral induction process and dormancy release in tulip at the morphological, physiological and molecular levels. Several genes have been identified that either negatively or positively correlate with these processes, such as *TgTFL1* with floral induction and *TGSQA* with floral initiation (Chapter 4). These two genes can be used as expression markers to adjust, for example, the temperature treatment of bulbs in storage, after lifting of the bulbs from the field. Instead of applying the commonly used 34°C treatment after lifting, bulbs can e.g. be stored at

a lower temperature (17-20°C) directly in order to prevent floral bud blasting in cultivars that are highly sensitive to ambient temperature changes in spring. Besides gene expression levels, glucose content displayed a good correlation with the length/growth of the various internal tissues during the winter period in the field. Further research is required to confirm the correlation in different years and cultivars, but it has the potential of being a marker for the chilling requirement in tulip. Besides the practical applications, a genome sequence of tulip would increase the quality of research from a fundamental perspective.

Concluding remarks

There are still many of unanswered research questions in relation to the development and flowering of tulip. In this thesis, the results of the first in-depth molecular studies have been presented. These analyses show that tulip shares various mechanisms with perennial plant species. Furthermore, at a tissue and organ level, surprising commonalities between bulbs and seeds have been elucidated. Altogether, this provides tulip breeders and growers with novel insights to modify and optimize their daily practice. For scientists this has created additional research questions to investigate.

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Summary

The ornamental geophyte *Tulipa gesneriana* is the most cultivated bulbous species in the Netherlands. It is widely grown in the field for vegetative propagation purposes and in greenhouses for the production of high quality cut flowers. Over the last decade, the tulip bulb industry is affected by the rapid climate change the world is facing. Temperature is rising and influences the vegetative to reproductive phase change (floral induction) inside the tulip bulbs in spring and processes that are occurring during winter, such as dormancy release.

In this thesis the two temperature-dependent processes related to tulip flowering, being floral induction and dormancy release, were investigated in detail with a special focus at the molecular level. Flowering time has been studied in a broad range of species, including the model species *Arabidopsis thaliana* and *Oryza sativa*. The current understanding of this process can be translated to non-model species, such as tulip, through a 'bottom-up' and 'top-down' approach (Chapter 2). For the 'bottom-up' approach conservation of molecular pathways is assumed and researchers make use of sequence homology searches to identify candidate genes. The 'top-down' approach starts from large scale data mining, such as RNA-sequencing (RNA-seq) data or microarrays, followed by the association between phenotypes, genes and gene expression patterns. Here, a comparison with data from model plant species is made at the end of the process and this also leads to the identification of candidate genes for a particular process.

Large scale genomics data mining in tulip is only possible via transcriptome analysis with RNA-seq derived data, because no full genome-sequence is present at this moment. Genome sequencing remains a challenge for species with a large and complex genome, containing probably a large number of repetitive sequences, which is the case for tulip and lily. In chapter 3 a high quality transcriptome of tulip and lily is presented, which is derived from a collection of different tissues. In order to obtain good transcriptome coverage and to facilitate effective data mining, different filtering

parameters were used. This analysis revealed the limitations of commonly applied methods used in de novo transcriptome assembly. The generated transcriptome for tulip and lily is made publicly available via a user friendly database, named the '*Transcriptome Browser*'.

The molecular regulation of the temperature-dependent floral induction was studied through the use of RNA-seq (Chapter 4). A better understanding of this process is needed to prevent floral bud blasting (dehydration of the flower) in the future. The development at the shoot apical meristem (SAM) was morphologically investigated in two contrasting temperature environments, high and low. Meristem-enriched tissues were collected before and during the start of flower development. The start of flower development is morphologically visible by rounding of the SAM and correlates with the up-regulation of *TGSQA*, an *AP1-like* gene. A 'top-down' approach was used to identify possible regulators of the floral induction in tulip. However, Gene ontology (GO)-enrichment analysis of the differentially expressed genes showed that the floral induction, maturation of the bulb and dormancy establishment are occurring around the same period in time. Therefore a 'bottom-up' approach was followed to identify specific flowering time regulators based on knowledge obtained from other species. Expression analysis in tulip, heterologous analysis in Arabidopsis and yeast two hybrid-based protein-protein interaction studies revealed that *Tulipa gesneriana TERMINAL FLOWER 1* (*TgTFL1*) is likely a repressor of flowering, whereas Tulipa gesneriana *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS-LIKE2* (*TgSOC1L2*) acts probably as a floral activator.

Another well-known flowering time regulator is *FLOWERING LOCUS T* (*FT*), which is a member of the PEBP gene family found in Arabidopsis and many more plant species. In tulip and lily, a total of four highly similar sequences to FT and HEADING DATA 3A (Hd3a) were identified (Chapter 5). Overexpression of *Lilium longiflorum FT* (*LlFT*) and *TgFT2* in

Arabidopsis resulted in an early flowering phenotype, but upon overexpression of *TgFT1* and *TgFT3* a late flowering phenotype was observed. The tulip PEBP genes *TgFT2* and *TgFT3* have a similar expression pattern during development, but show a different behaviour in Arabidopsis. Therefore the difference within the amino acid sequence was investigated, which resulted in the identification of two important amino acids for the FT function, which appeared to be mutated in TgFT3. Interchanging of these amino acids between TgFT2 and TgFT3 resulted in conversion of the phenotype, showing the potential importance of these positions in the protein and these specific amino acids for the molecular mode of action of these two proteins. Based on all the data, *LlFT* is considered to play a role in creating meristem competency to flowering related cues and *TgFT2* to act as a florigen involved in the floral induction. The function of *TgFT3* is not clear, but phylogenetic analysis suggests a bulb specific function.

After the floral induction and completion of flower development inside the tulip bulb, a period of prolonged cold is required for proper flowering in spring. Low temperature stimulates the re-mobilization of carbohydrates from the scale tissues to the sink organs, such as the floral stem, floral bud and leaves. Not many details are known about the molecular and metabolic changes during this cold period. In chapter 6, first insights are shown on the development of the different tissues inside the bulbs. The floral bud appears to be the least active tissue in comparison with the floral stem and leaves, suggesting a type of floral bud dormancy in tulip. However, metabolic changes are suggesting that the floral bud is still showing active cell division and/or preparation for elongation by turgor-driven cell wall extension. Dormancy of all tissues seems to be released ten weeks after planting and is correlated with the increase of glucose levels. In the leaves, from this same moment, photosynthesis related genes are up-regulated suggesting that the leaves are preparing for photosynthesis while still beneath the soil surface.

At the end of the thesis a glance is given at different perspectives of the tulips life cycle, categorizing tulip as a perennial, biennial or annual plant species, respectively. The perennial way of life is applicable when growing bulbs from seeds, while biennial and annual are more in relation to vegetative propagation. Also the importance of bulb size is highlighted, because it will determine if the bulbs are able to flower or not the following spring. Two scenarios are discussed related to availability of energy in the presence of carbohydrates and meristem incompetency to floral inducing signals. Throughout all research done for this thesis, it became clear that tulip bulbs and seeds have a lot in common. By combining the knowledge of processes in different plant species or developmental systems it is possible to understand how flowering and dormancy release are regulated and this provides us with novel insights how these processes are regulated in bulbous plant species, such as tulip.

Acknowledgements

During my bachelor studies at Avans University of Applied Science I could have never imagined doing a PhD. Even though at the time I always laughed when saying that the doctor's title sounds nice and it would be fun to get one. In New Zealand I was convinced by Alison Fisher, a female scientist that who doing her sabbatical at the lab of Richard Macknight. However, to start a PhD I had to first obtain my master degree. After two years of obtaining practical experience and following lectures I finished it and my former supervisor Ludmila Mlynarova told me about this particular PhD position. I remember entering for the first time in Richard's office and I was super nervous. This feeling disappeared quickly after talking for more than half an hour about the position and, of course, about tulips! Ludmila, but also Kim Boutilier, really supported me in getting this position and I am very thankful for that. The interview is a moment I will never forget. I still remember that Henk Hilhorst asked me during the interview the question 'What if you cannot extract RNA?' and that I kept saying 'well then we will try again!'. I am very thankful that both Richard and Henk were convinced enough to hire me for the job (hope you do not regret it :-p).

Before I started, I heard a lot of good stories about Richard and I must admit that people were completely right about him. Richard, I am grateful that you were my supervisor for the last four years and I really enjoyed the freedom you gave me in performing the research. I know we did not always agree on everything, but it did mean that we kept each other sharp on the particular subject. You really supported me to grow into an independent scientist. Thank you for that. Also many thanks to my promotor Henk Hilhorst for integrating the bulb group into the weekly meetings of the seed lab. In the beginning it did not make much sense to be part of the seed lab, but as time went on the research led us to a point that showed us that bulbs and seeds might not be that different. This is also something that I remember Henk saying. Really has been a pleasure to be part of the seed lab. Thank you Leónie and Wilco for your input during the whole project. It was nice to get suggestions from a different angle. Also during the meetings your comments helped me to think about the experiments and what would be a good next

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Nevertheless, in general I would like to thank all fellow PhD students for the nice time in Wageningen. During the PhD trip I had to miss some of the days, but it was great to spend time together scientifically but also outside of science. It was a pleasure to organize this PhD trip together with Bing, Maria Cecilia, Esmer and Beatriz. Science can be a pain, but everyone in the lab helped each other in times of need. I want to thank Natalia for her help, suggestions and discussions during this big challenge of understanding bulbs. Also Bas, you helped me a lot with the practical side of things, but also the discussions with you were nice and very helpful.

Every year bulbs were planted in the field and for the first two years this was done by Maarten Holdinga and I want to thank him for that. I had never worked with bulbs before, so I really learned a lot from Maarten but also from Alex Silfhout, who helped us with the bulbs in the last two years of the PhD. Some of the experiments were performed with equipment of the group Bioscience and I want to thank them for that. Without the microscope I could have never made those beautiful pictures of the shoot apical meristem inside the tulip bulbs. A special thanks to Martijn and Marco who helped me and provided plasmids for the yeast two hybrid experiment. Also big thank you to Froukje for growing Arabidopsis plants for the floral dipping and Leonie for the primers of the Arabidopsis reference gene. Also thank you Jeroen van Arkel and Roland Mumm for performing the GC-ToF-MS analysis and helping me with setting up the experiment. I have learned a lot and am very thankful for that. Another person to mention is Paul Arens. I am thankful that you were willing to share the 454-sequence data and help us finding FT sequences in your lily data. It was nice collaborating with you. Also Michele Zaccai, thank you so much for the oppertunity to combine our work into a nice paper, that hopefully will be accepted soon. Every six months we had a meeting with people of the sector and I would like to thank Carlo Randag, Bas Brandwagt, Sjaak Langeslag, Henk Gude, Paul Bijman and Danielle Kroes for their input and support. In addition, we received visits from growers and I am happy that they were able to travel to Wageningen and to discuss the project and how we could improve things.

During the last four years I had the pleasure to work with five bachelor students. Nick Snelders was my first student and I appreciate all his efforts he put in getting the Virus Induced Silencing (VIGS) to work in tulip. The FT work described in chapter 5 started as a side project with Twan Groot, who helped me with the cloning. It was funny to discover that we both collected euro coins of the different European countries. After a period of having no students, Juliette Silven en Judit Nadal Bigas visited the lab for a long period to help me out with the cloning of some genes to make overexpression lines of some flowering time genes. I must admit that it was a challenge having two students at the same time, but they both managed to help me take a step further in the research. Thank you Juliette for making the constructs related to the *TgFT-like* gene and thank you Judit for perfoming all the yeast two hybrid experiments. Last, but not least, Janna Damen came for the bachelor thesis in the last six months of my PhD. It was a very short period, but for a first internship I was impressed with what you did. Unfortunately the results were not what we expected, but for sure we both learned a lot from it.

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For sure I have forgotten to mention people and sorry if I did! It has been like a rollercoaster the last four years, but with the support of everyone I managed to complete this adventure. Thank you for that.

About the author

Curriculum vitae

Melissa (H.A.C.F) Leeggangers was born on the 18th of January 1989 in Oosterhout (NB), the Netherlands. She obtained her bachelor degree in Biological and Medical Laboratory Sciences at Avans University of Applied Sciences in 2010. During her bachelor studies she followed the minor Molecular Research and Bioinformatics and finished the studies with an internship at the University of Otago in Dunedin, New Zealand. After completion of the bachelor studies, she moved on to Utrecht University where she obtain her master degree in Molecular and Cellular Life Sciences in 2012. On the 1st of October 2012 she started as a PhD student at the endowed Chairgroup Physiology of Flower bulbs at the Laboratory of Plant Physiology in Wageningen.

Publications

Leeggangers, H.A.C.F.*, Moreno-Pachon*, N., Gude, H. and Immink R.G (2013). Transfer of knowledge about flowering and vegetative propagation from model species to bulbous plants. The International journal of developmental biology 57(6-8):611-20. Doi: 10.1387/ijdb.130238ri.

Todd, R.B., Zhou, M., Ohm, R.A., **Leeggangers, H.A.C.F.**, Visser, L. and de Vries R.P. (2014). Prevalence of transcription factors in ascomycete and basidiomycete fungi. BMC genomics 15:214. Doi: 10.1186/1471-2164-15-214

Leeggangers, H.A.C.F.*, Folta A.*, Murras, A., Nap, J.P. and Mlynarova, L. (2015). Reduced seed germination in Arabidopsis over-expressing SWI/SNF2 ATPase genes.Physiologia Plantarum 153(2):318-26. Doi 10.1111/ppl.12231.

Moreno-Pachon, N.*, **Leeggangers, H.A.C.F.***, Nijveen, H., Severing, E., Hilhorst, H.W.M, Immink, R.G.H (2016).Elucidating and mining the Tulipa and Lilium transcriptomes.Plant Molecular Biology 92(3):249-61. Doi: 10.1007/s11103-016-0508-1

Leeggangers, H.A.C.F., Nijveen, H., Bigas Nadal, J., Hilhorst, H.W.M, Immink, R.G.H. (2016/2017). Molecular regulation of temperature-dependent floral induction in Tulipa gesneriana. Plant Physiology, *under review*.

Hendrika A.C.F. Leeggangers*, Tamar Rosilio-Brami*, Judit Bigas-Nadal , Noam Rubin, Aalt D.J. van Dijk, Francisco F. Nunez de Caceres Gonzalez, Shani Saadon-Shitrit, Harm Nijveen, Henk W.M. Hilhorst, Richard G.H. Immink and Michele Zaccai. (2016/2017). Characterization of Tulipa gesneriana and Lilium Longiflorum genes from the PEBP family and their putative roles in flowering time control. Journal of Experimental Botany, *under review*.

** Equally contributed*

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: H.A.C.F. (Melissa) Leeggangers
Date: 31 January 2017
Group: Plant Physiology
University: Wageningen University & Research

1) Start-up phase	date
▶ **First presentation of your project**	
Floral transition and dormancy release in tulip	Oct 19, 2012
▶ **Writing or rewriting a project proposal**	
Floral transition and dormancy release in tulip	Oct 2012-Jan 2013
▶ **Writing a review or book chapter**	
Transfer of knowledge about flowering and propagation from model species to bulbous plants, Int.J.Dev. Biol.57. pp 611-620 (2013). DOI:10.1387/ijdb.130238ri	Jan 2013-Jun 2013
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	date
▶ **EPS PhD student days**	
EPS PhD student day, Leiden (NL)	Nov 29, 2013
EPS PhD student day 'Get2gether', Soest (NL)	Jan 28-29, 2016
▶ **EPS theme symposia**	
EPS theme 1 symposium 'Developmental Biology of Plants', Leiden (NL)	Jan 17, 2013
EPS theme 1 symposium 'Developmental Biology of Plants', Wageningen (NL)	Jan 24, 2014
▶ EPS theme 3 symposium 'Metabolism and Adaptation', Amsterdam (NL)	Mar 11, 2014
EPS theme 1 symposium 'Developmental Biology of Plants', Wageningen (NL)	Jan 21, 2016
▶ **Meeting 'Experimental Plant Sciences' (Lunteren) and other National Platforms**	
Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 22-23, 2013
Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 14-15, 2014
Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 13-14, 2015
Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	Apr 11-12, 2016
▶ **Seminars (series), workshops and symposia**	
Flying seminars	
Origin and Consequences of genetic and epigenetic variation in Arabidopsis thaliana (Detflef Weigel)	Feb 27, 2013
Genetics and epigenetics: a complex relationship (Ortrun Mittelsten Scheid)	Nov 19, 2014
Seasonal flowering in annual and perennial plants (George Coupland)	Jan 19, 2015
The evolutionary significance of gene and genome duplications (Yves van de Peer)	Feb 03, 2015
Regulation of root morphogenesis in tomato species in the face of a changing environment (Siobhan Brady)	Sep 09, 2015
Other individual seminars	
Metabolomics-based functional genomics - from Arabidopsis to crops and medicinal plants (CBSG seminar)	Apr 08, 2013
Using the non-model genus Passiflora to study the evolution of novelty in plant reproductive development (Marcelo Dornelas)	Jan 27, 2015
Symposia	
Mini symposium 'Developments in Arabidopsis Research', Farewell of Maarten Koornneef	Apr 11, 2013
The International Symposium on Genetic Variation of Flowering Time Genes and Applications for Crop Improvement	Mar 24-26, 2014
Sympsium Plant Biomass Utilization by Fungi (Utrecht, Oratie Dr. Ronald de Vries)	Feb 27, 2015
▶ **Seminar plus**	
Lunch discussion with George Couplant from Max Planck Institute (Cologne, Germany)	Jan 19, 2015
▶ **International symposia and congresses**	
Molecular mechanisms controlling flower development (France)	Jun 08-12, 2013
Floral transition in Arabidopsis (Tübingen, Germany)	Sep 16-18, 2013
Plant Dormancy symposium (Auckland, New Zealand)	Nov 04-07, 2013
▶ **Presentations**	
Poster: Identification of ambient temperature responsive genes involved in the floral induction of tulip	Apr 22-23, 2013
Poster: The effect of high temperature on the floral transition in tulip	Sep 16-18, 2013
Poster: The regulation of dormancy induction and release in tulip	Nov 04-07, 2013
Poster: Het effect van temperatuur op de bloem aanleg en het doorbreken van de winterrust in tulp	Jan 2014
Poster: To flower or not to flower: the floral transition in *Tulipa gesneriana*	Mar 24-26, 2014
Talk: Identification of flowering time genes in *Tulipa gesneriana*	Apr 22-28, 2015
Talk: The hot, the cold and the tulip: Identification of key flowering time genes	Jan 21, 2016
Talk: Flowering time control in tulip and Arabidopsis: Comparing apples and oranges?	Apr 11-12, 2016
▶ **IAB interview**	
▶ **Excursions**	
PhD trip Plant Physiology - visiting German and Swiss Universities and Companies	Apr 22-28, 2015
Subtotal Scientific Exposure	*20.0 credits**

3) In-Depth Studies	*date*
▶ **EPS courses or other PhD courses**	
CBSG genome mining, Wageningen (NL)	Dec 13, 2012
An Introduction to Mass Spectrometry-based Plant Metabolomics, Wageningen (NL)	Dec 09-13, 2013
The power of RNA-seq, Wageningen (NL)	Dec16-18, 2013
▶ **Journal club**	
Literature discussion at Plant Physiology	Oct 2012-Apr 2016
▶ **Individual research training**	
Subtotal In-Depth Studies	*5.7 credits**

4) Personal development	*date*
▶ **Skill training courses**	
Scientific Writing	Sep 16-Nov 18, 2014
Efficient Writing Strategies	Mar 11-Apr 22, 2015
Effective Behaviour in your Professional Surroundings (EB)	Apr 2015
Presenting with Impact	Dec 2016
▶ **Organisation of PhD students day, course or conference**	
PhD trip of Plant Physiology 2015	2014 - 2015
▶ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*6.9 credits**

TOTAL NUMBER OF CREDIT POINTS*	**46,1**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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