Modulation of Developmental Transitions by Trehalose-6-phosphate Signaling in *Arabidopsis thaliana*

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Summary

Appropriate timing of developmental transitions (both vegetative and reproductive) ensures that the plants produce flowers and fruits in the most favorable conditions, which is crucial for their survival. In addition to the known pathways, carbohydrate or energy status also plays an important role in the proper timing of phase transitions, as flowering and subsequent production of seeds are highly energy demanding. However, our knowledge regarding the mechanisms that integrate sugar or energy signals with the other known developmental pathways is still limited. In *Arabidopsis thaliana*, the status of sucrose – the main circulatory sugar and energy source – is conveyed by trehalose-6-phosphate (T6P), a signaling intermediate formed during the biosynthesis of disaccharide trehalose. Trehalose-6-phopshate synthase (TPS) enzyme catalyzes the production of T6P.

When homozygous mutants with a defective *TPS1* gene due to a transposon insertion (*tps1-2*) were rescued by introducing a dexamethasone (DEX)-inducible *TPS1* construct (*GVG:TPS1*), the resulting plants (*tps1-2 GVG:TPS1*) flowered extremely late even under long days (LD), which are normally conducive for flowering. This suggests that T6P / TPS1 pathway is necessary for the proper timing of flowering in *Arabidopsis thaliana*.

The results discussed in the first chapter of this thesis demonstrate that T6P / TPS1 pathway regulates the expression of key floral promoting genes such as, *FLOWERING LOCUS T* (*FT*) and *TWIN SISTER OF FT* (*TSF*) in the leaves of *Arabidopsis thaliana*. Furthermore, in the shoot apical meristem, T6P / TPS1 signaling regulates the expression of *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes (*SPL3*, *SPL4* and *SPL5*), partially via microRNA156 (miR156), which is an upstream regulator of *SPLs*. This dual role played by T6P / TPS1 pathway ensures that the

reproductive transition in plants occurs only when there are sufficient energy sources, despite the exposure to a favorable photoperiod.

In addition to an extreme delay in flowering, *tps1-2 GVG:TPS1* plants exhibit a prolonged juvenile phase or a delay in the vegetative phase transition. The experiments performed in the second chapter show that T6P / TPS1 signaling regulates vegetative phase change at least in part through the miR156-SPL module. Taken together, the results of the second chapter suggest that T6P, rather than sucrose might function as the age-dependent signal responsible for the repression of miR156, and promote the juvenile-to-adult phase transition and flowering in *Arabidopsis thaliana*.

The results of an ethyl methanesulfonate (EMS)-based suppressor screen performed in *tps1-2 GVG:TPS1* is described in the third chapter. A fast isogenic method based on next-generation sequencing was adopted to map the EMS-induced suppressor mutations. The candidate mutations responsible for the suppressor phenotype in the recessive mutant *160-1* include *akin10*, which forms a part of sucrose non-fermenting (SNF)-1 related protein kinase (SnRK1), a stress and energy-sensor that has attracted a lot of attention in recent years. Mapping and characterization of all the non-allelic EMS-induced mutants in *tps1-2 GVG:TPS1* is expected to unravel many additional signaling components in T6P / TPS1 pathway in *Arabidopsis thaliana* and thus hold much promise.

Zusammenfassung

Eine exakte zeitliche Abstimmung der Übergänge zwischen verschiedenen Entwicklungsphasen ermöglicht Pflanzen die Bildung von Blüten und Früchten unter bestmöglichen Bedingungen, was für ihren Fortbestand von entscheidender Bedeutung ist. Neben den bereits bekannten Signalwegen spielt auch der Zucker- oder Energie-Status eine wichtige Rolle beim präzisen Wechsel der Wachstumsphasen, da die Blüten- und Samenbildung große Energieresourcen erfordern. Es ist jedoch noch immer wenig darüber bekannt wie Zuckersignale in andere Entwicklungsprogramme integriert werden. In *Arabidopsis thaliana* wird der Gehalt an Saccharose, dem wichtigsten metabolischen Zucker und Energieträger, durch Trehalose-6-Phosphat (T6P) reflektiert, einem Zwischenprodukt in der Signalkette, das bei der Biosynthese des Disaccharids Trehalose entsteht. Die Produktion von T6P wird von dem Enyzm Trehalose-6-Phosphat Synthase katalysiert.

Rettet man homozygote Mutanten, in denen das *TPS1* Gen durch eine Transposon-Insertion inaktiv war (*tps1-2*), mit Hilfe eines durch Dexamethason (DEX) induzierbaren *TPS1* Konstrukts (*GVG:TPS1*), blühen die resultierenden Pflanzen sehr spät, auch unter Langtagbedingungen welche normalerweise das Blühen induzieren. Dies weist darauf hin dass der T6P / TPS1 Signalweg für die Blühinduktion in *Arabidopsis thaliana* nötig ist.

Im ersten Kapitel dieser Arbeit werden Ergebnisse diskutiert, die zeigen, dass der T6P / TPS1 Signalweg die Expression wichtiger blühinduzierender Gene wie *FLOWERING LOCUS T* (*FT*) und *TWIN SISTER OF FT* (*TSF*) in *Arabidopsis thaliana* reguliert. Zudem kontrolliert der T6P / TPS1 Signalweg die Expression von *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) Genen (*SPL3*, *SPL4* und *SPL5*) am Sprossmeristem - zum Teil durch microRNA156 (miR156), einem Regulator der *SPL* Gene. Diese duale Rolle von des T6P / TPS1 Signalwegs garantiert, dass der

Übergang zum reproduktiven Wachstum in Pfanzen nur erfolgt wenn genügend Energievorräte vorhanden sind, selbst unter vorteilhafter Photoperiode.

Zusätzlich zum stark verzögerten Blühzeitpunkt in *tps1-2 GVG:TPS1* Pflanzen zeigen diese auch eine verlängerte Julvenilphase, was einer Verzögerung des Wachstumsphasen-Übergangs im vegetativen Stadium entspricht. Die Experimente des zweiten Kapitels zeigen dass der T6P / TPS1 Signalweg diesen Übergang zumindest teilweise durch das miR156-SPL Modul steuert. Zusammenfassend legen die Experimente des zweiten Kapitels nahe, dass eher T6P als Saccharose als altersabhängiges Signal dient, welches die Repression von miR156 vermittelt und den Übergang von juvenilem zu adulten vegetativem Wachstum wie auch die Blühinduktion in *Arabidopsis thaliana* steuert.

Die Ergebnisse einer Mutagenese mittels Ethylmethansulfonat (EMS) von *tps1-2 GVG:TPS1* zur Sichtung von Pflanzen mit unterdrückten Phänotypen werden im dritten Kapitel beschrieben. Zur Kartierung der Mutationen wurde die Methode des fast isogenic mapping mittels next generation sequencing angepasst. Die möglichen mutierten Gene, die für den unterdrückten Phänotyp in der rezessiven Mutante *160-1* verantwortlich sind, beinhalten *akin10*, dessen Proteinprodukt als Unterheit von SUCROSE NON-FERMENTING (SNF)-1 RELATED PROTEIN KINASE (SnRK1) fungiert, einem Stress- und Energie-Sensor, der in den letzten Jahren grosse Aufmerksamkeit auf sich gezogen hat. Es wird erwartet dass das Kartieren und die Charakterisierung aller nicht-allelischen EMS-induzierten Mutanten in *tps1-2 GVG:TPS1* viele zusätzliche Signal-Komponenten im T6P / TPS1 Signalweg in *Arabidopsis thaliana* aufdecken wird.

1 Introduction

1.1 *Arabidopsis thaliana* as a model organism

Angiosperms or flowering plants are the most diverse and evolved group of land plants today. The number of species under this group has been estimated to range between 250,000 to 400,000. Based on phylogenetic estimates (Bell et al., 2010), angiosperms can be subdivided into eight major clusters, which include monocots and eudicots – the two groups with the highest economic importance. Monocots and eudicots can be either short-lived annuals and biennials or perennials, which live and produce flowers and seeds for many years. Most of the angiosperms have complex genomes, large size and long life cycle. Therefore in order to expedite plant research, it is important to have model organisms, which are easy to study and represent most of the diversity of flowering plants. Crop species like maize (*Zea mays*), tomato (*Solanum lycopersicum*), barley (*Hordeum vulgare*) and Petunia (*Petunia x hybrida*) were used before the 1980's as popular models for genetic experiments, which substantially improved our understanding of plant biology. Despite having long generation times, relatively large-sized and complex genomes with multiple ploidy levels, research on these species enabled efficient plant breeding, resulting in better crop varieties.

In 1943, Friedrich Laibach proposed *Arabidopsis thaliana* (L.) Heynh. (2n = 10), referred to as Arabidopsis throughout this thesis, as a model for plant biology research. Popularly known as thale cress or mouse-ear cress, Arabidopsis is a small weed plant belonging to the *Brassicaceae* family that is native to the northern hemisphere. However, this small plant did not immediately gain popularity among the plant researchers at that time. This was due to the facts that besides lacking any commercial value, Arabidopsis was not a well-suited model for carrying out cytological studies. Moreover there was difficulty in regenerating the whole plant from cells grown in tissue culture medium. But its advantages such as small size, short duration of life cycle, relatively small sized genome, large number of progeny per plant and amenability to various molecular biology techniques influenced a large

number of scientists from the early 1980's to adopt Arabidopsis as a model system for plant molecular genetics research.

Today, Arabidopsis is unarguably the most studied plant and favorite model organism used by plant researchers worldwide. It has a small genome of about 157 Mb (Bennett et al., 2003) and was fully sequenced in the year 2000 (Arabidopsis Genome, 2000). Despite its modest size, the Arabidopsis genome contains more than 27,000 protein-coding genes. The functions of a large number of these genes have been extensively studied over the last decades through genetic and molecular analyses. However, the function of the majority of the remaining genes is yet to be determined (Haas et al., 2005). Reverse genetics projects were successful in elucidating the functions of a number of unknown genes and as a result, a vast array of Arabidopsis mutants are available for research purposes (information on these can be found at http://www.arabidopsis.org/portals/mutants/findmutants.jsp). The last thirty years of Arabidopsis research have given us an enormous amount of knowledge and resources, which further reinforced the value of this weed as a plant model system.

1.2 Developmental stages in Arabidopsis

Higher plants undergo various stages of developmental transitions in their life cycle. After pollination and fertilization, a diploid zygote is produced, which marks the start of sporophytic phase of plant growth. The assemblage of stem cells established at opposite ends of the developing embryo forms the shoot apical meristem (SAM) and the root apical meristem (RAM). During seed germination, continuous division of RAM and SAM produces radicle and plumule respectively. RAM and SAM continue to divide and give rise to various organs, throughout the life cycle of plants. The shoot or aerial part of the plant originates from the SAM and undergoes more-or-less distinct developmental changes, which can be broadly divided into vegetative phase transition and floral transition (see details below).

1.2.1 Vegetative phase transition

After germination, plants produce leaves and grow rapidly. During this period, due to an increase in photosynthetic capacity, they accumulate biomass and size. The

time of vegetative growth can be divided further into a juvenile vegetative phase and an adult vegetative phase. In perennial species like cacti, these stages are often quite distinct (Mauseth, 2006), whereas in Arabidopsis, they are less conspicuous.

Plant shoots at the juvenile vegetative phase are only capable of producing vegetative organs like leaves. After juvenile to adult phase transition, the shoot acquires reproductive competence and can produce flowers once the environmental conditions are favorable. This phase transition affects multiple traits in the plant body such as size, shape, serration of leaves and trichome distribution on leaves. As a result, organs of distinct morphologies and identities can co-exist simultaneously in the same plant, a condition known as heteroblasty (Goebel, 1889; Poethig, 1990, 2010). The anatomic differences between juvenile and adult organs are due to distinct internal developmental programs as well as environmental factors.

The duration of vegetative phase in Arabidopsis varies between natural accessions. In summer-annual varieties, it lasts for weeks, whereas in winter-annual accessions it can take several months depending on the vernalization (prolonged exposure to cold) requirement (Amasino, 2010). Even though at first look, the changes in morphology of the juvenile and adult leaves in Arabidopsis are not very distinct, on careful observation it is possible to identify the differences (Telfer et al., 1997). Leaves with long petioles, small and round blades and smooth margins characterize the juvenile stage, i.e. leaves that were produced before the vegetative phase transition. Moreover, juvenile leaves produce trichomes only on the adaxial (upper) side, which is the most distinct and easiest to score feature that discriminate between the developmental phases of leaves (Telfer et al., 1997). On the contrary, adult leaves in Arabidopsis have shorter petioles, elongated and enlarged blades, serrated margins and trichomes on adaxial and abaxial (lower) sides. At the time of vegetative phase transition, some leaves may display a mix of juvenile and adult features. Presence or absence of abaxial trichomes is an easy method to score and this can be used to specify the number of juvenile leaves in Arabidopsis, which reflect the duration of vegetative phase (Chien and Sussex, 1996; Telfer et al., 1997).

Research in Arabidopsis has provided a deep insight into the genetic control of vegetative phase change in plants. As mentioned previously, both external and endogenous factors affect the vegetative phase transition. By growing Arabidopsis plants under different day length conditions, the duration of juvenile phase can be manipulated (Telfer et al., 1997). Mutant plants affecting various regulatory systems such as hormone signaling pathways can also exhibit differences in the duration of the juvenile phase. For example, Arabidopsis mutants insensitive to the phytohormone gibberellic acid (GA) show a delayed phase change phenotype (Chien and Sussex, 1996). In addition, mutants defective in micro-RNA (miRNA) and trans-acting small interfering RNA (ta-siRNA) biogenesis and regulation also display accelerated vegetative phase change, indicating that small RNAs might contribute to the regulation of vegetative phase change. Small RNAs in general are post-transcriptional regulators that exert their effects on targets through complementary base pairing and recruiting proteins that inhibit target accumulation. ta-siRNAs have been shown to influence heteroblasty by affecting leaf polarity through targeting a small set of auxin response factors (Fahlgren et al., 2006; Hunter et al., 2006; Allen and Howell, 2010), whereas effects of the miRNA biogenesis on vegetative phase change seem to be much more diverse (Rubio-Somoza and Weigel, 2011). The double stranded RNA binding protein HYPONASTIC LEAVES1 (HYL1) that is involved in miRNA biogenesis has been shown to regulate juvenile growth (Li et al., 2012b) by controlling the accumulation of *MIR156a*. Similarly HASTY1 (HST1), which is important for miRNA processing, influences the vegetative phase transition presumably by regulating the levels of miRNAs in general (Telfer and Poethig, 1998; Park et al., 2005; Matsoukas et al., 2013)

In Arabidopsis, two miRNAs – miR156 and miR172 – are known to regulate heteroblasty (Wu and Poethig, 2006; Wu et al., 2009; Huijser and Schmid, 2011). miR156 is among the most abundant miRNAs, especially at the seedling stage (Axtell and Bartel, 2005; Wang et al., 2009). When overexpressed constitutively from the *Cauliflower mosaic virus* (*CaMV) 35S* promoter, miR156 (*35S:miR156*) delays vegetative phase change and causes the production of a large number of juvenile leaves (Wu and Poethig, 2006). In addition, flowering is moderately delayed in *35S:miR156* plants (Schwab et al., 2005). miRNAs regulate the expression of

downstream target genes, which are often transcription factors. Use of mimicry constructs in sequestering the miRNAs and thereby perturbing their functions, provides an effective tool to study the downstream effects (Franco-Zorrilla et al., 2007). In Arabidopsis, the *MIR156a-f* genes (Xie et al., 2005) encode the primary transcripts of miR156. When mimicry-targets against miR156 (*MIM156*) were overexpressed to sequester miR156, the resulting plants completely skipped the vegetative phase and flowered extremely early after producing a few adult leaves (Todesco et al., 2010). This suggests that miR156 plays an important role in regulating the duration of the juvenile phase.

It has been shown that the levels of miR156 decline continuously from the seedling stage in an age-dependent manner (Wu and Poethig, 2006; Wang et al., 2009). Furthermore, organ ablation experiments have demonstrated that the miR156-suppressing factor originates from leaves (Yang et al., 2011). This age-dependent factor remained a mystery until recently, when two independent groups proposed that sugars such as sucrose, produced in plants as a result of photosynthesis, could repress miR156 (Yang et al., 2013; Yu et al., 2013). Sucrose was shown to repress *MIR156a* and *MIR156c*, which have dominant roles in the *MIR156* gene family (Yang et al., 2013). While initially it might seem surprising that a metabolite like sucrose regulates one of the most important developmental changes in the life cycle of plants, it should be remembered that sucrose increase as plants mature. Sucrose thus might very well serve as an indicator of a plant's age. However it is not clear whether sucrose-mediated suppression of miR156 is achieved directly or via other metabolic signaling components. Interestingly, mutant plants in which the starch metabolism is perturbed, such as *ADP glucose pyrophosphorylase1-1* (*adg1-1), phosphoglucomutase1 (pgm1), starch-excess1 (sex1), sex4* and *choloroplastic β-amylase3* (*bam3)* show a prolonged juvenile phase. In contrast, mutant plants defective in sugar signaling such as *glucose insensitive1* (*gin1-1), gin2-1* and *gin6* exhibit a shortened juvenile phase (Matsoukas et al., 2013).

The downstream targets of miR156 in Arabidopsis are the transcripts of the *SQUAMOSA PRMOTER BINDING PROTEIN-LIKE (SPL)* genes. Target regulation by miRNAs involves complementary base pairing between miRNA and the target

transcript, which occur either through transcript cleavage or translational repression. Out of 17 *SPL* genes, 11 are targeted by miR156 (Xie et al., 2005; Wang et al., 2008; Shikata et al., 2009; Wu et al., 2009; Ruth et al., 2010; Xing et al., 2010). Mutations in individual *SPL* genes do not show any noticeable change in vegetative phase transition, suggesting that they might be functionally redundant. Among the miR156-targeted *SPL* genes, *SPL3*, *SPL4* and *SPL5* are the smallest and the miR156 recognition site resides in their 3' UTRs (Gandikota et al., 2007). Besides promoting flowering, these genes have been shown to also accelerate vegetative phase transition (Gandikota et al., 2007; Wang et al., 2009; Wu et al., 2009). Transgenic plants produced by overexpressing miR156-resistant versions of *SPLs* - *rSPL3*, *rSPL4* and *rSPL5* - reduced the duration of juvenile phase and promoted early flowering (Cardon et al., 1997; Wu and Poethig, 2006).

The repressive activity of miR156 on phase transitions is counteracted upon by another miRNA, miR172, which has opposing functions. Interestingly, there seems to exist a complex intensive cross-regulation between these miRNAs and their targets. While the abundance of miR172 is regulated by miR156 through SPL9 and SPL10 (Chuck et al., 2007; Wu et al., 2009), expression of *SPL3*, *SPL4* and *SPL5* are also regulated by miR172 (Jung et al., 2011). Even though having developmentally distinct roles, the signals from miR156 and miR172 seem to converge at *SPL3*, *SPL4* and *SPL5* genes. This cross talk has been shown to be important for coordinating the developmental transitions in Arabidopsis (Jung et al., 2011).

SPL9 and *SPL15* are two closely related *SPL* genes in Arabidopsis that are also targeted by miR156. Analyses of double mutant plants for *SPL9* and *SPL15* indicated that these genes act redundantly to accelerate the vegetative phase change in Arabidopsis (Schwarz et al., 2008; Wang et al., 2009). Furthermore, plants overexpressing miRNA insensitive *SPL9* (*rSPL9)* did not produce any juvenile leaves (Wang et al., 2009; Wu et al., 2009). It was shown that SPL9 could promote the appearance of abaxial trichomes by activating miR172b (Wu et al., 2009). SPL9 also directly activates *TRICHOMELESS1* and *TRIPTYCHON*, two negative regulators of trichome formation and thereby affects the acropetal distribution of trichomes along the shoot (Yu et al., 2010). *SPL2*, *SPL10* and *SPL11* form another group of closely

related genes that are targeted by miR156 (Riese et al., 2007). However, these genes have only a weak effect on vegetative phase transition (Huijser and Schmid, 2011). Our knowledge regarding the roles of remaining miR156-targeted *SPLs* – *SPL6* and *SPL13* – is still limited (Huijser and Schmid, 2011).

Like miR156, miR172 and its target genes have also been proposed to have active roles in regulating the vegetative phase transition. Whereas the levels of miR156 are regulated by sugars or plant age, miR172 levels are under the control of photoperiod (Jung et al., 2007). In contrast to miR156, the levels of miR172 increase as the plants age (Figure 1, below). The targets of miR172 in Arabidopsis are six AP2-type transcription factors, which function as repressors of flowering (Aukerman and Sakai, 2003; Chen, 2004; Mathieu et al., 2009; Yant et al., 2010). Overexpression of miR172 and its targets produced contrasting phenotypes when compared to the overexpression of miR156 and its targets (Aukerman and Sakai, 2003; Chen, 2004; Jung et al., 2007; Mathieu et al., 2009). While miR172 overexpression caused a premature transition to the adult phase, *mir172a* mutant showed a delay in formation of abaxial trichomes (Wu et al., 2009). It has also been shown that *MIR172b* is a direct target of SPL9 (Wu et al., 2009). By activating miR172, SPL9 indirectly represses the AP2-type transcription factors and thereby accelerates phase transition (Wu et al., 2009). In short, the vegetative phase change in Arabidopsis, at least in part, is regulated through the complex interplay of antagonistic effects of miR156 and miR172 and their respective targets (Figure 1, below).

The embryonic master regulator FUSCA3 (FUS3), a transcription factor that regulates the biosynthesis and signaling of the phytohormone ethylene, is also involved in regulation of vegetative phase transition in Arabidopsis (Lumba et al., 2012). *fus3* loss-of-function mutants display accelerated phase transition and *FUS3* overexpression causes a delay in phase change, indicating that FUS3 promotes juvenile leaf identity (Lumba et al., 2012). Similarly, AKIN10, a catalytic subunit of SNF1-RELATED PROTEIN KINASE1 (SnRK1) protein kinase, which functions in energy signaling, also promotes juvenile leaf identity (Tsai and Gazzarrini, 2012a). Surprisingly, AKIN10 directly interacts with FUS3 (Tsai and Gazzarrini, 2012a),

implying that cross talk between energy and hormonal signaling might influence the timing of the vegetative phase transition in Arabidopsis.

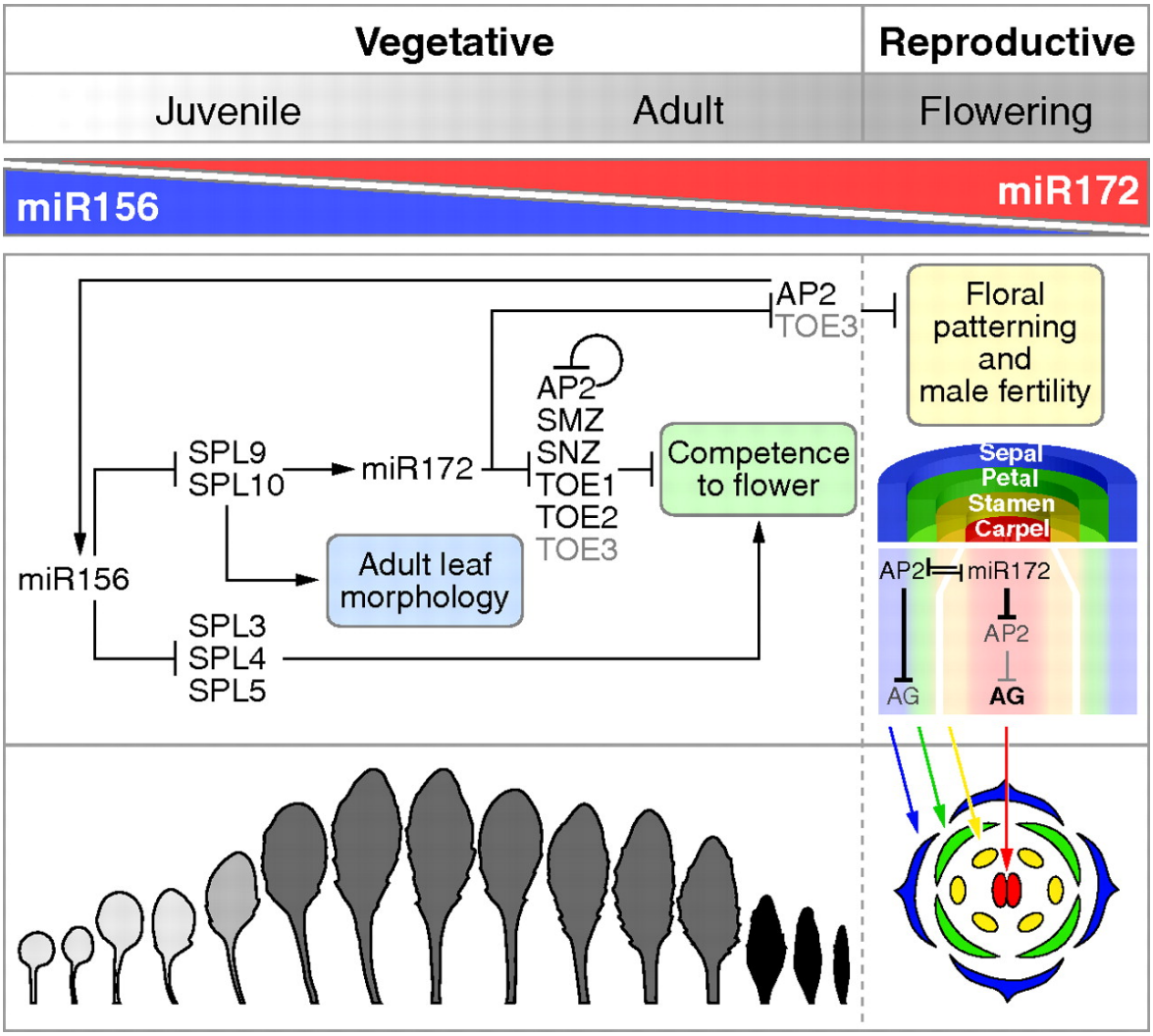

Figure 1: Regulation of phase change in Arabidopsis.

Juvenile leaves (light grey, lower left) are almost round in shape and exhibit trichomes only on their adaxial side. As the plant matures, the levels of miR156 steadily decrease, allowing for the production of SPL9 and SPL10 proteins that promote adult leaf traits (dark grey; elongated leaves with abaxial trichomes). At the same time, SPL9 and SPL10 directly induce the expression of *MIR172* genes. Increased levels of miR172 result in the downregulation of six AP2-like transcription factors that normally repress flowering. Abbreviations: AG, AGAMOUS; AP2, APETALA2; miR156, mature miRNA156; miR172, mature miRNA172; SMZ, SCHLAFMÜTZE; SNZ, SCHNARCHZAPFEN; SPL, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; TOE, TARGET OF EARLY ACTIVATION TAGGED (Huijser and Schmid, 2011).

Recently, the potent floral repressor FLOWERING LOCUS C (FLC) has also been implicated in regulating the vegetative phase transition (Willmann and Poethig, 2011). In plants with high levels of FLC (*FRI*;*FLC*), the abaxial trichome production is significantly delayed when compared to the *FRI;flc-3* mutant and wild type (WT) Columbia (Col-0) plants (Willmann and Poethig, 2011). Many of the upstream factors, which regulate flowering also influence vegetative phase change. For example, plants defective in floral repressor genes such as *TERMINAL FLOWER1* (*tfl1*) and *tfl2* reach adult phase earlier than their respective wild types (Matsoukas et al., 2013). In addition, Arabidopsis imitation switch (ISWI) proteins, CHROMATIN REMODELING 11 (CHR11) and CHR17 together with plant specific RINGLET (RLT) proteins, which are important in flowering, were also shown to be required for the maintenance of juvenility (Li et al., 2012a). Furthermore, certain other chromatin remodeling factors such as *Arabidopsis thaliana* BRAHMA (AtBRM) are also known to promote juvenile phase (Farrona et al., 2004).

1.2.2 Floral transition

After the vegetative phase transition, plants gain reproductive competence, which means that they have reached a state in which flowering can be induced, given the right circumstances. Although visible changes seem to occur only in the SAM, the whole plant responds to various endogenous and environmental signals, while transiting from the adult vegetative phase to reproductive development. Decades of research have unraveled significant parts of complex signaling networks (Figure 2, below) and underlying genetic and molecular mechanisms involved in flowering time regulation (Srikanth and Schmid, 2011).

Day length or photoperiod is one of the important environmental factors that affect the timing of flowering in many plant species. During the course of evolution, plants have evolved mechanisms to perceive changes in day length. The signaling cascade that regulates flowering in repose to day length is called the photoperiod pathway. Light perception in plants is facilitated through specialized photoreceptors such as phototropins, cryptochromes and phytochromes. While blue light is perceived by phototropins and cryptochromes, red / far-red perception occurs through phytochromes (Quail et al., 1995; Lariguet and Dunand, 2005; Li and Yang, 2007).

One way by which light regulates flowering is through the circadian clock, which is constantly reset through day/night cycles and acts as an internal timekeeper. At the same time, the expression of photoreceptors is controlled by the circadian clock, suggesting the existence of a regulatory loop which gates and resets the clock (Toth et al., 2001).

The circadian clock in turn controls the expression of CONSTANS (CO), a putative zinc finger transcription factor (Redei, 1962; Putterill et al., 1995). CO is instrumental in initiating the downstream signaling cascade in the photoperiod pathway. The expression of *CO* oscillates under the control of circadian clock, with maximum levels produced 20 hours after the dawn in short day (SD) conditions (Suarez-Lopez et al., 2001). In long day (LD), the expression of *CO* is transcriptionally regulated by the activity of three circadian regulated proteins – GIGANTEA (GI), FLAVIN-BINDING, KELCH REPEAT, F-BOX (FKF1) and CYCLING DOF FACTOR (CDF1) (Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009). Activity of these three proteins results in the peaking of *CO* expression, 16 hours after the dawn under LD. At the post-translational level, CONSTITUTIVELY PHOTOMORPHOGENIC (COP1) and members of the SUPPRESSOR OF PHYA-105 (SPA) protein family (Hoecker and Quail, 2001; Laubinger et al., 2006; Liu et al., 2008b) regulate CO protein stability and accumulation. The complex regulation of CO holds the key to the mechanisms by which plants sense photoperiod. Under SD conditions, *CO* expression coincides with the night, resulting in immediate degradation of the protein that is produced. In contrast, *CO* expression in the late afternoon of a LD allows for CO protein to accumulate and induce flowering.

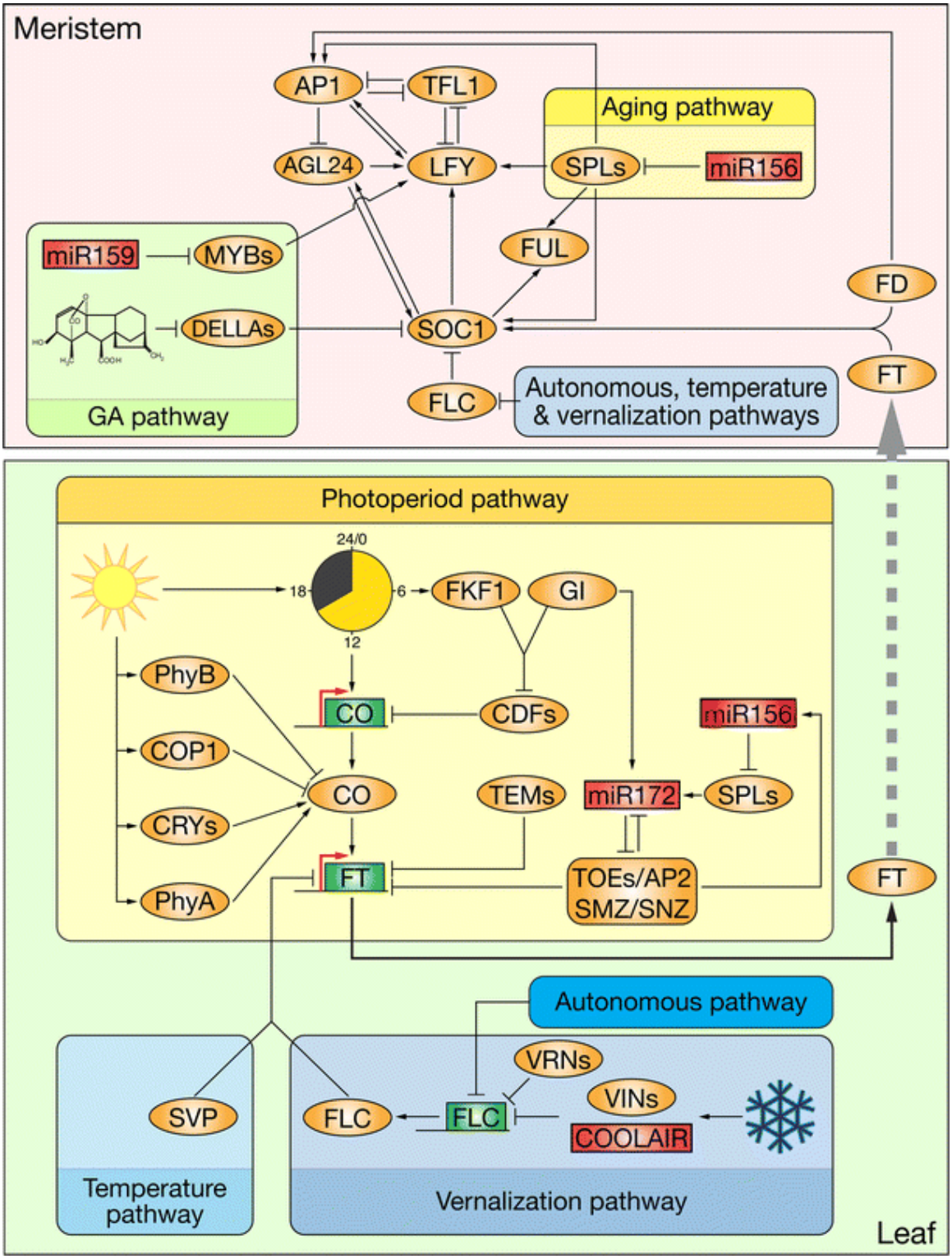

Figure 2: Cross-talk between different flowering time pathways.

All the genes are presented in green, microRNAs in red and the proteins in orange color (Srikanth and Schmid, 2011).

FLOWERING LOCUS T (FT) has been shown to be the primary transcriptional target of CO (Kobayashi et al., 1999; Wigge et al., 2005; Yamaguchi et al., 2005; Yoo et al., 2005). Apart from *FT* activation, CO also regulates the *FT* expression by facilitating periodic histone deacetylation through the Arabidopsis histone deacetylase (HDAC) complex to prevent precocious flowering under LD (Gu et al., 2013). Interestingly, all the above said signaling activities from light perception, regulation of CO protein to activation of *FT* happen in leaves. Several lines of evidences suggest that FT is at least a part of 'florigen', a hypothetical hormone like agent which travels from the leaves to the shoot apex as a long distance signal (Takada and Goto, 2003; Corbesier et al., 2007; Jaeger and Wigge, 2007; Mathieu et al., 2007; Notaguchi et al., 2008) and promotes flowering. At the SAM, FT supposedly interacts with FD, a meristem-specific bZIP transcription factor (Abe et al., 2005; Wigge et al., 2005) that has a significant role in floral induction. Thus the light signal, which is perceived in leaves, is transduced through complex signaling cascades to the SAM, where the actual flowering event occurs.

Many temperate plants such as the winter-annual accessions of *Arabidopsis thaliana* require a prolonged exposure to cold period (vernalization) to induce flowering in the next spring. The dominant locus *FRIGIDA* (*FRI*) has a major role in imparting vernalization requirement in these accessions (Amasino, 2005). In addition to FRI, the MADS-box protein FLC is also required for vernalization to take place (Lee et al., 1994; Maarten et al., 1994; Michaels and Amasino, 1999). FLC is a floral repressor and FRI functions by upregulating *FLC* expression (Geraldo et al., 2009). FLC directly represses certain flowering promoting genes, like *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* and *FT.* In winter-annual plants, high FLC levels contribute to vernalization requirement. A Loss of function in either of the two genes – *FLC* or *FRI* – results in early flowering without the need of vernalization. Initial silencing of *FLC* in response to vernalization involves VIN3, which is transiently induced in response to cold temperatures (Sung and Amasino, 2004; Bond et al., 2009). Subsequently, two important regulators, VERNALIZATION1 (VRN1) and VRN2 are required to maintain the epigenetic silencing of *FLC* (Gendall et al., 2001; Levy et al., 2002). In addition recent studies have shown that two noncoding RNAs, COOLAIR (cold induced long antisense intragenic RNA) and

COLDAIR (cold assisted intronic noncoding RNA) participate in early events of the epigenetic silencing of *FLC* (Swiezewski et al., 2009; Heo and Sung, 2011).

Throughout the vegetative stages of growth, plants experience ambient temperature differences, which also influence flowering. Elevated ambient temperatures (25 or 27^0C in our laboratory conditions) promote flowering also in otherwise non-inductive SD conditions in many Arabidopsis accessions (Balasubramanian et al., 2006). Accessions with non-functional alleles of *fri* or *flc* flower even earlier than they usually do under 23^0C LD. In these accessions, it is likely that the higher temperature substitutes LD. Responsiveness to higher temperatures has been suggested to involve a special histone variant, H2A.Z. These H2A.Z-containing nucleosomes seem to wrap DNA more tightly than normal nucleosomes (Kumar and Wigge, 2010). Deposition of this histone variant into nucleosomes requires ACTIN RELATED PROTEIN 6 (ARP6) (Kumar and Wigge, 2010), which is part of a SWR1 chromatin-remodeling complex. Higher temperatures can overcome the tighter wrapping of DNA by H2A.Z and this provides a possible mechanism by which the gene expression is regulated in a temperature-dependent manner (Kumar and Wigge, 2010). One prominent target of this regulatory module seems to *FT*, which has been shown to be activated by the PHYTOCHROME INTERACTING FACTOR 4 (PIF4) transcription factor in response to higher temperatures under SD (Kumar et al., 2012).

Under LD, flowering in response to changes in ambient temperature seems to be mediated by the two MADS-box proteins SHORT VEGETATIVE PHASE (SVP) and FLOWERING LOCUS M (FLM), the latter of which has sequence similarities with *FLC* (Hartmann et al., 2000; Balasubramanian et al., 2006; Lee et al., 2007). SVP and FLM have been shown to repress flowering and to interact genetically (Scortecci et al., 2003; Lee et al., 2007; Li et al., 2008). Alternate splice forms of *FLM* exist with respect to temperature, suggesting that splicing can be a mechanism by which flowering time is regulated (Balasubramanian et al., 2006). The FLMβ-SVP complex formed at low temperatures can repress the transcription of many floral promoter genes. At high temperature conditions, the *FLM-β* splice variant is downregulated (Lee et al., 2013; Pose et al., 2013) and instead, FLM-δ forms the major translated

splice variant. It acts as a dominant negative form of FLM, which also binds to SVP, but forms an inactive complex. This in turn might contribute to early flowering at higher temperatures, by reducing the amount of the active FLMβ-SVP complex (Pose et al., 2013). In addition, ambient temperature-dependent flowering has also been suggested to be regulated through the miR156-SPL3 module via *FT* in Arabidopsis (Kim et al., 2012).

A number of mutants in genes, like *LUMINIDEPENDENS (LD)*, *FCA*, *FY*, *FPA*, *FLOWERING LOCUS D (FD)*, *FVE*, *FLK* and *REF6*, which flower late independently of day length, constitute the so-called autonomous pathway of flowering (Simpson, 2004). All the above proteins repress the levels of *FLC* and activate flowering, by either acting as chromatin remodeling and maintenance factors or components that influence RNA processing (Lee et al., 1994; Macknight et al., 1997; He et al., 2003; Ausin et al., 2004; Lim et al., 2004; Liu et al., 2007; Hornyik et al., 2010).

Apart from environmental factors, a number of endogenous factors also regulate flowering time in Arabidopsis. The phytohormone GA (Hedden and Phillips, 2000) and the associated signaling cascade constitute a regulatory pathway, which controls floral transition. Initial experiments that employed *ga1-3*, a loss of function mutant in the early step of GA biosynthesis, demonstrated that GA is needed for flowering in SD, but not in LD (Wilson et al., 1992). However, it was later shown that GA perception, which in plants occurs through GIBBERELLIC INSENSITIVE DWARF1 (GID) receptors, is required to induce flowering also under LD. This is largely based on the observation that the Arabidopsis *gid1* triple mutant plants are extremely late flowering in LD, suggesting that GA regulates flowering in LD as well (Willige et al., 2007). Further support for this comes from SD to LD shift experiments coupled with GA-biosynthesis inhibitor Paclobutrazol treatments in *ga1-3*, which indicated a function for GA in promoting flowering under LD by regulating FT, in parallel to CO (Hisamatsu and King, 2008).

GA signaling involves the interaction between GID1 in its GA-bound state with DELLA proteins, which function generally as repressors of plant development (Sun, 2010). The interaction with GID1 promotes the ubiquitination of DELLA proteins by

E3 ubiquitin ligase, which facilitates their degradation via the 26S proteasome pathway (Murase et al., 2008). DELLA proteins are an important point of integration of the GA pathway and light signaling as they directly interact with PIF proteins (de Lucas et al., 2008). Apart from PIF proteins, GA also regulates GNC (GATA, NITRATE-INDUCIBLE, CARBON METABOLISM INVOLVED) and GNL (GNC-LIKE), which are targets of PIFs, in a DELLA-dependent manner (Richter et al., 2010). In addition, activation of *LEAFY* (*LFY)* by GA, at least in part is responsible for the floral induction by GAs (Blazquez et al., 1998a), indicating that the GA and photoperiod pathways converge at *LFY*. In SD, the reciprocal regulation of SOC1 and AGL24 via GA is important for floral induction (Liu et al., 2008a) and activation of *SOC1* by GA seems to occur via downregulation of floral repressors like SVP (Li et al., 2008). In LD, GA induces the expression of *FT* and *TSF*, independently of CO and GI. Furthermore, GA signaling also promotes flowering via photoperiod independent regulation of *SPL* genes (Galvao et al., 2012; Porri et al., 2012; Yu et al., 2012).

Besides GA, another endogenous factor that affects flowering time in plants is the energy or carbohydrates status conveyed through sugar signaling. The role of sugars in flowering time regulation will be introduced in detail in the following section.

1.3 Sugar signaling in plants

Like in all other organisms, sugars have a central role in plant metabolism and development. Besides their metabolic value, sugars have also acquired signaling functions, during the course of evolution. Environmental factors such as light, temperature, and biotic/abiotic stresses affect sugar metabolism and signaling in plants. The metabolism of sugars in plants is a highly dynamic process and the sugar concentration and signaling varies over the course of plant development.

1.3.1 Sugars in plant development

Sugars are so crucial in plant development that sugar signaling takes action right from embryogenesis. High amount of hexoses like glucose, produced as a result of enhanced activity of cell wall invertase (CW-INV) during seed development, ensure mitotic activity in the developing cotyledons and thereby promote normal growth of the embryo (Chen and Jones, 2004). Sugar-mediated activation of cell division in

seeds has been suggested to occur via CYCLIN D (CYCD) proteins in a cytokinin dependent manner (Dewitte et al., 2003).

The next developmental stage, when embryo transits from a mitotic to differentiation phase associated with an increase in cell growth and expansion, is marked by a large and transient increase in sucrose uptake from the cotyledons (Weber et al., 2005). The enhancement in growth and development at this stage is mediated by AP2-type transcription factors via modulation of sugar metabolism (Ohto et al., 2005). In addition, the interplay between sugar and abscisic acid (ABA) signaling, which varies at different developmental stages (Dekkers et al., 2008), ensures timely seed germination (Price et al., 2003). For example, sugar transporters such as vacuolar glucose transporter 1(AtVGT1) also have been shown to fulfill a crucial role in seed germination (Aluri and Büttner, 2007).

Interactions between sugar and ABA also have a critical role in regulating the post-germination developmental arrest of the young germinated embryos, which helps them cope with the new and adverse environmental conditions (Lopez-Molina et al., 2001). Several known sugar mutants such as *glucose insensitive5* (*gin5*) and *gin6*/*sucrose uncoupling6* (*sun6*)/*sugar insensitive5* (*sis5*) are allelic to *ABA INSENSITIVE 4* (*ABI4*) which encodes an AP2-type transcription factor (Leon and Sheen, 2003). In addition, ABA response element (ABRE) binding basic leucine zipper transcription factors such as ABF2, ABF3 and ABF4 are essential components in glucose sensitivity and signaling (Kang et al., 2002). Apart from ABA, ethylene is another phytohormone, which interacts with sugar signaling (Yanagisawa et al., 2003).

Further analyses regarding the role of sugars in plant development revealed a role for *STIMPY* (*STIP*) in meristem establishment (Wu et al., 2005; Skylar et al., 2011). Sucrose-dependent growth enhancement during the early stages of plant development also involves the PIF transcription factors (Liu et al., 2011; Stewart et al., 2011), which have also been implicated in the regulation of auxin biosynthesis by sugars (Sairanen et al., 2012). In addition, sugar signaling can affect clock genes and

a complex reciprocal interaction exists between metabolic signaling and the circadian clock (Bolouri Moghaddam and Van den Ende, 2013).

Sugar sensing and metabolism are also important in vegetative growth and development of plants. Proteins that are involved in the regulation of sugar metabolism, such as sucrose synthase (SUS), ADP-glucose pyrophosphorylase, and sucrose non-fermenting 1(snf1)-related kinases (SnRKs) have been implicated as markers in early leaf development of tomato (Pien et al., 2001). In addition, ATHB13, a homoeodomain leucine zipper (HDzip) transcription factor, regulates lateral expansion of epidermal cells and thereby controls the shape of cotyledon and leaves in a sucrose-dependent manner (Hanson et al., 2001). Sucrose signals also regulate the synthesis and metabolism of amino acids, possibly through repressing the transcription factor bZIP11 (Hanson et al., 2008), which is a target of SnRKs (Hummel et al., 2009). Apart from bZIP11, bZIP1 (AtbZIP1) (Kang et al., 2010) and bZIP63 (AtbZIP63) (Matiolli et al., 2011) have also been proposed to be involved in sugar signaling and affect plant growth and development.

The timing of the transition between various developmental stages such as shoot morphogenesis is also affected by the source (sugar exporting tissues and organs) strength or photosynthetic capacity (Tsai et al., 1997). Sugar mutants such as *gin1, gin2* and *gaolaozhuangren2* (*glz2*) show abnormal growth and development. Sucrose has also been proposed to be a part of the unknown factor that causes the age-dependent decrease in the expression of miR156, which is an essential process in the age-pathway of flowering (Yang et al., 2013; Yu et al., 2013).

In addition, AtSUC1 (*Arabidopsis thaliana* sucrose transporter 1) has been implicated in sucrose-dependent signaling during pollen germination and for normal functioning of the male gametophyte (Sivitz et al., 2008). Sucrose signaling, in co-operation with diverse hormones such as GA, jasmonic acid (JA) and ABA, regulates the synthesis of anthocyanins, which are physiologically important plant secondary metabolites (Loreti et al., 2008). Sugars also influence root growth in plants. For example, glucose has been proposed to interact with auxin signals, which are essential for the proper root growth and development (Mishra et al., 2009). Also,

SHORT ROOT (SHR) and SCARECROW (SCR), which belong to the GRAS family of transcription factors that regulate root growth and radial patterning in Arabidopsis, function by modulating sugar response pathways (Cui et al., 2012).

HEXOKINASE1 (HXK1), which functions upstream to GIN1 in the glucose response pathway (Zhou et al., 1998), is an important component of plant sugar signaling (Rolland et al., 2006). A major effect of HXK1 signaling in plants is the repression of genes involved in photosynthesis (Moore et al., 2003). During senescence, the sugar-mediated repression of photosynthetic genes is correlated with an enhancement in *HXK* expression. HXK1 signaling appears to involve extensive cross talks with various plant hormone response pathways possibly through the interaction with F-actin proteins (Smeekens et al., 2010). In addition, HXK-like 1 (HXL1), a member of the hexokinase gene family in Arabidopsis, also connects glucose and hormone response pathways and has been reported to be negatively influencing plant growth (Karve and Moore, 2009). In particular, HKL1 has been recently proposed to function as a part of an important node in the cross talk between sugar signaling and the plant hormone ethylene (Karve et al., 2012).

Plant sugar singling also involves a number of protein kinases and protein phosphatases. Apart from different calcium-dependent protein kinases (CDPKs), plants also encode a superfamily of SnRKs. In Arabidopsis, only two out of three members of these protein complexes are present, namely SnRK1 and SnRK2 (Bhalerao et al., 1999). The SnRK1 kinases are usually heterotrimeric complexes formed by a catalytic subunit and two regulatory subunits. The Arabidopsis SnRK1 has two catalytic subunits - AKIN10 and AKIN11 (Polge and Thomas, 2007). Upon activation by high cellular sucrose or low cellular glucose, SnRK1 can phosphorylate various plant specific enzymes, such as those involved in starch synthesis (Halford et al., 2003). SnRK1 is also implicated in sugar and ABA signaling pathways (Jossier et al., 2009). SnRK1 thus acts as a metabolic sensor, which modulates plant growth in order to meet the energy demand on a need-based manner (Polge and Thomas, 2007). SnRK1 also achieves part of this function through the regulation of gene expression. There are evidences of SnRK1-mediated regulation of gene expression in sugar/starch metabolism such as *SUCROSE SYNTHASE 4* (*SUS4*) (Patrick et al., 1998)

and *α-AMYLASE 2* (*αAMY2*) (Laurie et al., 2003). SnRK1 is also important in seed germination as it interacts with FUS3, which is a master regulator of seed maturation and development (Tsai and Gazzarrini, 2012b).

Glucose signaling interacts with the target of rapamycin (TOR) kinase pathway, which is an important integrator of energy, nutrients and stress signaling, and promotes growth and development in all eukaryotes (Xiong and Sheen, 2012). In Arabidopsis, glucose-TOR signaling regulates various developmental aspects such as root hair formation and has been implicated in transcriptome reprogramming and meristem activation in roots (Xiong et al., 2013).

Sugar signals, especially those mediated by sucrose, at least in part, are likely to be occurring via trehalose-6-phosphate (T6P), which is an intermediate formed during the biosynthesis of trehalose and has been shown to act as a signaling molecule. In Arabidopsis, T6P acts as a proxy for sucrose status (Lunn et al., 2006; Paul, 2008; Yadav et al., 2014). The disaccharide trehalose, its synthesis and signaling in plants will be addressed in detail in section 1.4. Mutant plants which are defective in disaccharide trehalose metabolism, like *trehalose-6-phosphate synthase1-2* (*tps1-2*) show impaired vegetative growth and extreme delay in floral transition (van Dijken et al., 2004). INTERMEDIATE DOMAIN transcription factor AtIDD8 modulates flowering time in Arabidopsis by regulating sugar transport and metabolism (Seo et al., 2011). The sugar signaling and reproductive transition in plants will be dealt in detail in the following section.

1.3.2 Sugars in flowering time regulation

At the time of flowering, a large and transient mobilization of sugars occurs from source leaves to the SAM (Corbesier et al., 1998; Corbesier et al., 2002). Surprisingly, many flowering time mutants such as *co, gi, fca, fpa* and *ld* could be rescued by growing them on media containing exogenous sucrose (Takashi and Yoshibumi, 1993; Roldan et al., 1999). Interestingly, the effect seems to depend on sugar concentration and the genetic background of the mutants, since flowering was promoted in plants grown on 1% sucrose while high concentrations of sucrose (>5%) had the opposite effect (Ohto et al., 2001). The delay in flowering induced by

high concentrations of sucrose can be attributed to an extension of the adult vegetative phase, rather than in the juvenile phase. Moreover, this delay seems to be caused by the effect of sugars on the metabolism rather than simple osmotic stress (Ohto et al., 2001). Clearly, the carbohydrate status and flowering related traits share a complex relationship (El-Lithy et al., 2010), which demands further in-depth research.

Several Arabidopsis mutants with defects in carbohydrate metabolism exhibit perturbed flowering time phenotypes. *glz2*, which is impaired in glucose signaling, exhibits a delay in flowering time, in addition to glucose insensitivity (Chen et al., 2004). *atvgt1*, which is defective in sugar compartmentalization to vacuoles, also shows a delayed flowering phenotype (Aluri and Büttner, 2007). On the other hand, *low-beta-amylase1* (*lba1*) (Yoine et al., 2006) and *suc9* (Sivitz et al., 2007) mutant plants show early flowering phenotypes, as does the Arabidopsis *sweetie* mutant, which also displays severe perturbations in carbohydrate metabolism (Veyres et al., 2008). Arabidopsis *amylase1* (*amy1*) mutant has enhanced levels of *CO* and *FT* and as a result exhibits an early flowering phenotype (Jie et al., 2009).

Many mutations in genes that functions upstream to sucrose synthesis, cause the plants to exhibit late flowering phenotypes. AtIDD8 is a transcriptional activator of *SUCROSE SYNTHASE* (*SUS*) genes and *idd8* mutants are late flowering (Seo et al., 2011). Similarly, the *nana* mutant in Arabidopsis, which is impaired in carbohydrate metabolism due to a T-DNA insertion in the promoter of a chloroplast localized protease, exhibits a dwarf phenotype and flowers significantly later than WT (Paparelli et al., 2012). The most extreme delay in flowering is, however, found in mutants with perturbed trehalose metabolism, such as *tps1-2,* which carries a transposon insertion in the *TPS1* gene (van Dijken et al., 2004). The disaccharide trehalose, its biosynthesis and the signaling properties of the intermediate product T6P, have recently attracted a lot of attention.

1.4 Trehalose biosynthesis, metabolism and functions

Trehalose is a disaccharide (composed of two glucose units linked by α, α-1, 1 bond), which is found throughout all kingdoms of life (Veluthambi et al., 1981). Trehalose

serves as carbon source, compatible osmolyte and forms part of the exoskeleton in bacteria, archea and fungi (Reviewed in Paul et al., 2008). In arthropods, trehalose is the main blood sugar. In addition, trehalose is the starting material for the biosynthesis of chitin, which forms the integral part of insect exoskeleton (Merzendorfer and Zimoch, 2003). Trehalose has been shown to function as a stress protectant, especially in xerophytic plants such as *Selaginella* (Jain and Roy, 2010). In other plants, trehalose was proposed to have roles in the defense against biotic stresses such as herbivory (Singh et al., 2011; Hodge et al., 2013) and pathogen attack (Brodmann et al., 2002). However, the majority of land plants contain only minute quantities of trehalose (Zentella et al., 1999), which point towards the possibility of alternate functions in plants.

Trehalose is produced via the intermediate compound T6P from glucose-6-phosphate (G6P) and UDP-glucose by TPS followed by the dephosphorylation of T6P to trehalose by trehalose-6-phosphate phosphatase (TPP) (Figure 3) (Cabib and Leloir, 1958). Even though various other pathways exist for trehalose biosynthesis in other organisms, the one described above is the only pathway present in plants. Arabidopsis has a large number of trehalose metabolism genes (Table 1) (Leyman et al., 2001; Vandesteene et al., 2012; Yang et al., 2012), which were identified based on homology with their yeast (*Saccharomyces cerevisiae*) counterparts. Of the 11 TPS proteins (Table 1), only TPS1 seems to have a demonstrable TPS activity (Blazquez et al., 1998b; Vandesteene et al., 2012). AtTPS6, a member of Class II TPSs (Table 1) was also shown to be enzymatically active through yeast mutant complementation assay (Chary et al., 2008). However two recent studies rule out this possibility (Ramon et al., 2009; Vandesteene et al., 2010). AtTPS1 contains an auto-inhibitory extension at the N-terminal region that restricts its activity in plants (Van Dijck et al., 2002). The enzymatic activity of N-terminal truncated AtTPS1 was shown to be higher in yeast complementation assays when compared to the full-length protein. In addition, mutagenesis of specific amino acids - 17 (arginine) and 27 (leucine) - at the N-terminal region resulted in an enhancement of AtTPS1 action, suggesting that N-terminus is a target for modulation of TPS activity in plants (Van Dijck et al., 2002).

Table 1: Trehalose metabolism genes in Arabidopsis.

(Ponnu et al., 2011)

Class	Gene name	AGI number
Class I TPSs	*TPS1*	At1g78580
	TPS2	At1g16980
	TPS3	At1g17000
	TPS4	At4g27550
Class II TPSs	*TPS5*	At4g17770
	TPS6	At1g68020
	TPS7	At1g06410
	TPS8	At1g70290
	TPS9	At1g23870
	TPS10	At1g60140
	TPS11	At2g18700
Class I TPPs	*TPP1/TPPC*	At1g22210
	TPP2/TPPD	At1g35910
	TPP3/TPPB	At1g78090
	TPP4/TPPE	At2g22190
	TPP5/TPPF	At4g12430
	TPP6/TPPG	At4g22590
	TPP7/TPPH	At4g39770
Class II TPPs	*TPP8/TPPI*	At5g10100
	TPP9/TPPA	At5g51460
	TPP10/TPPJ	At5g65140
Trehalase	*TRE1*	At4g24040

AtTPPA and *AtTPPB* are the only known genes among TPPs, which have been shown to rescue the yeast *tps2* mutant (Vogel et al., 1998), which lacks TPP activity (De Virgilio et al., 1993). In contrast to TPSs and TPPs, trehalase enzyme, which converts trehalose into two glucose molecules, seems to be encoded universally by a single gene (Table 1, above) (Leyman et al., 2001; John, 2007).

1.5 Trehalose-6-phosphate signaling in plants

Many studies demonstrate the capability of T6P as a signaling molecule. The important functions of T6P in various metabolic and developmental processes have been reviewed extensively (Paul et al., 2008; Ponnu et al., 2011). Many researchers have shown the importance of T6P as a central regulator of carbohydrate metabolism (Figure 3, below). In addition to T6P, trehalase, the enzyme responsible for cleaving trehalose into two molecules of glucose, also has been proposed to possess signaling properties (Barraza and Sanchez, 2013).

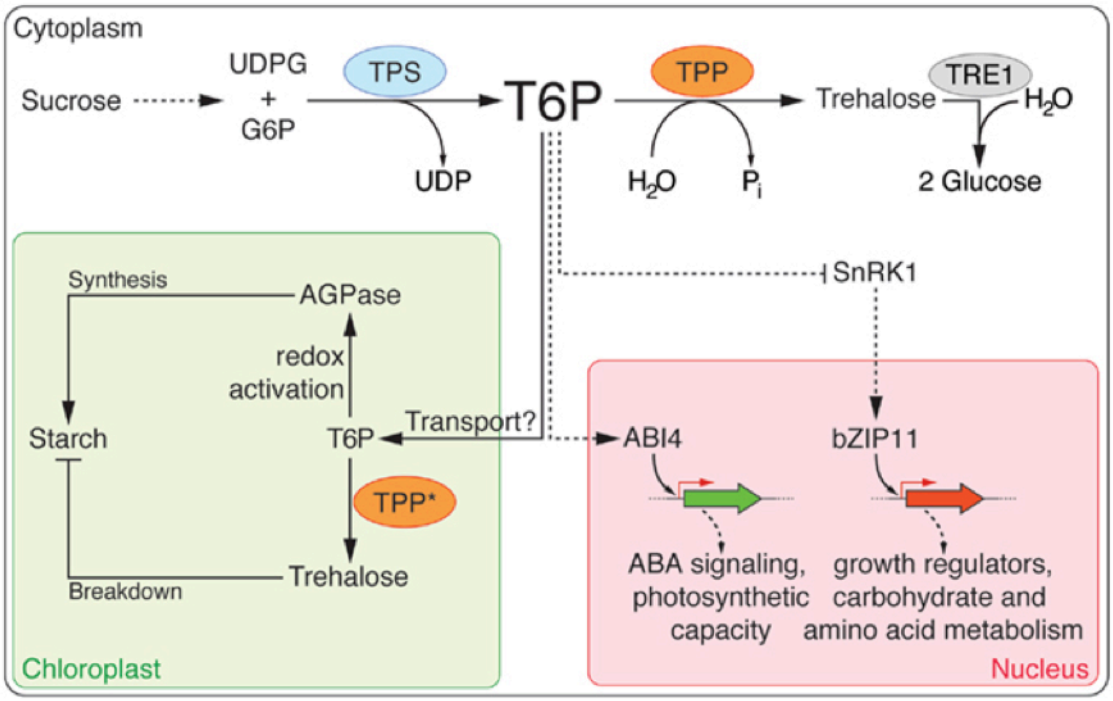

Figure 3: Biosynthesis of trehalose and central role of T6P in carbohydrate metabolism.

The precursors of T6P are derived from the sucrose metabolism. It has been suggested that T6P is transported by an unknown mechanism into plastids where it induces starch synthesis via thioredoxin-mediated activation of AGPase. T6P might be converted into trehalose, which has been shown to regulate starch breakdown in plastids. Several TPPs (marked with an asterisk) have been predicted to localize to plastids, but this still needs to be confirmed experimentally. SnRK1, which represses plant growth, is inhibited by T6P (Ponnu et al., 2011).

1.5.1 Trehalose-6-phosphate as a signaling molecule

T6P has been proposed several times to possess signaling properties. The ability of WT plants to utilize sucrose corresponds to the amount of T6P (Schluepmann et al., 2004). Based on this and other observations, it has been proposed that T6P conveys the sucrose status in plants (Lunn et al., 2006; Yadav et al., 2014). Moreover it was shown that the developmental defects associated with the overexpression or downregulation of *TPS1* have been attributed to changes in T6P content, rather than trehalose (Paul et al., 2008).

In particular, the effects of T6P as a signaling molecule in starch metabolism have been extensively studied (Figure 3). T6P is known to translocate into the chloroplast by an unknown mechanism to promote the action of ADP-glucose pyrophosphorylase (AGPase) - the major enzyme regulating starch synthesis - through a thioredoxin-mediated redox reaction (Kolbe et al., 2005). When the sucrose level rises, there is a concomitant increase in the level of T6P and it results in enhanced starch production via AGPase activation (Lunn et al., 2006). Thus T6P might also be a link between the cytosolic sugar status and plastidic storage of carbohydrates (Figure 3).

Furthermore, T6P was shown to inhibit SnRK1 in Arabidopsis (Zhang et al., 2009), and thereby promoting active biosynthetic processes in growing tissues (Figure 3). SnRK1 has been globally identified as a metabolic sensor, which is important in adapting metabolism in accordance with the demand and supply of energy (Polge and Thomas, 2007). SnRK1, when overexpressed, promotes the survival of plants under stress, especially in starvation and in low light conditions, besides affecting the inflorescence development (Baena-Gonzalez et al., 2007). It has been suggested that a T6P-specific regulatory loop might exist, which controls the carbon availability in actively growing cells (Delatte et al., 2011). It is also known that T6P/SnRK1 – mediated carbon signaling is important in growth recovery after relieving cold stress (Nunes et al., 2013a; Nunes et al., 2013b).

Recently bZIP11, a transcription factor implicated in amino acid synthesis has been shown to affect T6P levels and signaling. bZIP11 is repressed by sucrose via a translational inhibition mechanism (Hanson et al., 2008). Interestingly, bZIP11 overexpressing plants have significantly reduced levels of T6P (Ma et al., 2011). These plants also exhibit resistance to exogenous trehalose application, which is accompanied by a marked reduction in SnRK1 activity (Delatte et al., 2011). Taken together, the above results suggest that T6P plays a central role in carbohydrate metabolism.

It is known that T6P is essential for the initiation of senescence in plants, especially in response to carbon availability (Wingler et al., 2012). T6P signaling has also been shown to play a role in metabolic reprograming during pathogen attack (O'Hara et al., 2013). In addition, T6P pathway has also been proposed to have cross talks with hormonal pathways. For example, the brassinosteroid-responsive protein EXO is known to regulate extracellular carbon metabolism in plants. Interestingly, *exo* mutant plants could be partially rescued by trehalose feeding (Lisso et al., 2013). It has been shown that exogenous trehalose feeding results in impaired growth of WT plants due to the accumulation of T6P (Schluepmann et al., 2004). However it is not clear if the rescue of *exo* mutant by exogenous trehalose application is due to an enhanced T6P accumulation or an increase in glucose levels as a result of trehalase activity in these plants.

1.5.2 Trehalose-6-phosphate in plant development

A significant amount of research done on T6P in the past decade has revealed that this signaling molecule plays an important role in diverse developmental processes. Developmental effects of T6P were first observed when tobacco plants over-expressing bacterial *TPS1* and *TPP* genes (*OTSA* and *OTSB*, respectively) exhibited phenotypic abnormalities. Similar results were later obtained in Arabidopsis (Schluepmann et al., 2003).

The role of T6P signaling was also reported to contribute to the aberrant inflorescence branching and architecture phenotypes in the maize *RAMOSA* mutants (Satoh-Nagasawa et al., 2006). Meristem determinacy in maize is regulated by a

signaling pathway, which involves three RAMOSA (RA) proteins, RA1, RA2 and RA3. Interestingly *RA3* encodes a functional TPP enzyme.

Moreover, homozygous *tps1*-2 mutant Arabidopsis embryos aborted prematurely at torpedo stage, demonstrating the essential role of TPS1 in embryo maturation (Eastmond et al., 2002). Later analyses revealed that the cell cycle activity was perturbed in these mutants and the cell walls were thicker than those of WT embryos (Gomez et al., 2006). Attempts were made in rescuing the Arabidopsis *tsp1* homozygous embryo-lethal mutant by expressing *TPS1* under seed-specific *ABI3* promoter. The rescued plants were stunted and accumulated starch and soluble sugars, before dying prematurely (Gomez et al., 2010). Weak alleles of *tps1* isolated from a TILLING population were found to be ABA hypersensitive besides showing phenotypic abnormalities (Gomez et al., 2010). These results suggest that T6P signaling might interact with ABA in addition to carbohydrate metabolism to co-ordinate growth and development in plants.

Embryo-lethal phenotype of *tps1-2* mutant embryos were also rescued by introduction of a dexamethasone (DEX)-inducible construct (referred to as *GVG:TPS1*) (van Dijken et al., 2004). The DEX-inducible system (Aoyama and Chua, 1997) was modified for this purpose and *AtTPS1* gene was inserted behind the upstream activation of *UBIQUITIN10* promoter (Sun and Callis, 1997) to ensure the expression of *AtTPS1* at all the developmental stages upon DEX application. Screening of the plants obtained after transforming heterozygous *tps1-2* mutant with *GVG:TPS1* construct yielded four independent inducible lines with single transgene insertion. Line 201 which showed inducible expression (referred to as *tps1-2 GVG:TPS1* throughout this thesis) of *TPS1* upon DEX application displayed diverse developmental phenotypes right from the seedling stage to reproductive transition (van Dijken et al., 2004). In addition to the delayed growth of root and shoot, the rescued plants flowered extremely late even under inductive light conditions. These plants grew slowly compared to WT and often produced aerial rosettes, especially in later developmental stages. These abnormalities can almost completely be relieved by DEX-induced expression of *TPS1*, which suggests the role of T6P in normal growth and development in Arabidopsis.

2 Aims and objectives

This research work was mainly aimed at investigating the role of T6P / TPS1 signaling in the regulation of flowering time and vegetative phase change in Arabidopsis. A homozygous *tps1* mutant in the background of Col-0 accession with a DEX-inducible *TPS1* construct (*tps1-2 GVG:TPS1*) is used in most of the studies presented in this thesis. The primary objectives of this research work were:

1. Investigation of genetic and molecular causes for the extreme delay in flowering of *tps1-2 GVG:TPS1* plants and thereby placing T6P / TPS1 in the standard flowering time pathways in Arabidopsis.
2. Analysis of T6P / TPS1 function in the juvenile-to-adult phase transition of Arabidopsis by investigating the causes of prolonged juvenile phase in *tps1-2 GVG:TPS1* plants.
3. EMS mutagenesis in *tps1-2 GVG:TPS1* to identify and characterize the genes involved in T6P- / TPS1-mediated regulation of developmental transitions in Arabidopsis.

This thesis is divided into three parts. Chapter 1 deals with the regulation of flowering time in Arabidopsis by T6P / TPS1 signaling. Chapter 2 explains the role of T6P / TPS1 signaling in the vegetative phase transition of Arabidopsis. EMS mutagenesis in *tps1-2 GVG:TPS1* and mapping of the putative suppressor mutant plants are described in Chapter 3.

3 Chapter 1

Regulation of flowering time in Arabidopsis by trehalose-6-phosphate signaling

Part of this work was published in Science (2013) Vol.339, 704-707

Contributions to this chapter:

All experiments and their analyses described in this chapter have been carried out by myself, if not mentioned otherwise.

With Tobias Langenecker: Diurnal time course qRT-PCR to observe the expression levels of *CO*, *GI*, *FT* and *TSF* in *tps1-2 GVG:TPS1* compared to WT (Figure 6).

With Markus Schmid: Microarray analysis to observe the global gene expression difference in *tps1-2 GVG:TPS1* grown in SD 23^0C, compared to WT (Figure 12).

3.1 Abstract

Proper timing of flowering in plants ensures successful pollination, fertilization and seed production. Plants integrate various environmental and endogenous signals to time the reproductive transition. Metabolic signals such as plant carbohydrate status play an important role in determining the timing of flowering, as the associated processes like seed production are highly energy intensive. In Arabidopsis, sucrose or energy status is conveyed through trehalose-6-phosphate (T6P), an intermediate product formed during trehalose biosynthesis. Mutant Arabidopsis plants defective in the *TREHALOSE-6-PHOPHATE SYNTHASE1* (*TPS1*) gene, flower extremely late even under long day conditions even when sufficient sugar sources are available, suggesting the requirement of T6P / TPS1 signaling in floral transition. The experiments described in this chapter demonstrate that *FT*, which constitutes a vital component of the photoperiod pathway, is insufficiently expressed in this mutant. Moreover, induction of *TPS1* in *tps1-2 GVG:TPS1* plants by spraying dexamethasone, resulted in upregulation of *FT*, indicating that the T6P / TPS1 pathway is indispensable for the expression of *FT* in leaves. On the other hand, microarray analysis revealed that the T6P / TPS1 signaling regulates the expression of *SPL3*, *SPL4* and *SPL5* genes at the shoot apical meristem (SAM). This regulation seems at least in part via miR156 and independent of the photoperiod pathway. Thus T6P / TPS1 signaling links the energy status in plants to key developmental processes such as floral transition by regulating important genes in the leaves as well as in the SAM.

3.2 *tps1-2 GVG*:*TPS1* flower extremely late even under long day conditions

In order to characterize the *tps1-2 GVG:TPS1* mutant with respect to flowering under different day lengths and temperatures, plants were grown on soil under different conditions, such as LD 23˚C, SD 23˚C, SD 16˚C and SD 4˚C. Flowering time was determined by counting the number of leaves after bolting (when the inflorescence reached 1 cm high). *tps1-2 GVG:TPS1* plants grown under inductive day length and temperature (LD 23˚C), bolted very late after growing for more than 2 months (70-80 days after sowing or DAS) (Figure 4, below). Bolting was extremely delayed, both in terms of number of leaves produced at the time of flowering (Figure 5, A) and also the duration of growth.

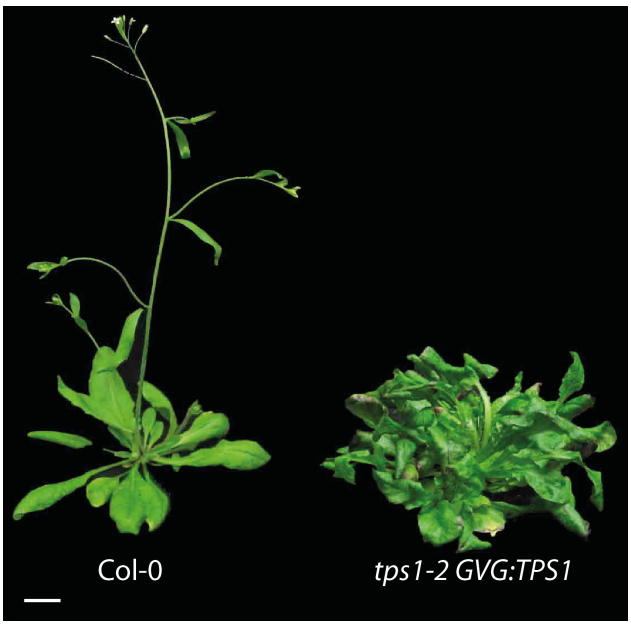

Figure 4: Phenotype of *tps1-2 GVG:TPS1* plants under LD conditions.

Plants were grown under LD 23˚C and images were taken at 20 days after sowing (DAS) and 50 DAS for Col-0 and *tps1-2 GVG:TPS1* plants, respectively. Scale bar: 1cm. Modified from Ponnu et. al., 2011.

Even though, and contrary to a previous publication (van Dijken et al., 2004), bolting could eventually be observed in *tps1-2 GVG:TPS1* under our growth conditions, most of the buds produced failed to develop into normal flowers since the immature buds turned red in color (presumably due to the high anthocyanin content) and died. Visual examination of the opened flowers (Figure S1) under a light microscope revealed that the development of pollen grains were failed or perturbed (data not shown) in the aborted flowers. However, all the other floral organs were appeared to be normally developed in those flowers. Thus pollination and fertilization failed

to occur in the opened flowers of *tps1-2 GVG:TPS1*, mostly due to abnormalities associated with the androecium.

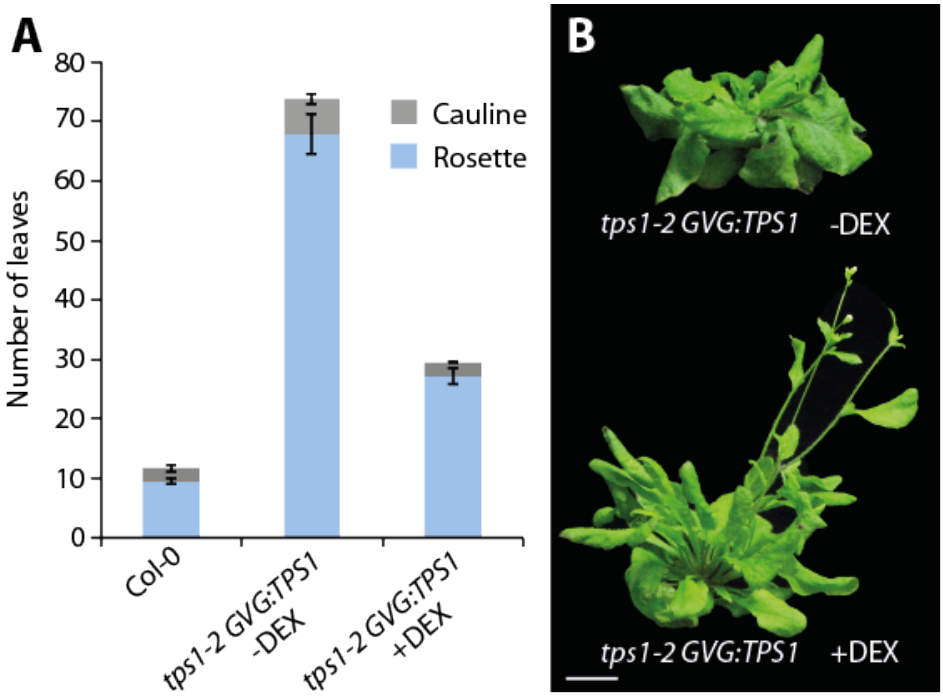

Figure 5: Effect of spraying dexamethasone on *tps1-2 GVG:TPS1* plants.

(**A** and **B**) Phenotype of *tps1-2 GVG:TPS1* plants sprayed (at 2-day intervals from 10 DAS) with 1µM DEX under LD 23˚C, compared to WT. Flowering time was calculated by counting the number of leaves at the time of bolting. Error bars indicate SD. n=20. Scale bar: 1cm.

On the other hand, all the essential floral organs were poorly developed in the unopened buds, which died prematurely. In addition, multiple inflorescences were often produced from the axillary meristems of *tps1-2 GVG:TPS1* plants that gave them a bushy appearance. Plants grown under SD 23˚C, SD 16˚C and SD 4˚C, did not even bolt and died after growing for several months. Other abnormalities such as short and weak root system and small stature of the plants were also observed in *tps1-2 GVG:TPS1* plants, as described in van Dijken et. al., 2004. Additionally, formation of aerial rosettes could be observed especially at later stages of the plant growth.

As described previously (van Dijken et al., 2004), most of the phenotypic abnormalities of *tps1-2 GVG:TPS1* plants including late flowering, could at least be

partially rescued by spraying 1µM DEX solution or watering with 5µM DEX solution (Figure 5, B). Nevertheless, the partial rescue still caused difficulties in agrobacterium-mediated plant transformation. Alternate spraying of 1µM DEX and 50µM GA could partially solve this problem. Spraying of GA solution seems to induce more flowers to open and improve fertility in *tps1-2 GVG:TPS1* plants (on visual observation).

3.3 Trehalose-6-phosphate signaling regulates the expression of *FT* and *TSF*

The fact that *tps1-2 GVG:TPS1* plants flower extremely late even under LD conditions, suggests a possible interaction of T6P signaling with the photoperiodic pathway. In addition, determination of T6P levels (In collaboration with Vanessa Wahl and Mark Stitt at Max Planck Institute for Molecular Plant Physiology at Golm, Germany) across a 72-hour diurnal time course in WT seedlings revealed that the levels of T6P changed diurnally and attained a maxima towards the end of the day (Wahl et al., 2013). This is in agreement with a previous report, which proposed that T6P levels follow the diurnal changes occurring in the sucrose levels (Lunn et al., 2006). Interestingly, expression of *FT*, the key regulator in the photoperiod pathway of flowering, also peaks at the end of the day as a result of induction by CO protein, which in turn is regulated by light and the circadian clock (Imaizumi et al., 2005).

3.3.1 *FT* and *TSF* are insufficiently expressed in *tps1-2 GVG:TPS1*

(Experiments in section 3.3.1 were performed together with Tobias Langenecker.)

To determine the diurnal changes in the expression levels of major genes involved in the photoperiod pathway, qRT-PCR analysis was performed using RNA isolated from whole seedlings collected across a 72-hour diurnal time course. Expression of *CO* and its upstream regulator *GI* in *tps1-2 GVG:TPS1* did not show any significant difference in the diurnal expression pattern, when compared to the WT (Figure 6, A and B). However, expression of *FT* at the end of the day was completely abolished in the *tps1-2 GVG:TPS1* mutant (Figure 6, C). Moreover, qRT-PCR on the rosettes collected from a developmental series (4-to 14 DAS) showed that the *FT* expression was insufficient at all the time during important developmental transitions in this mutant (Figure 7).

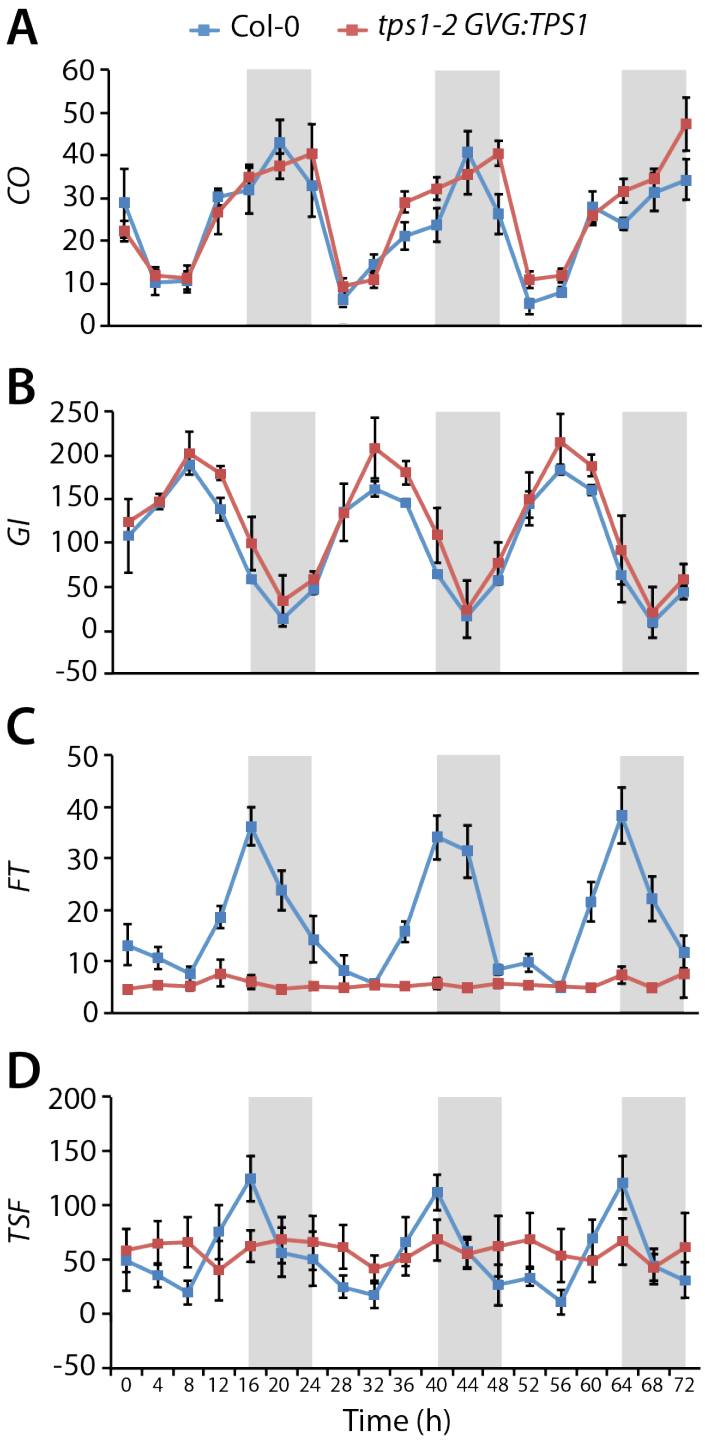

Figure 6: Diurnal time course of *CO*, *GI*, *FT* and *TSF* over 72 hours.

Expression of *CO* (**A**), *GI* (**B**), *FT* (**C**) and *TSF* (**D**) in 12 to 14 day old Col-0 and *tps1-2 GVG:TPS1.* Expression was determined by qRT-PCR using three biological replicates with three technical repetitions each and normalized to *TUB2*. Shaded areas indicate dark periods. Error bars indicate SD. Modified from Wahl et. al., 2013.

In addition to *FT*, CO protein is known to target *TWIN SISTER OF FT* (*TSF*), another flowering time gene that acts redundantly with *FT* (Yamaguchi et al., 2005). The induction of *TSF* at the end of the day was also substantially reduced in *tps1-2 GVG:TPS1* plants (Figure 6, D).

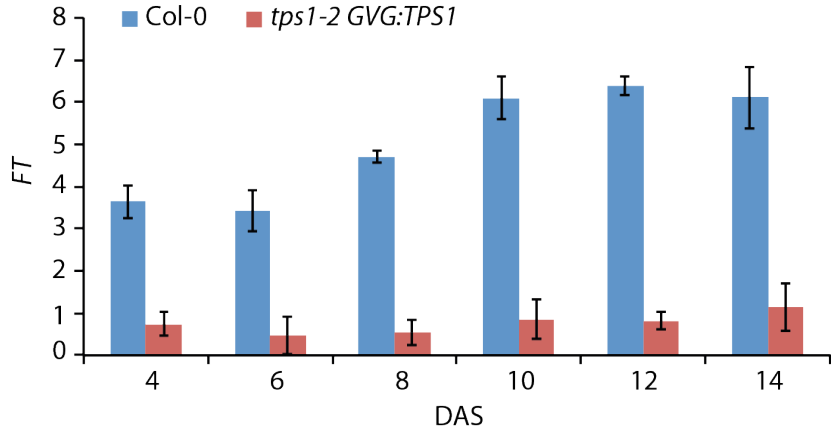

Figure 7: Expression of *FT* in *tps1-2 GVG:TPS1* during developmental transitions.

Expression of *FT* in 4 to 14 day old Col-0 and *tps1-2 GVG:TPS1* rosettes from plants grown under LD. Expression was determined by qRT-PCR using three biological replicates with three technical repetitions each and normalized to *TUB2*. Error bars indicate SD.

3.3.2 *FT* expression in *tps1-2 GVG*:*TPS1* plants could be induced by dexamethasone application

Insufficient expression of *FT* could be one of the reasons for the late flowering of *tps1-2 GVG:TPS1* plants. If loss of function of TPS1 causes the reduced expression of *FT* in these plants, then application of DEX and thereby inducing *TPS1* should possibly restore the normal levels of *FT*. Indeed, *FT* expression was found to be significantly induced in *tps1-2 GVG:TPS1* seedlings in response to spraying with 1 µM DEX (Figure 8, A and B), indicating that T6P signaling is required for the expression of *FT* (and *TSF*) under inductive photoperiods.

3.3.3 Misexpression of FT complements the late flowering of *tps1*-2 *GVG:TPS1*

Attempts were made to rescue the late flowering phenotype of *tps1-2 GVG:TPS1* mutant plants by misexpressing *FT*. To constitutively express *FT* in *tps1* mutant background, *tps1-2 GVG:TPS1* plants were crossed with an *FT* over-expressing line (*35S*:*FT*) (Mathieu et al., 2007). The resulting plants homozygous for both transposon insertion in *TPS1* and the transgene *35S*:*FT* (*35S:FT tps1-2 GVG:TPS1*) were identified by genotyping (see methods). These plants flowered early (average

total leaf number (TLN) – 5.85), complementing the late flowering phenotype of *tps1-2 GVG:TPS1* mutant (Figure 9, A and G).

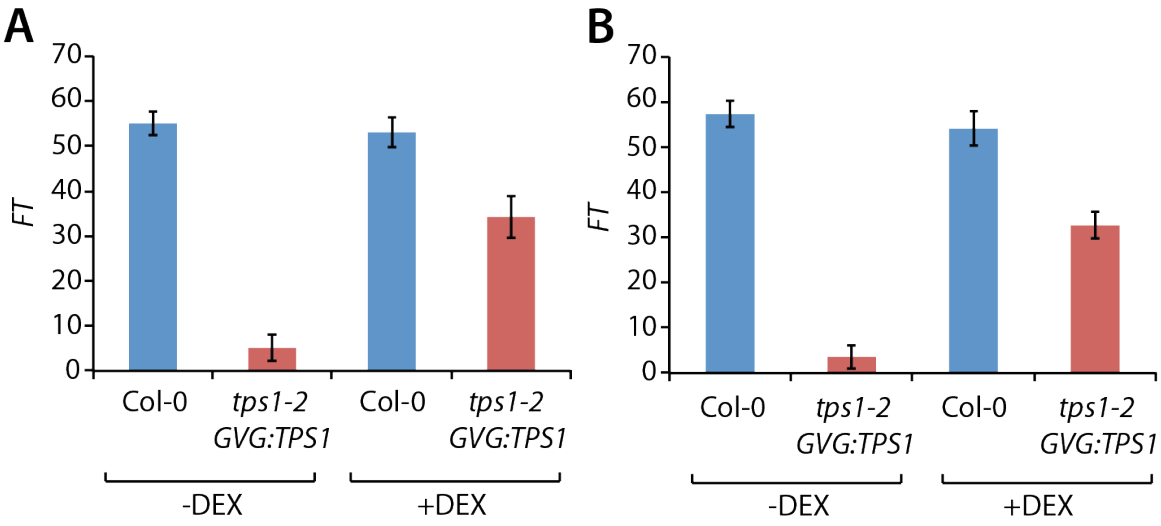

Figure 8: *FT* expression upon dexamethasone (DEX) application.

13 day-old LD 23˚C-grown Col-0 and *tps1-2 GVG:TPS1* plants were treated with 1 µM DEX (+DEX) or mock (-DEX) and *FT* expression was measured (**A**) 24 h and (**B**) 48 h after induction in rosettes leaves harvested at ZT=16 h (end of day). Error bars indicate the upper and lower limit of SD of three biological replicates with three technical repetitions each. Modified from Wahl et. al., 2013.

The promoter derived from *AtSUC2* sucrose symporter gene can direct expression of transgenes into phloem companion cells (Truernit and Sauer, 1995) where *FT* is usually expressed. The double homozygous plants obtained after a cross between *tps1-2 GVG:TPS1* plants with a *SUC2:FT* line (Mathieu et al., 2007) (*SUC2:FT tps1-2 GVG:TPS1*) also exhibited an early flowering phenotype (TLN – 4.75, Figure 9, E and H) under LD. This confirms that T6P signaling is acting upstream of *FT* in the photoperiod pathway. This also suggests that TPS1 and/or T6P are not required for the long-distance transport of the FT protein from leaves to the SAM.

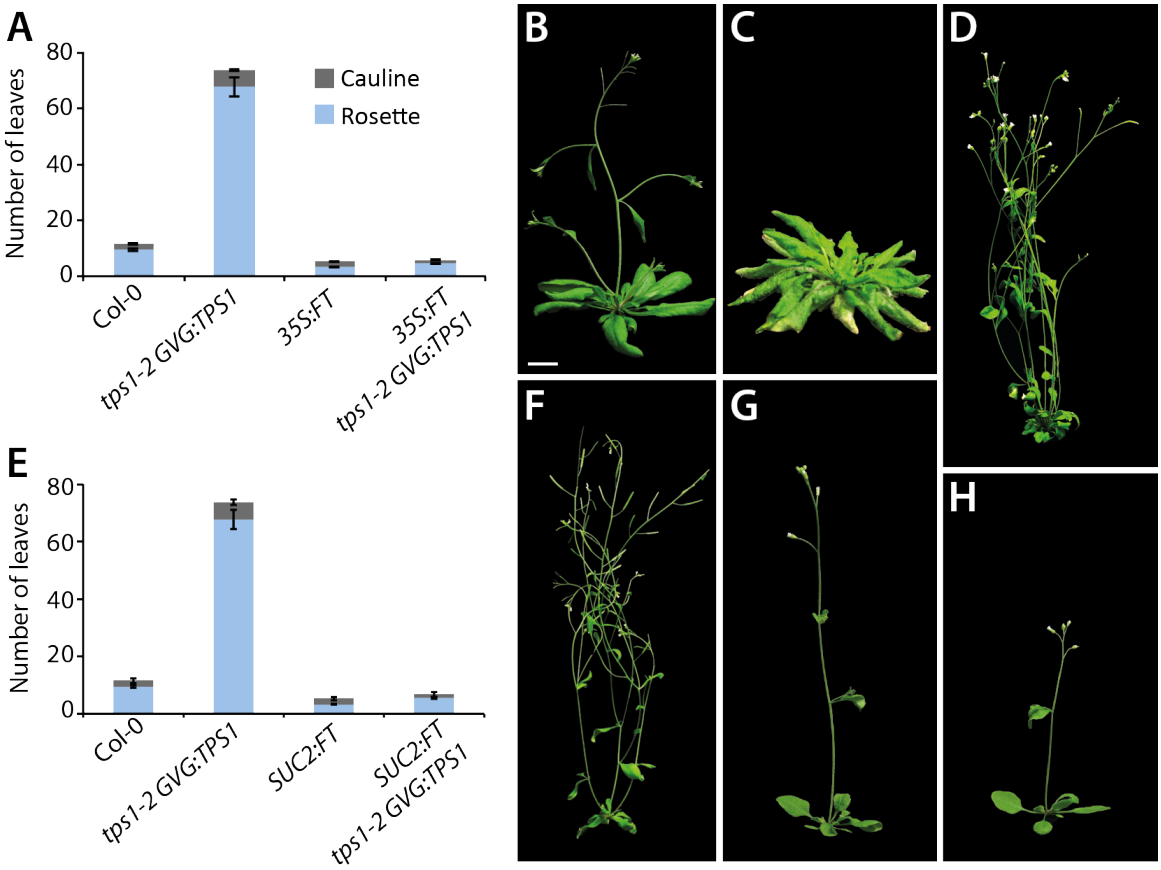

Figure 9: Flowering phenotype of *35S:FT tps1-2 GVG:TPS1* and *SUC2:FT tps1-2 GVG:TPS1* plants.

Phenotype of *35S:FT tps1-2 GVG:TPS1* (**A**, **G**) and *SUC2:FT tps1-2 GVG:TPS1* (**E, H**) plants grown under LD 23˚C in comparison with the controls - WT (**B**), *tps1-2 GVG:TPS1* (**C**), *35S:FT* (**D**) and *SUC2:FT* (**F**). Flowering time was calculated by counting the number of leaves at the time of bolting. Error bars indicate SD. Scale bar: 1cm.

3.3.4 *ft-10 tps1-2 GVG:TPS1* double mutants flower only marginally later than *tps1-2 GVG:TPS1* plants under LD

To observe the effect of loss of function of *FT* in *tps1* mutant background, *tps1-2 GVG:TPS1* was introduced into *ft-10*, a strong T-DNA insertion mutant (Yoo et al., 2005). The resulting double homozygous plants (*ft-10 tps1-2 GVG:TPS1*) flowered only marginally later than *tps1-2 GVG:TPS1* plants under LD conditions, implying that the two genes act largely in the same pathway (Figure 10). Taken together, the above results demonstrate the essential role of T6P signaling in the regulation of *FT*

expression and indicate a tight interconnection between the photoperiod pathway and carbohydrate signaling in the control of flowering.

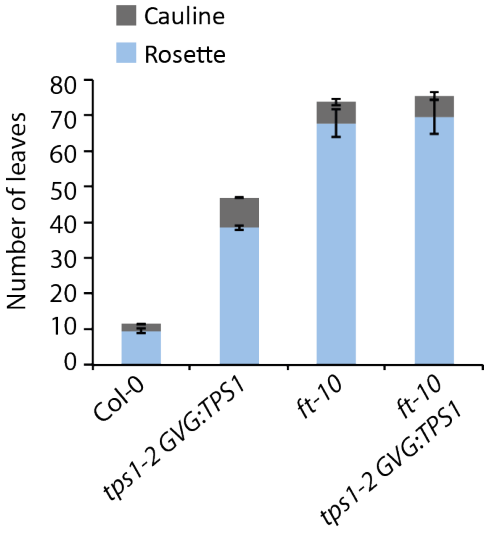

Figure 10: Flowering time of *ft-10 tps1-2 GVG:TPS1* plants.

Phenotype of *ft-10 tps1-2 GVG:TPS1* plants grown under LD 23˚C, in comparison with *ft-10*, *tps1-2 GVG:TPS1* and WT. Flowering time was calculated by counting the number of leaves at the time of bolting. Error bars indicate SD.

3.4 T6P- / TPS1-mediated flowering time regulation is partially independent of FLC

FLC is a key repressor of flowering and it acts by promoting vernalization requirement in winter annual accessions of Arabidopsis (Michaels and Amasino, 1999). Vernalization modifies the chromatin structure of *FLC* and thereby reduces its transcript and protein levels. This provides the vernalized plants with the necessary competence to flower (Robertson et al., 2008). FLC represses flowering mainly by directly suppressing flowering time genes such as *FT* and *SOC1* (Lee, 2000). To determine the interaction between the FLC-mediated vernalization pathway and T6P signaling in flowering time control, the *flc-3* allele (Michaels and Amasino, 2001), which is an early-flowering deletion mutant of *FLC*, was introduced into the *tps1-2 GVG:TPS1* background. The double mutant plants (*flc-3 tps1-2 GVG:TPS1*) flowered with an intermediate TLN of 56.7 (Figure 11, A and B). This indicates that T6P pathway affects flowering, at least partially independent of FLC-mediated vernalization pathway.

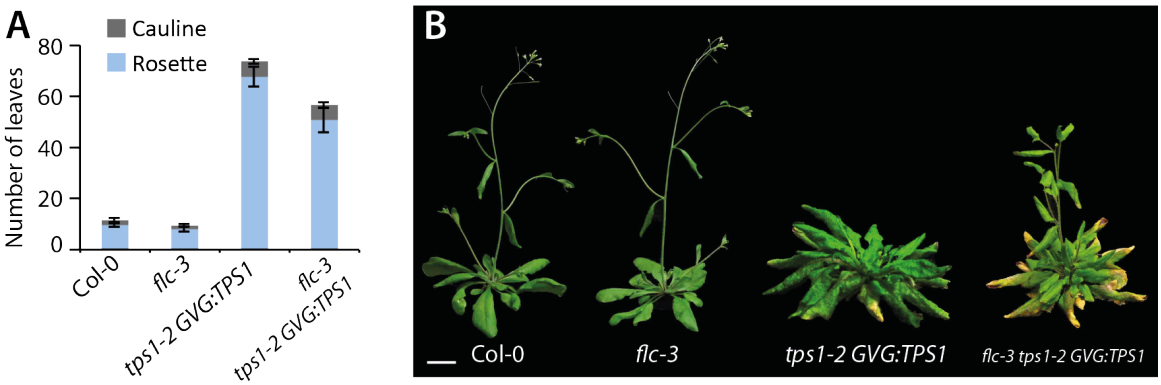

Figure 11: Flowering phenotype of *flc-3 tps1-2 GVG:TPS1* plants.

(**A** and **B**) Phenotype of *flc-3 tps1-2 GVG:TPS1* plants grown under LD 23°C, in comparison with *flc-3*, *tps1-2 GVG:TPS1* and WT. Flowering time was calculated by counting the number of leaves at the time of bolting. Error bars indicate SD. Scale bar: 1cm.

3.5 Potential targets of T6P / TPS1 signaling at the shoot apical meristem

(Microarray experiments in section 3.5 were performed together with Markus Schmid.)

The non-induced *tps1-2 GVG:TPS1* plants (grown without DEX spraying) flowered extremely late, irrespective of day length. In SD conditions, these plants almost never flowered (TLN >100). This suggests that T6P pathway interacts with signals other than the photoperiod pathway as those other signals account for floral induction in those conditions. It is likely that these signals originate from different parts of the plants, not necessarily from just leaves. But all those signals should ultimately converge at SAM, where the actual event of flowering occurs.

3.5.1 *SPL3* is insufficiently expressed in *tps1-2 GVG:TPS1*

To identify the potential additional target genes of T6P- / TPS1-mediated flowering at the SAM, microarray analyses were performed on the apical meristems collected from 21-day-old non-induced *tps1-2 GVG:TPS1* and Col-0 plants grown under SD. No significant changes were observed in *tps1-2 GVG:TPS1* samples on the expression levels of genes involved in photoperiod (Figure S2), ambient temperature,

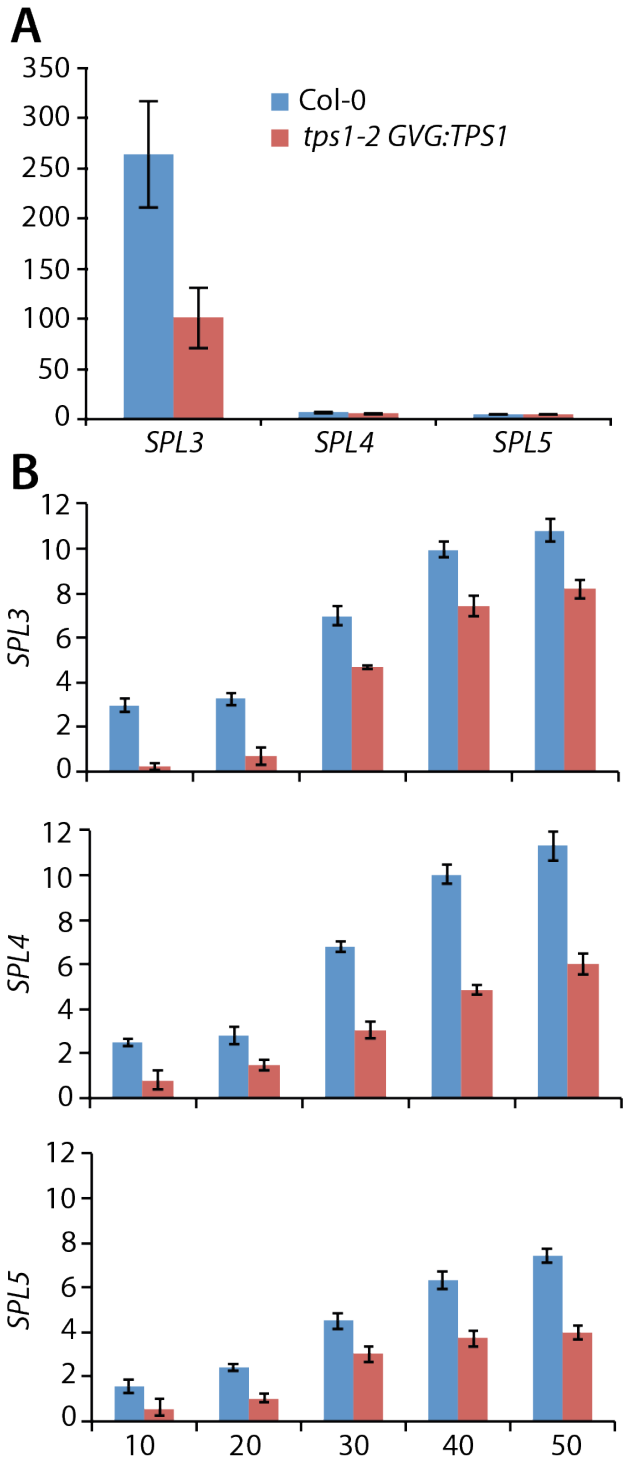

Figure 12: Potential targets of T6P signaling in the SAM.

(**A**) Expression of *SPL3*, *SPL4*, and *SPL5* in apices of 21-day-old SD-grown Col-0 and *tps1-2 GVG:TPS1* plants as determined by microarray analysis. Error bars indicate minimum and maximum values of two biological replicates. (**B**) Expression of *SPL3*, *SPL4* and *SPL5* by qRT-PCR in SD-grown Col-0 and *tps1-2 GVG:TPS1* plants 10, 20, 30, 40 and 50 days after germination (DAG). Data represent two biological replicates with three technical repetitions each. *TUB2* was used as control. Error bars indicate SD. Modified from Wahl et. al., 2013.

vernalization (Figure S3) and GA signaling (Figure S4) when compared to WT. However, *SPL3*, which is involved in miR156-mediated age pathway of flowering showed a drastic reduction (60%) in *tps1-2 GVG:TPS1* mutant when compared with Col-0 control plants (Figure 12, A).

3.5.2 *SPL3*, *SPL4* and *SPL5* are potential targets for T6P signaling at SAM

To test if the expression of *SPL3* varied over the course of plant development in *tps1-2 GVG:TPS1* in comparison with WT, apical meristems from 10-to 50-day-old plants were dissected and used for qRT-PCR analyses. Expression of *SPL3* was reduced (Figure 12, B) as expected from the microarray results. This experiment also identified two closely related genes – *SPL4* and *SPL5* – as additional targets of T6P signaling at the SAM. Expression levels of these genes were below the detection level in the microarray experiment, but both genes were readily detectable by qRT-PCR.

3.5.3 miR156 is insufficiently expressed in shoot apical meristems of *tps1-2 GVG:TPS1* plants

SPLs and their upstream regulator miR156 form the core components involved in age-pathway of flowering (Wang et al., 2009). This is a fail-safe mechanism to ensure that the plants will eventually flower, even in the absence of inductive signals (Wang et al., 2009). This is achieved by a gradual decline in the miR156 levels independently of light and other external factors, with a simultaneous increase of miR156-targeted *SPLs* in the leaves. *SPL3*, *SPL4* and *SPL5* form a clade of related genes, which are targeted by miR156. Since the levels of these transcripts were strongly reduced in *tps1-2 GVG:TPS1* plants, the levels of mature miR156 were measured by qRT-PCR at different time points between 10 and 50 days after germination, in comparison with WT (Figure 13). *tps1-2 GVG:TPS1* plants maintained slightly but consistently higher levels of miR156 between 10 and 30 days after germination, which is consistent with the low levels of *SPL3*, *SPL4* and *SPL5* during this period. However, between 40 and 50 days after germination, the levels of miR156 declined to a similar level in both the genotypes. Nevertheless, the expression of the *SPL* genes remains low at these later time points, indicating that T6P pathway regulates the expression of *SPL3*, *SPL4* and *SPL5* also independently of miR156.

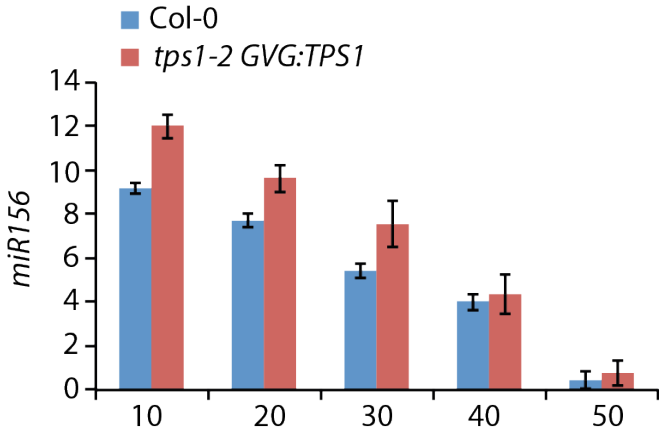

Figure 13: Expression of mature miR156 in *tps1-2 GVG:TPS1* plants.

Expression levels of mature miR156 as measured by qRT-PCR in the RNA extracted from the apices of SD-grown Col-0 and *tps1-2 GVG:TPS1* plants, 10 to 50 days after germination (DAG). Error bars indicate upper and lower limits of SD of three biological replicates with three technical repetitions each. *TUB2* was used as control. Modified from Wahl et. al., 2013.

3.5.4 Constitutive expression of *MIM156* complements the late flowering phenotype of *tps1-2 GVG*:*TPS1* plants

miRNA activity in plants can be disrupted by introducing a target mimic, which will sequester and render the miRNA inactive (Franco-Zorrilla et al., 2007). In order to reduce the miR156 levels, a mimicry construct (*MIM156*) was introduced in *tps1* mutant background by crossing *tps1-2 GVG:TPS1* plants with a mimicry-overexpressing line (*35S:MIM156*). The resulting double homozygous plants (*35S:MIM156 tps1-2 GVG:TPS1*) restored the flowering in *tps1-2 GVG:TPS1* (Figure 14, A and B). This suggests that T6P / TPS1 signaling acts in part through the miR156-mediated age pathway of flowering. Taken together the above results demonstrate that the T6P pathway contributes to the regulation of the miR156-SPL module, but that is not sufficient by itself to explain the repression of *SPL3*, *SPL4*, and *SPL5* at the SAM.

3.5.5 Reduced expression of *FT* is not as a result of insufficiency of SOC1 and FUL

It is known that SPL proteins can promote the expression of *FT* in leaves in part by regulating *SOC1* and *FRUITFUL* (*FUL*) – two MADS-box transcription factors that have been shown to play important roles in regulating flowering time and flower and fruit development (Torti and Fornara, 2012). To investigate if a reduction in the expression levels of *SOC1* and *FUL* causes the insufficient expression of *FT*, qRT-PCR

analysis on whole rosettes from *tps1-2 GVG:TPS1* and WT plants collected 10 to 40 days after germination was performed (Figure 15). The expression levels of *SOC1* and *FUL* before flowering, did not change in *tps1-2 GVG:TPS1* plants in comparison with WT (Figure 15). Hence these results suggest that in the leaves, T6P / TPS1 pathway regulates flowering time, largely independent of *SOC1* and *FUL*.

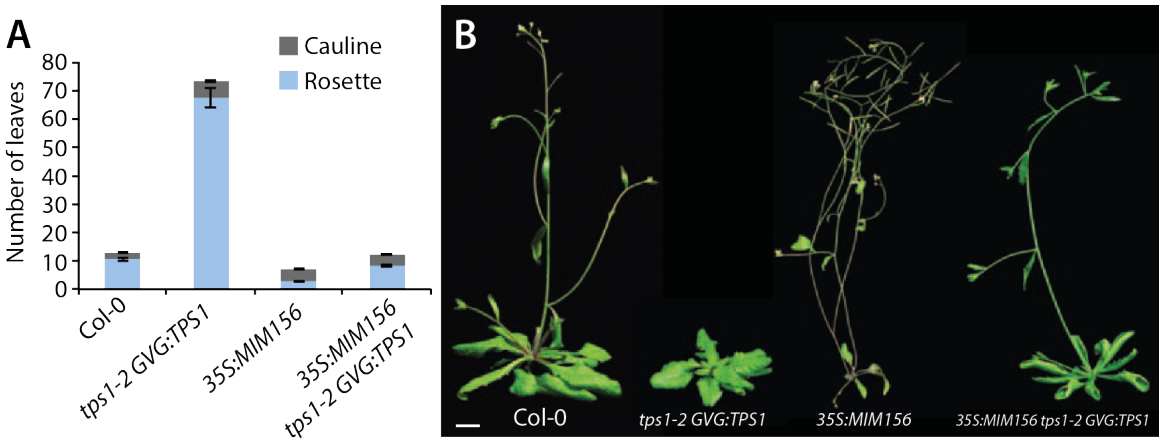

Figure 14: Flowering phenotype of *35S:MIM156 tps1-2 GVG:TPS1* plants under LD.

(**A** and **B**) Phenotype of *35S:MIM156 tps1-2 GVG:TPS1* plants grown under LD 23°C, in comparison with *35S:MIM156*, *tps1-2 GVG:TPS1* and WT. Flowering time was calculated by counting the number of leaves at the time of bolting. Error bars indicate SD. Scale bar: 1cm. Modified from Wahl et. al., 2013.

3.6 Discussion

Flowering time in plants is a complex phenomenon that integrates signals from various environmental and endogenous cues. Decades of research in Arabidopsis have provided us with an in-depth knowledge of major factors influencing flowering time, such as light, temperature and hormones (Srikanth and Schmid, 2011). However the information on how plants integrate physiological signals like carbohydrate or energy status into the flowering pathway is still limited. The results presented here provide an inroad into how metabolic signals conveyed by T6P, a proxy for sucrose status in plants, influence the reproductive transition in Arabidopsis at the molecular and gene-regulatory level.

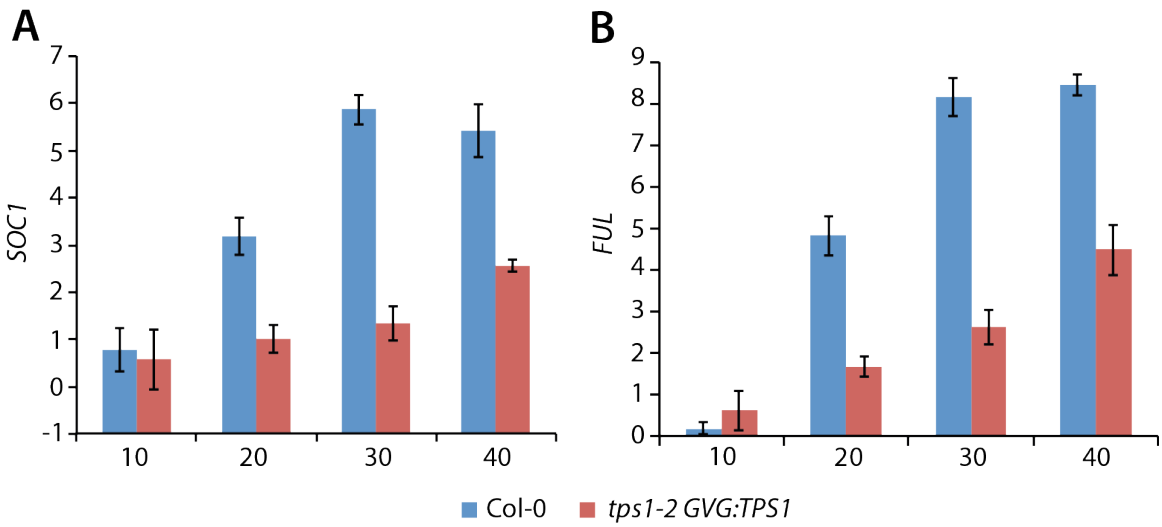

Figure 15: Expression of integrator genes in *tps1-2 GVG:TPS1.*

Expression of (**A**) *SOC1* and (**B**) *FUL* was determined by qRT-PCR in RNA extracted from 10-day-old whole rosettes and leaves of 20 to 40-day-old LD-grown Col-0 and *tps1-2 GVG:TPS1* plants. Note that flowering in wild-type Col-0 occurs at about 10 days in LD. Error bars represent upper and lower limits of the SD of the mean of three biological replicates with three technical repetitions each. Modified from Wahl et. al., 2013.

It has previously been demonstrated that sugars are mobilized from leaves and are transported to the SAM during floral transition (Roldan et al., 1999; Corbesier et al., 2002) in Arabidopsis. However at that time, it was not clear whether the sugars act as signals and regulate the floral transition or whether they merely function as energy source to support the energy-demanding processes of flowering and seed set. In this context, it is interesting that many of the mutants with perturbed carbohydrate metabolism (*adg1-1, pgm1, sex1, bam3, gin1-1*) are also abnormal in terms of flowering time (Matsoukas et al., 2013). One such mutant with a defect in trehalose metabolism (*tps1-2 GVG:TPS1*) due to a transposon insertion in the *TPS1* gene, exhibits an extreme delay in flowering time even under otherwise inductive LD conditions (van Dijken et al., 2004).

Day length influences the timing of floral transition, mainly by modulating the diurnal expression and accumulation of CO protein via the circadian clock (Suarez-Lopez et al., 2001). Expression levels of *FT* - a direct target of CO - also change diurnally. 72-hour diurnal time course analysis (Figures 6 and 7) indicates the

possibility that the production of T6P is diurnally regulated in WT plants. The levels of T6P peak at the end of the day, presumably reflecting the sucrose levels produced as a result of photosynthesis (Wahl et al., 2013). This peaking of T6P can be correlated with a previous study on diurnal changes of sucrose (Lunn et al., 2006). The end of the day is exactly the time when circadian-regulated *CO* also shows the highest expression (Imaizumi et al., 2005). Interestingly, qRT-PCR on whole rosettes collected from a 72-hour diurnal time course (Figure 6, C) showed that *FT*, but not *CO* and *GI,* is insufficiently expressed in *tps1-2 GVG:TPS1* plants especially at the end of the day. In addition, *FT* expression could be induced by DEX application in these plants, demonstrating the essential role of T6P signaling for the induction of flowering. *TSF*, another flowering time gene acting redundantly with FT (Yamaguchi et al., 2005), also showed a reduced expression in *tps1-2 GVG:TPS1* at the end of the day (Figures 6, D and 7). Expression of *FT* and *TSF* at the end of the day was also abolished in another *tps1* knockdown line created by using artificial-microRNA technology (*35s:amiR-TPS1*) (Wahl et al., 2013). Furthermore, expressing *FT* constitutively (using *35S:FT*) (Figure 9) or in phloem companion cells (using *SUC2:FT*) (Figure 9) which induces flowering independent of day length, was sufficient to completely suppress the late flowering in *tps1-2 GVG:TPS1* (Figure 9) and *35S:amiR-TPS1* plants (Wahl et al., 2013). Moreover, *ft-10 tps1-2 GVG:TPS1* flowered only marginally later than *tps1-2 GVG:TPS1* under LD conditions (Figure 10) suggesting that T6P signaling and FT act largely in the same pathway.

As mentioned before, FT has been shown to act genetically downstream of CO and GI. But in contrast to *FT*, expression of these two genes was not altered in the *tps1-2 GVG:TPS1* mutant when compared to WT (Figure 6, A and B), suggesting that T6P signaling integrates with the photoperiod pathway downstream of CO. However, at present the possibility of T6P signaling affecting CO post-translationally cannot be discarded. In such a scenario, loss of TPS1 would affect CO protein accumulation, which would result in reduced expression of its targets - *FT* and *TSF*. It would be interesting to observe the diurnal changes of CO protein levels in *tps1-2 GVG:TPS1*. This is difficult at present, since there is no CO-specific antibody available, which could be utilized to monitor the levels of native CO through protein immunoblot analysis. Another option would be to introduce any reliable epitope tagged *CO*

constructs driven by the native *CO* promoter (such as *CO*:*HA-CO* or *CO*:*GFP-CO*) in the *tps1-2 GVG:TPS1* background and to quantify the changes in CO protein levels in a diurnal manner. Efforts were also made to rescue the late flowering phenotype of *tps1-2 GVG:TPS1* plants by misexpressing *CO*. Preliminary results suggest that expressing CO in the phloem companion cells (using *SUC2:CO*) of *tps1-2 GVG:TPS1* plants is sufficient to complement the delay in flowering (data not shown), but further analyses are required. Taken together, these experiments raise the possibility that T6P might regulate the expression of *FT* by influencing the activity of upstream regulators such as CO. Further experiments are needed to elucidate the interaction between T6P signaling and the upstream components of *FT*.

From the experiments described, it is evident that T6P levels fluctuate in a diurnal manner (Wahl et al., 2013). Both previous and recent studies have shown that T6P serves as readout of the sucrose status in plants and convey this information to the other signaling pathways (Lunn et al., 2006; Yadav et al., 2014). Since the production of sucrose also broadly followed the same diurnal pattern (Lunn et al., 2006), this raises the question, whether the observed peak in the levels of T6P merely represents the status of sucrose or the T6P pathway itself is under the control of the circadian clock. It was recently shown that the major metabolic output of circadian clocks in plants is the production of sugars by photosynthesis and, similar to light and temperature, sugar signals can also entrain circadian rhythms in plants (Haydon et al., 2013). Evidence for this comes from the analysis of PSEUDORESPONSE REGULATOR 7 (PRR7), an important component of the core oscillator in Arabidopsis. Analysis of *prr7* mutants in Arabidopsis showed that sucrose levels provide the feedback to the clock via PRR7 (Haydon et al., 2013). It would be interesting to see if the gene expression of any of the important clock components is being regulated by T6P pathway either through PRR7 or independent of it.

The circadian clock is buffered against the changes in environment by entraining the rhythms. Different environmental and endogenous signals affect the circadian regulator for the entrainment to occur. It is known that circadian clock receives output from the photoperiod pathway (Hayama and Coupland, 2003) to regulate

floral transition. Temperature is another important environmental signal that is implicated to be modulating the clock (Eriksson and Millar, 2003).

Studies on natural variation in Arabidopsis indicate that the genes involved in the vernalization pathway, such as *FLC,* can also affect the circadian clock in addition to influencing floral transition (Salathia et al., 2006). FLC and FRI make up the core of the vernalization pathway of flowering. FLC is a MADS-box transcription factor that acts as a floral repressor while FRI is required to upregulate FLC (Amasino, 2005). FLC is a part of the repressor complex that directly targets the flowering time genes such as *FT* and *SOC1* and as a result, flowering is delayed in non-vernalized winter-annual accessions of Arabidopsis. Cold treatment or vernalization reduces the transcript and protein levels through epigenetic silencing of FLC. To investigate the role of FLC in T6P-mediated flowering, double mutant plants were created with mutations in *TPS1* and *FLC* (*flc-3 tps1-2 GVG:TPS1*). These plants exhibited an intermediate flowering time phenotype when compared to the single mutant plants (Figure 11), implying that at least a part of the floral repressive function of T6P / TPS1 seems to occur via FLC. In WT plants, the expression levels of FLC are low due to the loss of function mutation in *FRI* (Michaels and Amasino, 2001). However, microarray analysis using the apical meristems collected from SD grown plants did not show any significant expression difference of FLC in *tps1-2 GVG:TPS1* mutant in comparison with WT (data not shown). These experiments indicate that T6P / TPS1 signaling influences the flowering time at least partially independent of FLC.

tps1-2 GVG:TPS1 mutants flower extremely late irrespective of the day length conditions (Figure 4) (Wahl et al., 2013). This raises the possibility that the TPS1/T6P might also interact with pathways other than the photoperiod pathway. After light perception in leaves, the circadian-regulated CO activates its downstream target *FT*, which forms part of the florigen complex that moves towards the shoot apex. Thus the most obvious non-leaf tissue, where T6P signaling can have its regulatory functions is the SAM. Interestingly, *TPS1* is expressed in cells that encircle the center of the SAM, as revealed by RNA *in situ* hybridization (Wahl et al., 2013). In addition, T6P levels increased in apical meristems of LD grown WT plants, especially during the floral transition. This could also be observed in SD grown plants shifted

to LD (Wahl et al., 2013). This rise in T6P levels also corresponds to an increase in sucrose levels, indicating that T6P might act as a proxy for sucrose status in the SAM as well. When *TPS1* was expressed under the control of stem cell specific *CLV3* promoter (*CLV3:TPS1*), the plants exhibited an extremely early flowering phenotype under LD and SD, confirming the role of T6P signaling at the SAM. It is interesting to note that *TPS1* expression was observed in the flanking regions of SAM, but not in the *CLV3* expression domain (predominantly in L1 and L2 layers). This would raise the possibility that either the functionality of TPS1 protein is maintained in this domain or T6P / TPS1 might possess non-cell autonomous properties and affect the adjacent cell layers in Col-0 accession. In contrast, expression of the bacterial TPP gene *otsB* under *CLV3* regulatory sequences (*CLV3:otsB*) had the opposite effect (Wahl et al., 2013). Moreover, the expression of *CLV3:TPS1* was sufficient to complement the late flowering phenotype of *ft-10* plants (Wahl et al., 2013), indicating that T6P signaling can act largely independent of FT to induce flowering at the SAM.

Expression levels of major genes involved in processes that regulate floral transition such as photoperiod, temperature, vernalization and gibberellic acid, were essentially unchanged in *tps1-2 GVG:TPS1* apical meristems in comparison with WT, as revealed through microarray analysis (Figures S2, S3 and S4). However *SPL3,* a known component of miR156-mediated age pathway of flowering was significantly reduced in *tps1-2 GVG:TPS1* meristems (Figure 12, A), which was again confirmed by qRT-PCR. Gene expression analysis on dissected meristems from 10 to 50 day-old *tps1-2 GVG:TPS1* and WT plants also identified *SPL4* and *SPL5*, two genes that are closely related to *SPL3,* as additional potential targets of T6P / TPS1 signaling at the SAM (Figure 12, B). These *SPL* genes are regulated by various signals that control flowering, such as age of the plants. The age-pathway ensures that plants will ultimately make the transition to flowering, even in the absence of inductive signals (Wang et al., 2009).

SPL3, *SPL4* and *SPL5* form a sub-clade among the SPL genes that are regulated by miR156 (Gandikota et al., 2007). During the seedling stage, the levels of miR156 are high, which represses *SPL* genes and prevent precocious flowering. But as plants

age, a gradual decline in miR156 ensures the sufficient expression of *SPL* genes which promote flowering (Wang et al., 2009). Between 10 and 30 DAG under SD, *tps1-2 GVG:TPS1* plants maintained significantly higher levels of mature miR156, when compared to WT (Figure 13). This might at least in part explain the insufficient levels of *SPL3*, *SPL4* and *SPL5* during this stage. But as the plants age, the expression levels of miR156 decline in a similar manner in both *tps1-2 GVG:TPS1* and WT plants (Figure 13), while *SPL3*, *SPL4*, and *SPL5* levels remain low. These results clearly show that T6P signaling controls the expression of *SPL3*, *SPL4* and *SPL5* at the SAM, at least in part via miR156 and partly independent of miR156-mediated age-pathway.

In addition, the down-regulation of *TPS1* (*35S:amiR-TPS1*), coupled with constitutive overexpression of a MIR156 gene (*35S:MIR156b*) makes the plants (*35S:MIR156b 35S:amiR-TPS1*) fail to flower in both LD and SD, due to an additive effect (Wahl et al., 2013). Moreover, expressing a mimicry construct against miR156, which captures and renders the miRNA inactive (*35S:MIM156*) in *tps1-2 GVG:TPS1*, was sufficient to completely complement its late flowering phenotype (Figure 14). This shows that miR156-mediated age pathway regulates flowering time, partly independent of T6P pathway. *SPL* genes are known to promote the expression of *FT* by regulating the expression of *SOC1* and *FUL* in leaves (Yamaguchi et al., 2009). This could imply that the repression of *FT* observed in *tps1-2 GVG:TPS1* plants (Figure 6 and 7) may be due to the reduced expression of *SOC1* and *FUL*. However, *tps1-2 GVG:TPS1* and *35S:amiR-TPS1* plants showed no difference in the expression of *SOC1* and *FUL*, especially before flowering (Figure 15) (Wahl et al., 2013). This rules out the possibility that the observed repression of *FT* in *tps1-2 GVG:TPS1* plants is because of the insufficient expression of *SOC1* and *FUL* and this effect is rather due to a direct influence of T6P signaling on *FT* expression.

So far our results demonstrate that T6P signaling regulates floral transition in spatially separate tissues – at the leaves (Figures 6, 7 and 8) and at the SAM (Figure 16). Firstly *TPS1* signaling is essential for the induction of *FT* in leaves, even under LD conditions (Figures 6, 7 and 8). This mechanism likely ensures that metabolic

signals such as sugar status are integrated with environmental signals like day length. This helps the plants to ensure that flowering only commences when

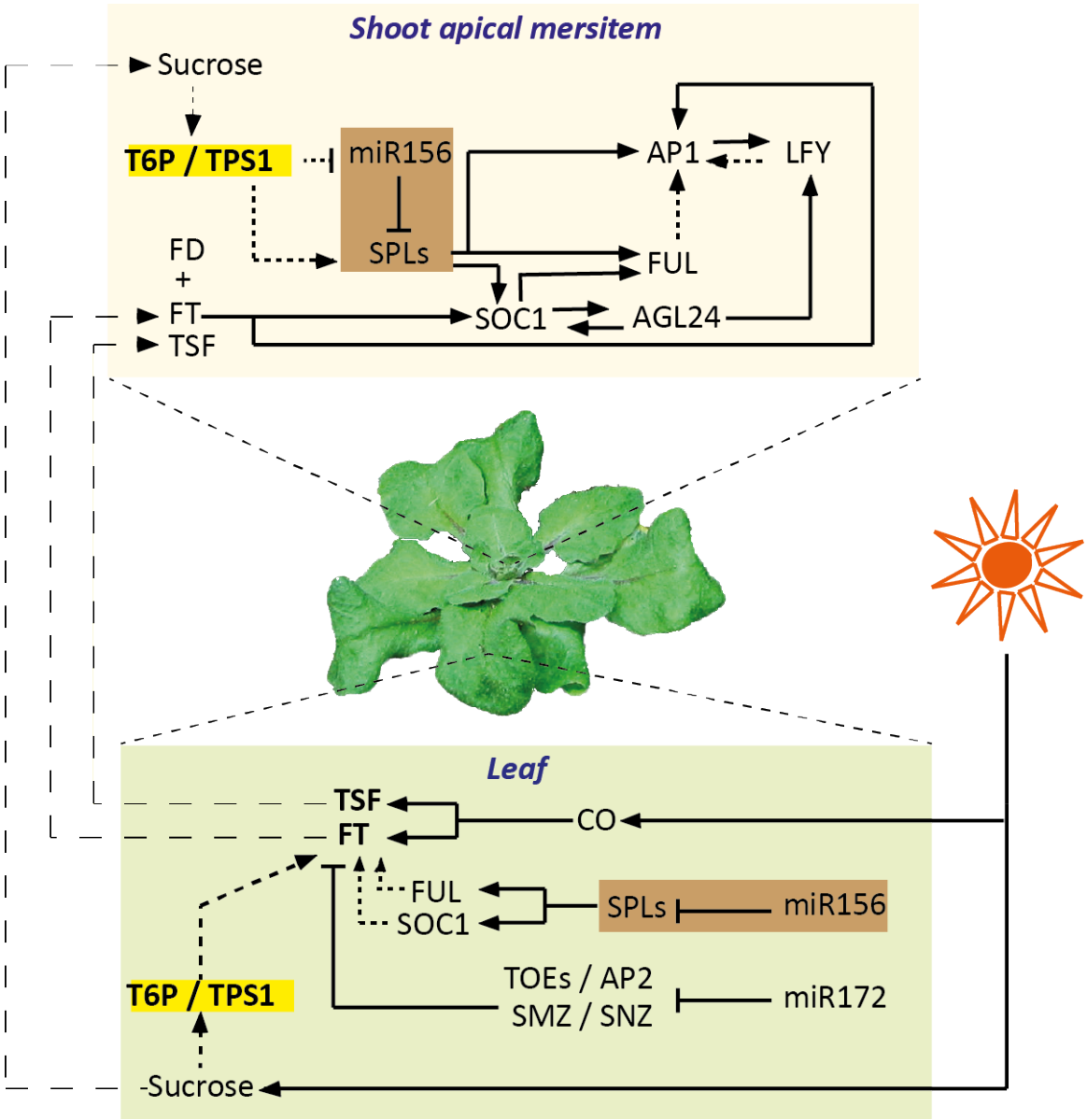

Figure 16: A minimal model explaining the dual role T6P pathway in regulating flowering time in Arabidopsis.

Solid lines indicate direct interactions and dashed lines indicate indirect interactions. Transport of FT protein (florigen) and sucrose from leaves to the shoot apical meristem is indicated. Modified from Wahl et. al., 2013.

sufficient carbohydrates are available to meet the energy demand and also when the light conditions are conducive. On the other hand, T6P signaling influences the expression of major flowering time and floral-pattering genes at the SAM via

regulating the expression of miR156-targeted *SPL* genes, independent of the photoperiod pathway (Figure 12). This provides a way for plants to integrate the carbohydrate status at the SAM to make important developmental decisions.

In addition to regulating flowering in an age-dependent manner, the miR156-SPL module also plays an important role in the transition from juvenile-to-adult phase in plants. As the *tps1-2 GVG:TPS1* plants maintain slightly higher levels of mature miR156 compared to WT (Figure 13), it would be interesting to investigate if the effect of enhanced miR156 also influences the vegetative phase change in Arabidopsis. Moreover, *35S:amiR-TPS1* plants maintain a higher level of sucrose, despite being insufficient in T6P (Wahl et al., 2013). Vegetative phase change in *tps1-2 GVG:TPS1* plants and the part played by miR156-SPL module will be discussed in the next chapter.

4 Chapter 2

Trehalose-6-phosphate signaling regulates vegetative phase change in Arabidopsis

Contributions to this chapter:

All experiments and their analyses described in this chapter have been carried out by myself, if not mentioned otherwise.

4.1 Abstract

Vegetative phase change in Arabidopsis is influenced by various endogenous and environmental factors. Among the endogenous factors, the micro-RNA 156 (miR156)- SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) module plays an active role in regulating this important phase transition. Arabidopsis seedlings maintain high levels of mature miR156, which keeps the expression of its downstream targets –*SPL* transcripts – to a minimum. As the plants age, the levels of miR156 get gradually reduced, allowing the *SPLs* to accumulate and to promote the juvenile-to-adult phase transition. Recently it has been proposed that sugars such as sucrose and glucose could act as age-dependent signals that repress *MIR156* genes to trigger the vegetative phase change in plants. Sugar status in Arabidopsis is conveyed in part through trehalose-6-phosphate (T6P), which is an intermediate signaling compound produced during the biosynthesis of disaccharide trehalose. *tps1-2 GVG:TPS1* mutant plants defective in *TREHALOSE-6-PHOSPHATE SYNTHASE1* (*TPS1*), exhibit a prolonged juvenile phase when compared to WT even under inductive long day conditions. qRT-PCR and double mutant analyses demonstrate that the T6P / TPS1 pathway regulates vegetative phase change at least in part through the miR156-SPL module and largely independent of FLC. In addition, exogenous supplementation of even 4% sucrose did not accelerate vegetative phase change in WT plants grown under long days. Since T6P has been proposed to act as a proxy for sucrose status in plants, our results suggest that T6P rather than sucrose, might function as the age-dependent signal responsible for the repression of miR156, which promotes the juvenile-to-adult phase transition and subsequently flowering in Arabidopsis.

4.2 *tps1-2 GVG*:*TPS1* plants exhibit a delay in vegetative phase transition

Plants with defective carbohydrate metabolism usually show a drastic change in developmental transitions, as indicated by early (such as *lba1*, *atsuc9*, *sweetie* and *amy1*) and late (such as *glz2* and *atvgt1*) flowering sugar-mutants in Arabidopsis (Rolland et al., 2006). Many of the above-mentioned plants have abnormalities in the perception, signaling or compartmentalization of sugars such as sucrose and glucose. In plants, sugar status is thought to be conveyed through T6P (Lunn et al., 2006; Yadav et al., 2014), which plays a crucial role in sugar signaling at different stages throughout the plant development (reviewed in Ponnu et al., 2011).

A transposon insertion knocks down the expression of *TPS1* gene in *tps1-2 GVG:TPS1* plants, which makes them extremely late flowering even under LD conditions (Figure 4) (van Dijken et al., 2004). At the seedling stage, these plants showed retarded growth and produced more round leaves with anthocyanin accumulation in leaf margins (visual observation), when compared to WT. These defects can be rescued almost completely through the induction of *TPS1* by spraying DEX (Figure 5) (van Dijken et al., 2004). Apart from the long petiole, lack of abaxial trichomes and smooth margin, presence of round leaf blade is also one among the criteria, which determines the developmental stage of a leaf (Telfer et al., 1997). Juvenile leaves generally have round blades when compared to the adult leaves (Telfer et al., 1997). In Arabidopsis, miR156 plays an important role in maintaining the juvenility (Huijser and Schmid, 2011). WT seedlings have higher levels of miR156, which get reduced as the plants age. It is important to note here that when compared to WT, *tps1-2 GVG:TPS1* plants show enhanced levels of miR156 in the SAM from 10 to 30 DAS (Figure 13). The presence of many leaves with round blades and higher levels of miR156 during seedling stage implicate that these plants might also show a defect in vegetative phase transition.

4.2.1 *tps1-2 GVG*:*TPS1* plants grow more slowly compared to WT

To investigate the growth dynamics of *tps1-2 GVG:TPS1* plants in comparison with WT, the number of leaves and rosette diameters (in cm), which represents the plant size were measured in LD-grown seedlings from 4 to 14 days after germination

(DAG) (Figure 17). The WT plants produced approximately 3 times more number of leaves than *tps1-2 GVG:TPS1* mutants at 14 DAG. This clearly shows that the leaf production rate is delayed in *tps1-2 GVG:TPS1* plants (Figure 17, A) in comparison with Col-0. The individual leaves of the mutant plants were also visibly smaller. The delay in leaf production coupled with the smaller leaf blades ultimately resulted in a reduced rosette-size (Figure 17, B) in *tps1-2 GVG:TPS1* plants. Taken together these results indicate that overall growth and development are reduced in the *tps1-2 GVG:TPS1* plants.

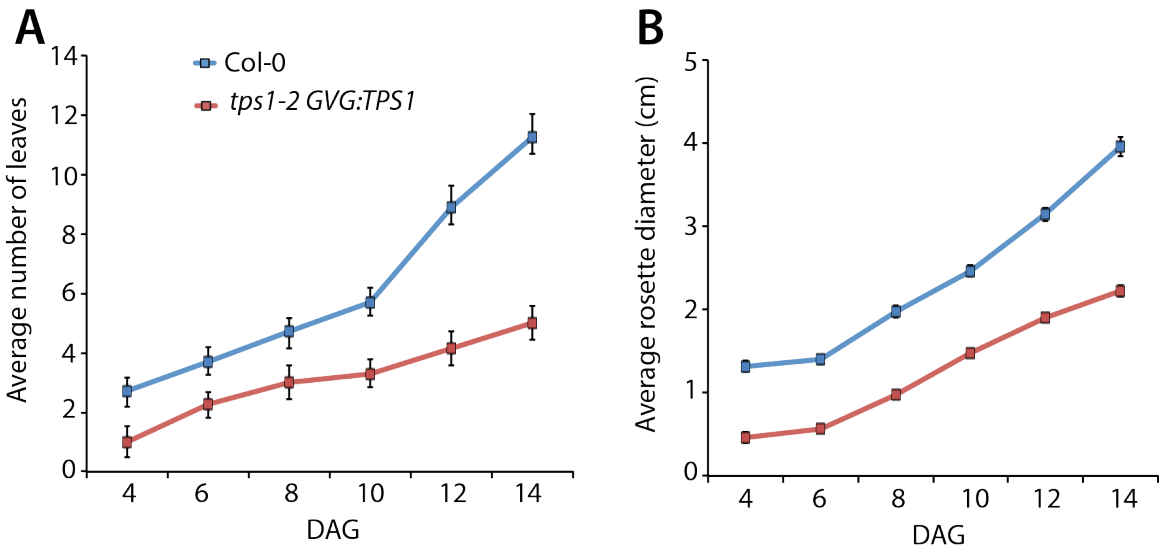

Figure 17: Growth of *tps1-2 GVG:TPS1* is delayed when compared to WT.

Average total leaf number (**A**) and average rosette diameter (**B**) in LD-grown *tps1-2 GVG:TPS1* plants when compared to WT. Number of visible leaves was counted and rosette diameters were measured from 4 to 14 DAG, at 2-day intervals. Error bars represent SD. n=20. DAG= days after germination.

4.2.2 *tps1-2 GVG:TPS1* plants produce more juvenile leaves than WT

The number of juvenile leaves produced represents the length of juvenile phase in plants (Chien and Sussex, 1996). Juvenile leaves in Arabidopsis are characterized by long petioles, round blades, smooth margins and complete absence of abaxial trichomes (Telfer et al., 1997). The presence or absence of the abaxial trichomes is a reliable measure to judge the juvenility of leaves (Chien and Sussex, 1996). In order to test if the length of juvenile phase varied between *tps1-2 GVG:TPS1* and WT,

plants were grown under LD and the number of juvenile leaves were determined based on the presence or absence of abaxial trichomes (Figure 18). Based on this criterion it was observed that the *tps1-2 GVG:TPS1* plants produced approximately double the number of juvenile leaves than WT (Average juvenile leaf number (JLN) = 9.75) under LD conditions (Figure 18, A). In addition, *tps1-2 GVG:TPS1* plants produced more roundish leaves than WT (Figure 1 B and C). Only the leaf morphology of juvenile leaves are depicted in Figure 18, B and C, since *tps1-2 GVG:TPS1* mutants produce >75 leaves before bolting under LD (Figure 5). Taken together the above results (Figures 17 and 18) show that *tps1-2 GVG:TPS1* plants exhibit a delay in vegetative phase change or an enhanced juvenile phase when compared to WT.

4.3 DEX-induced expression of *TPS1* rescues the delayed vegetative phase change phenotype of *tps1-2 GVG:TPS1*

DEX-inducible expression of *TPS1* has been shown to effectively rescue the embryo-lethality and growth abnormalities in *tps1-2 GVG:TPS1* plants (Figure 5) (van Dijken et al., 2004). Furthermore, spraying 1 µM DEX solution is sufficient to induce the expression of *FT* and thereby triggering flowering (Figure 7). To investigate if DEX-inducible expression of *TPS1* could also rescue the delayed vegetative phase change phenotype in *tps1-2 GVG:TPS1*, seedlings were grown under LD conditions on soil soaked with 5µM DEX solution starting from day five after germination (5 DAG) (Figure 19). DEX application to soil was continued till the emergence of first two adult leaves. Soil application or soaking with 5µM DEX eliminates the possibility of uneven wetting of leaves, which usually is a problem with spraying. In addition, soaking ensures that DEX is available all the time during the plant growth and *TPS1* is continuously expressed.

4.4 Leaves of *tps1-2 GVG:TPS1* plants maintain higher levels of miR156 during the time when vegetative phase transition occurs in WT

miR156 has been shown to play a prominent role in the regulation of the vegetative phase transition in plants (Wu and Poethig, 2006). In particular it has been demonstrated that high levels of miR156 promote juvenility. In agreement with this idea, WT plants maintain high levels of miR156 at the seedling stage, which get

reduced in an age-dependent manner and overexpression of miR156 has been shown to delay vegetative phase change in Arabidopsis plants (Huijser and Schmid, 2011). Interestingly, *tps1-2 GVG:TPS1* plants have higher levels of mature miR156 in the SAM from 10 to 30 DAS (Figure 13).

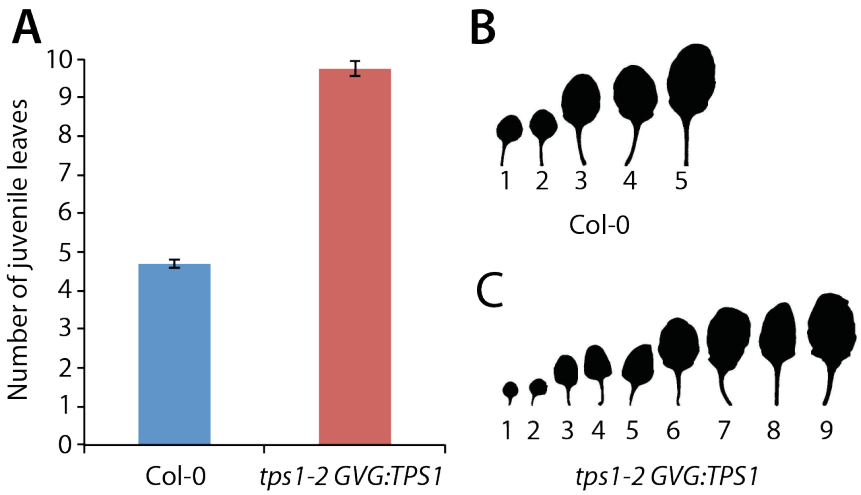

Figure 18: Juvenile phase is prolonged in *tps1-2 GVG:TPS1* plants under LD.

Number of juvenile leaves produced in *tps1-2 GVG:TPS1* plants compared to WT, grown under LD conditions (**A**). Juvenility of the leaves was determined by verifying the complete absence of abaxial trichomes. Error bars represent SD. n=20. Morphology of juvenile leaves of Col-0 and *tps1-2 GVG:TPS1* are depicted in **B** and **C**, respectively. Only the juvenile leaves are represented here, as the *tps1-2 GVG:TPS1* plants produce >75 leaves under LD conditions. Numbers represent leaf positions on the rosette. Leaves were detached from the plants, soon after the emergence of 1 to 2 adult leaves.

In WT plants, vegetative phase change usually occurs within 10 DAG (Figure 17). To test if T6P / TPS1 signaling interacts with miR156 to influence the vegetative phase change, mature miR156 levels were measured in the leaves of SD-grown *tps1-2 GVG:TPS1* and Col-0 plants at 10 and 20 DAS by qRT-PCR analysis (Figure 20). Mature miR156 levels were significantly higher in the leaves of *tps1-2 GVG:TPS1* plants at 10 and 20 DAS when compared to WT (Figure 20), demonstrating that the delay in vegetative phase change exhibited by *tps1-2 GVG:TPS1* could be caused at least in part due to higher levels of mature miR156 within 20 DAS.

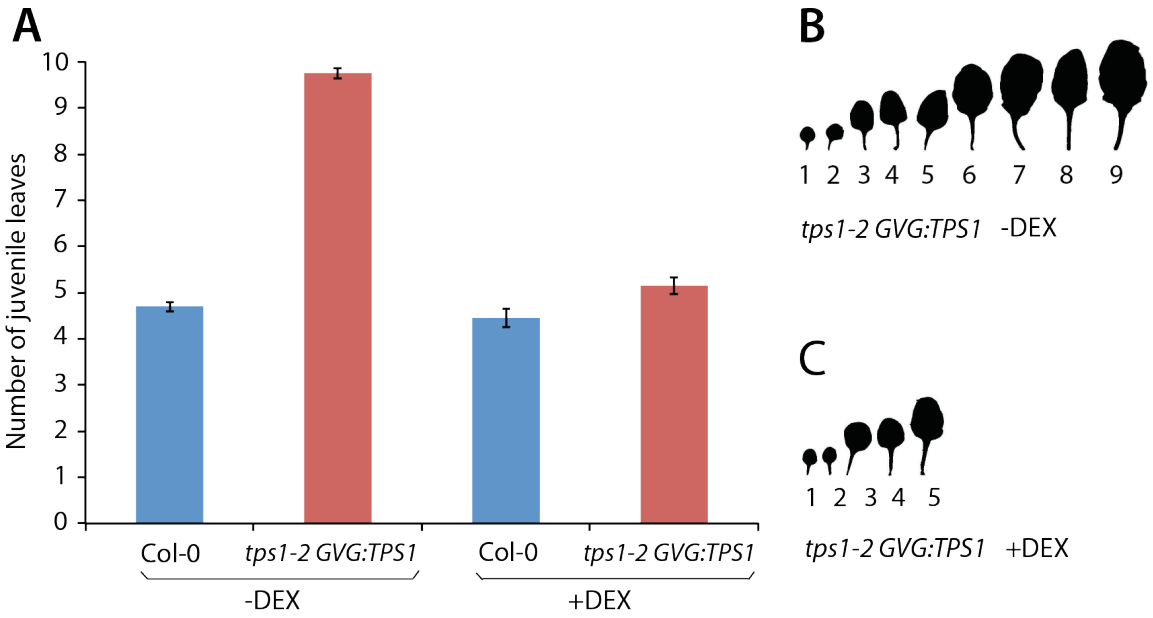

Figure 19: Inducible expression of *TPS1* complements the delayed vegetative phase change phenotype of *tps1-2 GVG:TPS1*.

Number of juvenile leaves produced in *tps1-2 GVG:TPS1* and Col-0 under LD conditions with and without DEX application. To induce the expression of *TPS1*, plants were grown on soil soaked with 5µM DEX starting from 5 DAG until the emergence of first two adult leaves. Error bars represent SD. n=20. Leaf morphology of juvenile leaves of *tps1-2 GVG:TPS1* with and without DEX application are depicted in **B** and **C** respectively. Numbers represent leaf positions. Leaves were detached from the plants soon after the production of first two adult leaves.

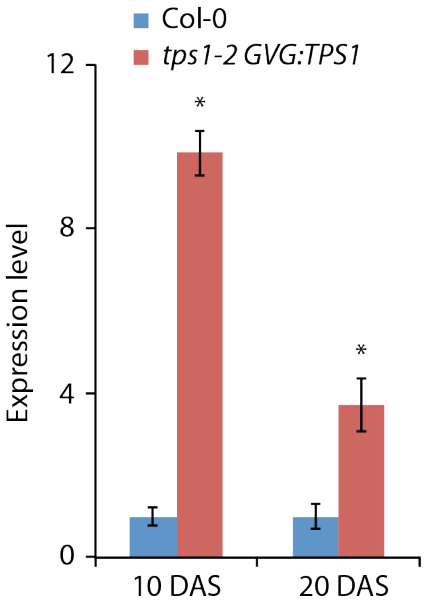

Figure 20: Levels of mature miR156 are elevated in *tps1-2 GVG:TPS1* leaves.

Leaves of SD grown plants were harvested at 10 and 20 DAS. Levels of mature miR156 were measured by qRT-PCR on two biological replicates with three technical replicates each. Expression levels in Col-0 are set to 1. *TUB2* was used as control. * represents significant difference from WT, Student t-test, $p<0.001$. Error bars indicate SD.

4.5 Constitutive expression of *MIM156* complements the late vegetative phase change phenotype of *tps1-2 GVG:TPS1*

An effective way of reducing the activity of a miRNA is by introducing a target mimic to sequester and make it inactive (Franco-Zorrilla et al., 2007). To test whether the enhanced levels of miR156 in *tps1-2 GVG:TPS1* contribute to the delay in vegetative phase change, a mimicry construct against miR156 (*MIM156*) was introduced into the *tps1* mutant background by crossing *tps1-2 GVG:TPS1* plants with a *MIM156* overexpressing line (*35S:MIM156*) (Schwab et al., 2005). The resulting double homozygous plants (*35S:MIM156 tps1-2 GVG:TPS1*) were grown under LD and vegetative phase change was observed by counting the number of juvenile leaves (Figure 21). Only two out of twenty *35S:MIM156* plants produced a juvenile leaf, whereas four out of twenty produced a juvenile leaf in the case of *35S:MIM156 tps1-2 GVG:TPS1*. The remaining plants produced no juvenile leaves at all. Thus reducing the activity of miR156 by introducing *35S:MIM156*, almost completely suppressed the juvenile phase in *tps1-2 GVG:TPS1* plants (Figure 21). This result suggests that the T6P / TPS1 pathway acts through miR156 or the signaling from both the components converges at same target/s.

4.6 miR156- targeted *SPL2, SPL4* and *SPL15* are insufficiently expressed in *tps1-2 GVG:TPS1* seedlings

miRNAs exert their effects by regulating their downstream targets either through mRNA degradation or translational inhibition. miR156 is known to regulate 11 out of 17 *SPL* genes in Arabidopsis (Huijser and Schmid, 2011). In order to investigate if the enhanced levels of miR156 in the leaves of *tps1-2 GVG:TPS1* plants also result in differential expression of miR156–targeted *SPLs*, qRT-PCR analyses were carried out on leaves collected from 10-day old plants grown under SD conditions. *SPL2*, *SPL4,* and *SPL15* were significantly down-regulated in *tps1-2 GVG:TPS1* plants when compared to WT, whereas the expression of *SPL3*, *SPL10* and *SPL11* were not changed significantly (Figure S5). The above results (Figures 20, 21 and 22) suggest that T6P / TPS1 signaling regulates the vegetative phase change in Arabidopsis through influencing the expression of *SPL2*, *SPL4* and *SPL15*, at least in part through miR156.

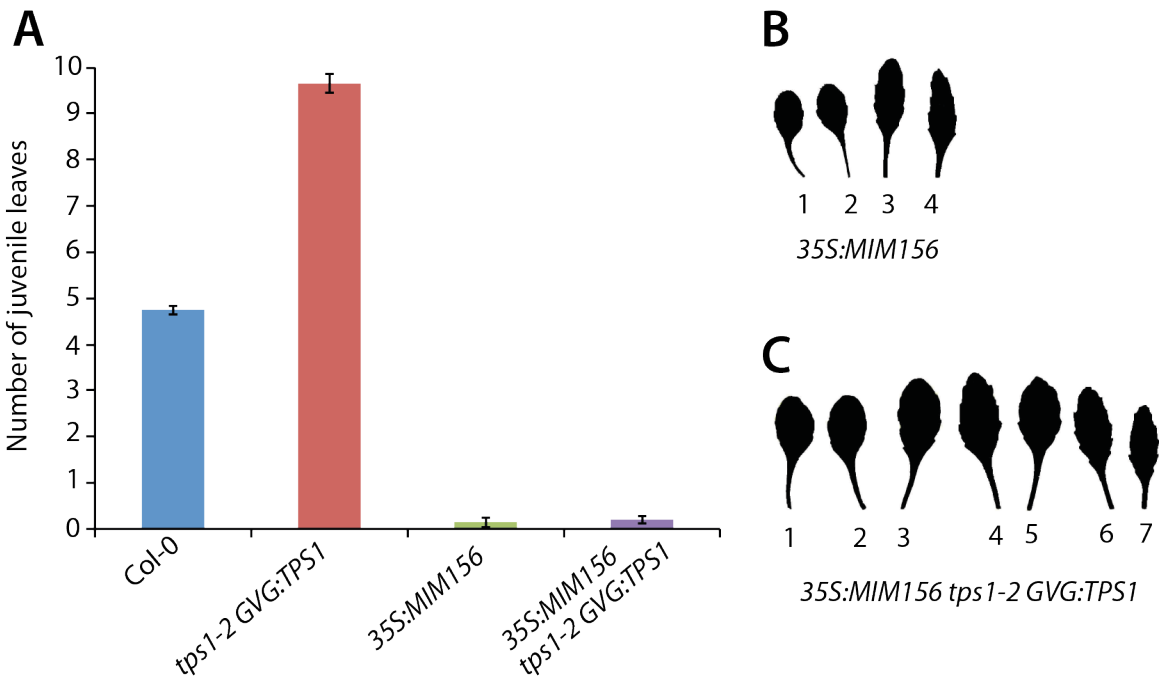

Figure 21: Vegetative phase change in *35S:MIM156 tps1-2 GVG:TPS1* plants under LD.

Number of juvenile leaves produced in *35S:MIM156 tps1-2 GVG:TPS1* compared to WT, *35S:MIM156* and *tps1-2 GVG:TPS1* plants grown under LD conditions (**A**). Juvenility of the leaves was determined by verifying the complete absence of abaxial trichomes. Error bars represent SD. n=20. Leaf morphology of adult leaves of *tps1-2 GVG:TPS1* and *35S:MIM156 tps1-2 GVG:TPS1* are depicted in **B** and **C** respectively. Numbers represent leaf positions. Leaves were detached from the plants soon after bolting.

4.7 Effects of T6P signaling on vegetative phase transition is largely independent of FLC

FLC, a floral repressor is also known to regulate vegetative phase transition in Arabidopsis (Willmann and Poethig, 2011). To investigate the role of FLC in regulating T6P / TPS1 mediated vegetative phase transition, an early flowering deletion mutant of *FLC* (*flc-3*) was introduced into *tps1* mutant background by crossing. *flc-3* plants produced a similar number of juvenile leaves under LD, when compared to WT (Figure 23) consistent with the previous finding (Deng et al., 2011). In contrast, the double homozygous plants (*flc-3 tps1-2 GVG:TPS1*) produced significantly fewer juvenile leaves (JLN – 8.2) than *tps1-2 GVG:TPS1* (JLN – 9.75) (Figure 23). This implies that FLC does contribute to the delay in vegetative phase

change observed in *tps1-2 GVG:TPS1* plants only to some extent, as the introduction of *flc* mutation caused the decrease of only 1.3 juvenile leaves (Figure 23). This suggests that the T6P / TPS1 pathway regulates vegetative phase change largely independent of FLC.

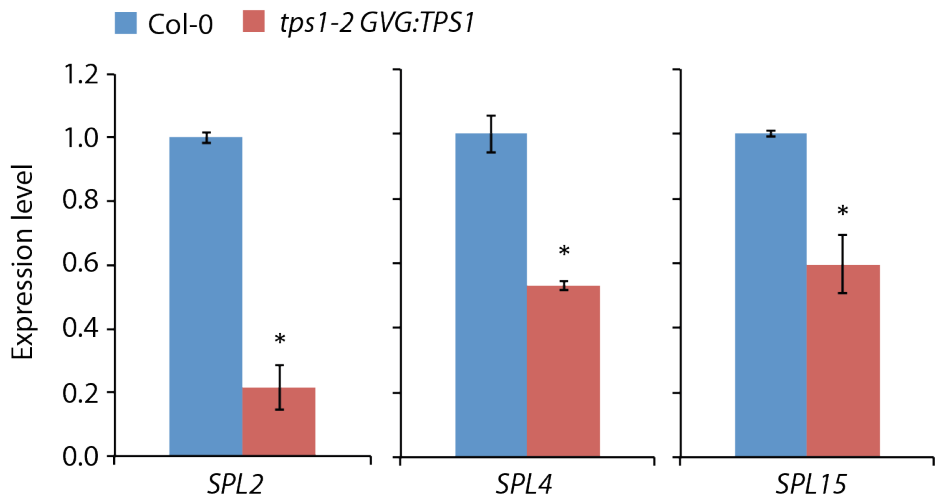

Figure 22: Expression of *SPL2*, *SPL4* and *SPL15* in 10-day old *tps1-2 GVG:TPS1* leaves compared to WT under SD.

Leaves of SD-grown plants were harvested at 10 DAS and expression of SPLs were measured by qRT-PCR on two biological replicates with three technical repetitions each and normalized to Col-0 using *TUB2* as control. * represents significant difference from WT, Student t-test, $p<0.05$. Error bars indicate SD.

4.8 Exogenous sucrose supplementation has negligible influence on juvenility in Arabidopsis

Recently it has been shown that miR156 in Arabidopsis is repressed by sugars such as sucrose and glucose produced via photosynthesis (Yang et al., 2013; Yu et al., 2013) in an age-dependent manner. This sugar-mediated repression of *MIR156a* and *MIR156c* was suggested to promote the vegetative phase change (Yang et al., 2013; Yu et al., 2013). At the seedling stage, plants accumulate high levels of mature miR156, which ensure that the downstream targets such as *SPLs* remain repressed during the juvenile phase. As the plants age, more and more sugars are produced in the source leaves and exported to sink tissues. These sugars in turn repress miR156 and indirectly enhance the expression of *SPLs*, triggering the vegetative phase

transition. Thus sugar-mediated repression of miR156 might be a part of age-sensing mechanism in plants (Yang et al., 2013; Yu et al., 2013).

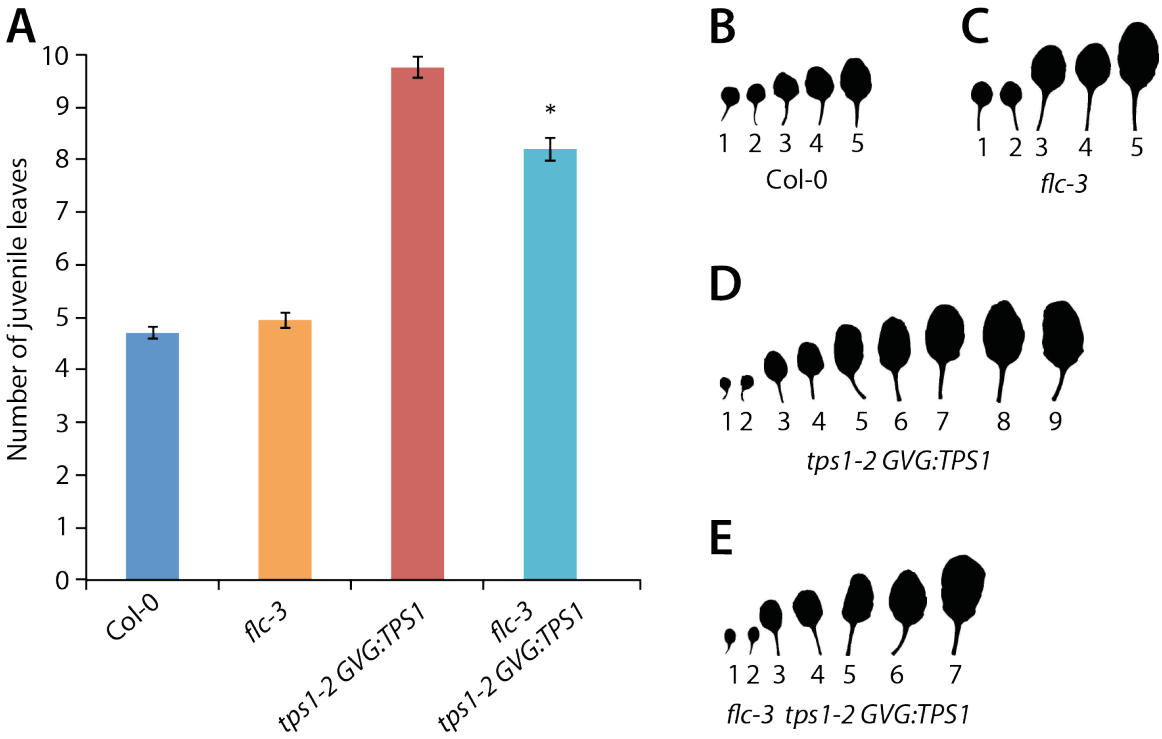

Figure 23: Vegetative phase change in *flc-3 tps1-2 GVG:TPS1* plants under LD.

Number of juvenile leaves produced in *flc-3 tps1-2 GVG:TPS1* compared to WT, *flc-3* and *tps1-2 GVG:TPS1* plants grown under LD conditions (**A**). Juvenility of the leaves was determined by verifying the complete absence of abaxial trichomes. Error bars represent SD. n=20. Leaf morphology of juvenile leaves of Col-0, *flc-3*, *tps1-2 GVG:TPS1* and *flc-3 tps1-2 GVG:TPS1* are depicted in **B**, **C**, **D** and **E** respectively. Numbers represent leaf positions. Leaves were detached from the plants soon after the emergence of first two adult leaves. * represents significant difference from *tps1-2 GVG:TPS1*, Student t-test, $p<0.05$.

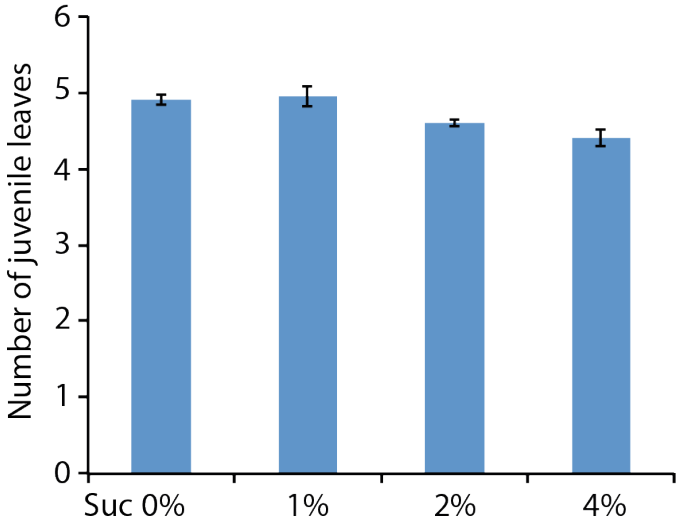

Figure 24: Effect of exogenous sucrose on vegetative phase change in LD-grown Col-0 plants.

Col-0 plants were grown in half MS plates supplemented with 0%, 1%, 2% or 4% sucrose and the number of juvenile leaves was determined after the plants had produced at least two adult leaves. Error bars represent SD. n=20.

A previous study showed that sugars affect the adult vegetative phase rather than the juvenile phase (Ohto et al., 2001). Growing *glabra1* (*gl1*) mutant (which has trichomes present only in the late adult leaves) (Larkin et al., 1994) in 2% or 5% sucrose induced the production of more number of adult leaves with trichomes. High levels of sugar (5%) delayed flowering in Arabidopsis, whereas 1% sucrose has induced a slight delay in floral transition (Roldan et al., 1999; Ohto et al., 2001). However, King and Bagnall (Rod and David, 1996) reported that the addition of sucrose in the growth medium (0.5 to 2%) significantly reduced the number of days to flower in L*er*. The reasons for the discrepancy between these results remain unclear. In this context, it is interesting to see if exogenous sucrose supplementation could accelerate vegetative phase transition presumably by the repression of *MIR156* genes. To investigate the effect of exogenous sucrose on vegetative phase change, Col-0 plants were grown on half Murashige and Skoog (MS) plates supplemented with 1%, 2% and 4% sucrose under LD conditions and allowed to grow until vegetative phase change had occurred (Figure 24).

Interestingly exogenous supplementation of even 4% sucrose did not induce any significant acceleration of vegetative phase change in Col-0 plants. This implies that either exogenous sucrose plays no or negligible role in vegetative phase transition in Arabidopsis or the plants compensate the effects of exogenous sucrose supplementation by reducing the photosynthesis. However, high amount of sucrose supplementation (such as 4%) made the plants produce more dark green and

smaller leaves than the ones which were grown on lower or no sucrose media (data not shown). This indicates that exogenous sugar supplementation can have more complex effects than previously thought. If the plants compensate the exogenous sugar by reducing the photosynthesis, the amount of sucrose in the plants in all the treatments (0 to 4% sucrose) should be similar. Analysis of the internal sugar concentrations in the plants grown with different amounts of exogenous sucrose might give us a better picture.

4.9 Discussion

Vegetative phase change in plants is an important event, which provides the necessary maturity to the shoots to produce adult leaves and subsequently flowers (Wu and Poethig, 2006). Until this stage Arabidopsis plants produce only juvenile leaves, which are characterized by long petioles, round blades, smooth margins and lack of abaxial trichomes. After the vegetative phase transition, plants produce adult leaves, which have a shorter petiole, serrated margins and elongated blades with abaxial trichomes (Telfer et al., 1997).

Both intrinsic and environmental factors affect the process of vegetative phase transition in plants (Willmann and Poethig, 2011). Important regulatory systems such as the biogenesis and accumulation of miRNAs play a significant role in vegetative phase change. Recent research on Arabidopsis has unraveled the roles of miR156 and miR172 in juvenile-to-adult phase transition in plants (Huijser and Schmid, 2011). In order to prevent precocious flowering, plants during the early stages maintain significantly higher levels of miR156, which keeps its downstream targets in a repressed state (Wang et al., 2009). As plants age, the levels of miR156 get reduced, resulting in the upregulation of *SPLs*. Activation of many of the miR156-targeted *SPLs* such as *SPL3*, *SPL4*, *SPL5*, *SPL9* and *SPL15* are known to promote adult phase in Arabidopsis (Wu and Poethig, 2006; Gandikota et al., 2007; Schwarz et al., 2008; Wang et al., 2009; Wang et al., 2008; Wu et al., 2009). Most recently, metabolic sugars such as sucrose and glucose have been proposed to be a part of age-dependent signal that causes the reduction of miR156 levels in plants (Yang et al., 2013; Yu et al., 2013). Sucrose and glucose produced in the pre-existing leaves were shown to repress expression of the miRNA genes, *MIR156a* and *MIR156c* and thus

might promote vegetative phase change in Arabidopsis (Yang et al., 2013; Yu et al., 2013).

T6P, an intermediate of the trehalose biosynthesis pathway, has recently been suggested to serve as a signal that conveys the information on sucrose level to other signaling pathways in Arabidopsis (Lunn et al., 2006; Yadav et al., 2014). *tps1-2 GVG:TPS1* mutants, which are defective in T6P / TPS1 signaling, exhibit a prolonged juvenile phase when compared to WT (Figure 18). Interestingly, an artificial *TPS1* micro-RNA line (*35S:amiR-TPS1*), which also showed a delay in vegetative phase change (pers. communication Vanessa Wahl, unpublished), maintained elevated levels of sucrose, despite having significantly low amounts of T6P (Wahl et al., 2013). This implies that sucrose might not be acting as a direct signal to regulate vegetative phase transition in Arabidopsis. Instead T6P could be acting downstream of sucrose and influencing the vegetative phase transition. In agreement with this hypothesis, DEX-inducible expression of *TPS1* in *tps1-2 GVG:TPS1* mutants almost completely rescued their late vegetative phase change phenotypes, clearly demonstrating that T6P / TPS1 pathway plays a significant role in regulating the juvenile-to-adult phase transition (Figure 19).

Recently we have shown that the T6P / TPS1 pathway modulates levels of miR156 and its targets *SPL3*, *SPL4* and *SPL5* to integrate the carbohydrate status with other signals during floral transition (Wahl et al., 2013). *tps1-2 GVG:TPS1* plants maintain higher levels of miR156 at the SAM, which is at least in part responsible for the late flowering phenotype in these mutants. qRT-PCR analyses showed that these plants also have higher levels of miR156 in leaves (Figure 20) especially in young seedlings when vegetative phase transition normally occurs in WT. Subsequently *SPL2*, *SPL4* and *SPL15* were shown to be insufficiently expressed in the leaves of SD-grown *tps1-2 GVG:TPS1* plants (Figure 22), suggesting that the T6P / TPS1 pathway interacts with miR156-SPL module to trigger the transition towards adult phase in Arabidopsis.

Introduction of a mimicry construct against miR156 (*35S:MIM156*) in *tps1* mutant background (*35S:MIM156 tps1-2 GVG:TPS1*) completely suppressed the production

of juvenile leaves (Figure 21) indicating that the T6P / TPS1 signaling pathway acts largely through miR156 in promoting the adult phase. On the other hand, analysis of *flc-3 tps1-2 GVG:TPS1* double mutant plants, which have an intermediate juvenile phase compared to the single mutants (Figure 23), indicates that *FLC* does play only a minor role in regulating T6P / TPS1-mediated vegetative phase. Based on our experiments we propose a model (Figure 25) in which T6P acts downstream to sucrose and conveys the carbohydrate signal through miR156 and indirectly induces the accumulation of *SPL* genes to promote the vegetative phase transition in Arabidopsis. As the late vegetative phase change phenotype of *tps1-2 GVG:TPS1* is pronounced even under LD conditions, analysis of miR156 and its targets in leaves of LD-grown plants will be much more informative. Since it is known that *SPL9* and *SPL15* play active roles in promoting vegetative phase change (Schwarz et al., 2008) and *SPL15* is insufficiently expressed in *tps1-2 GVG:TPS1* (Figure 22), it would also be worthwhile to investigate their interaction with T6P / TPS1 signaling through the analysis of a *tps1-2 spl9 spl15* triple mutant.

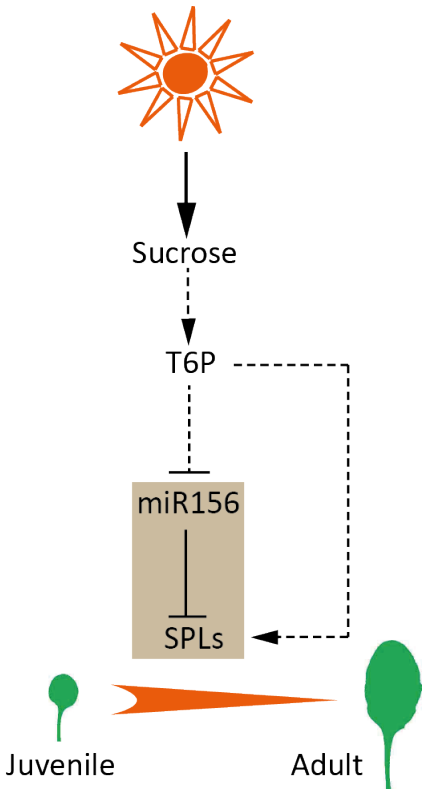

Figure 25: A model depicting the role of T6P / TPS1 pathway in regulating vegetative phase change in Arabidopsis.

Solid line indicates direct interactions and dashed lines indicate indirect interactions. Sucrose-mediated reduction of miR156 levels is achieved through T6P, which indirectly activates *SPLs* and triggers vegetative phase transition.

Even though sugars have been shown to suppress the expression of *MIR156* genes (Yang et al., 2013; Yu et al., 2013), it has not been conclusively proven that sugar

mediated repression of miR156 is sufficient for the vegetative phase transition to occur. To investigate the role of sucrose in promoting adult phase, Col-0 plants were grown on different concentration of sucrose (0%, 1%, 2% and 4%) and juvenile leaves were counted after the emergence of first two adult leaves (Figure 24). However any significant acceleration in vegetative phase transition could be observed even with 4% exogenous sucrose (Figure 24). This confirms a previous research (Ohto et al., 2001), which suggests that exogenous sucrose delays the late vegetative phase and have no noticeable influence on early vegetative phase. However the contradicting data available on the effects of exogenous sucrose concentration on developmental transitions in Arabidopsis demand further in-depth study. While the observations of King and Bagnall (Rod and David, 1996) suggest that exogenous sucrose (0.5 to 2%) supplementation has an accelerating effect on Arabidopsis development and floral transition, independent studies from two other groups show that exogenous sucrose has an acceleratory effect on adult vegetative phase, but an inhibitory effect on flowering (Ohto et al., 2001; Roldan et al., 1999).

Moreover, there is not much known regarding the effects of exogenous sucrose supplementation on plant metabolism and vital processes like photosynthesis. It is also possible that the plants compensate the exogenous sucrose supplementation with reducing or altering the photosynthesis. In that case, providing exogenous sucrose might not result in higher sucrose levels in the plants. Besides, since sugars were shown to repress miR156 (Yang et al., 2013; Yu et al., 2013), it would be interesting to see if exogenous sugars could repress the *MIR156* genes or mature miRNA in WT plants. Expression analysis of miR156 and miR156-targeted *SPLs* coupled with T6P and sucrose measurements in exogenous sucrose-supplemented plants might give a better understanding of the scenario.

HXK1 is a metabolic sensor that, in addition to its enzymatic function, has been proposed to transduce sugar signals and to have regulatory functions (Cho et al., 2006). However it is unclear whether HXK1 plays an active role in sucrose-mediated repression of miR156 (Yang et al., 2013; Yu et al., 2013). Indeed it has been suggested that *TPS1*, which is largely responsible for the production of T6P, acts

downstream to *HXK1* (Avonce et al., 2004), suggesting that *HXK1* might act through *TPS1*.

On the other hand, T6P has been shown to inhibit the protein kinase SnRK1, in actively growing plant tissues (Zhang et al., 2009; Debast et al., 2011; Delatte et al., 2011; Nunes et al., 2013b). SnRK1 performs central regulatory functions in the plant cells in response to the endogenous energy status (Baena-Gonzalez et al., 2007). Recently, a model has been proposed in which T6P-SnRK1 interaction regulates the active growth processes during energy-scarce situations (Ma et al., 2011). During a carbon stress or scarcity of sucrose (for example induced by prolonged darkness), SnRK1 inhibits the genes involved in the active growth processes in plants and induces those genes involved in survival response against stress (Baena-Gonzalez et al., 2007). After the plants are relieved from the stress, the plentiful supply of sucrose produced through photosynthesis results in the synthesis or mobilization of T6P. The T6P then inhibits the activity of SnRK1, which results in blocking the activity of genes that are involved in stress response and inducing the genes that promote active growth (Ma et al., 2011).

Interestingly, SnRK1 has been proposed to be involved in vegetative phase transition in Arabidopsis as well since *AKIN10* overexpressing plants display a prolonged vegetative phase (Tsai and Gazzarrini, 2012b). Surprisingly, the interaction between AKIN10 and the seed maturation master regulator FUS3 seems to be important for this effect (Tsai and Gazzarrini, 2012a). Given these results it would be interesting to analyze the role of SnRK1 / AKIN10 in T6P / TPS1-mediated regulation of vegetative phase change in Arabidopsis.

5 Chapter 3

A suppressor screen in *tps1-2 GVG:TPS1* identifies novel targets of T6P / TPS1 signaling that regulate developmental phase transitions in *Arabidopsis thaliana*

Contributions to this chapter:

All experiments and their analyses described in this chapter have been carried out by myself, if not mentioned otherwise.

With Tobias Langenecker and Jörg Hagmann: Fast isogenic mapping of the EMS-induced putative mutant *160-1* (Figure 32 and Tables 3 and 4).

5.1 Abstract

In Arabidopsis, the trehalose-6-phospate (T6P) / TREHALOSE-6-PHOSPHATE SYNTHASE1 (TPS1) pathway is integral in coordinating metabolism with development. Our previous studies demonstrate that T6P / TPS1 pathway is essential for the proper timing of vegetative and reproductive phase transitions. In order to identify additional signaling components involved in T6P / TPS1-mediated control of plant development, an EMS-based suppressor screen was performed in homozygous *tps1* mutant (*tps1-2 GVG:TPS1*). More than 100 EMS-induced putative mutant plants, in which the late flowering phenotype of *tps1-2 GVG:TPS1* was suppressed, were obtained from the screening of 300 M2 pools under long day (LD) conditions. Several of these plants also showed a shortened juvenile phase in comparison with the non-mutagenized *tps1-2 GVG:TPS1* plants. After grouping and allelism tests, 15 putative non-allelic mutant complementation groups were obtained and mapping populations were generated by backcrossing them to the parental *tps1-2 GVG:TPS1* line. Mapping was performed for one of the suppressors, *160-1*, using the next-generation sequencing-based fast isogenic method. Non-synonymous EMS-type mutations were identified in 9 genes at the top of chromosome 3, including *KIN10*, a known stress and sugar-signaling factor.

5.2 Suppressor screen in *tps1-2 GVG:TPS1* through EMS mutagenesis

The results described earlier (Chapter 1 and 2) demonstrate that T6P / TPS1 signaling interacts with the photoperiod as well as the age pathway of flowering. In order to identify additional genes that participate in the T6P / TPS1 signaling in regulating flowering time and vegetative phase change, *tps1-2 GVG:TPS1* seeds were mutagenized using EMS (ethyl methanesulfonate) to identify individuals in which flowering was restored in the absence of DEX treatment (Figure 27). EMS mutagenesis is a powerful forward genetics tool, which can be employed to find new genotypes responsible for given phenotypes (Page and Grossniklaus, 2002). After EMS treatment, approximately 12,500 M1 plants were grown under LD. These plants were regularly sprayed with DEX to induce flowering and subsequent production of viable seeds. M2 seeds were collected as 300 pools of approximately 40-50 M1 plants each. Approximately 500 M2 plants were grown from each of the 300 M2 pools under LD and screened for suppressor mutants without spraying DEX (Figure 26).

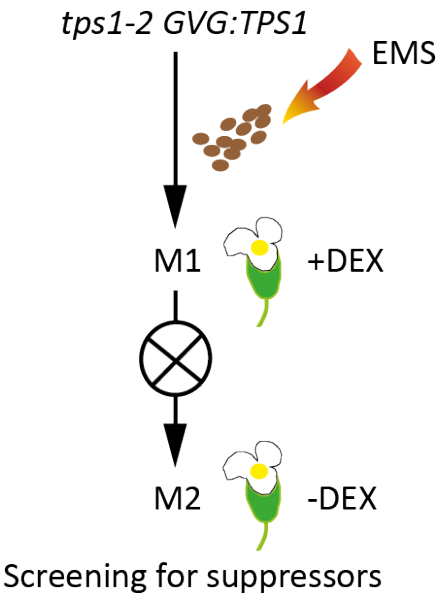

Figure 26: Suppressor screen in *tps1-2 GVG:TPS1.*

Seeds of *tps1-2 GVG:TPS1* (M0) were treated with EMS (M1). Approximately 12,500 M1 plants were grown under LD and sprayed with DEX to induce flowering and seed production. M2 seeds were collected as 300 pools with each pool containing seeds from about 40-50 M1 plants. Approximately 500 M2 plants from each pool were sown under LD 23°C and screened for suppressor mutants in which flowering was restored without spraying DEX.

5.2.1 A number of putative EMS-induced suppressors rescue the late flowering phenotype of *tps1-2 GVG:TPS1*

Screening of M2 seeds under LD 23°C without DEX spraying yielded 127 putative mutants, which suppressed the late flowering phenotype of *tps1-2 GVG:TPS1.* However despite rescuing the late flowering phenotype, 21 of these plants did not produce any viable seeds. The remaining 106 plants suppressed both the late flowering and sterility of *tps1-2 GVG:TPS1* (Figure 27). Out of these, 50 lines were selected for further analysis from those plants that showed a uniform phenotype in the M3 generation.

5.3 Genotyping confirms the transposon insertion at the *TPS1* locus in the putative suppressor plants

In order to rule out the possibility of contaminations in seed stock or accidental outcrossing, the 50 selected putative suppressors were grown under LD and genotyped for the presence of homozygous *tps1-2* mutant allele. Genotyping PCR was performed using two gene specific primers (G-22756 and G-22758, see Table S2) and a *dSpm* primer (G-19968) specific for the transposon insertion in *TPS1* (Figure 29, A). Gene specific primers amplified a 547 bp region of *TPS1* in WT plants, but failed to produce any amplification in *tps1-2 GVG:TPS1* (Figure 28, B). In contrast, PCR analysis with the *dSpm* specific primer and gene specific reverse primer (G-22758) amplified a portion of the transposon insertion and the flanking *TPS1* gene in *tps1-2 GVG:TPS1* plants but failed to produce any amplicon in WT. Plants heterozygous for *tps1-2* showed amplifications with both the sets of primers. In summary, the PCR analyses confirmed the presence of homozygous *tps1-2* transposon insertion in all the 50 selected candidate mutants.

5.4 Initial characterization of *tps1-2 GVG:TPS1* suppressor mutants into complementation groups based on phenotypes and genetic crosses

The 50 selected putative mutants, in which the presence of the transposon insertion at the *TPS1* locus had been confirmed by PCR-based genotyping, were grown under LD 23°C and grouped according to their additional phenotypes. Distributing the putative suppressors into phenotypic classes was necessary, because allelism test between all the 50 individual candidates were simply not possible. The criteria

chosen were a similar flowering time and the general appearance of the suppressor mutant in comparison with WT and *tps1-2 GVG:TPS1*. The phenotypic dissimilarities in the general appearance of the mutants could be attributed to the second site mutations. In order to see if the suppressor mutations lie within the same gene, allelism tests were performed first within the members of each group and then between the groups.

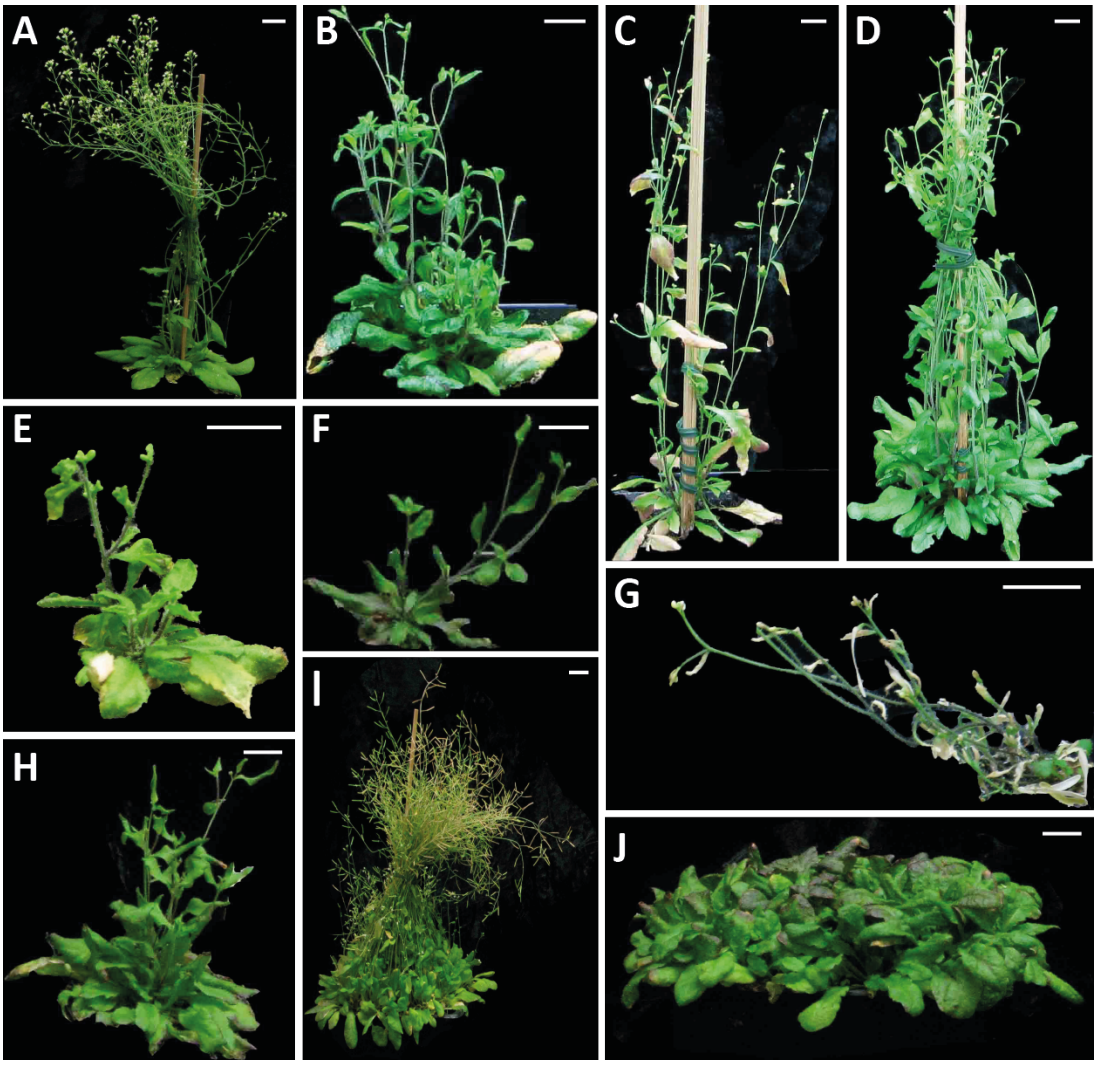

Figure 27: Putative mutant plants that suppress the late flowering phenotype of *tps1-2 GVG:TPS1*.

Phenotypes observed among the EMS-induced mutants of *tps1-2 GVG:TPS1* under LD conditions. **A** to **D**: Examples of suppressor plants which produced normal flowers and siliques. **E** to **H**: Suppressor mutants that rescued the late flowering phenotype of *tps1-2 GVG:TPS1*, but failed to produce any viable seeds. **I** and **J**: Col-0 and *tps1-2 GVG:TPS1* respectively. Scale bar: 1cm.

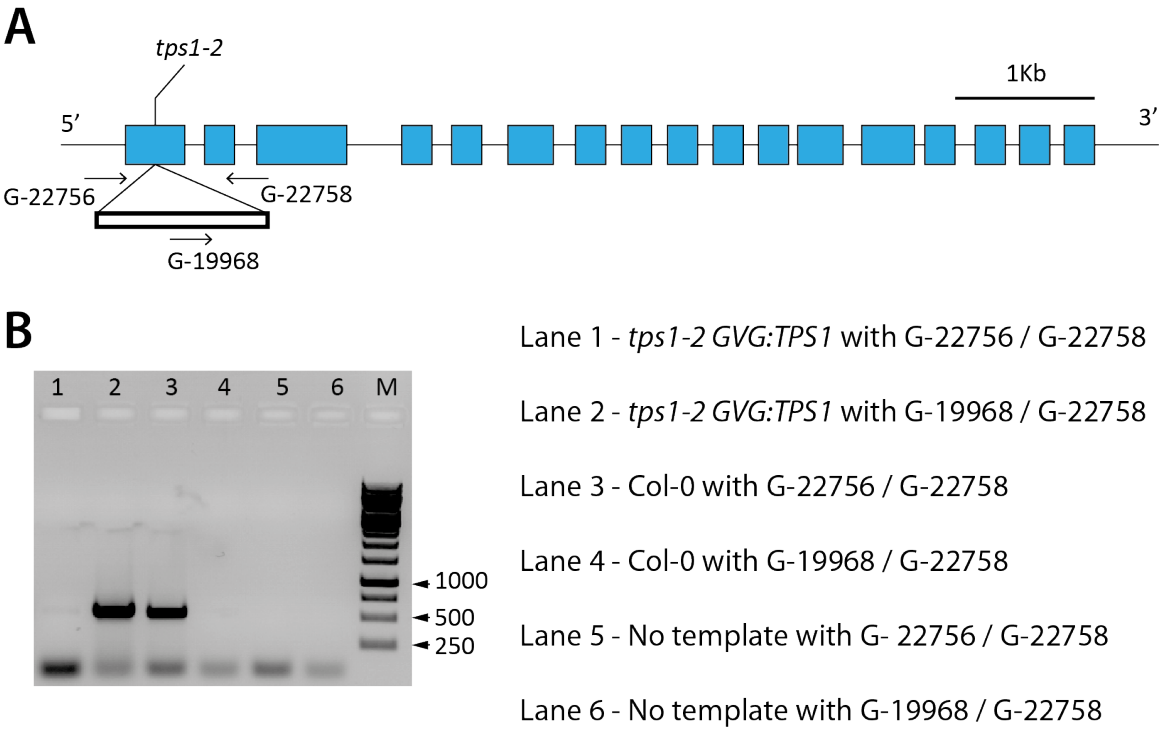

Figure 28: Genotyping of *tps1-2 GVG:TPS1* to ensure the intactness of transposon insertion.

Schematic structure of the *TPS1* gene with the *tps1-2* transposon insertion in the 1st exon (**A**). Boxes and lines represent exons and introns respectively. Primers G-22756 and G-22758 are gene specific and amplify 547 bp in WT plants. Primer G-19968 is specific to *dSpm* insertion and amplifies 550 bp with G-22758 in *tps1-2 GVG:TPS1* plants (**B**).

Allelism test were based on the analysis of the progeny of crosses between the suppressor mutants. If the progeny of a cross between two candidate mutants abolished the suppressor phenotype and exhibited the late flowering nature of *tps1-2 GVG:TPS1*, then the parent mutant plants were grouped as non-allelic. If the progeny showed the early flowering suppressor phenotype, the two candidate mutants were grouped as allelic. Another possibility when all the F1 plants show the early flowering suppressor phenotype is when one of the mutant candidate harbor a dominant mutation. In such cases the F1 plants always showed the suppressor phenotype. Many mutants showed a reduced seed set phenotype and crosses involving these plants were not always successful. In most of the cases spraying DEX before and after crossing was essential for the successful production of F1 seeds. The putative non-allelic candidates were backcrossed with non-mutagenized *tps1-2*

GVG:TPS1 to test for the inheritance nature of the EMS induced mutations (see Table 3). Based on the inter- and intra-group allelism tests, the putative suppressor plants were divided into 15 putative non-allelic complementation groups (Table 2).

Table 2: Genetic characterization of *tps1-2 GVG:TPS1* suppressor mutants.

'Pool' specifies the number of individual pool of M2 seeds (from 40-50 M1 plants), from which the mutant is derived. 'Nr.' denotes the number of the mutant plant isolated from the specific pool.

Complementation group	M2 Identifier		Description	
	Pool	**Nr.**	**Additional phenotype**	***tps1-2****
A	30	19	Bigger leaves, flower phenotype	-/-
	30	23	is like Col-0	-/-
	32	2		-/-
	30	26		-/-
	33	1		-/-
B	41	18	Like *tps1-2 GVG:TPS1*, but flowers normally	-/-
C	55	21	Phenotype is like *tps1-2 GVG:TPS1*, but flowers normally; increased apical dominance and branched inflorescence	-/-
D	25	1	More crinkled and rounded leaves; still very late flowering	-/-
E	88	3	Like *tps1-2 GVG:TPS1,* but	-/-
	79	8	flowers normally	-/-
F	2	1	Small rosette diameter, slight	-/-
	1	3	leaf serrations and normal flowering	-/-
G	271	1	Like *tps1-2 GVG:TPS1*, but much bigger plants; still late flowering	-/-
H	107	2	Looks different from both WT and *tps1-2 GVG:TPS1*	-/-

I	140	2	Intermediate phenotype; in-	-/-
	107	3	between WT and *tps1-2*	-/-
	106	2	*GVG:TPS1*	-/-
	185	1		-/-
J	11	7	Big plants with small siliques	-/-
K	55	12	WT leaves with low apical	-/-
	55	6	dominance while flowering	-/-
L	77	3	Like *tps1-2 GVG:TPS1*, but flowers normally	-/-
M	128	1	Looks different from WT and *tps1-2 GVG:TPS1*	-/-
N	160	1	Crinkled leaves with small siliques	-/-
	161	1		-/-
O	250	5	Looks different from WT and *tps1-2 GVG:TPS1*; unspecified	**-/-**

*Confirmed by genotyping.

5.5 Some *tps1-2 GVG:TPS1* suppressors also rescue the transition from juvenile-to-adult phase

In order to test if the suppressor mutants also rescued the juvenile phase defect of *tps1-2 GVG:TPS1* in addition to the late flowering, plants from each of the complementation groups were grown under LD and the juvenile leaves were counted. Five complementation groups exhibited an early transition to adult phase when compared to *tps1-2 GVG:TPS1* plants under LD (Figure 29).

5.6 EMS mutagenesis did not cause unwanted activation of *GVG:TPS1* in the putative non-allelic suppressor mutant plants

Since the *tps1-2 GVG:TPS1* seeds used for EMS mutagenesis carry a DEX-inducible *TPS1* construct (*GVG:TPS1*), there is a possibility that the suppressor phenotype observed in at least some of the putative mutants was caused by inadvertent activation of *GVG:TPS1*. In order to rule out this possibility, qRT-PCR was performed on the LD-grown mutant rosettes to test for *TPS1* expression (Figure 30). None of

the non-allelic mutants showed an unwanted expression of *TPS1*, confirming that *GVG:TPS1* was not activated in these lines (Figure 30).

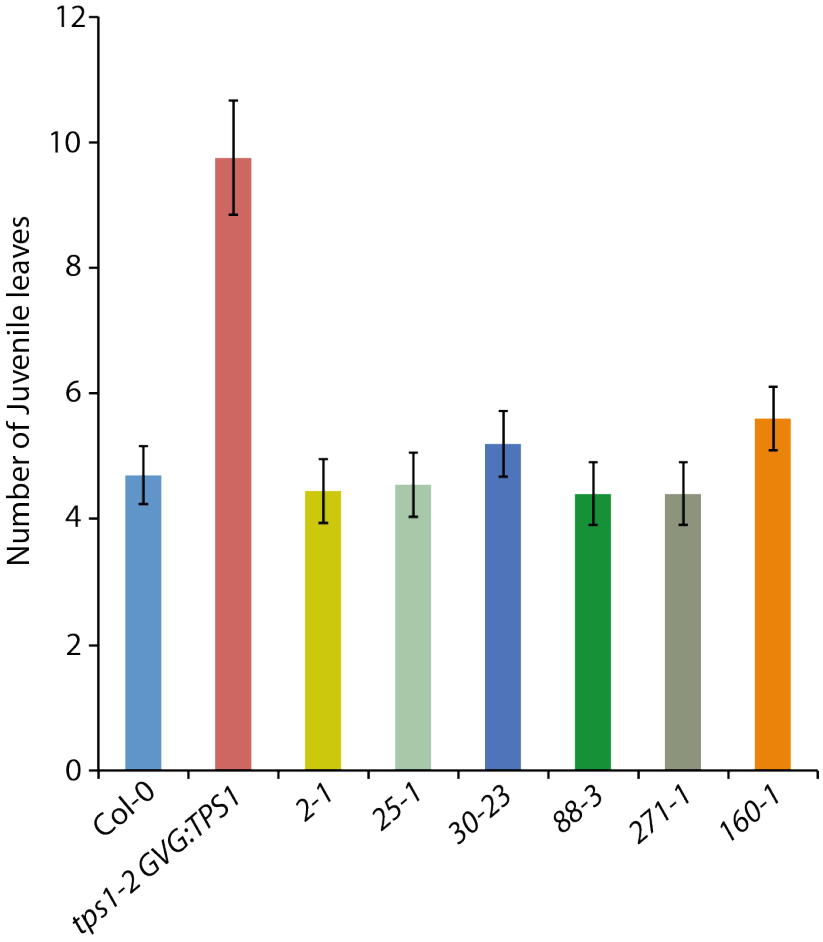

Figure 29: *tps1-2 GVG:*TPS1 suppressor mutants rescue the delay in vegetative phase change.

Plants were grown under LD at 23°C and the number of juvenile leaves was counted after the emergence of first two adult leaves. Error bars represent SD. n=20. Suppressor plants are named according to M2 pool identifier number and the line number.

5.7 Backcrossing identifies the genetic nature of EMS-induced mutations in putative suppressor plants

(Experiments in section 5.7 were performed together with Tobias Langenecker)

In order to identify the genetic nature of EMS-induced mutations in the suppressor candidates, backcrosses were performed between the mutants and *tps1-2 GVG:TPS1* plants. Based on whether the F1 progeny of these backcrosses exhibited late flowering like *tps1-2 GVG:TPS1* or flowered like the suppressor without DEX application, the mutations were grouped as recessive or dominant (Table 3). Out of the 15 putative non-allelic complementation groups, 6 were grouped as dominant and 9 were grouped as recessive based on the backcross analysis (Table 3).

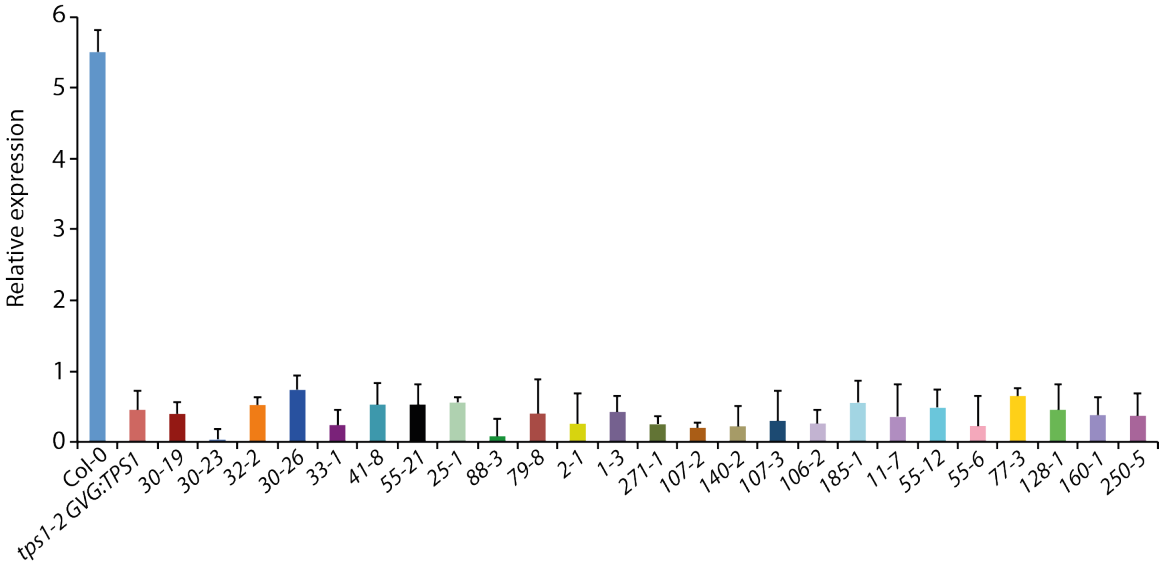

Figure 30: Expression of *TPS1* in EMS-induced suppressors of *tps1-2 GVG:TPS1.*

Expression of *TPS1* in rosettes of 15-day old LD-grown plants. Error bars represent SD. Expression was determined by qRT-PCR using three technical repetitions and was normalized to *TUB2*. Error bars represent SD.

Table 3: Genetic nature of EMS-induced suppressors of *tps1-2 GVG:TPS1*.

M2 Identifier		Description	
Pool	**Nr.**	**Type**	**Complementation group**
30	23	Dominant	A
41	18	Recessive	B
55	21	Recessive	C
25	1	Dominant	D
88	3	Dominant	E
2	1	Dominant	F
271	1	Recessive	G
107	2	Recessive	H
185	1	Recessive	I
11	7	Recessive	J
55	6	Recessive	K
77	3	Dominant	L

128	1	Dominant	M
160	1	Recessive	N
250	5	Recessive	O

5.8 Fast isogenic mapping of the suppressor mutant plants by next-generation sequencing

(Experiments in section 5.8 were performed together with Tobias Langenecker. Jörg Hagmann analyzed the next-generation sequencing mapping data)

Forward genetic screens such as enhancer and suppressor screens are powerful tools to identify new components in a signaling pathway of interest and to characterize their function (Page and Grossniklaus, 2002). Recently, next-generation sequencing has been introduced as a fast and convenient method to identify the causal SNPs (single-nucleotide polymorphism) in EMS induced mutants (Schneeberger et al., 2009). This reduces the labor and time involved in traditional mapping, but still requires crosses with a diverged accession and subsequent selection of plants with the suppressor phenotype in F2 to create suitable mapping populations.

Since *tps1-2 GVG:TPS1* is in the Col-0 accession, according to the above mentioned method crosses have to be done with a different accession such as Landsberg *erecta* (L*er*). Unfortunately there are no *tps1* mutants available outside Col-0 accession. As a consequence, crosses between *tps1-2 GVG:TPS1* (Col-0) and a different accession (i.e. L*er*) would create a line which would segregate not only for the suppressor mutation but also for *tps1-2* and the DEX-inducible *GVG:TPS1* rescue construct. This would make creating a mapping population tedious. However, more recently another method called fast isogenic mapping has been introduced in *Arabidopsis thaliana*, which relies on the sequencing of pooled DNA from a bulk segregant F2 population, resulting from a backcross with the non-mutagenized parent (Hartwig et al., 2012). This method eliminates the need to introgress the *tps1* mutation in any other accessions. A brief illustration showing the different steps involved in fast isogenic mapping of suppressor mutants in *tps1-2 GVG:TPS1* is depicted in Figure 31.

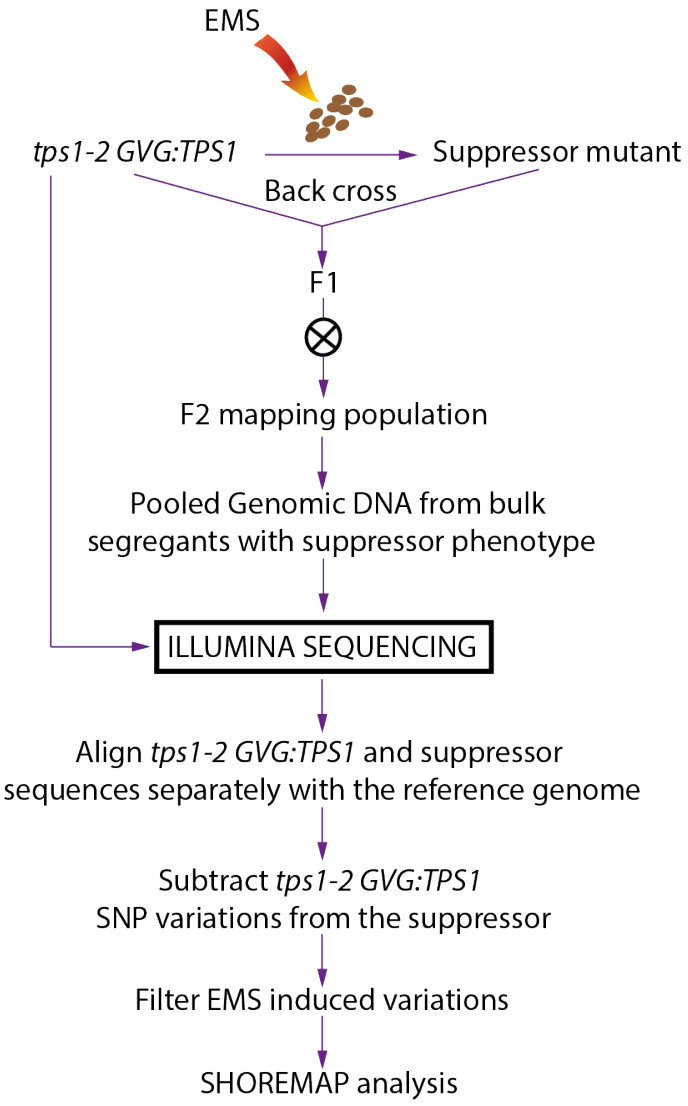

Figure 31: Schematic illustration of fast isogenic mapping in suppressor mutants of *tps1-2 GVG:TPS1*.

Putative recessive non-allelic suppressor mutant plants were backcrossed to the non-mutagenized *tps1-2 GVG:TPS1* parent. In the F2 generation, segregating individuals that displayed the suppressor phenotype were bulked and the pooled genomic DNA extracted was subjected to high-throughput sequencing. All EMS-induced mutations unique to the suppressor plants were selected for SHOREmapping analysis.

5.8.1 Identification of EMS-induced candidate mutations in *160-1* using SHOREmapping

The putative non-allelic suppressors in *tps1-2 GVG:TPS1* were subjected to fast isogenic mapping. Backcrosses were made between suppressors and the non-mutagenized *tps1-2 GVG:TPS1* plants. The F1 plants were treated with DEX to induce the expression of *TPS1* for proper flowering and seed production. The F2 plants were grown under LD conditions and individuals that flowered and set viable seeds were selected from the segregating population.

For the recessive line *160-1*, leaf samples from 180 individual F2 segregant plants showing the suppressor phenotype (BC1F2) were bulked and genomic DNA was extracted. This pooled DNA was then subjected to next-generation genome sequencing (Figure 32). Genomic DNA from non-mutagenized *tps1-2 GVG:TPS1* was sequenced in parallel as a control. Out of about 40 million high quality reads, >95% were aligned to the Col-0 reference sequence and yielded an average genome coverage of approximately 50-fold. The optimum coverage for the mapping-by-sequencing approach from a backcross population as suggested from the simulation experiments is 50-fold (James et al., 2013). Using SHOREmap (Ossowski et al., 2008), SNPs between both the *160-1* line and the parental *tps1-2 GVG:TPS1* to the reference genome (Col-0) were identified separately. SNP analysis was performed in the data obtained from the segregating BC1F2 *160-1* plants to identify all EMS-induced mutations with an allele frequency higher than 25% to distinguish fixed from non-fixed mutations. From these SNP set, all SNPs that were identified in a comparison between the *tps1-2 GVG:TPS1* and Col-0 were subtracted, since these mutations were already present in the non-mutagenized parent. This ensures that only the novel EMS induced changes specific to the selected BC2F2 plants were considered further. This analysis revealed a strong increase in the frequency of EMS-induced SNPs at the top of chromosome 3 in the BC2F2 of *160-1* (Figure 32). Within this region nine SNPs (Table 4) with mutant allele frequency higher than 90% that would all cause non-synonymous amino acid changes could be identified (Table S1).

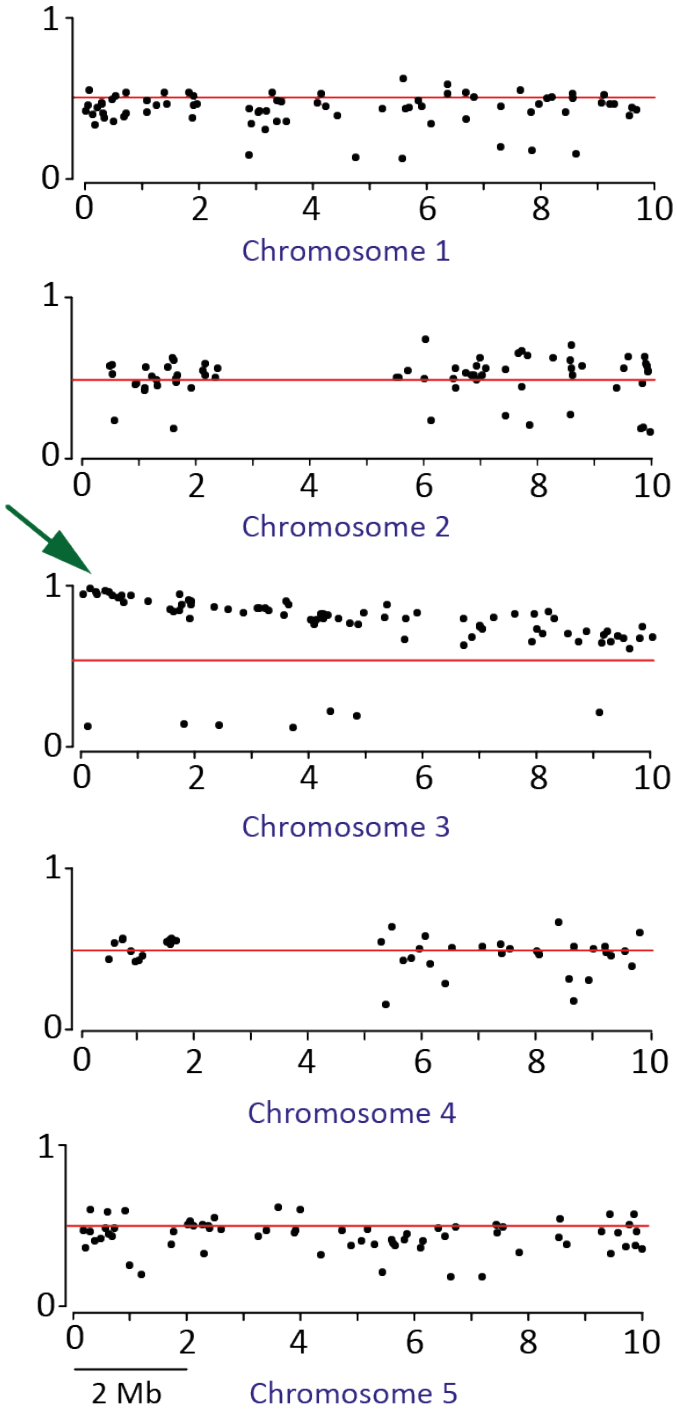

Figure 32: Allele frequency EMS-induced mutations in *160-1*.

Allele frequency estimations of EMS-induced mutations in the 160-1 mapping population across all five chromosomes. 10Mb region from the beginning of each chromosome is shown. Allele frequencies were estimated as fractions of reads supporting the mutant allele divided by the number of all reads aligning to a given SNP. Only the base calls with a quality score of more than 25 were considered to reduce the sequencing errors. Green arrow indicates the allele frequency distortion at the beginning of chromosome 3 in comparison with the remaining regions of the genome.

Table 4: Linked potential candidate genes for *160-1* suppressor phenotype.

Nr.	Read percentage supporting the alternate allele	ATG Number	Name
1	0.939759	AT3G01090	AKIN10, KIN10, SNF1 KINASE HOMOLOG 10, SNF1-RELATED PROTEIN KINASE 1.1, SNRK1.1
2	0.948718	AT3G01720	Unknown protein
3	0.948718	AT3G01770	ATBET10, BET10, BROMODOMAIN AND EXTRATERMINAL DOMAIN PROTEIN 10
4	0.927273	AT3G02930	Unknown protein
5	0.942308	AT3G03120	ADP-RIBOSYLATION FACTOR B1C, ARFB1C, ATARFB1C
6	0.897436	AT3G03220	ATEXP13, ATEXPA13, ATHEXP ALPHA 1.22, EXP13, EXPA13, EXPANSIN 13, EXPANSIN A13
7	0.931035	AT3G03630	CS26, CYSTEINE SYNTHASE 26
8	0.916667	AT3G06230	ATMKK8, MAP KINASE KINASE 8, MKK8
9	0.896104	AT3G06380	ATTLP9, TLP9, TUBBY-LIKE PROTEIN 9

Mutations in any of the above genes could potentially cause the suppressor phenotype of *160-1*. All of the SNPs were non-synonymous and occurred at the coding regions. For the type of mutation and predicted amino acid change, see Table S1.

5.9 Discussion

The T6P / TPS1 pathway plays an essential role in determining the timing of developmental transitions in Arabidopsis. In the leaves of juvenile plants, T6P / TPS1 signaling is involved in the age-dependent reduction of miR156 levels and subsequent upregulation of *SPL* genes, which are necessary for vegetative phase transition (Chapter 2). In addition, the T6P / TPS1 pathway is absolutely required

for the induction of *FT* in leaves. At the SAM, T6P / TPS1 integrates the energy or metabolic signals with the various flower promoting pathways by interacting with miR156-SPL node (Chapter 1).

In order to identify additional genes that participate in T6P / TPS1 signaling in regulating flowering time and juvenile-to-adult phase transition, an EMS suppressor screen was carried out in *tps1-2 GVG:TPS1* (Figure 26). A number of putative mutant candidates which rescued the late flowering and prolonged juvenile phase phenotype of *tps1-2 GVG:TPS1* were obtained from the screen (Figure 27 and 29). In total, more than 100 candidate mutants were obtained from 300 M2 pools. Allelism tests among the initial 50 mutants identified 15 non-allelic complementation groups (Table 2). These lines did not show enhanced *TPS1* expression when compared to the non-mutagenized *tps1-2 GVG:TPS1*, ruling out the chances of unwanted activation of *GVG:TPS1* (Figure 30). The genetic nature of EMS-induced mutations in the putative mutant plants were analyzed by backcrossing with non-mutagenized *tps1-2 GVG:TPS1* and out of 15 complementation groups tested, 9 were found to be recessive (Table 3). Crosses involving *tps1-2 GVG:TPS1* sometimes result in self pollination or no pollination at all due the problems associated with the pollen grains. There is a possibility that this would distort the genetic characterization of mutant candidates into complementation groups.

EMS-based suppressor or enhancer screens were instrumental in revealing the functions of many previously unknown genes and signaling components in the past (Reviewed in Page and Grossniklaus, 2002). Even though the mutagenesis itself is simple to perform, further downstream analyses like mapping the EMS-induced mutations can be cumbersome and time taking. Fortunately, recent advances in sequencing technologies and data analysis makes identification of EMS-induced mutations much easier and faster than the traditional methods (Ossowski et al., 2008). Sequencing-based mapping methods developed in the past years (Austin et al., 2011; Cuperus et al., 2010; Ossowski et al., 2008; Schneeberger et al., 2009) do not differ from the traditional mapping technologies until after the selection of phenotypes in the segregating F2 populations. This means that for these methods it is still necessary to cross the putative mutants with plants belonging to a diverged

accession in order to create the mapping population. In the EMS-screens using the WT plants, this might not pose a problem since many different accessions are available in Arabidopsis. However, problems might arise if the screen was performed in a mutant, and there are no mutations available outside that specific accession for that particular gene. In this case introgression of the initial mutation into the diverged accession through 7 to 8 backcrosses is the only practical, but time consuming, way to create the parent plant required for crossing. Even then, mapping an EMS-induced mutation that is close to the original mutation (here: *tps1-2*) can be problematic, since the two mutations will be genetically linked and only few recombination events will be recovered.

Recently a fast isogenic mapping method was described (Hartwig et al., 2012) that involves creation of mapping population by performing backcrosses between the EMS-induced mutants and the non-mutagenized parent. This method was suited to map the putative candidates of *tps1-2 GVG:TPS1*, as there are no known mutant plants for *TPS1* outside the Col-0 accession. Employing fast isogenic mapping to the *160-1*, a recessive EMS-induced putative mutant of *tps1-2 GVG:TPS1,* yielded 9 potential causal non-synonymous SNPs (Table 4). Since T6P is known to repress SnRK1 (Zhang et al., 2009), an obvious one among the 9 candidate genes to cause the suppressor phenotype in *160-1* is *AKIN10*, (Table 4), which is a catalytic subunit of SnRK1 protein kinase (Baena-Gonzalez et al., 2007). In Arabidopsis, 3 genes - SnRK1.1 or AKIN10, SnRK1.2 or AKIN11 and SnRK1.3 (Polge and Thomas, 2007) - encode SnRK1. It is a metabolic sensor, which trigger various metabolic and transcriptional changes to restore homoeostasis especially when the plants experience different kinds of stresses (Baena-Gonzalez et al., 2007). SnRK1.1 or AKIN10 overexpression (*35S:AKIN10-HA*) causes a delay in floral transition, (Tsai and Gazzarrini, 2012a), while *akin10* T-DNA insertion RNA-null mutant (SALK_127939) shows a flowering time phenotype similar to Col-0 (data not shown). It is possible that the suppression of active growth processes by SnRK1 overexpression also results in delayed phase transitions in *35S:AKIN10-HA* plants. In addition to causing a delay in flowering, *AKIN10* overexpressing plants also showed a prolonged juvenile (Tsai and Gazzarrini, 2012a) phase. Interestingly, the EMS-induced mutant *160-1* suppresses the delayed vegetative transition in *tps1-2*

GVG:TPS1 (Figure 29). If the causal mutation in *160-1* turns out to be in *SnRK1.1* or *AKIN10*, it is possible that the delay in phase transitions observed in *tps1-2 GVG:TPS1* is at least partially due to the active SnRK1 complex in these plants, as T6P is required to suppress SnRK1 in normal growth conditions.

Another probable candidate for *160-1* is *BROMODOMAIN AND EXTRATERMINAL DOMAIN PROTEIN 10* (*AtBET10*) (Table 4). This protein belongs to a bromodomain containing transcriptional regulator family (fsh/Ring3 class) and proposed to have a role in chromatin remodeling (Florence and Faller, 2001). AtBET10 protein has been shown to interact with a novel class of Ca^{2+} / Calmodulin proteins, whose expression and confirmation are altered by signal molecules like Ca^{2+} and H_2O_2 (Du and Poovaiah, 2004). Preliminary mapping of two other suppressor mutants in *tps1-2 GVG:TPS1* also has chromatin remodeling factors as candidate genes (data not shown). This would indicate that T6P / TPS1 signaling can have possible roles in the chromatin remodeling. In that case it would be interesting to study how the mutation in *TPS1* affect the chromatin landscape in Arabidopsis.

Various strategies can be employed to find the EMS-induced causal mutations in the *tps1-2 GVG:TPS1* suppressor plants. Deep sequencing of the amplified region around the putative mutations (dCARE) could potentially reduce the number of putative candidate SNPs (Hartwig et al., 2012). Recapitulating the suppressor mutant phenotype in *tps1-2 GVG:TPS1* by crossing with T-DNA insertion mutant lines for all the putative candidate genes on *tps1-2 GVG:TPS1* plants will also confirm the actual causal gene for the suppressor phenotype. Then complementation with the genomic rescue constructs could be utilized for further proof. Alternatively, overexpressing the artificial micro-RNAs (amiRs) in *tps1-2 GVG:TPS1* plants against the genes in which the putative candidate SNPs were located, and observing if the resulting plants recapitulate the suppressor phenotype can be a strategy. Sequencing of the additional alleles available from the complementation group and looking for the common SNPs among the allelic lines will also help to narrow down the EMS-induced putative suppressor mutations.

T6P / TPS1 is a signaling molecule which plays an integral role in diverse developmental processes in plants (Ponnu et al., 2011). Signaling steps and components involved in T6P / TPS1 pathway in regulating the developmental processes in plants is still obscure. In this context, further studies on the putative mutants obtained through the suppressor screen in *tps1-2 GVG:TPS1* hold much significance.

6 Materials and methods

6.1 Plant growth conditions

Columbia (Col-0) accession was used in all the plant work mentioned in this thesis. Initially the line *tps1-2 GVG:TPS1* was referred to as *ind-TPS1* #201 (van Dijken et al., 2004). Mutant plants and transgenic lines such as *ft-10*, *35S:FT*, *SUC2:FT* and *35:MIM156* were described elsewhere (Supplementary text). The genotypes were confirmed by PCR (Table S2).

The growth chambers used for growing plants were maintained at a temperature of 23°C and a relative humidity of 65%. White and Gro-Lux Wide Spectrum fluorescent light bulbs with a fluence rate of 125 to 175 μmol m^{-2} s^{-1} (in plant growth area) or F17T8/TL741 bulbs (Philips Electronics, Eindhoven, Netherlands) were used (in Percival Scientific, Perry, IA, USA) in these chambers. Long day (LD) and short day (SD) are described as 16h light / 8h dark and 8h light / 16h dark respectively.

6.2 Flowering time measurements

Seeds were stratified in 4°C with 0.1% Agarose (Roth, Germany) for 3 days and then sown on soil. To measure the flowering time, rosette and cauline leaves were counted after the plants were bolted when the inflorescence reached at least 2 cm height. The flowering time data were expressed as the number of leaves (rosette and cauline) at the time of flowering. Errors were represented as standard deviation of the mean.

6.3 Measurement of juvenile phase

Plants were grown as described above. The juvenility of a leaf is determined by the presence or absence of abaxial (lower side of the leaf) trichomes (Telfer et al., 1997). Presence of even one abaxial trichome qualifies a leaf to be considered as in adult stage. Leaves without abaxial trichomes were counted as juvenile leaves. The juvenile leaves were counted after at least two adult leaves were produced,

especially in the late flowering mutants like *tps1-2 GVG:TPS1*. The juvenility of the plants was expressed as the number of juvenile leaves produced. Errors were represented as standard deviation of the mean.

6.4 Microarray analysis

Apical meristems of 20-day old Col-0 and *tps1-2 GVG:TPS1* plants grown under SD and shifted to LD for 5 days, were collected in the morning just after the lights in the chambers were switched on. The samples were collected zeitgeber (ZT) 0-2h to minimize the differences in the expression of circadian or diurnally regulated genes and snap frozen in liquid nitrogen.

The total RNA was extracted from these samples using Plant RNeasy Mini kit (Quiagen, Hilden, Germany). Biotinylated cRNA was synthesized using the MessageAmpTm II-Biotin Enhanced, Single Round aRNA Amplification Kit (Ambion / Life Technologies, Darmstadt, Germany) following the manufacturer's instructions. 15 µg biotinylated cRNA was fragmented and 2 µl from the fragmented cRNA was used in the gel electrophoresis to assess the quality. The fragmented cRNA samples were then hybridized to GeneChip Arabidopsis ATH1 arrays (Affymetrix, Santa Clara, USA) according to the manufacturer's instructions. EukGe-WS2_v4 protocol on an Affymetrix GenChip Fluidics Station 450 was used to wash and stain the GeneChip arrays. The stained array chips were scanned using an Affymetrix GeneChip Scanner 3000.

gcRMA package (Wu et al., 2004) implemented in R (http://www.r-project.org/) was used to normalize the array data (.CEL files) and the differentially expressed transcripts were identified by RankProducts analysis (Breitling et al., 2004). The microarray data have been deposited with EBI ArrayExpress (E-MEXP-3727).

6.5 EMS mutagenesis and suppressor screen in *tps1-2 GVG:TPS1*

6.5.1 EMS treatment of *tps1-2 GVG:TPS1* seeds

Around 15000 *tps1-2 GVG:TPS1* seeds (600 µl) (M0) were stratified in 4°C for 3 days. These were then treated with 25 ml of 0.4% (v / v) ethyl methanesulfonate (EMS) (Sigma – Aldrich, Steinheim, Germany) in a 50 ml Falcon tube, sealed with Parafilm and rotated in a tube rocker for 15 hours. Then the seeds were allowed to settle in the bottom and the EMS solution was pipetted out. The seeds were then repeatedly washed (8-10 times) with distilled water with mixing and rocking. In the last wash step, the tube was allowed to settle for an hour for the EMS to diffuse out of the seeds. The EMS treatment was carried out in a special hood kept for the purpose of mutagenesis. The washed M1 seeds were sown directly on soil.

6.5.2 Selection of suppressor mutant plants

M1 plants were grown under LD (23°C) conditions in green house and sprayed with 1µM dexamethasone (DEX) (Sigma - Aldrich, Steinheim, Germany) solution (with 0.02% Tween-20, Sigma - Aldrich, Steinheim, Germany) at 2-day intervals from 10 days after sowing (DAS). This was essential for the proper flowering and seed set in M1 plants. M2 seeds were collected as 300 pools with each pool representing seeds from 40 – 50 M1 plants. Approximately 500 M2 plants were grown from each of the 300 M2 pools under LD 23°C in the plant growth chambers and screened for suppressor mutants (those plants which flowered) without spraying DEX. *tps1-2 GVG:TPS1* and Col-0 plants were used as controls. Bulked seeds from the potential mutant plants obtained in the screen were screened again (M3) under LD conditions for genotyping and phenotyping. First 50 uniform flowering EMS candidate lines were used for further genetic characterization such as allelism and test for genetic nature of the mutation.

6.5.3 Mapping of putative suppressor candidates

6.5.3.1 Creation of mapping population

Mapping population was created according to the method described for fast isogenic mapping (Hartwig et al., 2012). One member from each of the 15 putative non-allelic complementation groups obtained after allelism tests, was used to perform back crosses (BC1) with non-mutagenized *tps1-2 GVG:TPS1* plants. 1µM DEX was sprayed at 2-day intervals to both the parents from 15 DAS. The F1 seeds (BC1F1) were sown in soil and DEX spraying was done for flowering and seed set. F2 seeds (BC1F2) from the individual BC1F1 lines were sown under LD conditions and screened for plants with the suppressor phenotype (early flowering compared to non-mutagenized *tps1-2 GVG:TPS1* plants). Leaf samples were pooled from the BC1F2 plants, which showed the suppressor phenotypes, and used for fast isogenic mapping. Samples from the non-mutagenized *tps1-2 GVG:TPS1* plants were also separately bulked for genome sequencing.

6.5.3.2 Fast isogenic mapping

6.5.3.2.1 Genomic DNA extraction

From approximately 900 BC2F2 plants, around 200 plants were selected based on their suppressor phenotype and the leaf samples were pooled. Genomic DNA was extracted from these bulked samples using DNeasy Plant Mini Kit (Quiagen). The concentrations of the DNA extracted were measured using a Nanodrop (Peqlab). High quality DNA samples (260:280 ratio of 1.8) with a total of 1 µg were used for sequencing.

6.5.3.2.2 Library preparation for sequencing

Quality check of the DNA samples were performed with an Agilient 2100 bio-analyzer and libraries were generated using Illumina genomic DNA kit according to the manufacturer's instructions. The concentrations of the amplified libraries were measured and the samples were sequenced using Illumina Genome Analyzer in a 96-bp paired end run.

6.5.3.3 SHOREmapping

The reads obtained from the candidate mutants and non-mutagenized *tps1-2 GVG:TPS1* plants were independently aligned to the Col-0 reference genome using GenomeMapper (Ossowski et al., 2008; Schneeberger et al., 2009). SHORE consensus was applied after correcting the paired-end alignments to find out the variations between mutants and the reference. The SNPs specific to *tps1-2 GVG:TPS1* were then removed and the EMS-induced SNPs were filtered out. Allele frequency was calculated as the ratio of reads of mutant alleles divided by all the reads at that specific locus. The Arabidopsis Information Resource 10 (TAIR) genome annotation was used to identify the effect of sequence change in the mutated genes.

6.6 Standard techniques and buffers

All the chemicals were purchased from Sigma (Munich, Germany), Bio-Rad (Munich, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Roche (Mannheim, Germany). Restriction endonucleases were purchased from Fermentas (Burlington, Canada) and New England Biolabs (Ipswich, MA, USA). DNA polymerases like Pfu, Taq and Phusion were purchased from Fermentas and Finnzyme (Espoo, Finland). Oligo nucleotides were ordered from MWG (Ebersberg, Germany).

6.6.1 Genomic DNA extraction

Leaf bits / whole leaves / whole rosettes collected were immediately frozen in liquid nitrogen and ground to fine powder using a mortar and pestle or in micro-centrifuge tubes with micro pestles. To about 100 mg of this powder, 200 µl of CTAB solution (1.4M NaCl, 0.1M Tris pH 8.0, 20 mM EDTA, 2% CTAB, 1% polyvinylpyrrolidone, 1µg/µl RNAse A) was added and incubated for one hour at 65°C. 200 µl of chloroform:isoamylacohol 24:1 solution was then added and mixed vigorously by vortexing. The tubes were centrifuged for 15 minutes at 14000 g. The supernatants were transferred to new tubes and mixed with 150 µl of isopropanol. After 15 minutes of centrifugation at 14000 g, the supernatants were discarded and the pellets were washed with 200 µl of 75% ethanol. Centrifugation at 14000 g was

repeated and the pellets were dried for 30 minutes at 37°C. After 15-20 minutes, the pellets were resuspended in 50 µl of deionized water and stored at -20°C. For PCR analyses, two µl out of 1:10 dilution solutions were normally used.

For DNA extraction with 96-well plates, the above protocol was slightly modified. A metal bead was added into each well of the 96-well plates. Leaf bits were then directly collected into the wells. The whole plate was then frozen at -80°C and shaken using a Retsch MM 300 homogenizer (Retsch GmbH, Haan, Germany) to grind the samples into a fine powder. The powder from each well was then resuspended by adding 500 µl of modified CTAB solution (1.42M NaCl, 0.1M Tris pH 8.0, 20 mM EDTA, 2% CTAB, 0.2% beta-mercaptoethanol, 1µg/µl RNAse A - pre-heated at 65°C) and incubated at 65°C for one hour. After addition of 500 µl of chloroform:isoamylacohol 24:1, the samples were centrifuged at 4000 g for 20 minutes. 200 µl of the supernatants were transferred to new plates and mixed with 0.7 volumes of isopropanol. The samples were then incubated at -20°C for at least 30 minutes, and centrifuged for 15 minutes at 4000 g. The pellets obtained were washed with 100 µl of 75% ethanol, air dried and resuspended in 250 µl of deionized water. One µl of this DNA solution was directly used as template for PCR analysis.

6.6.2 RNA extraction, cDNA synthesis and qRT-PCR analysis

Phenol / Chloroform extraction using TRIzol® Reagent (Life technologies, Darmstadt, Germany) was performed to extract the total RNA from plant samples. This was followed by sodium acetate / Glycogen assisted ethanol purification to improve the quality of RNA. Using 1µg of total RNA as template, first strand cDNA synthesis primed with oligo-(dT) 18 was carried out using RevertAid kit (Fermentas / Thermo Scientific, St. Leon-Rot, Germany) in 20 µl reaction volumes. The single stranded cDNA obtained was diluted to 5-fold and 4 µl were used as a template per PCR reaction.

For the quantification of mature miR156 (Figure 13), the total RNA extracted was reverse transcribed using RevertAid kit (Fermentas / Thermo Scientific, St. Leon-Rot, Germany) following the manufacturer's protocol with the exception that a 1:1

mixture of oligo-(dT)18 and the miR156-specific stem-loop RT primer (G-30607 - GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC; Table S2) was used in the priming step. qRT-PCR analysis for the expression difference in mature miR156 was performed according to a previously described protocol (Varkonyi-Gasic and Hellens, 2011). qRT-PCR was performed on an Opticon DNA engine (MJ Research / Biozym, Hessisch Oldendorf, Germany) using SYBR® Green I (Life Technologies) reaction mixture. The thermal profile of the reactions were:

Step 1: 95°C for 5 min

Step 2: 95°C for 30 sec

Step 3: 60°C for 30 sec

Step 4: 72°C 20 sec

Step 5: Go to step 1 and repeat 39 cycles

Step 6: 72°C for 7 min

Gene expression was normalized to *TUBULIN BETA CHAIN2* (*TUB2*) (At5g62690) and the expression differences were calculated by $2^{-\Delta\Delta Ct}$ method. Errors were given as upper and lower limits of standard deviation of the mean.

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8 Appendix

8.1 Supplemental materials

8.1.1 Supplemental figures

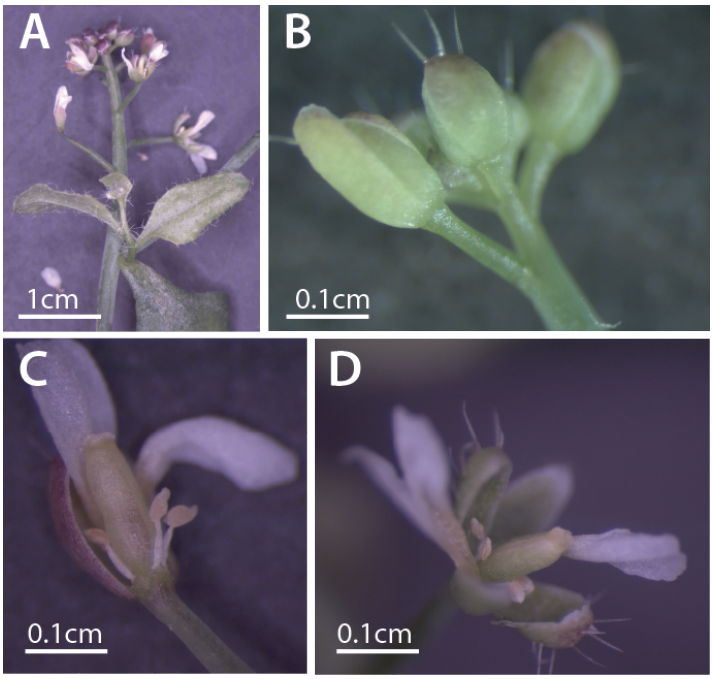

Figure S1: Flowers of non-induced *tps1-2 GVG:TPS1* plants.

A branch from the inflorescence of non-induced (without DEX spraying) *tps1-2 GVG:TPS1* plant (A). Unopened, aborted buds (B) and opened flowers with under developed stamens (C and D).

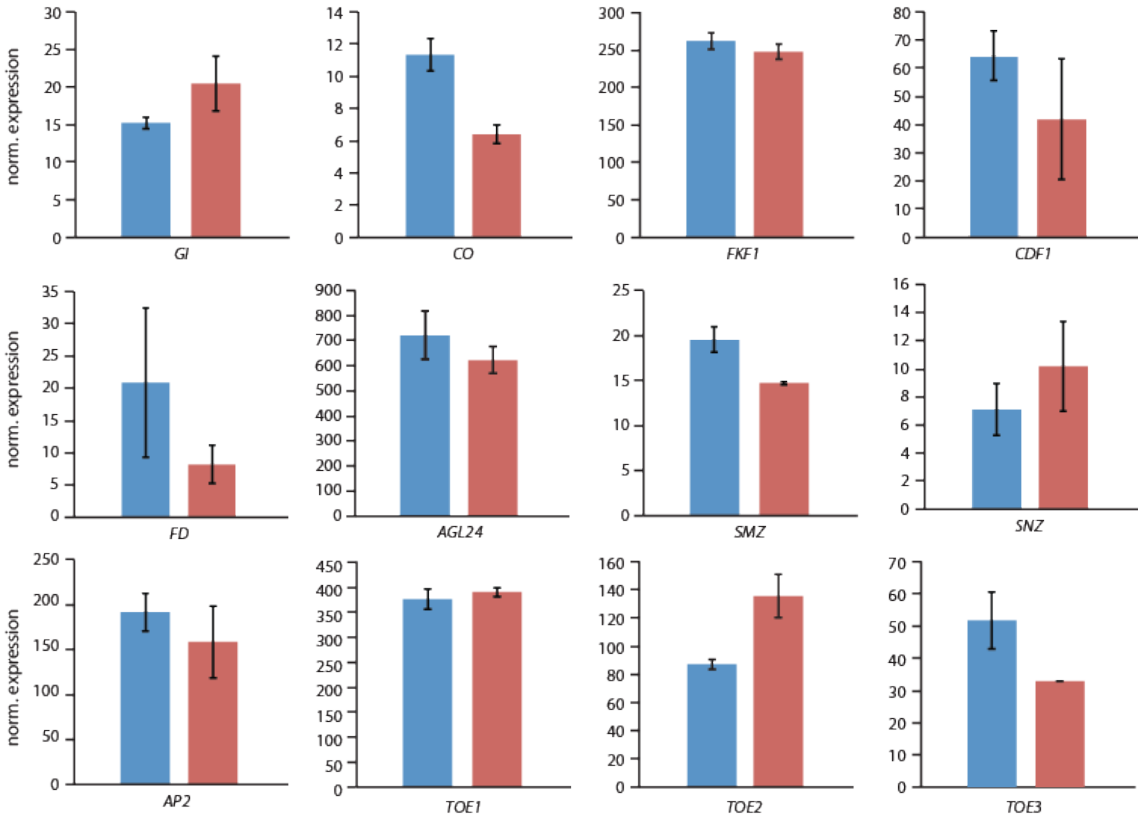

Figure S2: Expression of genes involved in photoperiod pathway as revealed by microarray analysis.

Total RNA was isolated from manually dissected shoot apical meristems of 21-day-old SD-grown Col-0 (blue) and *tps1-2 GVG:TPS1* (red) plants, converted into biotinlyated cRNA and hybridized to Affymetrix Arabidopsis Genome ATH1 arrays. The transcripts for two important photoperiod pathway genes, FT and SOC1, were not detectable by microarray in meristems of SD-grown plants. Minimum and maximum values obtained by hybridization of two biological replicates, from which the mean expression was calculated, are indicated (Modified from Wahl et. al., 2013).

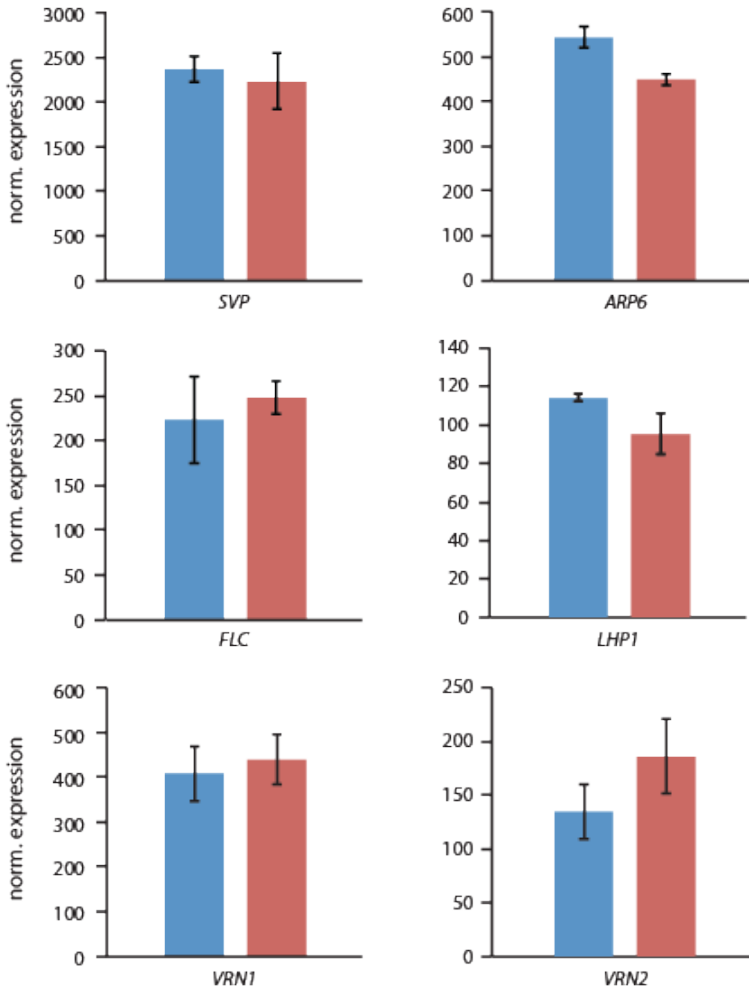

Figure S3: Expression of genes involved in ambient temperature and vernalization as revealed by microarray analysis.

Total RNA was isolated from manually dissected shoot apical meristems of 21-day-old SD-grown Col-0 (blue) and *tps1-2 GVG:TPS1* (red) plants, converted into biotinlyated cRNA and hybridized to Affymetrix Arabidopsis Genome ATH1 arrays. The transcript for *FRI* was not detectable by microarray in meristems of SD-grown plants. Minimum and maximum values obtained by hybridization of two biological replicates, from which the mean expression was calculated, are indicated (Modified from Wahl et. al., 2013).

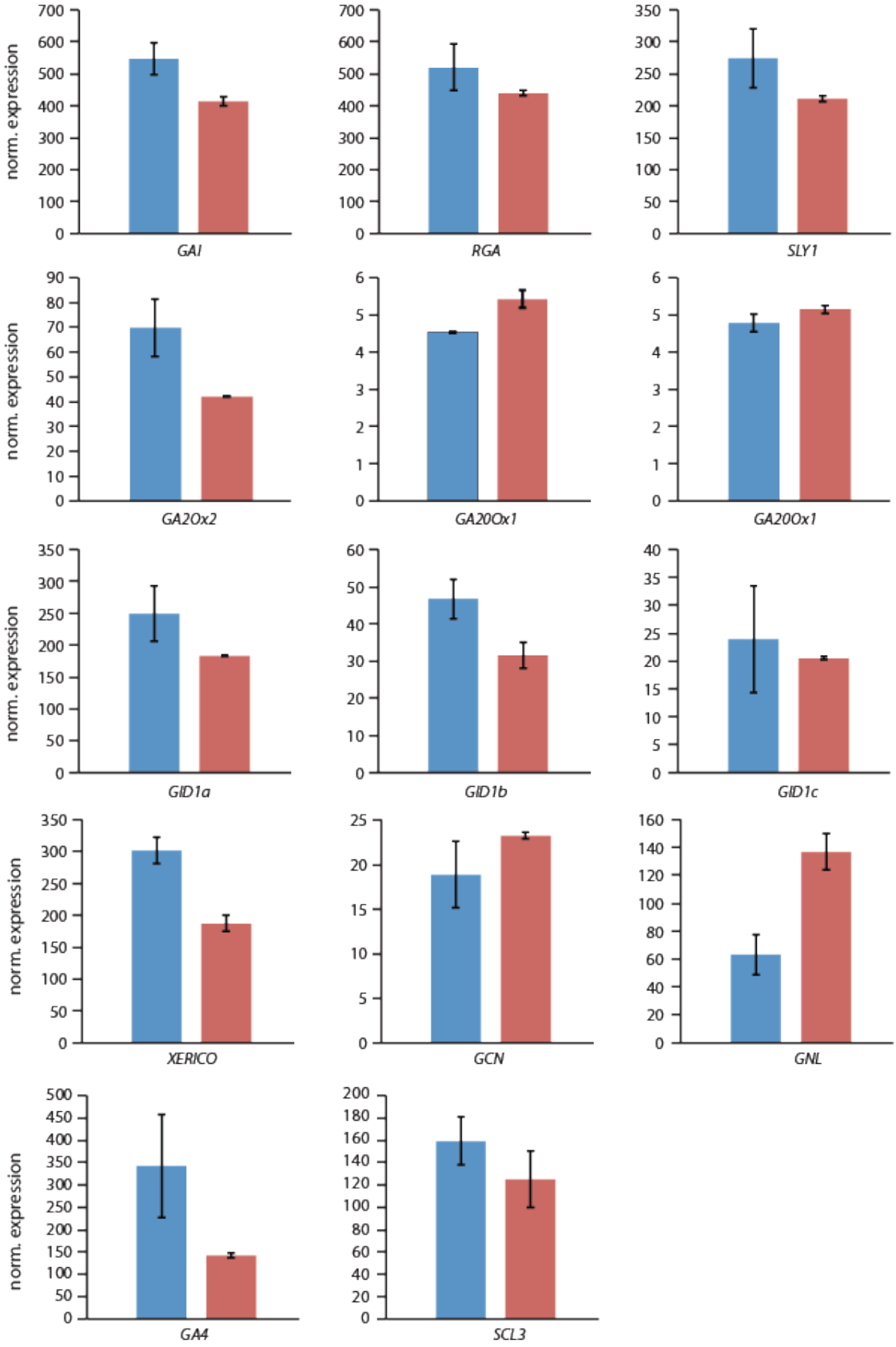

Figure S4: Expression of genes involved in giberellic acid biosynthesis as revealed by microarray analysis.

Legend continued.

Total RNA was isolated from manually dissected shoot apical meristems of 21-day-old SD-grown Col-0 (blue) and *tps1-2 GVG:TPS1* (red) plants, converted into biotinlyated cRNA and hybridized to Affymetrix Arabidopsis Genome ATH1 arrays. The transcript for *GA1* was not detectable by microarray in meristems of SD-grown plants. Minimum and maximum values obtained by hybridization of two biological replicates, from which the mean expression was calculated, are indicated (Modified from Wahl et. al., 2013).

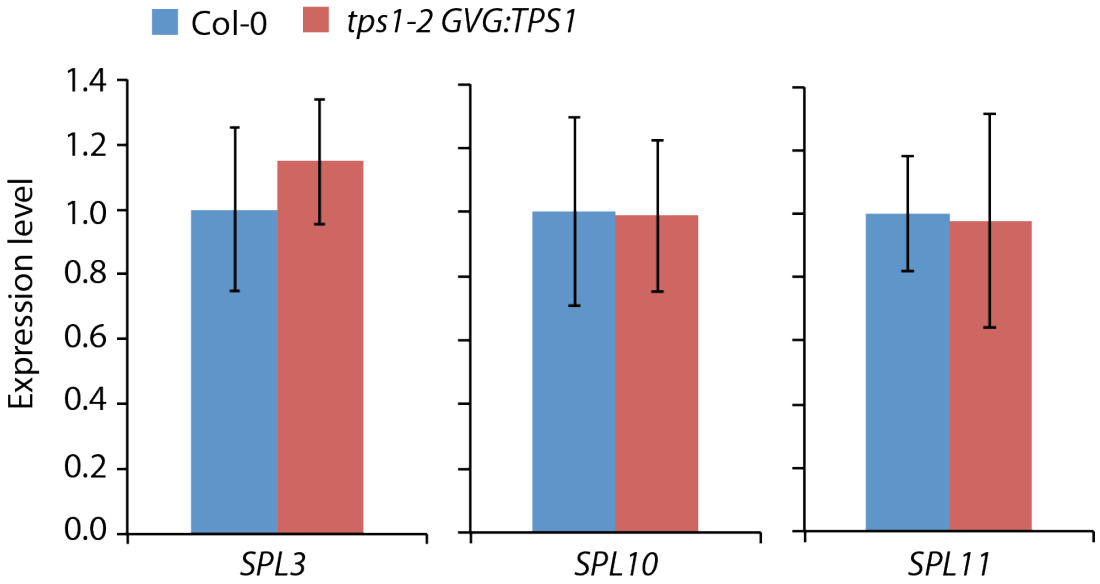

Figure S5: Expression of *SPL3*, *SPL10* and *SPL11* in 10-day old *tps1-2 GVG:TPS1* leaves compared to WT under SD.

Leaves of SD-grown plants were harvested at 10 DAS and expression of *SPLs* were measured by qRT-PCR on two biological replicates with three technical repetitions each and normalized to Col-0 using *TUB2* as control. Expression levels in Col-0 are set to 1. Error bars indicate SD.

8.1.2 Supplemental tables

Table S1: Linked potential candidate genes for the suppressor phenotype of *160-1* at the beginning of chromosome 3.

Gene ID	Position	Reference base	Alternate base	Percentage of reads supporting the alternate allele	SNP quality (Max. = 40)	Intergenic or not	Synonymous / non-Synonymous (S / NS)	Alternate codon	Reference amino acid	Alternate amino acid	Gene strand
AT3G01090	33220	C	T	0.939759	38	CDS	NS	Gga	G	R	-
AT3G01720	263216	G	A	0.948718	40	CDS	NS	Ctt	L	F	-
AT3G01770	276000	G	A	0.948718	40	CDS	NS	gCt	A	V	-
AT3G02930	656556	G	A	0.927273	40	CDS	NS	Gct	A	T	+
AT3G03120	717372	G	A	0.942308	40	CDS	NS	Gat	D	N	+
AT3G03220	743905	G	A	0.897436	40	CDS	NS	tCa	S	L	-
AT3G03630	879018	G	A	0.931035	40	CDS	NS	cCt	P	L	-
AT3G06230	1885817	G	A	0.916667	40	CDS	NS	Gca	A	T	+
AT3G06380	1937703	G	A	0.896104	40	CDS	NS	cGc	R	H	+

Table S2: List of oligonucleotides used

Gene ID	Name of the oligo	Sequence (5' -> 3')	Purpose
TPS1	G-22756	GACACTTGGTTTCTTGATATGTCCTG	Genotyping
At1g78580	G-22758	GCTGTCTTGGATACTGAACCAGT	
tps1-2	G-19968	GAGCGTCGGTCCCCACACTTCTATAC	Genotyping
At1g78580	G-22758	GCTGTCTTGGATACTGAACCAGT	
35S:MIM156	G-0474	AGAACACGGGGGACGAGCT	Genotyping
	G-2225	CGCATATCTCATTAAAGCAGG	
FT	G-34967	TAAGCTCAATGATATTCCCGTACA	Genotyping
At1g65480	G-34968	CAGGTTCAAAACAAGCCAAGA	
ft-10	G-34969	CCCATTTGACGTGAATGTAGACAC	Genotyping
At1g65480	G-34968	CAGGTTCAAAACAAGCCAAGA	
flc-3	G-0474	AGAACACGGGGGACGAGCT	Genotyping
AT5G10140	G-0868	AAAATATCTGGCCCGACGAAG	
35S:FT	G-0426	TTGGAGAGAACACGGGGGACG	Genotyping
	IK020	ACTGTTTGCCTGCCAAG	
SUC2:FT	G-7549	CCACTCTTCCTCTTCCTCCACC	Genotyping
	IK020	ACTGTTTGCCTGCCAAG	
TUB2	N-0078	GAGCCTTACAACGCTACTCTGTCTGTC	qRT-PCR
AT5G62690	N-0079	CACCAGACATAGTAGCAGAAATCAAG	
FT	G-30966	CCCTGCTACAACTGGAACAAC	qRT-PCR
At1g65480	G-30967	CACCCTGGTGCATACACTG	
TSF	G-33510	TGCCACCACTGGAAATGCC	qRT-PCR
AT4G20370	G-33511	CGTTTGTCTTCCGAGTTGCC	
CO	G-30962	CACTACAACGACAATGGTTCC	qRT-PCR
AT5G15840	G-30963	GGTCAGGTTGTTGCTCTACTG	
GI	G-30970	AGCAGTGGTCGACGGTTTATC	qRT-PCR
AT1G22770	G-30971	ATGGGTATGGAGCTTTGGTTC	
miR156	G-30606	GTGCAGGGTCCGAGGT	qRT-PCR
	G-30608	GCGGCGGTGACAGAAGAGAGT	
SOC1	G-30998	AAACGAGAAGCTCTCTGAAAAG	qRT-PCR
AT2G45660	G-30999	AAGAACAAGGTAACCCAATGAAC	

FUL	G-00654	TTGCAAGATCACAACAATTCGCTTCTC	qRT-PCR
AT5G60910	G-00655	GAGAGTTTGGTTCCGTCAACGACGATG	
SPL2	G-35317	TTGGGACTTCCAGTGACTCTGGCTTC	qRT-PCR
AT1G54150	G-35318	CATCAAACTCAGAGAGACAGTGGAACC	
SPL3	G-30976	CTCATGTTCGGATCTCTGGTC	qRT-PCR
AT2G33810	G-30977	TTTCCGCCTTCTCTCGTTGTG	
SPL4	G-30978	CTCTCAGGACTTAACCAACGC	qRT-PCR
AT1G53160	G-30979	CAGAGCTCTTCCTTCTTCGC	
SPL5	G-31000	AAGGCATCTGCTGCGACTGTTG	qRT-PCR
AT3G15270	G-31001	TCCTCCTCCTCTCATTGTGTCC	
SPL10	G-35327	GTGGGAGAATGCTCAGGAGGC	qRT-PCR
AT1G27370	G-35328	GAGTGTGTTTGATCCCTTGTGAATCC	
SPL11	G-31006	ATGTTCTCTACATCTCAAACCTC	qRT-PCR
AT1G27360	G-31007	GACTCCTGAGTATTCTCCCAC	
SPL15	G-31009	TCGAGTCGAAACCAGAAGATG	qRT-PCR
AT3G57920	G-31008	ACAACAATAGCACAGATTCAAGC	

8.2 Lists

8.2.1 List of figures

8.2.2 List of tables

9 Acknowledgements

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10 Curriculum vitae

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