MicroProteins in the regulation of flowering time

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Zusammenfassung

Pflanzen sind als sessile Organismen darauf angewiesen, sich Veränderungen in ihrer Umwelt anpassen zu können. Um dies zu bewerkstelligen habe sie in ihrer Evolution bemerkenswert vielseitige molekulare Netzwerke entwickelt, die es ihnen erlauben Veränderungen in ihrer Umgebung wahrzunehmen und sich diesen anzupassen. Transkripitionsfaktoren sind Proteine, welche die Aktivität von Genen regulieren und sind ein wesentlicher Bestandteil dieser molekularen Netzwerke. Oft interagieren sie mit anderen Proteinen anhand von spezifischen Interaktionsdomänen und bilden größere Proteinkomplexe.

MikroProteine, eine Klasse von kleinen Proteinen die lediglich aus einer solche Interaktionsdomäne bestehen, interagieren spezifisch mit anderen Proteinen und verhindern so die Bildung eines funktionellen Komplexes. Diese Funktionsweise ermöglicht es ihnen die Aktivität von Transkriptionsfaktoren in regulatorischen Netzwerken zu beeinflussen. Mehrere Beispiele für solche durch MikroProteine regulierten Prozesse wurden in den letzten Jahren beschrieben.

Um die Rolle von MikroProteinen in Entwicklungsprozessen besser zu verstehen, haben wir in der Modellpflanze *Arabidopsis thaliana* systematisch nach Proteinen mit den Eigenschaften von MikroProtein gesucht. Unter anderem fanden wir dabei zwei kleine B-Box Proteine, im Folgenden miP1a und miP1b genannt, die wir genauer untersucht haben. Die Überexpression der beiden Proteine bewirkt spätes Blühen unter normalerweise blühinduzierenden Wachstumsbedingungen wohingegen die künstliche Reduktion ihrer Expression leicht früheres Blühen bewirkt. Die beiden Proteine sind in der Lage mit CONSTANS, einem anderen B-Box Protein welches maßgeblich an der Blühinduktion beteiligt ist, zu interagieren. Wir konnten zeigen, dass miP1a und miP1b CONSTANS in seiner Aktivität inhibieren. Die beiden MikroProteine weisen eine zyklische Expression im Verlauf des Tages mit einem Expressionsmaximum während der Nacht auf. Vornehmlich sind sie in der Vaskulatur der oberirdischen Pflanzenteile exprimiert.

Zusätzlich konnten wir zeigen, dass miP1a und miP1b mit TOPLESS, einem transkriptionellem Ko-Repressor interagieren und CONSTANS mit diesem zusammen in einem trimeren Komplex binden. Diese Entdeckung beschriebt eine neue Funktion von CONSTANS, dass somit nicht nur als Blühinduzierender Faktor sondern auch als reprimierender Faktor fungieren kann, wenn es durch miP1a und miP1b in einem Komplex zusammen mit TOPLESS eingebunden wird.

Abstract

Plants are, as sessile organisms, highly dependent on their ability to adapt to an ever changing environment. In order to do so they developed a remarkable variety of regulatory networks which enable them to perceive, integrate and adapt towards their surroundings on a molecular level. Transcription factors as regulators of gene activity are an essential component of those networks. They often interact *via* specific interaction domains and form high order complexes with further proteins. MicroProteins, small proteins with a single protein-protein interaction domain, function as negative regulators of protein complex formation by sequestering their target proteins in a non-functional state. Their mode of function enables them to modulate the activity transcription factors in regulatory networks and in the past years several examples for processes in which microProteins play important roles have been described.

In order to learn more about the role of microProteins in development, we performed a computational screen to identify proteins with microProtein characteristics in the model plant *Arabidopsis thaliana*. Among the identified proteins where two small B-Box proteins – subsequently named miP1a and miP1b- which we further characterized. Overexpression of miP1a/b in *Arabidopsis* causes late flowering under inductive long day conditions whereas artificial reduction of their expression causes plants to flower slightly earlier. Both microProteins are able to interact with the flower promoting B-Box protein CONSTANS and we characterized miP1a/b as negative regulators of CONSTANS activity. Analysis of the temporal and spatial expression of miP1a and miP1b revealed a diurnal pattern of expression with high mRNA levels in the night period and a vascular expression of miP1a and miP1b.

Finally, we characterized the interaction of miP1a/b with the transcriptional co-repressor TOPLESS and showed, that the two microProteins are able to engage CONSTANS and TOPLESS in a trimeric complex. Our findings point towards a novel role for CONSTANS, not only as a flowering promoting factor, but also as a repressor of the floral transition in the presence of miP1a and miP1b, were it becomes engaged together with TOPLESS in a trimeric complex.

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List of abbreviations

3-AT	3-Amino-1,2,4-triazole
aa	amino acid
AD	activating domain
bHLH	basic helix-loop-helix
bp	base pairs
cDNA	coding DNA
COL	CONSTANS like
Da	Dalton
DBD	DNA binding domain
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DTT	Dithiotritol
EDTA	Ethylenediaminetetraacetic acid
et al.	et altera
EtOH	Ethanol
Fig	Figure
FLIM	Fluorescence lifetime imaging
g	Standard gravitational force
GFP	Green fluorescent protein
LiAc	Lithium acetate
MES	2-(N-morpholino)ethanesulfonic acid
MIGS	micro RNA induced gene silencing
miP	microProtein
miR	micro RNA
mRNA	messenger RNA
MS	Murashige & Skooge
OD	Optical density
OX	over expressor
P1/2	Primer 1/2
PAGE	Polyacrylamid Gel Electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylen glycol
RNA	ribonucleic acid
SAM	Shoot apical meristem
SDS	Sodium dodecyl sulfate
sec	second
Suppl	Supplemental
Taq	*Thermophilus aquaticus* polymerase
TPL	TOPLESS
TPR	TOPLESS related
ZPR	Little Zipper

Aim of the Thesis

Plants possess a remarkable developmental plasticity that allows them to adapt to an ever changing environment in an optimal manner. The basis of this plasticity lies in the regulatory molecular network that sense and integrate internal and environmental stimuli into adapted genetic activity. A species of small proteins – termed microProteins – function as modulators within these networks by specifically interacting with other proteins and hindering them from engaging in functional complexes. Also several examples for microProteins have been identified in plants already, little is known about the general extent to which microProteins influence developmental networks. In order to identify more such proteins and their respective targets, we performed a computational screen in the model plant *Arabidopsis thaliana*.

Using the given criteria of a microProtein we identified several new proteins with those characteristics, amongst them two small B-Box containing proteins. As so far no examples for microProteins from that protein family existed, we chose these two B-Box proteins to verify the microProtein character and investigate their function further.

We found that the two small B-Box proteins can interact with the flowering time regulator CONSTANS, itself a B-Box protein, and have a negative effect on the initiation of flowering under long day conditions. We further analyzed the temporal and spatial expression of the two B-Box proteins. Key experiments under varying growth conditions, analysis of plants with reduced expression and genetic crosses allowed us to define the two B-Box proteins as microProteins affecting CO activity.

Structural analysis of the two B-Box proteins revealed a conserved amino acid motif at the C-terminus. We found this motif to mediate interaction with transcriptional co-repressors from the TOPLESS/TOPLESS-RELATED family. Furthermore the two microProteins are able to engage the transcription factor CONSTANS in a complex together with TPL/TPR proteins, suggesting a flower repressing effect for this complex.

1. INTRODUCTION

MicroProteins in plant development

Plants are, like every organism on earth, depending on and influenced by other organisms and abiotic factors in their environment. However, as sessile organisms, plants cannot easily escape unfavorable environmental conditions so they have developed an impressive morphological plasticity to cope with them.

This plasticity ranges from very fast adaption of the photosynthesis machinery in response to light intensity changes towards complex changes in the development and morphology of the plant regulated by genetic networks. The complex genetic networks underlying these developmental decisions involve very finely adjusted interactions of transcriptional activators and repressors that tightly control the expression and activity of regulatory genes and proteins which finally convert the environmental input into a developmental outcome. Involved in the coordination of these complex networks is a species of small proteins, termed microProteins (miPs) that function as negative regulators of larger protein complex formation (Staudt and Wenkel, 2011; Graeff and Wenkel, 2012; Eguen et al., 2015). As flexible modulators of protein-complex formation and activity, these types of proteins are emerging as versatile regulators of genetic networks.

Definition

The first and maybe most disputed question to answer is: what is a microProtein?

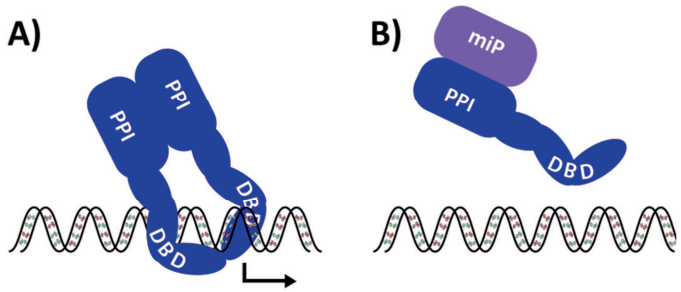


Figure 1. 1 Mechanism of microProtein function
Many transcription factors require dimerization for DNA binding, often mediated via specific Protein-Protein Interaction domains **(A)**
A miP containing this PPI domain prevents dimerization and DNA binding, sequestering the monomer in a non-functional state **(B)**

Different publications in the last years have tried to define this species of proteins, applying more (Staudt and Wenkel, 2011; Eguen et al., 2015) or less stringent criteria (Seo et al., 2011a; Magnani et al., 2014). All the existing definitions agree, that the group of regulatory proteins termed microProteins is characterized not by a common origin or direct similarity in their sequence but by a common mode of function. They seldom have more than 120 amino acids

and possess a protein-protein interaction (PPI) domain as their main functional feature. Via this PPI-domain they form heterodimers with other proteins that have similar PPI-domains and so prevent the formation of a functional protein complex. The ability to interact with its protein partners via specific PPI-domains is often a prerequisite for a protein to function correctly (Nooren and Thornton, 2003; Reichmann et al., 2007). If this formation is interrupted by a miP binding the PPI-domain and preventing the interaction with the functional interaction partner the activity of the protein complex is disturbed (Fig. 1.1). All the known miPs show a relatively strong sequence similarity towards their target proteins, as specific PPIs often occur between domains of the same type. This observation has been used to identify new true and potential miPs in the past (Wenkel et al., 2007; Seo et al., 2011a; Magnani et al., 2014; Straub and Wenkel, unpublished).

The disaccord over the definition of miPs lies in the question whether small proteins that function as negative regulators with more than one domain, and larger proteins functioning by sequestrating other large proteins in a non-functional state, should be termed miPs (Seo et al., 2011a; Magnani et al., 2014)or not (Staudt and Wenkel, 2011; Eguen et al., 2015).

The strict definition of Eguen et al. seems more suited to define the species of microProteins. As the prefix 'micro' suggests, the mode of function described for miPs has similarities to microRNAs as dominant-negative inhibitors and implies a small size for these proteins. The definitions of Seo et al. or Magnani et al include proteins like the DELLAs (56 – 64 kDa), most JAZ proteins (19 -37 kDa) and most Aux/IAAs (18 – 37 kDa) (Lamesch et al., 2012) due to their mode of function. These proteins cannot be called small and some of them contain domains which are not only involved in PPI but are necessary for the repressive character of the proteins (Osmont and Hardtke, 2008; Perez and Goossens, 2013). In addition the definition of Eguen et al. allows proteins not involved in transcriptional regulation also to be classified as miPs, although there are few examples such as as inhibitors of ion channel formation (Miller, 2000; Hsu et al., 2004; Lewis et al., 2004; McCrossan et al., 2009; Roy et al., 2014). Nevertheless, most so far described miPs are involved in transcriptional regulation.

Transcriptional repression as an important part of genetic regulation

Transcriptional regulation, hence the control of a gene's transcription to mRNA that is then translated to a protein, is a key prerequisite for the correct formation, metabolism and development of an organism. Some enzymes directly or indirectly, via their substrates or products, influence the expression of some genes, the

vast majority of the transcriptional processes though is regulated by transcription factors (TFs). The genomes of different plant species seem to contain a relatively high number of proteins with TF ability; current estimation state that around 7-10% of the total proteins in plants are TFs or are due to their amino acid sequence similarity grouped together with them in a protein family (Riano-Pachon et al., 2007; Mitsuda and Ohme-Takagi, 2009).

The classical TF is a protein that recognizes a specific DNA-sequence motif in the promoter region of a gene and then promotes the recruitment of the RNA-Polymerase II and associated proteins to this locus, so that the transcription of this gene can begin (Lee and Young, 2000). On the other hand some TFs also prevent the formation of the transcriptional complex at a gene locus and thereby function as transcriptional repressors. The specificity of the DNA-binding is caused either directly by the structure of the DNA-binding region or caused by the formation of a homo- or heteromeric complex that gives rise to the protein structure necessary for recognizing a specific DNA-element.
Many TF families contain a lot of members that can interact and whose different possible combinations show different DNA-binding and sequence specificities. This gives rise to a plethora of potential complexes and is the foundation of the plasticity and versatility of these genetic networks.
Other TFs are known to influence epigenetic modification of a target locus. These modifications on the DNA-associated histone molecules or the DNA itself influence the density and accessibility of a genomic region and in this manner regulate its transcription. A class of proteins called co-repressors falls into this category (Liu and Karmarkar, 2008). They are themselves not able to bind DNA but interact with TFs. Directed to a promoter region by this interaction they recruit histone modifiers like de-acetylases and methyltransferases, causing the formation of heterochromatin at the respective locus. The targeted promoter-region becomes transcriptionally inactive (Krogan and Long, 2009).

Furthermore, some TFs are not able to bind DNA themselves but rather function by interacting with DNA-binding proteins when these bind to the respective target locus and can influence their activity positively or negatively. The mechanism of miP function, as long as they are regulators of transcription factor complex formation, belongs in this class even though miPs represent a special category within this group of negative regulators.

Examples for microProteins in animals and plants

The first identified miP was the protein Id, a regulator of bHLH transcription factors involved in neuronal and muscular differentiation. This widespread class of transcription factors forms dimers via the HLH-domain and interacts with specific DNA regions via the basic-domain. Id lacks the basic-domain and when it interacts with other bHLHs it prevents binding to DNA (Benezra et al., 1990; Ruzinova and Benezra, 2003).

MiPs of the Id-type can also be found in plants, although there seems to be no direct conservation suggesting they originated from convergent evolution (Eguen et al., 2015). In *Arabidopsis thaliana* the KIDARI-protein (KDR) interacts with HFR1, an atypical bHLH-protein, which in turn negatively regulates the bHLH TF PIF4 that is involved in photomorphogenesis and hypocotyl elongation in the shade. This inhibition of a negative regulator makes KDR itself a positive regulator of PIF4 activity (Hyun and Lee, 2006; Hong et al., 2013).

Another example are the three BANQUO proteins, which are also negative regulators of HFR1 activity, but additionally function as regulators of organ development in flowers, the BRI1 signaling network and cell elongation (Zhang et al., 2009b; Mara et al., 2010; Bai et al., 2012; Ikeda et al., 2012). The mechanism here is very similar to the function of KDR as the BANQUO proteins bind and sequester an atypical bHLH TF in a non-functional state, so that other bHLH proteins can work undisturbed. Ikeda et al. coined the fitting term 'triantagonistic' for this mode of operation.

Further miPs can be found as regulators of the MYB family of transcription factors (TRY, CPC, ETC1/2/3, TCL1/2 which regulate root hair and trychome formation (Tominaga-Wada and Wada, 2014)), MEINOX-homeodomain TFs (KNATM which represses BELL-TALE protein activity in leaf proximal-distal patterning (Magnani and Hake, 2008)) and ZF-homeodomain proteins (Minifinger 1-3 which negatively regulate larger zinc-finger homeodomain proteins involved in leaf and meristem identity definition (Sicard et al., 2008; Hong et al., 2011; Hu et al., 2011)). Another example are the LITTLE ZIPPERs (ZPRs), small leucine-zipper domain containing proteins that negatively regulate the activity of class III HD-ZIP transcription factors (Wenkel et al., 2007; Brandt et al., 2013). The class III HD-ZIP proteins are important regulators of the shoot apical meristem (SAM) identity, leaf patterning and vasculature formation. In all these processes they are affected by the ZPRs (Wenkel et al., 2007; Kim et al., 2008). The ZPRs also play an important role in fine-tuning the complex interplay between REVOLUTA (REV), KANADI1 and HD-ZIP II TFs in the process of leaf polarity organization where the activation of ZPR expression by REV forms a negative-feedback loop (Bou-Torrent et al., 2012; Brandt et al., 2012; Brandt et al., 2013).

The described miPs are similar in their mode of function but diverse in their PPI domains. Some miPs like the ZPRs and KDR can be found in all angiosperms where they fulfill similar functions. In ancient plants no true miPs have been identified so far but the evolutionary origin of many miPs like the ZPRs and the MYB-type miPs can be traced back to ancestors in mosses and algae (Du et al., 2013; Floyd et al., 2014; Eguen et al., 2015). Taken together there are many developmental processes in plants in which miPs play an important role as negative regulators of protein activity.

Molecular titration

Negative regulation appears to be a very prominent and important aspect in the transcriptional control of eukaryotic genes. The thorough analysis of *Arabidopsis thaliana* transcription factors has revealed that approximately 30% of all TFs are indeed transcriptional repressors that actively or passively repress the transcription of genes to whose promoter region they bind (Kagale and Rozwadowski, 2011) and other passive repressors like the miPs can be found in most developmental and signaling networks (Krogan and Long, 2009). The antagonistic function of transcriptional repressors towards transcriptional activators seems at first glance to be a complicated and wasteful mechanism, as both activating and repressing proteins have to be transcribed and translated in order to function and their activity and abundance has to be precisely regulated, which often requires additional regulatory factors.

A closer look at the mechanism shows that the interplay of transcriptional activators and repressors allows a level of regulation that cannot be achieved by regulating the activator frequency alone. Besides, the usage of repressive complexes that are recruited by specific and often small proteins allows the re-use of these complex-proteins for further and different applications and the expression and the degradation of their recruiting proteins resembles fast and specific regulation of their activity (Liu and Karmarkar, 2008; Osmont and Hardtke, 2008). Regarding this, the usage of transcriptional repressors becomes less wasteful and in the case of the miPs, their small size makes them an effective and efficient way of regulation.

MiPs are additionally interesting as negative regulators that function by sequestering their target proteins in anon-functional state. Experiments in yeast and elaborate modeling approaches have shown that such sequestering proteins are not only negative regulators but also can modulate the dose-response behavior of their targets. This idea, termed molecular titration, bases on the assumption that a protein usually responds in a linear manner proportional to its activating signal. If an inhibitor molecule blocks the activity of the protein, a

certain threshold of the activating signal would have to be reached in order to overcome the inhibitory effect of the repressor, shifting the dose-response from a linear one towards a more sigmoidal course (see Fig. 1.2). The amount and interaction strength of the repressor here mark the threshold value that needs to be overcome in order to reach an active state of the regulatory unit (Buchler and Louis, 2008; Buchler and Cross, 2009). As a linear activity curve is not optimal for responding to in- or external stimuli the proposed model for regulating protein activity represents an elegant mode to modulate the activity of such proteins like receptors or transcription factors. Observations and experiments indicate that miPs can function as such molecular titrators (Wenkel et al., 2007; Magnani and Hake, 2008).

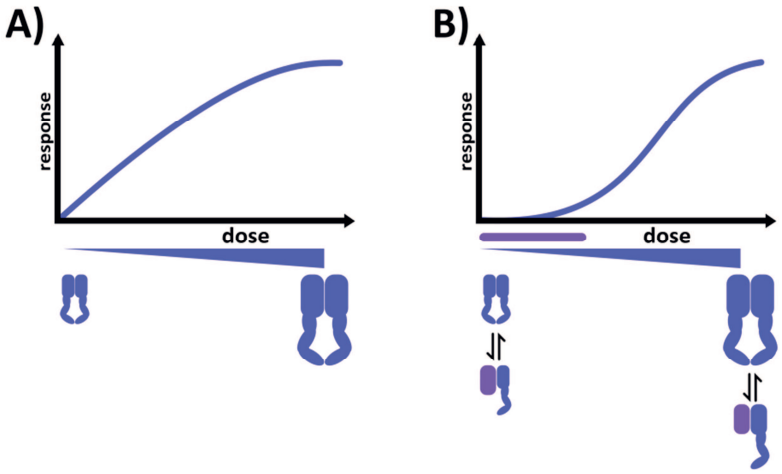


Figure 1. 2 Molecular titration
The response increases linear with the amount and activity of protein **(A)**
A specific inhibitor like a microProtein buffers the target proteins activity and defines a threshold, where little response is measurable. The response increases non-linear, after the threshold is overcome **(B)**

Mechanism of miP function

MicroProteins have as interaction partners with larger proteins the ability to modify the function of those proteins in different manners. The most common mechanism is a simple sequestration of the respective target protein in a non-functional state, preventing it from forming a complex. This, more or less passive mode of action, can be observed for many described miPs like KIDARI (Hyun and Lee, 2006) and the ZPRs (Wenkel et al., 2007). A further mechanism of regulation can be observed for the mini Zn-finger (MIF) proteins which, in addition to preventing DNA-binding of their target ZHD5, also prevent the import of the protein into the nucleus (Hong et al., 2011).

MiPs targeting TFs have so far only been shown to prevent their binding to DNA (Benezra et al., 1990; Wenkel et al., 2007; Hong et al., 2011), however the miP-TF heterodimer might function as a repressive complex rather than a transcriptional activator. This mechanism has been described for the Aux/IAA or JAZ proteins that

connect transcriptional co-repressors and the usually activating TFs to a repressive complex (Szemenyei et al., 2008; Perez and Goossens, 2013). The Aux/IAAs and JAZ proteins are not miPs under the strict definition, but miPs that in this manner might exist.

miP biogenesis

Most known miPs are coded as independent genes, so that their transcription and translation is not per se linked to the expression of their target proteins. These kinds of miPs are termed trans acting miPs or *trans*-miPs (Magnani et al., 2014; Eguen et al., 2015).For some of them though, like the ZPRs, it has been shown that their expression is also controlled by their target REVOLUTA. In this way a negative feedback loop regulating the activity of REV is established (Wenkel et al., 2007; Brandt et al., 2012; Brandt et al., 2013). In contrast to this independent origin are the *cis*-miPs which are truncated versions of a full length protein and are directly derived from their target proteins gene locus. They can originate from alternative transcription start sites, alternative polyadenylation sites or alternative splicing. As only little is known about the extent of alternative transcription and polyadenylation, this miP origin remains hypothetical so far. Few examples exist for *cis*-miPs derived from alternative splicing and in *Arabidopsis thaliana* these do not fit the strict miP characteristics as they are just slightly shorter than their respective target.

One example is the a splice variant of the floral regulator gene FLM, FLMδ, which functions as a negative regulator of the full length splice variant FLMβ. Interestingly the ratio of FLMβ to FLMδ seems to be influenced by ambient temperature which either promotes the activity of the floral repressor protein SVP in cold temperatures or inhibits it in a warmer climate; FLMδ both prevents FLMβ interaction with SVP as well as sequesters SVP when bound to it (Lee et al., 2013; Pose et al., 2013). Other known examples are inhibitory splice variants of TFs like IDD14 (Seo et al., 2011b), PIF6 (Penfield et al., 2010) or CCA1 (Seo et al., 2012) with truncated DNA-binding domains(Seo et al., 2013). These splice variants can scarcely be considered miPs due to their size.

However, c*is*-miPs are particularly interesting as their transcription is directly linked to the transcription of the full length version, as it occurs from the same gene locus at the same time, and differences in abundance are mostly due to post-transcriptional modification, e.g. alternative splicing. In this way they would allow a mode of regulation which is directly linked to the regulation of the target genes expression.

Outlook

This part provided some overview about what is known so far about microProteins in plants and other eukaryotes. Although only around 40 proteins that clearly qualify as miPs are known in plants (Graeff and Wenkel, 2012; Eguen et al., 2015), most of them in *Arabidopsis thaliana*, this may not be due to the rare occurrence of these kind of regulators but rather because they have been overlooked so far. Small proteins are often hard to characterize, loss of function mutants are often not available or have no obvious phenotype as many miPs described so far have homologs (Eguen et al., 2015). The recent approaches on identifying potential miPs indicate that there are probably many miPs among the small proteins in the proteome of plants, as many of this small proteins have miP characteristics (Seo et al., 2011a; Straub and Wenkel, unpublished). A close and systematic look upon this large and poorly described pool of proteins will most likely result in the identification of a variety of new plant miPs, involved in many interesting regulatory processes.

The photoperiodic regulation of flowering time

Plants are highly dependent on the environmental conditions they grow in and have therefore developed complex regulatory mechanisms to synchronize their development with these conditions. One of the most crucial decisions during a plants life is to find the right time to initiate flowering, as the production of flowers and seeds consumes many of a plant's resources. This transition needs to be well adjusted to environmental conditions like the availability of water and nutrients or the surrounding temperature as well as to endogenous factors like the organisms' age or overall constitution.

Additionally, the plant not only has to perceive and integrate environmental signals to assess the current state of its environment, but it also has, to a certain extent, to foresee how the environmental conditions are going to be during flowering, seed maturation and seed release (Amasino, 2010). In summary, the plant needs to sense its environment and anticipate seasonal changes. The molecular genetic network that functions as an endogenous timekeeper, allowing the plant to synchronize its metabolism and development with the daily and seasonal changes in its environment is referred to as the circadian clock (Song et al., 2010). MicroProteins are involved in the modulation of complex genetic networks and in this we identified and characterized two miPs involved in the process of photoperiodic flower initiation. But first a short overview about this complex regulatory pathway will be given.

Photoperiodic regulation of flowering

The developmental adaption of plants is controlled on the lower cellular level by complex genetic circuits which integrate the environmental stimuli. One of the most important environmental factors for a photosynthetic organism is the amount, the duration and the quality of light (Fankhauser and Christie, 2015). Plants evolved many mechanisms and a set of proteins that changes their confirmation and activity in response to light of a certain quality. These proteins therefore work as photorecptors. Many developmental processes are controlled and influenced by the activity of these light responsive proteins (Christie et al., 2015; Fankhauser and Christie, 2015).

The duration of the photoperiod has a great influence on the development and reproduction of plants and has therefore been extensively studied for almost 100 years (Garner and Allard, 1920). Experiments with modulated length of light and dark phases made clear, that plants respond differently towards light in the morning or the evening and those responses were linked towards the circadian rhythm (Bünning, 1936). A model was postulated which stated that flowering is induced by light, as an external cue, when perceived during a certain time of the internal circadian rhythm in which the plant is sensitive to this (Pittendrigh and Minis, 1964). This external coincidence model laid the foundation for the discovery of the molecular components regulating the transition from vegetative growth to flowering (Searle and Coupland; Amasino, 2010).

Arabidopsis thaliana is a facultative long day plant, meaning flowering is initiated when the light period of the day becomes longer then the dark period. Many plants in temperate climate zones developed such a flowering behavior, as increasing day length coincides with beneficial growth conditions in the spring and early summer. *Arabidopsis* was established as a model organism to study this behavior (Searle and Coupland, 2004). In other climate zones with hot and dry summers, plants in are in the initiation of their floral transition sensitive to the shortening of the light period that occurs in autumn.

A screen of mutagenized *Arabidopsis thaliana* plants for mutants with 'increased viability' –e.g. stronger vegetative growth- resulted in the identification of the *CONSTANS* (co) and the *GIGANTEA* (gi) locus. The discovered mutaitons are insensitive to photoperiodic changes and behave like plants grown under short day conditions. Crossings between these and other mutant plants revealed a major role for both loci in the photoperiodic transition to flowering (Koornneef et al., 1991).

GIGANTEA was identified as a gene under direct regulation of the circadian clock components CCA1 and LHY and is now considered as a key mediator between the genetic networks responsible for circadian rhythm and flower initiation (Fowler et al., 1999; Searle and Coupland, 2004). GI is located in the nucleus and mediates the interaction of several factors involved in circadian clock and light signaling (Song et al., 2013); it also has DNA binding and transcriptional activation abilities (Sawa and Kay, 2011). However, GI is in its expression not severely affected by the change and the duration of the photoperiod and therefore not the regulatory hub of the circadian and photoperiodic flower inducing pathway.

CONSTANS and the B-Box family of transcription factors

The other identified locus named CONSTANS was mapped to a gene coding a Zn-finger containing protein of the B-Box family with assumed transcription factor characteristics (Putterill et al., 1995). The B-Box domain is found in many eukaryotic proteins. In animals, B-Box domains are usually associated with a RING domain and a coiled-coil domain. Many of these RBBCC/TRIM (for RING B-BOX Coiled-Coil/Tripartite Motif) proteins are involved in developmental processes, where they regulate transcription, interact with other proteins and are involved in ubiquitination (Gangappa and Botto, 2014). In plants, B-Box containing proteins do not possess the other two motifs found in animals but some have additional domains important for their function. In the model plant *Arabidopsis thaliana,* 32 proteins containing B-Box domains were identified. The B-box can usually be found at the N-terminus of the protein. It is assumed to mediate homotypic protein-protein interactions between B-Box proteins and is an essential feature for the correct function of the respective B-box protein (Robson et al., 2001). The

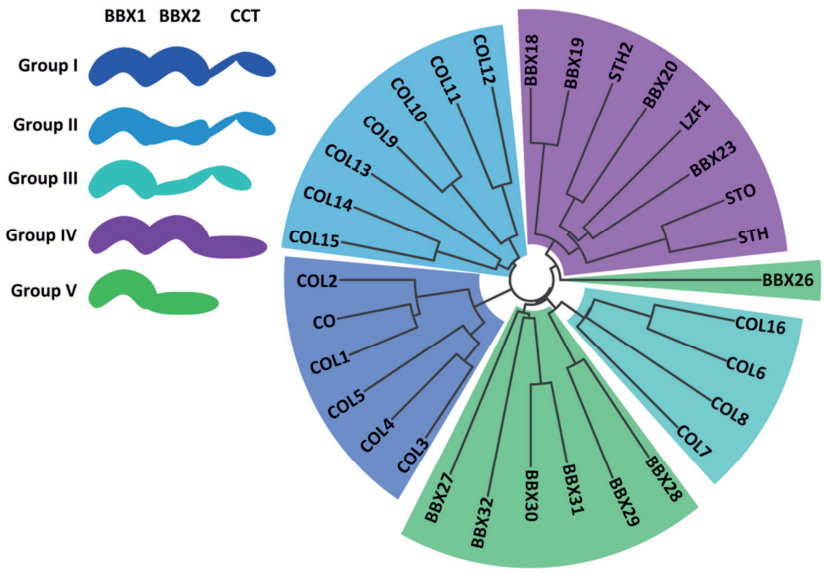


Figure 1. 3 B-Box family tree in Arabidopsis thaliana
The 32 protein are categorized into 5 groups, according to their number of B-Box domains and additional domains. A ClustalW alignment was used to build this tree

members of the B-Box family are classified in five groups due to their structural features (Figure 1.3) (Khanna et al., 2009).

Group I has two B-Box domains at the N-terminus and a C-terminal CCT (for CONSTANS CONSTANS-LIKE TOC1) domain. Group II is similar to group I but the second B-Box is slightly different in its sequence compared to group I. Group III has only the first B-Box and a CCT domain. Due to the shared structure with B-Box domains and a CCT domain at the C-terminal end the proteins of group I-III are also referred to as CONSTANS-LIKE proteins.
The proteins of the group IV are different as they have two N-terminal B-Box domains but lack a C-terminal CCT domain. The Group V B-Box proteins have only one B-Box and no further annotated domains (Khanna et al., 2009; Crocco and Botto, 2013).

Role and regulation of CONSTANS

Today our understanding of photoperiodic flowering time regulation has put the focus on the activity of CONSTANS in the leaf vasculature. CO activity is therefore regulated on several levels.

The first level of regulation is transcriptional. *CONSTANS* shows a diurnal pattern of expression. In the early morning only low amounts of *CO* mRNA can be found. The levels increase during noon and reach a high abundance 14 to 16 h after the beginning of the light period. In light periods of more than 12 h, which corresponds to summer day length in temperate climate zones, the levels of *CO* mRNA increase even further during the begin of the dark period (Suarez-Lopez et al., 2001). In the late night they drop down and reach their minimum again in the morning of the next day. This cyclic expression of *CO* is regulated via the interplay of GI and FKF1, two proteins regulated in their activity by core components of the circadian clock (Park et al., 1999; Shim and Imaizumi, 2015) with a group of transcriptional repressors called Cycling DOF Factors (CDFs). The CDF proteins repress the expression of *CO* in the morning. Blue light activates FKF1 and facilitates its interaction with GI, which is more abundant in the afternoon than in the morning or night, (Sawa et al., 2007; Fornara et al., 2009; Song et al., 2013) and the GI-FKF1 complex promotes the degradation of the CDF proteins in a light-dependent manner (Sawa et al., 2007). The interplay of activating and repressing factors causes the changing levels of *CO* mRNA during the day (Fig 1.3 A).

However, transcriptional regulation of *CO* is not sufficient to explain, why flowering is initiated only during long light periods, as *CO* mRNA shows a diurnal expression during long and short light periods (Suarez-Lopez et al.,

2001; Imaizumi et al., 2003; Valverde et al., 2004; Imaizumi et al., 2005). The key to the different effects of the CO regulatory module lies in in the regulation of CO at the protein level. CO protein accumulation is different during long and shot days.

Only low amounts of CO can be detected in plants grown under short day conditions in contrast to long day grown plants and CO protein seems to be only stable during the light period of the day (Valverde et al., 2004). This observation explains why it is only during the long day -when the high expression of CO occurs during the light period- that CO dependent initiation of flowering can be observed (Searle and Coupland, 2004; Amasino, 2010). The understanding of the molecular mechanisms regulating CO expression and stability explains therefore the external coincidence hypothesis, formulated more than fifty years ago (Pittendrigh and Minis, 1964).

The accumulation of CO protein under long day conditions is dependent on two different classes of photoreceptors (Fig. 1.3 B). Far red light, activating Phytochrome A (PhyA) and blue light, perceived by FKF1 and the Cryptochrome photoreceptors (CRY), promotes CO stability. In the dark CO is bound by SPA1, 3 and 4 proteins, that also interact with COP1 a RING-finger E3 ubiquitin ligase (Laubinger et al., 2006; Jang et al., 2008). CO becomes ubiquitinated and degraded via the proteasomal machinery. Activated CRY1 and CRY2 interact with the SPA proteins preventing the formation of the CO-SPA-COP1 complex and the degradation of CO. Blue light additionally helps to stabilize CO protein via FKF1, which when activated, binds and stabilizes CO in the late afternoon, during long days when FKF1 is predominantly expressed (Song, Smith et al., 2012). Far red light, activating PhyA, promotes CO accumulation as PhyA stabilizes CO in the afternoon, whereas red light, perceived via PhyB has the opposite effect on CO in the morning (Valverde et al.).The mechanism behind this process has recently been elucidated: HOS1, an E3 ubiquitin ligase like COP1, is activated by PhyB in the morning and suppressed in its activity in the afternoon *via* PhyA (Lazaro et al., 2015).

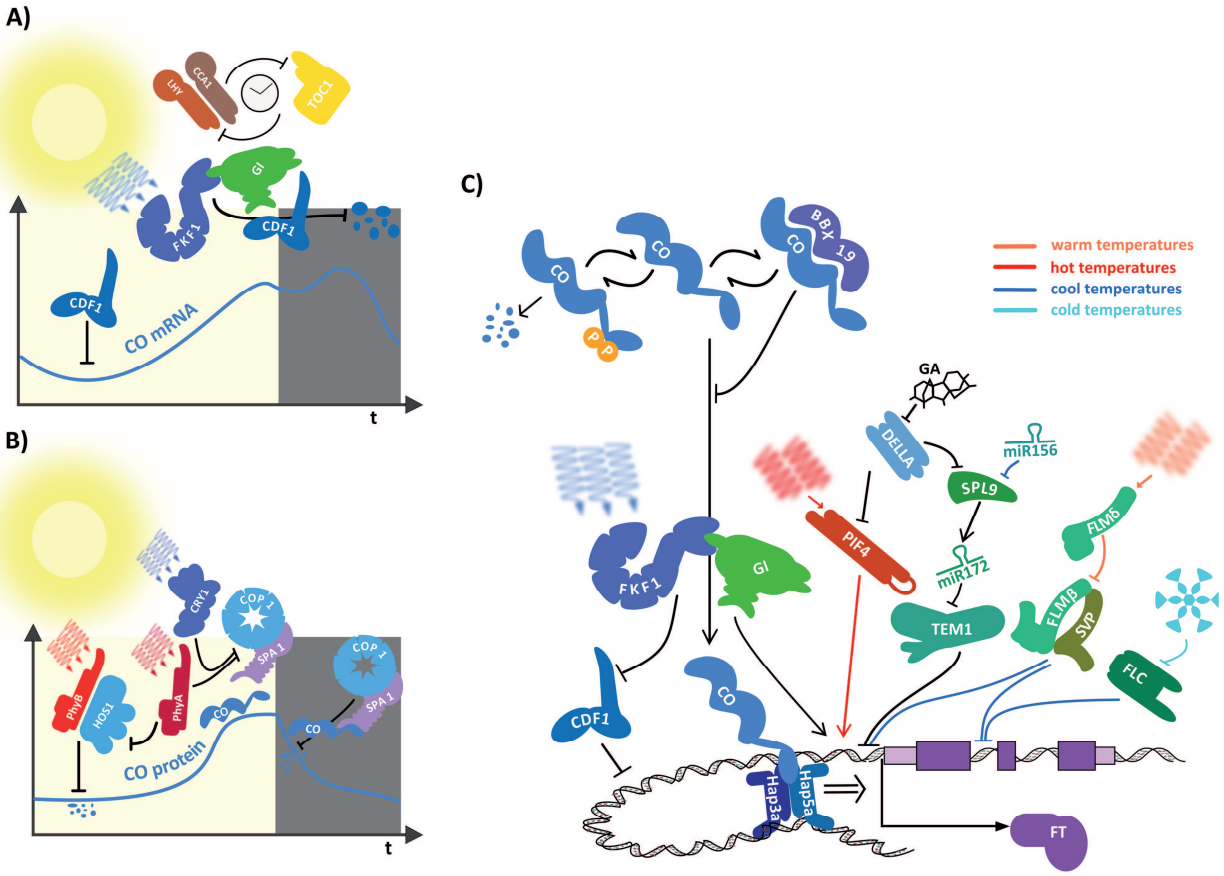


Figure 1.4
A) CONSTANS expression is suppressed via CDFs in the morning and increases when these are degraded by FKF1-GI during the day
B) CO protein is de-stabilized by activated PhyA and HOS1 in the morning and COP1-SPA1 in the dark. Activated PhyB and CRY1/2 inhibit COP1-SPA1 during the day, allowing CO accumulation in the afternoon of long days
C) CO is post-transcriptionally regulated by phosphorylation and interaction with BBX19. Interaction with Hap3a/5a at the *FT*-locus activates *FT* expression. Multiple other factors are involved in the regulation of *FT*. FLC and FLMß-SVP inhibit *FT* expression at cool temperatures (). FLMδ is predominant at warm (red arrows) temperatures, inhibiting SVP-dependent FT repression. FLC is repressed after vernalization. TEM1 and the other AP2-like repressors are targeted by miR172 in adult leaves. PIF4 induces *FT* expression at hot temperatures. FKF1-GI promotes *FT* expression by degrading CDF1 and actively. GA causes the degradation of DELLA-repressors, which has positive effects on PIF4 and miR172. The main activator of miR172, the SPLs, are targeted by miR156 in the cold and in young leaves
(Modified from Andrés and Coupland 2012; Song, Ito and Imaizumi 2013; Shim and Imaizumi 2015)

The discovery of the mechanisms by which red and blue light influence CO protein stability and the transition to flowering shows that not only the light duration but also the light quality is important for seasonal changes in flowering regulation; although the roles of those under environmental conditions are not yet well understood.

CO has recently been shown to be phosphorylated in the C-terminal region. The ratio of phosphorylated to unphosphorylated CO varies over the course of the day. Furthermore, phosphorylated CO is the preferred version of the protein targeted by SPA-COP1 and therefore the less stable one (Sarid-Krebs et al., 2015) .The discovery of this additional mechanism modifying CO stability shows that besides twenty years of research on

CONSTANS, the function and regulation of this essential genetic pathway integrator still offers many surprises and insight into how plants adapt to environmental stimuli.

Flowering locus T as a central hub during flowering time regulation

Early during the investigation of CO function it became clear, that it is a transcription factor which regulates merely the transcription of one single gene called Flowering Locus T (FT) (Putterill et al., 1995; Simon et al., 1996; Kardailsky et al., 1999; Yoo et al., 2005). CONSTANS is expressed in the phloem companion cells of the leaf and, when stabilized during long day, it causes the expression of *FT* in the same tissue. The FT protein is then transported via the phloem towards the shoot apical meristem (Corbesier et al., 2007; Kobayashi and Weigel, 2007; Tamaki et al., 2007; Turck et al., 2008). There it causes together with Flowering Locus D (FD) and 14-3-3 proteins the activation of *SOC1* and *APETALA1*. SOC1 as the primary activator of *LEAFY* expression and LEAFY together with APETALA1 is sufficient to mediate the transition of the shoot apical meristem to an inflorescence meristem (Amasino, 2010). As this decision ultimately defines the transition to flowering meristem and in a perennial plant like *Arabidopsis* also the fate of the whole organism, it explains why such a complex network of regulatory systems evolved to guarantee the initiation of flowering only under favorable conditions.

Research on the regulation of *FT* expression has in the past years broadened our understanding of how this important gene is regulated and has also revealed the high complexity of the regulatory networks involved. Binding sites for various regulatory factors have been identified within the *FT* genetic locus (Fig 1.3 C).
CO seems to be the prominent activator of *FT* transcription but there are also strong repressors involved in its regulation. The best understood of those is Flowering Locus C (FLC), a MADS-box transcription factor that interacts with the EMF1 polycomb repressive complex and directs it towards a binding site in the first intron of *FT*, thus causing repression of *FT* expression more or less independent of CO.

FLC expression is itself repressed by a polycomb complex in response to vernalization (Bastow et al., 2004; Heo and Sung, 2011; Marquardt et al., 2014). The expression of FLC is suppressed by the PRC2 mediated histone methylation during prolonged exposure to cold temperatures and therefore FLC integrates an additional environmental signal -the prolonged cold temperatures as an indication for winter- into the regulation of flowering (Searle and Coupland, 2004).

The mechanism of FLC function shows, how the *FT* gene serves as a integrational hub at which several regulatory networks involved in flowering time control submerge to enable flowering under beneficial environmental conditions.

CDF1 has recently been identified to influence *FT* expression not only via repression of *CO* expression in the morning but also by binding to the *FT* promoter and repressing its transcription. Its degradation via FKF1-GI in the afternoon is an additional regulatory level on which the circadian clock influences the transition to flowering (Song et al., 2012).

Further repressors of *FT* are the AP2-like proteins SMZ, SNZ and TOE1-3 which are regulated by the two antagonistic microRNAs miR172 and miR156. Those show alternating expression at different developmental ages and therefore prevent the transition to flowering in plants that are too young. This mechanism is not very dominant in *Arabidopsis* but plays an important role in perennial plants like the *Arabidopsis* relative *Arabis alpina* (Bergonzi et al., 2013). Involved in the age-dependent or autonomous flowering pathway are the SPL (Squamosa Promoter Binding Protein Like) proteins - repressed by miR156 in young leaves or buds - which promote the expression of miR172 in the leaves and important floral transition regulators in the shoot apical meristem (Wang et al., 2009; Wu et al., 2009). MiR172 directly targets the previously mentioned AP2-like repressors and so promotes the expression of *FT*.

The regulation of flowering *via* the activity of *FT* is a central feature that can be found in many plant species (Golembeski et al., 2014). However, it is not the only key regulator that integrates environmental signals. FLC for examples is not only regulating *FT* expression in leaves but also represses the activation of downstream targets of *FT* in the apical meristem and therefore the potency of a plant to flower (Searle et al., 2006; Andres and Coupland, 2012).

Temperatures are affecting flowering via different pathways. FLC responds to cold winter temperatures and regulation via miR172/156, SVP/SMZ and the TEM1/TOE1/2 proteins is influence *FT* expression and activity at cool temperatures. MiR172 targets the mRNA of the AP2-like repressors SVP/SMZ/TEM1/TOE1/2 and is itself regulated in a temperature-dependent manner (Cho et al., 2012; Jung et al., 2012). Warm and high temperatures are known to induce flowering. The induction of *FT* due to high temperatures is mediated by PIF4 and temperature-dependent histone modifications at the *FT* locus are important for this induction (Wigge et al., 2005; Kumar et al., 2012). PIF4 induces flowering mainly under high temperatures, whereas the induction at warm temperatures seems to be more dependent on GA signaling and factors involved in this process (Galvao et al., 2015).

An interesting further regulator is the previously already described FLM, protein that forms a complex with SVP and represses flowering in this manner (Scortecci et al., 2001). Different splice variants of this protein exist and a non-functional protein is made from a splice variant that is prevalent in warmer temperatures. The non-functional protein can still interact with SVP and inhibit it from binding to *FT* and other floral regulators and in this way promotes flowering under the inductive temperatures.
The regulation of flower initiation at the shoot apical meristem offers many further possibilities to control this process, and several of the key components are regulates by factors known to influence flowering. This level of regulation is downstream of the regulation of *FT* and can therefore induce or repress flowering even when the photoperiodic pathway and the circadian clock induce *FT* expression via CONSTANS.

CO as the main activator of *FT*

The so far described mechanisms of *FT* regulation are more or less independent of CO activity but they give an impression of the additional regulatory networks which influence *FT* expression via different mechanisms.

CO is a transcription factor that binds at the *FT* promoter and promotes the recruitment of the transcriptional machinery, allowing the transcription of the *FT* gene (Wigge et al., 2005).
Proximal to the transcription start site of *FT,* several CORE motifs -for CO responsive element- have been identified with which CO associates *in vitro*. These sites are evolutionary conserved and seem to be important for the activation of *FT* by CO (Adrian et al., 2010; Tiwari et al., 2010) However, CO possesses relatively poor DNA binding capabilities compared to other transcription factors (Tiwari et al., 2010). Additional factors with which CO interacts might influence its binding affinity and activity at the *FT* promoter. Via its C-terminal CCT-domain CO interacts with HAP3a and HAP5a, factors that are part of the HAP complex which is known to bind CCAAT elements in eukaryotic promoters and activate transcription (Edwards et al., 1998). This interaction is important for the activation of *FT*, as the HAP complex binds towards a region in the distal *FT* promoter (Cao et al., 2014). The interaction between the HAP complex and CO causes the formation of a loop between the distal and the proximal regions of the FT promoter and this structure may have a stabilizing effect on the binding of CO to the COREs and therefore promote *FT* expression. The stabilization of CO at the right region of the *FT* promoter seems to be an important step in the regulation of its expression.

B-Box protein in the regulation of flowering

Other B-Box proteins have been shown to influence flowering behavior of plants, although they do that to a much smaller extent than CO or in different ways. The closest homologues of CO among the BBX-family are COL1 and COL2, both showing a high degree of similarity especially in the B-Boxes and the CCT-domain (Ledger et al., 2001). However, the overexpression of these two proteins in *Arabidopsis* wild type plants has no detectable effect on the transition to flowering (Ledger et al., 2001) and causes only a slightly earlier flowering in a *co* mutant background (Kim et al., 2013). Domain swaps between CO and COL1/2 showed, that the slight variations in the B-Box are responsible for these different effects (Kim et al., 2013).

COL3, another B-Box protein with high similarity towards CO, affects flowering in an opposite manner to CO. The *col3* mutant flowers earlier under short and long days but as COL3 has been identified as a modulator of COP1 and DET1 during photomorphogenesis, the flowering time phenotype of *col3* indicates that COL3 influences the function of COP1 during light signaling rather than to regulate the floral transition (Datta et al., 2006).

COL5, another group I B-Box protein, promotes flowering under short days or in a *co* mutant background, but a loss of function has no effect on flowering (Hassidim et al., 2009).

From the structure group II proteins, which contain a different second B-Box domain and a C-terminal CCT domain, COL9 is the only member known to affect flowering. Overexpression causes late flowering under long day conditions, apparently by reducing CO expression (Cheng and Wang, 2005). A slightly earlier flowering of the *col9* mutant supports this idea.

The group III proteins are not known to influence CO-activity or flowering in general. Several of them respond in their expression to abiotic stresses like drought, heat or cold but little is known about the function of these proteins in plants (Gangappa and Botto, 2014).

STO and its homologues STH1, 2 and 3 have two B-box domains but lack the CCT-domain and are therefore classified as group IV B-Box proteins. Some members of this group have been shown to influence the flowering behavior when overexpressed or mutated (Sarmiento, 2013).

BBX19 is a member of the group IV of the B-Box protein family. Originally described as a regulator of photomorphogenesis and hypocotyl elongation in response to the circadian clock it was recently shown that BBX19 can also influence the activity of CO. Overexpression of BBX19 causes late flowering in *Arabidopsis* and reduced BBX19 expression causes plants to flower slightly earlier then the wild type (Wang et al., 2014a). The effect on flowering can be linked to BBX19's ability to interact with CO *via* its first B-Box domain, resulting in a reduction of *FT* expression. Like *CO* and many other B-Box proteins *BBX19* is expressed in a diurnal manner

with a peak of expression in the morning, where *CO* expression is detectable but *FT* is not expressed. This finding suggests a further mechanism of CO activity regulation by another B-Box protein which interacts with CO and prevents it from activating FT expression. However, more recent findings imply a more prominent role for BBX19 as an integrator of the circadian clock signals into PIF4/5 dependent hypocotyl elongation (Wang et al., 2015).

STO has been shown to promote flowering by increasing *FT* and *SOC1* expression and reducing *FLC* expression, all independent from CO (Li et al., 2014). The role of STO as a regulator of flowering has just emerged and as STO and its relatives are also regulators of photomorphogenesis (Indorf et al., 2007; Chang et al., 2008; Gangappa et al., 2013b; Gangappa et al., 2013a) by interacting with COP1 and modulating its function, the STO-like proteins might be better characterized as general regulators of light-affected developmental processes.
Last BBX32, a member of group V with only one B-Box domain and no further annotated domains, causes slightly later flowering when overexpressed in Arabidopsis. BBX32 is linked to the regulation of flowering via EMF1, with which it interacts and the interaction is necessary for its flower-delaying function (Park et al., 2011). However, BBX32 was also described as a modulator of STO/STH function in photomorphogenesis and its main role seems to lie in this process (Holtan et al., 2011a; Gangappa and Botto, 2014).
Interaction and regulation via COP1 seems to be a common feature among B-Box proteins. Many share a VP-rich region close to their CCT-domain or the C-terminus, the COP1-SPA complex most likely interacts via this region (Holm et al., 2001; Crocco and Botto, 2013). The BBX-SPA-COP1 module might be the plant's equivalent of the RBBCC/TRIM containing proteins, only that in plants the different domains are found in separate proteins (Gangappa and Botto, 2014).

Taken together, BBX19 is the only B-Box protein with a strong effect on CO activity described so far and the roles of other B-Box proteins besides CO in flowering time regulation seem to be minor (Gangappa and Botto, 2014). This is surprising, considering the high sequence similarity that the proteins share among each other, especially in the group I. As the flowering time effects for most other B-Box proteins where minor or observed only in *co* mutants or rather under conditions where CO is not active, CO can be considered as the major B-Box protein regulating the floral transition in the *brassicacea* family (Simon et al., 2015).

Findings and outlook

We identified the two other B-Box family proteins BBX30 and BBX31, subsequently named miP1a and miP1b, in a computational screen for new potential microProteins in *Arabidopsis*. Their overexpression causes a late flowering phenotype, similar to *co* loss of function mutant plants and we showed that these two B-BOX proteins can directly interact with CO *in vitro* and *in vivo*. They affect CO in its ability to activate *FT* expression and qualify therefore as microProteins in the strict sense (Eguen et al., 2015). We analyzed their temporal and spatial expression and found them to be expressed predominantly in the vasculature in a diurnal manner. Further investigation of their structural features revealed a C-terminal motif of five amino acids, conserved strongly among orthologs of miP1a and miP1b, which mediates interaction with members of the TOPLESS and TOPLESS-RELATED transcriptional co-repressor family. The flowering repressive function of the microProteins is depending on the C-terminal TPL-interaction motif and as miP1a can interact with CO and TPL/TPR proteins simultaneously, we assume that miP1a and miP1b are not passive repressors of CO activity but engage it in a trimeric complex that actively represses *FT* expression and therefore the floral transition.

2. MATERIALS AND METHODS

MATERIALS

General Materials

The chemicals and materials used for the work in this thesis were mainly purchased from Carl Roth, Sigma Aldrich, Applichem, GE Healthcare, Duchefa unless otherwise stated.
Sanger sequencing was performed by Macrogen.
DNA oligomers were synthesized by TAG Copenhagen A/S

Kits

Name	Manufacturer	Catalogue Nr.
GeneJET Plasmid Miniprep Kit	ThermoScientific	K0503
GeneJET Plant RNA extraction Kit	ThermoScientific	K0802
pENTR-SD-TOPO	Invitrogen	K2420-20

Enzymes

Name	Manufacturer	Catalogue Nr.
BP-clonase	Invitrogen	11789-100
BsrGI	New England Biolabs	R3575
DNase I	Applichem	A3778,0100
DNase I	ThermoScientific	EN0525
KAPA SYBR Fast qPCR Mastermix	Kappa Biosystems	KK4608
LR-clonase	Invitrogen	11791-100
Phusion-DNA polymerase	New England Biolabs	M0491S
Proteinase K	Sigma	P8044
Revertaid	ThermoScientific	EP0441
RiboLock	ThermoScientific	EO0382
Taq-polymerase	home made	

Antibodies

Name	Manufacturer	Catalogue Nr.
αFLAG	Sigma Aldrich	F3165
αGST-HRP	GE Healthcare	RPN1236
αHIS Tetra	Qiagen	34670
αHIS-HRP	Sigma Aldrich	A7058-1VL
αMBP-HRP	New England Biolabs	E8038S
αMouse-HRP	Sigma Aldrich	A9044

Vectors

Name	Purpose		Resistance	Source
pDEST15	N-terminal GST-tag	E.coli protein expression	Amp	Addgene
pDEST17	N-terminal 6xHIS-tag	E.coli protein expression	Amp	Addgene
pDONR201	Gateway™ Entry vector		Kan	Invitrogen
pDONR207	Gateway™ Entry vector		Gent	Invitrogen
pDRf1-GW	lac-promoter	Yeast protein expression	Amp	Addgene
pEarlyGate100	p35S expression	Plant expression	Kan	(Earley et al., 2006)
pEarlyGate104	p35S::mCherry-tag	Plant expression	Kan	(Earley et al., 2006)
pENTR-SD-TOPO	Gateway™ Entry vector		Kan	Invitrogen
pGADGWT7	N-terminal GAL4-AD	Yeast two hybrid	Amp	Addgene
pGBKGWT7	N-terminal GAL4-DBD	Yeast two hybrid	Kan	Addgene
pJAN33	p35S::FLAG-tag	Plant expression	Amp	(Wenkel et al., 2006)
pK7FWG2	p35S::GFP-tag	Plant expression	Spec	(Karimi et al., 2002)
pMAL2c	N-terminal MBP-tag	E.coli protein expression	Amp	Addgene
pMDC162	GUS-vector	promoter activity studies in plants	Kan	(Curtis and Grossniklaus, 2003)
pMDC32	p35S expression	Plant expression	Kan	(Curtis and Grossniklaus, 2003)
pSUC2	expression from SUC2 promoter	Plant expression	Spec	(Wenkel et al., 2006)
pTNT	T7-promoter	E.coli protein expression	Amp	Promega
pZEO	Gateway™ Entry vector		Zeo	Invitrogen

Antibiotics

Name	Abbreviation	Concentration
Ampicillin	Amp	100 µg/mL
Carbenicillin	Carb	50 µg/mL
Kanamycin	Kan	50 µg/mL
Streptomycin	Strep	50 µg/mL
Spectinomycin	Spec	100 µg/mL
Rifampicin	Rif	50 µg/mL
Chloramphenicol	Cam	34 µg/mL

Antibiotics - continued

Name	Abbreviation	Concentration
Gentamycin	Gent	30 µg/mL
Hygromycin B	Hyg	30 µg/mL

Bacteria and Yeast strains

Organism	**Name**	**Application**	**Resistance**
E.coli	TOP10	Cloning	Strep
E.coli	BL21 Rosetta 2	Protein Expression	Cam
E.coli	DB3.1	ccdB vector amplification	Strep
Agrobacterium tumefaciens	GV3101 pMP90	Plant transformation	Rif, Gent
Agrobacterium tumefaciens	GV3101 pMP90 RK	Plant transformation	Rif, Gent, Kan
Saccharomyces cerevisiae	Matchmaker Gold	Yeast-two hybrid	Ura^+
Saccharomyces cerevisiae	YM2471a	Yeast mating type A	-
Saccharomyces cerevisiae	PJ69-4α	Yeast mating type α	-

Oligonucleotides

Cloning

Name	5‘-3‘ Sequence	AGI code
COF	ggggacaagtttgtacaaaaaagcaggctgcATGTTGAAACAAGAGAGTAACG	At5g15840.1
COR	ggggaccactttgtacaagaaagctgggtcGAATGAAGGAACAATCCCAT	
COSBBr	ggggaccactttgtacaagaaagctgggtcTCACCCTGCTGCGTTATGGG	At5g15840.2
miP1b F	ggggacaagtttgtacaaaaaagcaggctgcATGTGTAGAGGGTTTGAGAA	At4g15248.1
miP1b R	ggggaccactttgtacaagaaagctgggtcTCAGAGAAACACAAAGGGAA	
miP1a F	ggggacaagtttgtacaaaaaagcaggctgcATGTGTAGAGGCTTGAATAA	At3g21890.1
miP1a R	ggggaccactttgtacaagaaagctgggtcTCAGAGAAAAACAAACGGAAC	
miP1a R -PFVFL	TCAAACCTCATGATTATCTTGTT	
miP1b R -PFVFL	TCACATAGTAGTGATCACAAAATT	
pmiP1af	ggggacaagtttgtacaaaaaagcaggctgctgtagagaaatgtcgtgggtttt	
pmiP1ar	ggggaccactttgtacaagaaagctgggtctgaggaaagaagatttgggaat	
pmiP1bf	ggggacaagtttgtacaaaaaagcaggctgcgaacctataaagaatatttctcgaatg	
pmiP1br	ggggaccactttgtacaagaaagctgggtctctttctttgtctctcttgtgttca	
TPLf	CACCATGTCTTCTCTTAGTAGA	At1g15750.1
TPLr	TCTCTGAGGCTGATCAGATGCA	
STO F	ggggacaagtttgtacaaaaaagcaggctgcATGAAGATACAGTGTGATGT	At1g06040.1
STO_BBX R	ggggaccactttgtacaagaaagctgggtcTTACATATAGTTGAGGTCAGAGC	
COL9 F	ggggacaagtttgtacaaaaaagcaggctgcATGGGTTACATGTGTGACTT	At3g07650.1
COL9_BBX R	ggggaccactttgtacaagaaagctgggtcTCAAATGGAAGCGAGTTCTGAG	
COL16 F	ggggacaagtttgtacaaaaaagcaggctgcATGATGAAAAGTTTGGCGAA	At1g25440.1
COL16_BBX R	ggggaccactttgtacaagaaagctgggtcTCAGTGGTTGCTATGCTTTA	

Cloning - continued

Name	5‘-3‘ Sequence	AGI code
miR173ts BP F	ggggacaagtttgtacaaaaaagcaggctgcGTGATTTTTCTCTACAAGCGAA	
MIGS miP1a 1F	GTGATTTTTCTCTACAAGCGAAATGTGTAGAGGCTTGAATAA	
MIGS miP1b 1F	GTGATTTTTCTCTACAAGCGAAATGTGTAGAGGGTTTGAGAA	
MIGS C-miP1a/b 1F	GTGATTTTTCTCTACAAGCGAAAATTTTCTAGCTNGGAGACA	
TPR1 BP 1F	ggggacaagtttgtacaaaaaagcaggctgcATGTCTTCTCTGAGCAGAGA	At1G80490.1
TPR1 BP 1R	ggggaccactttgtacaagaaagctgggtcTCATCTCTGAGGCTGGTCAG	
TPR2 BP 1F	ggggacaagtttgtacaaaaaagcaggctgcATGTCGTCTTTGAGCAGAGA	At1G04130.1
TPR2 BP 1R	ggggaccactttgtacaagaaagctgggtcTTACCTTTGAATCTGATCCG	
TPR3 BP 1F	ggggacaagtttgtacaaaaaagcaggctgcATGTCGTCGTTGAGTCGAGA	At5G27030.1
TPR3 BP 1R	ggggaccactttgtacaagaaagctgggtcTCATCTTTGTAACTGTTCTG	
TPR4 BP 1F	ggggacaagtttgtacaaaaaagcaggctgcATGTCGTCACTCAGCAGAGA	At3G15880.1
TPR4 BP 1R	ggggaccactttgtacaagaaagctgggtcCTACGAATCACTCGGTTGTT	

qPCR

Name	5'-3' Sequence	AGI code
At2g26400f	TTTGGACAAACTTGCAGAGC	At2g26400.1
At2g26400r	CGAGGCAGTAACGGATCTCT	
At3g49340f	CGAGGAATTTAAGGCGAGGT	At3g49340.1
At3g49340r	ATCTTCGTCATGCCTTCCAC	
FULf	GGTCATTTCAGGGTTGTCGT	At5g60910.1
FULr	CGAAGAGTTTGCCTTTGGAA	
MAF5f	TTCAGGATCTCCGACCAGTT	At5g65080.1/2
MAF5r	GACGGAGGATCCACAGAGAA	
qCO.1 1R	GGATGAAATGTATGCGTTATGG	At5g15840.1
qCO.1/2 1F	AAACTGCAGCGTACCACAGA	At5g15840.1/2
qCO.2 1R	CTGCTGCGTTATGGGAAGAT	At5g15840.2
qFTf	CAGGAATTCATCGTGTCGTG	At1g65480.1
qFTr	AGCCACTCTCCCTCTGACAA	
qGI 1F	GCTTGTGGAACTCCTTCGAG	At1g22770.1
qGI 1R	TTCAATGGTTGCTTCTGCTG	
qmiP1a 1F	GCAGAAGAAGTGACGGAGGA	At3g21890.1
qmiP1a 5'UTR F	TTTCCTCAATATCACCCAGAAGA	
qmiP1a R	CGCGTGAGTTTCTGACAAGA	
qmiP1a* 1R	GAGTTTCTGGGCAGAAGTGG	
qmiP1b 1R	TGCTATCATCCTTATCTCCGGT	At4g15248.1
qmiP1b 5'UTR F	GAACACAAGAGAGACAAAGAAAGAG	
qmiP1b R	ACGAGTTAGCTTCCGACAGG	
QQSf	TTTCGATCTGTCAGCCATTG	At3g30720.1
QQSr	CTGGTCGCTGTGGAGAAAAT	
ZAT7f	GGGAGATGAACGTGTTTTCC	At3g46090.1
ZAT7r	TCTCCTCATGTGACCACCAA	

Genotyping

Name	5'-3' Sequence
attB1 F	CAAGTTTGTACAAAAAAGCaggc
attB2 R	ACCACTTTGTACAAGAAAGCTggt
attR1 R	CAAAAAAGCTGAACGAGAAACG
attR2 F	ACAAGAAAGCTGAACGAGAAAC
CO 4F	AGAGAACAACAGGGCACGAC
FLAG F	GACTACAAGGATGACGATGACAAG
gi-2 mut 1F	CGCATTTTGACTCATTACAATTTAT
gi-2 WT 1F	CTCATTACAACCGTCCCATTT
GUS 1F	TTTCACGGGTTGGGGTTTCT
HygR F	GTGCTTGACATTGGGGAGTT
HygR R	GATGTTGGCGACCTCGTATT
M13_rev_(-29)	caggaaacagctatgac
M13_uni_(-21)	tgtaaaacgacggccagt
MIGS F	GTGATTTTTCTCTACAAGCGAA
p35S2	cgcacaatcccactatcctt
pCSA110 LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
pSUC2 1F	cactcgctcggatcgaaat
qamiR 1F	GTTTTCCCAGTCACGACGTT
qamiR 1R	TCACGACCTGTGAACAAAGC
qeGFP 1F	CACATGAAGCAGCACGACTT
qeGFP 1R	AGTTCACCTTGATGCCGTTC
qmCherry 1F	CCTGTCCCCTCAGTTCATGT
qmCherry 1R	CCCATGGTCTTCTTCTGCAT
SelA	TCGCGTTAACGCTAGCATGGATCTC
SelB	GTAACATCAGAGATTTTGAGACAC
T7 uni F	TAATACGACTCACTATAGGG

Additional Materials

Name	Manufacturer	Catalogue Nr.
Amylose beads	New England Biolabs	E8035S
Criterion™ TGX Stain-FREE™ precast protein gels	Biorad	5678114
DNA size marker	Invitrogen	10787-026
Gel filtration columns	emp Biotech	CP-0110
GelRed	Biotium	#41003
GSH-beads	Promega	V8600
Ni-NTA agarose	Machery nagel	745400
Protein size marker	Biorad	161-0363

Additional Materials - continued

Name	Manufacturer	Catalogue Nr.
Sigma protease inhibitor	Sigma	P9599
Super Signal West Extended Duration substrate	ThermoScientific	34076

Software

Name	**Application**
Biorad CFX manager	qPCR analysis
Biorad Image Lab	GelDoc Image analysis
CLC Main Workbench 7	DNA and Protein sequence analysis
Endnote X7	Citation management
GIMP 2	Image processing
Inkscape	Ilustrations
MEGA 6	DNA and amino acid sequence alignments
Microsoft Excel	Calculations
RobiNA	RNAseq data analysis

METHODS

Gel electrophoresis

DNA and RNA electrophoresis

DNA and RNA used for this thesis were tested in an agarose gel electrophoresis. Therefore a 1% - 2% agarose gel was prepared with 1x TAE-buffer (40 mM Tris, 1 mM EDTA, 20 mM Acetate). The DNA or RNA samples mixed 1:5 with a loading buffer containing 0.02% GelRed and loaded into the pockets of the agarose gel. The fragments were separated at 80V – 150 V and the gels photographed under UV (405 nm) illumination. To estimate the size of the DNA/RNA fragments 5 µL of the DNA size marker were run together with the samples.

Protein electrophoresis and membrane transfer

Protein samples were mixed 1:5 with protein loading buffer (100 mM Tris pH 6.8, 5 M Urea, 10% SDS, 25% Glycerol, 50 mM DTT, 0.1% Bromphenol blue) and separated on a precast stainfree SDS-Polyacrylamid gel in the appropriate running buffer (Laemmli, 1970). The gels were photo-activated with the Biorad GelDoc system, according to the manufacturer's recommendations. Proteins were transferred from the gels on a Nitrocellulose membrane using a semi-dry blotting with the appropriate buffer (Towbin et al., 1979) for 10 min at 2.5 A. Afterwards the protein transfer was controlled using the GelDoc imaging system.

PCR

Taq-polymerase PCR

For genotyping and primer testing the following PCR-reaction was prepared

2x Taq MM: 20 mM Tris pH 8.3, 3.5 mM $MgCl_2$, 100 mM KCl, 2 mM dNTPs, 0.1 µL Taq/µL, 0.6% Sucrose, 0,2% Orange G

PCR reaction	(12 µL)
6µL	2x Taq-MM
0.25 µL	10 µM P1
0.25 µL	10 µM P2
1 µL	template DNA
4.5 µL	water

The PCRs were performed in a Sensoquest Labcycler with initial heating to 95°C for 1 min. The thermal cycle was composed of 10 sec at 95°C, 10 sec at the respective annealing temperature of the primer (50°C- 60°C) and 60 sec per kbp of the expected fragment at 72°C.This cycle was repeated 30-40 times, followed by a final extension at 72°C for 2 min.

Phusion-polymerase PCR

For amplification of DNA fragments for cloning purposes

PCR reaction	(50 µL)
10 µL	5x HF-buffer
2.5 µL	10 µM P1
2.5 µL	10 µM P2
1 µL	10 mM dNTPs
0,5 µL	Q5 polymerase
1-5 µL	template DNA
(1 µL	DMSO)
32.5-37.5 µL	water

DMSO was added if the PCR reactions have proven themselves to be complicated to increase the binding affinity of the used primers. The PCR reactions were performed in a Sensoquest Labcycler with initial heating to 98°C for 1 min. The thermal cycle was composed of 10 sec at 98°C, 10 sec at the respective annealing temperature of the primer (50°C- 60°C) and 60 sec per kbp of the expected fragment at 72°C.This cycle was repeated 35-40 times, followed by a final extension at 72°C for 5 min.

Bacterial work

Cloning and genotyping

DNA amplification and purification

Primers to amplify the desired DNA fragment were designed and the PCRs with a gradient in the annealing temperature were performed to fin the optimal annealing temperature. A Phusion PCR was prepared and run at the respective PCR parameters. The 50 µL PCR was mixed with 150 µL TE-buffer (10 mM Tris pH 8) and 100 µL 30% PEG8000/30 mM $MgCl_2$. The mix was centrifuged for 15 min at 20,000 *g* to pellet DNA larger than 200 bp. The pellet was washed with 70% EtOH, dried and solubilized in 15 – 30 µL water. The DNA concentration

was determined using a Nanodrop spectrophotometer and 100 – 500 ng of the DNA were separated and analyzed on a agarose gel.

BP and TOPO cloning into Gateway™ entry vectors

50 to 200 ng of the PCR fragment were cloned either in the pENTR-SD vector, using the pENTR-SD-TOPO cloning kit or into the pDONR or pZEO entry vectors using a BP reaction. The cloning into the pENTR-SD vector was performed according to the manufacturer's manual, in a final volume of 2 µL instead of the described 20 µL. For the BP-cloning, the following reaction was prepared

BP-cloning reaction (2 µL)
Up to 1.4 µL PCR fragment
0.2 µL pDONR/ZEO vector (100 – 200 ng/µL)
Water to 1.6 µL
0.4 µL BP-clonase

The reaction was incubated at room temperature for 4 to 16 h and stopped with the addition of 0.4 µL Proteinase K and incubation at 37°C for 10 min.

Chemically competent *E.coli* cells were transformed with either the TOPO- or the BP-cloning reaction.

Transformation of chemically competent *E.coli*

Competent cells, stored at -80°C were thawed on ice and mixed with 1 µL plasmid or cloning reactions. The mix was incubated on ice for 30 min, heated to 42°C for 45 sec and chilled on ice for 2 min. 500 µL SOC -medium (2% Tryptone, 0.5% Yeast extract, 10 mM NaCl, 20 mM Glucose, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) were added and the cells incubated at 37°C for 60 min, pelleted and plated on LB-agar (1% Trypton, 0.5 % yeast extract, 0.5 % NaCl, 1% agar) with the appropriate antibiotics to select for the respective plasmid. The plates were incubated at 37°C overnight. Colonies were picked using a sterile pipette tip to inoculate 5 mL of liquid LB (1% Trypton, 0.5 % yeast extract, 0.5 % NaCl) with the respective antibiotics. The cultures were incubated overnight in a 37°C shaker at 200 rpm. 4 mL of the cultures were pelleted and used to isolate plasmids.

Bacterial DNA isolation

Plasmids from *E.coli* or *Agrobacteria* were prepared using the GeneJET Miniprep kit according to the manufacturer's recommendations. The DNA was eluted from the columns using 50 µL of water and the concentration was determined with a Nanodrop spectrophotometer.

Genotyping of isolated plasmids

To verify correct DNA fragment insertion into a Gateway™ vector, 1 µg of isolated plasmid was digested with BsrGI.

BsrGI digestion reaction (10 µL)
1 µL NEB CutSmart buffer
0.2 µL BsrGI-HF
1 µg Plasmid DNA
Water to 10 µL

The digestion was incubated at 37°C for 60 min and analyze on an agarose gel.
The sequence of plasmids with correct digestions patterns were analyzed with Sanger sequencing (Sanger et al., 1977)

PCR genotyping and colony PCR

Alternatively to genotyping by BsrGI-digestion, the correct assembly of the vectors was analyzed using PCR. Plasmids were therefore diluted to a concentration of 5 ng/µL and 1 µL of this dilution was used in a PCR with a vector specific forward primer, e.g. M13 F for the pENTR vector, SelA for the pDONR vectors or p35S2 for p35S-plant vectors, and a gene-specific reverse primer. Appropriate conditions for the PCR were used and afterwards analyzed with agarose gel electrophoresis. To analyze bacterial colonies for the correct plasmids, colonies were picked and diluted in 10 µL water. 1 µL of bacterial solution was used in a PCR with a plasmid specific forward and a gene specific reverse primer. The initial heating at 95°C was for a colony-PCR extended to 5 min, followed by the normal cycling steps at the respective temperatures. The fragments were, as priory mentioned, separated and analyzed on an agarose gel.

LR reaction into a Gateway™ destination vector

To create a Gateway™ destination vector, an entry vector containing the respective gene or DNA-fragment of interest was used. The entry vector and the desired destination vector were used in a molar ration of 3:1.

LR reaction (1.5 µL)
Entry vector up to 0.8 µL
0.4 µL destination vector (150 ng/µL)
0.3 µL LR clonase

The reaction was incubated for 4 to 16 h at room temperature. 0.3 µL Proteinase K were used to stop the reaction at 37°C for 10 min. Chemically competent *E.coli* were transformed with the terminated LR reaction.

Recombinant protein preparation

For expression of recombinant protein in *E.coli*, a gene cloned from *Arabidopsis* cDNA into an entry vector was recombined into bacterial protein expression vector in a LR reaction. The desired vectors containing the gene of interested with or without a protein tag and under the expressional control of the T7-promoter were used to transform chemically competent BL21 *E.coli* cells. Correct plasmid uptake was verified with colony PCR.

Three colonies harboring the correct plasmid were used to inoculate 5mL LB with the appropriate antibiotics to create an overnight culture. With 300 µL from the overnight cultures 30 mL TB medium (1.2 % Peptone, 2.4% Yeast extract, 0.4% Glycerol, 100 mM KPO_4-buffer pH7.5) + antibiotics were inoculated. The cultures were grown to an OD_{600nm} of 0.5 and protein expression was induced with the addition of 1 M IPTG to a final concentration of 0.1 – 1 mM. The cultures were incubated at 28°C at 200 rpm. 2 mL of the culture were pelleted before and every 2 h after the addition of IPTG as induction control. The pelleted cells were frozen at -80°C. Later the pellets were dissolved in 100 – 200 µL of protein loading buffer, boiled at 95°C for 10 min and used samples were separated and analyzed using SDS-PAGE.

The determined optimal incubation time and conditions were used to grow and incubate a 500 mL culture. The cells were pelleted, dissolved in 5 mL PBS buffer (500 mM NaCl, 3 mM KCl, 80 mM Na_2HPO_4, 17 mM KH_2PO_4) and 1 mL samples were frozen at -80°C.

Cell lysis and protein purification

For lysis the bacterial cells were thawed and pelleted again. The pellet was dissolved in 500 µL lysis buffer (20 mM Tris pH 7.7, 150 mM NaCl, 0.1% Tween-20, 1 mM DTT, 1 mg/mL Lysozym, 0.1 mg/mL DNaseI, 1% Sigma plant protease inhibitor cocktail). The pellet was sonicated 4x for 15 sec at 10% intensity while kept on ice to prevent overheating of the lysate. Afterwards the cells were incubated 30 min at room temperature.

For purification of MBP- or GST-tagged proteins, the lysate was incubated with either Amylose- (for MBP-tagged proteins) or GSH-(for GST-tagged proteins) magnetic beads at 4°C for 2 to 6h while rotating. The beads were prepared for usage according to the manufacturers recommendations. After the incubation the beads were captured in a magnetic rack and washed 3 times with washing buffer (20 mM Tris pH 7.7, 150 mM NaCl, 0.1% Tween-20, 1 mM DTT, 1% Sigma plant protease inhibitor cocktail). Protein was eluted from the beads

with 2x beads volume of the appropriate elution buffer, either containing 50 mM Maltose or 50 mM GST-peptide. Glycerol to a final concentration of 20% was added to the eluted protein and stored at -80°C for further use.

Proteins with a 6xHIS-tag were purified using Ni-NTA-agarose. A bed volume of 200 µL Ni-NTA agarose was washed 3x with 3 bed volumes of NPI10 buffer. The bacterial lysate was pelleted by centrifugation for 5 min at 10,000 g, the supernatant was added to the Ni-NTA agarose and incubated for 4h at 4°C while rotating. The solution was loaded on a filter column with a pore size of 0.2 µm and centrifuged for 1 min at 5,000 *g*. The Ni-NTA agarose remains in the column while the solution passes the filter. The agarose was three times washed with 600 µL NPI20. HIS-tagged protein was eluted with 2x 100 µL of NPI250. Imidazole that might interfere with the further reactions was removed from the protein solution using desalting columns. The proteins were eluted from those with 250 µL protein storage buffer (20 mM Tris pH 7.7, 150 mM NaCl, 0.1% Tween-20, 1 mM DTT, 20% glycerol, 1% Sigma plant protease inhibitor cocktail)

***In vitro* pull down assays**

To analyze protein-protein binding *in vitro*, bacterial lysates or purified proteins were mixed in equal volumes. The volume of the mixture was adjusted to 800 µL using binding buffer (20 mM Tris pH 7.7, 150 mM NaCl, 0.1% Tween-20, 1 mM DTT, 1 mM EDTA, 1% Sigma plant protease inhibitor cocktail).50 µL were taken as input control sample. 200µL Amylose- or GSH-beads, depending on the respective protein tags, were prepared according to the manufacturers recommendations and taken up in 200 µL binding buffer. The beads were added to the protein lysates and incubated at 4°C for 4h while rotating. The beads were captured in a magnetic rack, washed three times with binding buffer. Proteins were eluted from the beads using 2 x 75 µL binding buffer with 50 mM Maltose or GST, respectively. Afterwards residual protein was eluted from the beads by incubating 5 min at 65°C with 50 µL protein loading buffer. Input and elution sample were separated in a SDS-PAGE, blotted on nitrocellulose and an immune-detection with the respective antibodies was performed.

Protein detection

For immune-detection of proteins after blotting onto a nitrocellulose membrane, the membrane was washed with PBS buffer and incubated in a 5% solution of non-fat dry milk in PBS (or 3% BSA in PBS for HIS detection) for 1h at room temperature. Afterwards the antibody solution was added.

Antibody	concentration	Solution
αMBP-HRP	1:8,000	5% milk powder in PBS
αGST-HRP	1:5,000	5% milk powder in PBS
αHIS-HRP	1:10,000	3% BSA in PBS
$\alpha FLAG_{mouse}$	1:5,000	5% milk powder in PBS
αmouse-HRP	1:5,000	5% milk powder in PBS
αGFP_{rabbit}	1:5,000	5% milk powder in PBS
αrabbit-HRP	1:8,000	5% milk powder in PBS

The blot was incubated in the antibody solution for 4-16h at 4°C and afterwards washed three times for 5-10 min with PBS. If incubation with a secondary was necessary, the blot was incubated for 2 to 4h at 4°C with the appropriate secondary antibody.

For detection of HRP-bound antibodies on the blot, the membrane was placed in the GelDoc system and photographed to detect the protein blotted on the membrane. Afterwards 800 µL of a prepared SuperSignal West substrate were evenly distributed on the membrane. Luminescence signals were detected in the GelDoc system with the appropriate exposure settings and photos taken for later analysis.

Yeast work

Transformation

For yeast (*Saccharomyces cerevisiae*) transformation, a colony of a freshly grown strain was used to inoculate 5 mL YPD (2% Peptone, 1% Yeast extract, 2% Glucose) or appropriate SD media and incubated overnight while shaking at 28°C at 200 rpm. This overnight culture was used to inoculate 500 mL of YPD. The culture was incubated at 28°C while shaking and grown to an OD_{600nm} of 0.5-0.7. The culture was pelleted, washed with sterile TE or water and pelleted again. Thereafter the cells were resuspended in TE+100 mM LiAc and kept at room temperature for further use.

100-500 ng of the desired yeast transformation vectors were mixed with 100 µg of sheared salmon sperm DNA and 600 µL TE/100 mM LiAc/80% PEG4000. 150 µL of the prepared yeast cells were added and everything vortexed to mix properly. The suspension was incubated at 30°C for 30 min while shaking. Then 1/10 volume (75 µL) of DMSO were added and the cells heated to 42°C for 15 min. Afterwards the cells were chilled for 2 min on ice, pelleted for 1 min at 4,000 *g* and washed with sterile water. Finally the cells were resuspended in

150 µL water and then plated on media containing the appropriate SD medium (2% Glucose, 0.67% Yeast-N base, 1.2% agar, appropriate amino acid mix). The plates were incubated at 28°C for 2-3 days

Genotyping

To control the uptake of the correct plasmid in the yeasts, colonies were picked for each transformation and resuspended in 15 µL water. 5 µL of the cell suspension was transferred into a new tube and 5 µL of 40 mM NaOH added. The cells in NaOH were heated to 95°C for 10 min. 1 µL of this lysed cells was used as template in 12 µL PCR with a vector specific forward and a gene specific reverse primer. The PCR was analyzed on an agarose gel and positive colonies were used to inoculate 5 mL of the appropriate selection media.

Yeast-two hybrid studies

To investigate protein-protein interactions in the yeast system, yeast-two hybrid studies were performed (Fields and Song, 1989), the genes of interest were cloned into the pGADGWT7, for expression in yeast with an N-terminal GAL4-AD fusion, or the pGBKGWT7 vector, expression I yeast with N-terminal GAL4-DBD fusion. The respective vectors were transformed into a suitable yeast strain (Matchmaker Gold, YM4271a or PJ69-4α) and selected on minimal medium without Leucine and Tryptophan. Positive transformands were genotyped using PCR and used to inoculate 5 mL of SD -L/-W liquid medium, which were grown 1-2 days while shaking at 28°C. The OD_{600nm} of the different cultures was adjusted to 1 and a dilution series - 1:1, 1:10, 1:100, and 1:1,000 - of each culture prepared. 1.5 µL from the dilution series of the different cultures were dropped on SD -L/-W, SD -L/-W/-H, and SD -L/-W/-H with different concentrations of 3-AT (1 mM, 5 mM, 10 mM, 20 mM). The plates were incubated at 28°C for 2-3 days and then analyzed for yeast growth.

Yeast-three hybrid studies

To analyze higher order protein complex formation in yeasts, the genes of interest were cloned into the pGADGWT7 and pGBKGWT7 vectors. The proteins mediating the interaction were cloned into the pDRf1-GW vector that allows yeast expression without any added tags. A mating approach was used to create a yeasts harboring all three plasmids. Therefore the pGBKGWT7 vector were transformed into the pJ69-4α strain while the pGADGWT7 and the pDRf1-GW plasmids were co- or consecutively transformed into the YM4271a strain. Positive colonies of the different strains were used to inoculate liquid cultures with the appropriate minimal medium (either -W for the PJ69-4α strains containing the pGBKGWT7 plasmids or -L/-U for the YM4271a with the pGADGWT7/pDRf1-GW vectors). The cultures were grown overnight at 28°C while shaking. The PJ69-4α and the YM4271a cultures were adjusted to the same OD_{600nm} and 500 µL of the cultures were mixed in a tube

and incubated at 28°C for two days. Afterwards the cells were pelleted, washed with water, plated on -L/-W/-U minimal medium and incubated for 2-3 days at 28°C. Positive colonies harboring all three plasmids were selected using colony PCR. A dilution series was prepared similar like for the yeast-two hybrid and plated on -L/-W/-U, -L/-W/-U/-H and -L/-W/-U/-H + additional 3-AT to screen for tripartite complex formation.

Microscopy

Tobacco leaf infiltration

The behavior of proteins in planta was studied by transient expression of fluorescent protein tagged proteins in tobacco leaf epidermis cells (Sparkes et al., 2006). The genes of interest were either cloned into the pK7FWG2 vector (Karimi et al., 2002), for expression from the viral p35S-promoter with an N-terminal GFP tag, or into a modified pEarlyGate104 vector (provided by Sabine Müller/Dorothee Stöckle, ZMBP Tübingen), for expression from the viral p35S-promoter with an N-terminal mCherry-tag. *Agrobacterium* cells of the GV3101 pMP90 strain were transformed with the respective vectors and positive clones selected by colony PCR. Overnight cultures of these clones were used to inoculate 40 mL of LB with antibiotics and these were grown for 6h to overnight at 28°C while shaking. Additionally 40 mL of LB medium were inoculated with Agrobacterium transformed with the p19 protein expressing vector. The cultures were pelleted, washed with water and finally resuspended in 10 mL leaf infiltration buffer (10 mM Na-PO_4 buffer pH 7.5, 5 mM MES, 0.8% Sucrose). The OD_{600nm} of the different cultures used were adjusted to 4 with infiltration buffer and transformation mixes with equal parts of the vectors for expression studies and p19 were prepared. Two leaves of at least two 3-4 week old tobacco plants (*Nicotiana benthamiana*) were carefully infiltrated with the *Agrobacterium* solution using a syringe without a needle. Afterwards the plants were transferred to a growth cabinet with 12h light/12 h dark conditions for 2-3 days. Leaf discs of the transformed tobacco leaves were taken for microscopy studies.

Co-locolaization

To study subcellular co-localization of fluorescent-protein tagged proteins, tobacco leaf epidermis cells were transformed with the respective vectors as previously described. After two and three days, leaf disks of infiltrated leaves were cut out and used for localization studies under a laser scanning confocal microscope (Leica TCS SP5) with a 40 x lens. Sequential scans were performed using a white light laser to exclude overlapping emission spectra, with GFP excitation at 488 nm and emission capture between 500 nm and 540

nm, while mCherry was excited at 540 nm and the emission recorded from 600 nm to 680 nm. The fluorescence intensity in the pictures was analyzed with the Leica LAS X software.

FRET/FLIM studies

In order to investigate protein-protein interactions in planta, the gene of interest was recombined into the pK7FWG2 gateway destination vector (Karimi et al., 2002) to be expressed with an N-terminal GFP tag. The sequences that code for the potential interaction partners were recombined into a modified pEarlyGate104 vector to express N-terminal RFP-fusions in planta. Image and data acquisition was obtained with a Leica TCS SP8, combined with a PicoHarp 300 TCSPC Module and a Sepia Multichannel Picosecond Diode Laser (PDL 808-SC) (Pico-Quant). The samples were excited with a 470 nm pulsed laser (10 MHz) intensity regulated via a Thorlabs Laser Combining Unit (PBH51502/SS/SPL-S6). The emission was recorded from 500 nm to 560 nm in 128 x 128 pixels images with at least 2000 counts/pixel. The fluorescence lifetime measurements were analyzed using the PicoQuant SymphoTime Software (ver. 5.3.2.2). For each nucleus average fluorescence decay profiles were plotted and lifetimes were estimated by fitting the data with a mono-exponential decay function.

Plant work

Growth conditions

Arabidopsis thaliana plants were grown either under short (8h light/16h dark) or long day conditions (16h light/8h dark). Suitable growth cabinets, growth chambers or green houses with a light intensity of 70 – 120 µEinstein, a day temperature of 22°C and a night temperature of 18°C were used to grow plants. Seeds were sawn either on soil or on solid 0.5 MS medium (1.54 mM MES, 4.3% MS-salt, 0.8% Sucrose, 0.8% Agar) and stratified at 4°C for 3-5 days before transfer to the climate chambers.

Agrobacterium transformation

For Agrobacterium mediated plant transformation, the gene of interest was cloned into the desired plant expression vector. Chemically competent *Agrobacterium tumefaciens* cells of either the GV3101 pMP30 or the GV3101 pMP90 RK strain were transformed with 0.1-1µg of plasmid DNA. Cells were therefore thawed on ice, mixed with the plasmid DNA and incubated for further 15 min on ice, flash frozen in liquid nitrogen and heated to 37°C for 5 min. Afterwards they were again chilled on ice for 2 min and 500 µL of LB media were added. The

cells were incubated at 28°C for 2-3h while shaking and then plated on LB-media containing the appropriate antibiotics.

Transformation

Arabidopsis thalaian plants were transformed with Agrobacteria using a modified floral dip protocol (Clough and Bent, 1998). Seeds of Col-0 plants were sawn in 10 cm diameter pots with soil, stratified and grown under long day conditions for 4 weeks until inflorescences emerged and grew on average 10 cm high. An overnight culture of Agrobacteria transformed with the desired vector was used to inoculate 400 mL of LB with antibiotics and this was incubated at 28°C while shaking for 4-8 h until an approximate OD_{600nm} of 2-3. Cells were pelleted, washed once with water and resuspended in 200 mL transformation solution (1.54 mM MES, 4.3% MS salt, 0.5% sucrose). Flowers of the Arabidopsis plants were dipped into the Agrobacterium solution for 15 sec and gently swirled. The plants were kept within a plastic bag under short day conditions for 1-2 days and then transferred back to long day conditions. The seeds were collected, sawn on soil, stratified and after one week selected for positive transformed plants with spraying of a 0.1% glufosinate solution. Resistant plants were transferred to single pots and genomic DNA was prepared to verify the insertion of the T-DNA via PCR.

Genomic DNA isolation

Genomic DNA from plants was prepared using a modified Edwards protocol (Edwards et al., 1991). 100 µg of plant material were collected and grinded in 500 µL Edwards buffer (200 mM Tris pH 7.5, 25 mM EDTA, 250 mM NaCl, 10% SDS). The ground tissue was centrifuged for 5 min at 20,000 g and 400 µL of the clear supernatant transferred into a new collection tube. 280 µL of 2-propanol were added, mixed well and the tube was centrifuged for 7 min at 20,000g. The supernatant was discarded and the pellet washed twice with 70% EtOH, then dried and resuspended in 50 µL water. For genotyping by PCR 1 µL of this genomic DNA was used as template in the respective reaction.

GUS staining

To analyse promoter activities in plants, the respective promoter was cloned into the pMDC162 vector, which put the ß-glucoronidase under the control of the respective promoter.

Arabidopsis plants transformed with this vectors were selected on MS media containing 40 µg/mL Hygromycin. Leaves of 2-3 week old plants from the T_2-generation were used for the GUS staining. Plants were grown on MS media for three weeks and incubated in GUS staining solution (100 mM $NaPO_4$-buffer pH 8.0, 10 mM EDTA, 0.5 mM $\{Fe(CN)_6\}^{-3}$, 0.5 mM $\{Fe(CN)_6\}^{-4}$, 1% Triton-X 100, 0.3 mg/mL X-Glc) for 16h to 24h at 37°C. Plants were

destained in 100% ethanol and pictures of the stained plants were taken under a binocular with 8-10x magnification.

RNA preparation

Leaves of 4 week old plants grown on soil or 15 day old seedlings were used to isolate plant RNA. The plant material was flash frozen in liquid nitrogen and stored at -80°C until usage. The plant material was grinded using glass beads (5 mm diameter) and a Tissue Lyser from Retsch for 30 sec with a frequency of 30 shakes per second. Plant total RNA was isolated from the ground tissue using the GeneJET Plant RNA extraction kit according to the manufacturer's recommendations. RNA was eluted from the columns with 50 µL of RNase free water. 2 µL were analyzed on an agarose gel to check the RNA's integrity and the concentration was determined using a Nanodrop spectrophotometer. The RNA was stored at -80°C afterwards.

DNase digestion and cDNA synthesis

0.5-1.5 µg of total plant RNA were used for cDNA synthesis. The volume of the RNA was adjusted to 7.5 µL with nuclease free water and 2.5 µL of DNase digestion mix (1 µL DNase I (1 u/µL), 1 µL 10x DNase buffer + $MgCl_2$, 0.5 µL RiboLock) were added. The reaction was mixed and incubated for 30 min at 37°C. 1 µL of 50 mM EDTA were added to inactivate the DNase and the reaction was heated to 65°C for 10 min. Afterwards 1 µL of 10 µM oligodT primer were added and the reaction again incubated at 65°C for 5 min. Finally, 8 µL of RT mix were added (1 µL Reverse transcriptase, 2 µL 10 mM dNTPs, 4 µL 5x RT-buffer, 1 µL water) and the reaction was incubated at 42°C for 1h followed by 10 min at 70°C. The 20 µL reaction was afterwards diluted 1/10 with nuclease free water and used for qPCR analysis or cloning.

qPCR

For expression analysis using qPCR, the obtained diluted cDNA was used. An appropriate amount of each cDNA sample used in the analysis was mixed and used in a dilution series to assess the efficiency of the used primers. The cDNA was therefore diluted 1:1, 1:5, 1:25 and 1:125. The KAPA SYBR Fast 2x qPCR Mastermix was used for qPCR analysis and the reactions were prepared in with 4 technical replicates as followed

qPCR	(8µL)
4 µL	KAPA SYBR Fast 2x qPCR Mastermix
0.16 µL	10 µM P1
0.16 µL	10 µM P2
1.68 µL	water
2 µL	template DNA

The cDNA was pipetted into a 384 well plate suitable for qPCR analysis and afterwards 6 µL of the mastermix containing primers and water were added. The plate was spun down for 30 sec at 3,000 rpm, placed in the CFX384 touch Biorad qPCR thermal cycler and the following program was initiated

1 min initial heating to 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C, after every cycle the SYBRgreen fluorescent dye was excited with 497 nm and emission was recorded at 520 nm. After the 40 cycles the samples was gradually heated from 65°C to 95°C in steps of 0.5°C for 5 sec, fluorescence being recorded after each step.

The results were analyzed suing the Biorad CFX manager software and Excel. Gene expression levels were determined using the ΔCt method (Tolkien and Tolkien, 1977; Vandesompele et al., 2002).

RNAseq

Two samples for each plant type (Col-0, *35S::FLAG:miP1a*, *35S::FLAG:miP1b* and *co-SAIL*) were sequenced using Illumina HiSeq2000 and basecalls were performed using HiSeq Control Software v2.0.12.0 (Illumina). For each sample 2.5 to 3.2 Gbp were obtained. The analysis of the RNAseq data was performed by Daniel Straub.

Reads were quality checked with RobiNA v1.2.4_build656 and first 10 bases were clipped using Trimmomatic v0.32 (Lohse et al., 2012). In each sample more than 98% of reads passed the trimming. 63-65% of the surviving reads were successfully mapped to A. thaliana TAIR10 genome sequence and annotation (TAIR) using RobiNA's Bowtie (Langmead et al., 2009) allowing maximal two mismatches in the seed region. The normalization and statistical evaluation of differential gene expression has been performed using edgeR v2.6.12 (Robinson et al., 2010) with a minimum fold change of 4 and a FDR cut-off of 0.001 and using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995) for multiple testing correction. The raw data was normalized according to the default procedure and the dispersion was estimated using the auto setting of edgeR. Raw read data and output of statistical analysis were submitted to Gene Expression Omnibus (GSE56811).

3. RESULTS

Identification of potential microProteins and their interaction partners in *Arabidopsis*

In order to identify new microProteins matching the previously described criteria of Eguen et al., we performed a systematic search for such proteins in the proteome of *Arabidopsis thaliana* (Fig. 3.1).

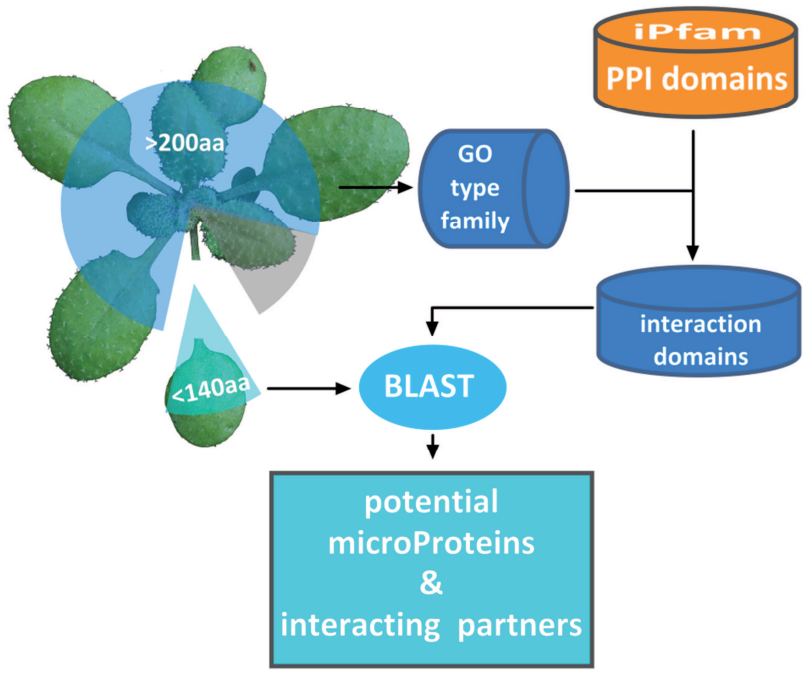


Figure 3. 1 Schematic depiction of the microProtein identification approach (modified after a figure from Daniel Straub)

Proteins were grouped in three categories according to their sequence length. The group with proteins of less than 140 aa was considered to contain the potential miPs, as all miPs described so far are within this size range. The potential interaction partners were searched within the group of proteins containing more than 200 aa. Proteins in the range from 140 to 200 aa were excluded from the analysis to avoid the generation of false-positive matches.

The domains of the large proteins were compared with the iPfam database and proteins containing protein-protein interaction domains were identified in this group. A filter for any annotated feature of a protein entered before the iPfam comparison would allow focusing on miPs and their targets fulfilling this criteria. For the carried out identification approach all annotated proteins were used.

The large proteins containing a PPI domain were used to create a database, against which the small proteins were aligned to, using the BLAST algorithm. A small protein showing high similarity towards the PPI domain of a large protein is considered a potential miP and the large protein as its potential interacting partner.

The search resulted in the identification of all known *Arabidopsis thaliana* microProteins and 32 (Supp. Table 1) new potential miPs targeting transcription factors. As a proof of concept, the two B-Box proteins within this group were further characterized, because so far no member of this family was known to possess miP qualities.

MiP1a and miP1b are microProteins that contain a B-Box motif and share a high similarity with COL proteins

The two B-Box proteins BBX31 and BBX30 - subsequently named miP1a and miP1b – are proteins of 121 aa and 117 aa in length. So far their function has not been annotated in the TAIR database (Lamesch et al., 2012). The B-Box is a domain involved in PPI that can be found in 32 *Arabidopsis* proteins, of which some are involved in the regulation of important developmental processes. The two potential B-Box miPs seemed therefore to be promising candidates for further characterization.

Phylogenetic analysis of the first B-Box from all 32 Arabidopsis B-Box proteins reveals that miP1a and miP1b are closely related to each other and cluster with both CONSTANS and CONSTANS-like proteins of group III (Fig. 3.2 A). The homology towards the COL branch of the family suggests a role in the regulation of those proteins rather than the STO-type B-Box proteins. Interestingly, both miP genes are physically located in the direct vicinity of *COL* genes. These findings suggest that *miP1a/b* genes evolved during one of the genome-amplification events (whole genome duplication or

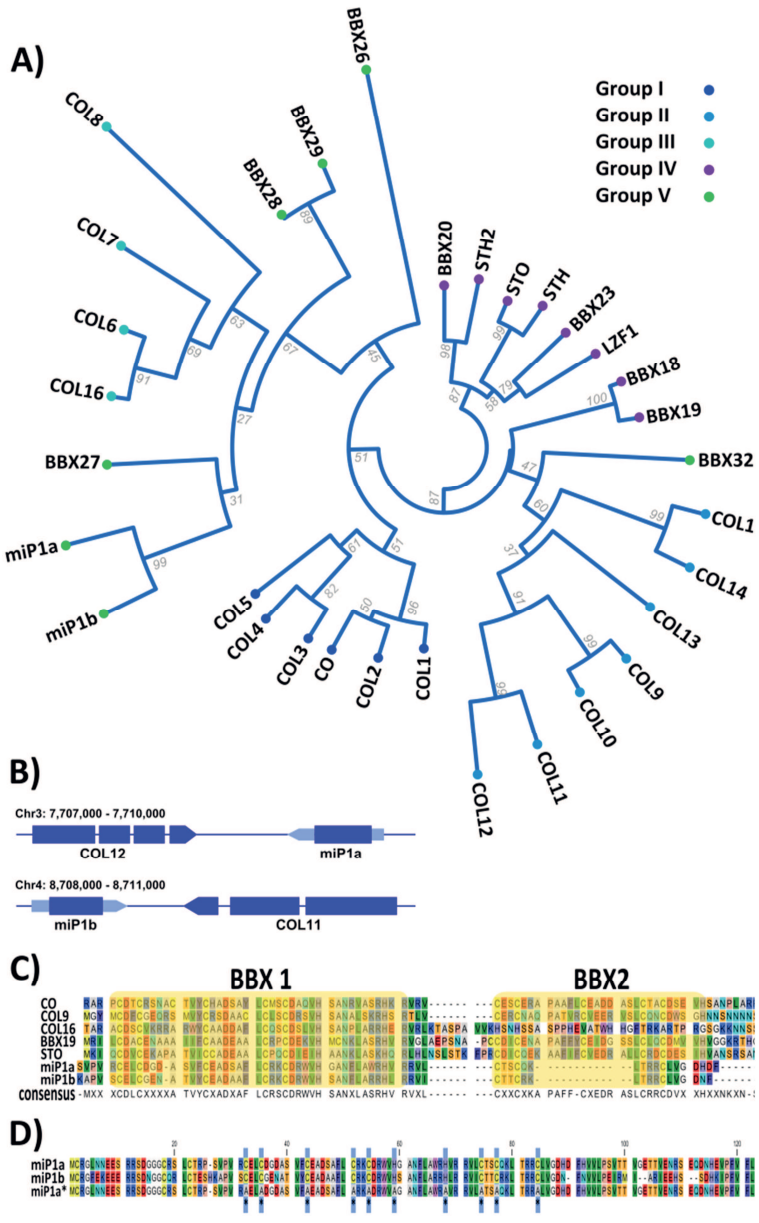


Figure 3. 2 A) Phylogenetic tree of the aligned B-Boxes from Arabidopsis thaliana B-Box proteins; **B)** genomic location of miP1a and miP1b **C)** MUSCLE alignment of the N-terminal region of CO, COL9, COL16, BBX19, STO , miP1a and miP1b. The B-box domains are highlighted in yellow **D)** Alignment of miP1a, miP1b and miP1a*, where the ten conserved Cys and His residues were replaced with Ala

tandem duplication), which enlarged the *COL* gene family (Fig 3.2 B). Alignment of all COL B-Box domains with the B-Box domains of miP1a/b reveals that miP1a/b have one full B-Box domain and remnants of a second B-Box-domain (Fig 3.2 C), setting them apart from other B-Box proteins like COL16 and BBX32. These findings point towards a role of miP1a/b as potential interaction partners of COL proteins. Based on the identification of the conserved elements, we created a mutated version of miP1a, in which the conserved cysteine and histidine residues of the B-Box domain were replaced by alanine (Fig. 3 D). This mutated protein version referred to as miP1a* was used to investigate the importance of the B-Box for the functionality of our microProteins.

MiP1a and miP1b interact with the flower promoting factor CONSTANS

Based on the structure of the B-Box domains of both miP1a/b and CO, we postulated that miP1a/b function by forming heterodimeric complexes, that sequester CO/CO-like (COL) proteins into non-functional complexes. To test whether CO physically interacts with miP1a/b, we performed directed yeast-two-hybrid studies. The coding sequences of CO and the B-Boxes of CO were fused in frame to the Gal4-activation domain (AD; pGADT7) and used as prey. The prey proteins were tested in yeast against the empty pGBKT7 vector expressing the Gal4-DNA binding domain (BD) and in frame fusions of miP1a, miP1b and miP1a*. We observed that CO and the CO B-Box-domain are able to interact with both miP1a and miP1b in yeast (Fig. 3.3 A). As predicted, no interaction was observed with the miP1a* protein, confirming that an intact Zn-finger B-Box is essential for this interaction.

To verify that the interactions of miP1a/b with CO, which were initially observed in yeast can also occur in a different heterologous system, we tested if miP1a and CO expressed and purified from *E. coli* cells, can be co-immunoprecipitated. We expressed fusions of CO to the maltose binding protein (MBP) and fusions of miP1a to the glutathion-S-transferase tag (GST). As a negative control we fused the LITTLE ZIPPER3 (ZPR3) protein, a small leucine-zipper miP to a GST-tag. All fusion proteins were expressed under the inducible T7 promoter in *E. coli* BL21 cells. After cell lysis, soluble protein fractions of either GST-miP1a and MBP-CO or GST-ZPR3 and MBP-CO were mixed and incubated with amylose-coated magnetic beads. After precipitation and washing, immune complexes were released by boiling in SDS-loading buffer and separated by SDS-PAGE. CO was able to physically interact with miP1a (Fig. 3.3 B) whereas no binding of GST-ZPR3 to MBP-CO was observed (Fig. 3.1B). This further supports the idea that miP1-type microProteins act by binding to the CO protein and that this binding does not require other accessory proteins.

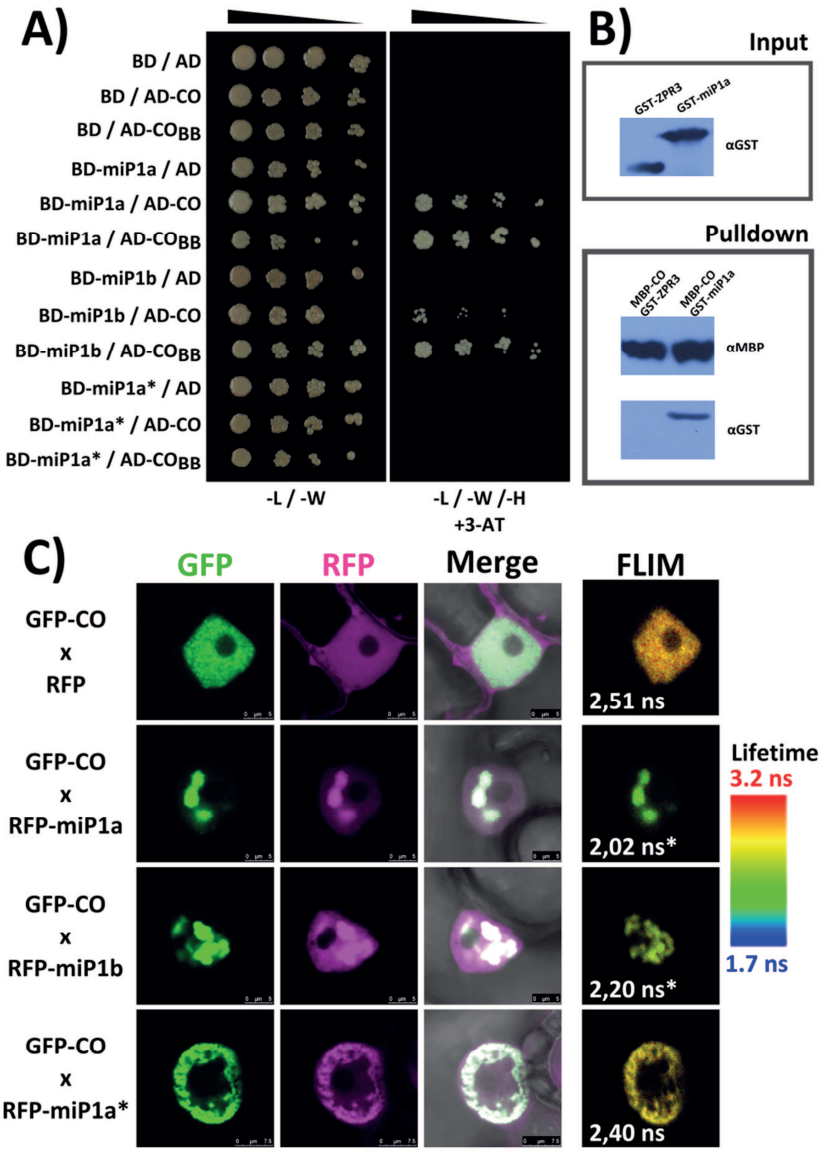


Figure 3. 3 Protein interaction studies
A) Yeast-two-hybrid assay with DBD-CO/COBB and AD/-miP1a/-miP1b/-miP1a*, growing on selective media without L/W and without L/W/H + 3-AT; **B)** αMBP and αGST immunoblot of an *in vitro* pull down of GST-miP1a/GST-ZPR3 with MBP-CO;
C) Fluorescence pictures nuclei of tobacco leaf epidermis cells transiently transformed with GFP-CO and RFP-miP1a/-miP1b/-miP1a*, fluorescence lifetime imaging (FLIM) of GFP in the respective nuclei, average lifetime in the left lower corner. Asterisks indicate significant lifetime changes

MiP1a/b could either inhibit CO by preventing its nuclear import, or by attenuating DNA-binding of CO. To determine whether miP1a/b can retain CO in the cytoplasm, we transiently co-transformed tobacco leaves with fusions of CO to the green fluorescent protein (GFP) and fusions of miP1a, miP1b and miP1a* to the red fluorescent protein (RFP). We observe that both miP1a and CO and miP1b and CO co-localize in small speckles in the nucleus (Fig. 3.3 C). Little fluorescence is observed in the cytoplasm, excluding the possibility that miP1a/b act by preventing nuclear import of CO. To test whether CO and miP1a/b also physically interact *in planta*, we performed FRET/FLIM experiments and detected significant lifetime changes of the GFP fluorophore in the speckles in which CO and miP1a/b co-localize. No significant lifetime changes were observed in nuclei co-expressing free RFP or RFP-miP1a*. Taken together, these results demonstrate that miP1a/b and CO are able to physically interact *in planta* through their B-Box domains and that these interactions do not inhibit nuclear localization of CO.

Overexpression of either miP1a or miP1b delays flowering under inductive long day conditions

MicroProteins have a dominant-negative effect on the activity of their target protein. Therefore we expected miP1a and miP1b to affect the flowering behavior of plants and tested this hypothesis by expressing them in plants under the control of the viral CamV35S promoter. The coding sequences of miP1a/b were isolated by PCR and recombined in the pJAN33 vector (Weigel et al., 2003) harboring a tandem-*CaMV35S* promoter for high-level ectopic expression. For each construct (*pJAN33-miP1a* and *pJAN33-miP1b*), we isolated (15 and 25 respectively) individual T1 transgenic lines that showed resistance to the herbicide glufosinate. The majority (about 80%) of the recovered transgenic plants showed severely delayed flowering when grown in long day conditions. To exclude an effect of the herbicide glufosinate, we selected three independent homozygote transgenic lines and tested the flowering behavior under controlled inductive long day conditions. This analysis revealed that the transition to flowering of transgenic miPOX plants is extremely compromised under inductive long-day conditions when compared to wild type Col-0 plants (Fig. 3.4 A, B).

Furthermore, overexpression of *miP1a/b* caused a severe decrease in the levels of *FT* mRNA in leaves of long day grown plants (Fig. 3.4 C), explaining the molecular nature of the observed late flowering phenotypes. Phenotypically and molecularly, *miP1a/b* overexpression plants strongly resemble plants carrying loss-of-function mutations in either *CO* or *FT*. These findings support our predictions and indicate that ectopic expression of miP1-type microProteins renders CO non-functional, resulting in attenuation of *FT* expression, which seems causal for the observed late flowering phenotypes. The ectopic expression of the mutant miP1a* protein does not cause an alteration in the flowering behavior of transgenic plants and *FT* mRNA levels are similar to the wild type (Fig 3.4 A,B,C), indicating that a functional zinc-finger B-Box domain is required for the observed late flowering phenotype of miP1a.

Overexpression of miP1a or miP1b did not cause flowering time changes when transgenic plants were grown under short day conditions (Fig 3.4 C). Since CO is inactive in short days our findings suggest that the most likely mode of miP1a/b action is rendering CO inactive in long day conditions and they further suggest that miP1a/b affect CO but not other flowering-promoting factors.

To further exclude any effect on other floral regulatory factors, we treated *co*, *ft* and miP1a/bOX plants growing under long day conditions with gibberillic acid (GA_3), a plant hormone that promotes flowering independently off the photoperiodic flowering regulatory pathway (Galvao et al., 2012). The GA_3 treated *p35S::miP1a* and *p35S::miP1b* plants behaved similar to GA treated *co* and *ft* plants (Fig. 3.4 D), indicating that

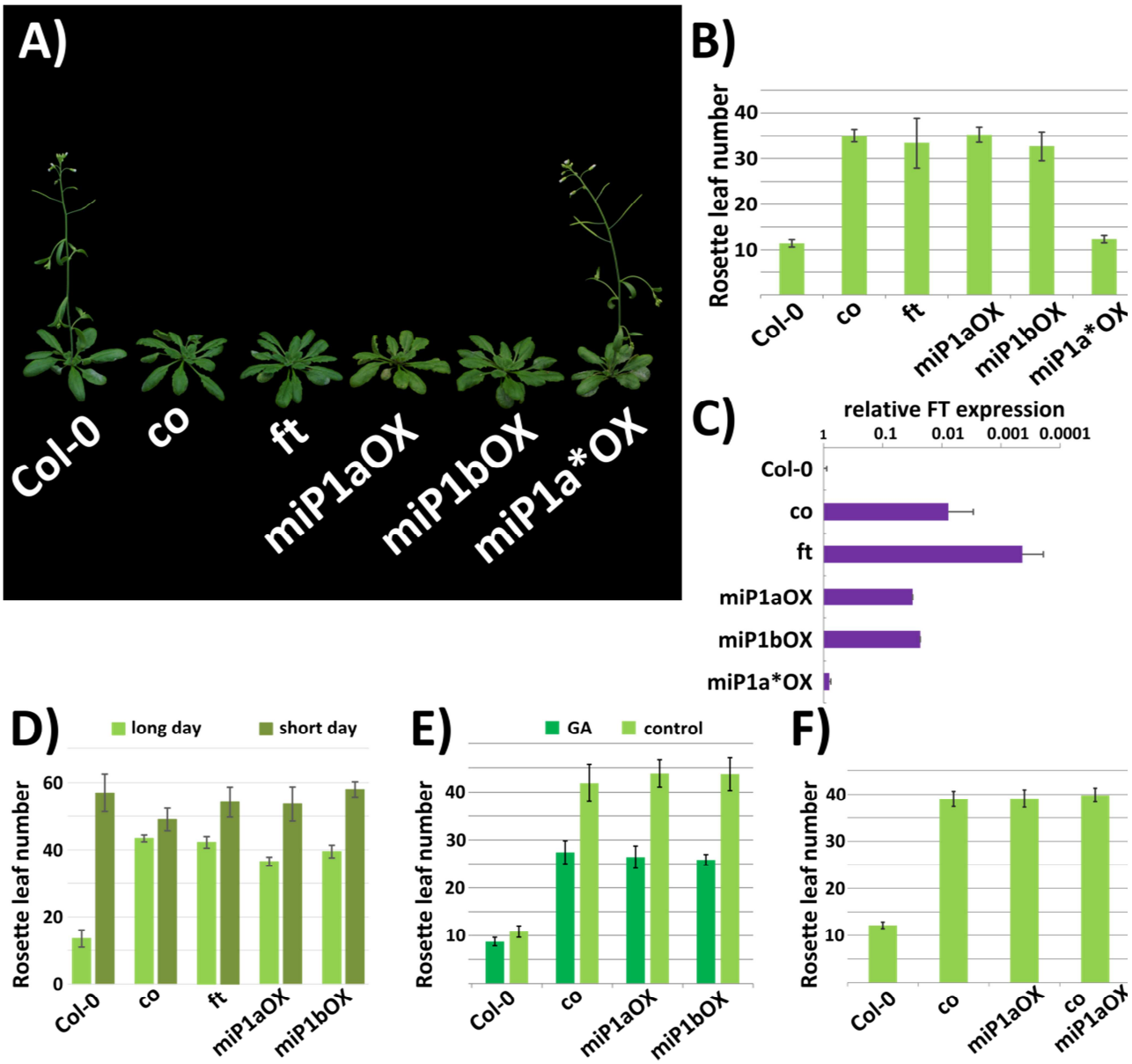


Figure 3. 4 Flowering time analysis of plants with overexpressing *miP1a/1b*
A) pictures of 4 week old Col-0, *co/ft* loss of function mutants and plants expressing *miP1a/miP1b/miP1a** from a viral *35S*-promoter; **B)** leaf number at bolting of plants grown under long day conditions (16h light / 8h dark); **C)** relative *FT* expression in 2 week old plants **D)** leaf numbers of Col-0, *co*, *ft*, and *p35S::miP1a/1b* plants at bolting grown under long day or short day (8h light / 16h dark) conditions; **E)** leaf number at bolting of plants grown under long day conditions treated either with a mock solution or 50 µm GA_3 until flowering; **F)** Leaf number at bolting of Col-0, *co*, *p35S::miP1a* and *co x p35S::miP1a* plants grown under long day conditions

the miP1 proteins do not affect the floral transition at the shoot meristem (Galvao et al., 2012; Romera et al., 2014). Finally is the dependence of the miP1a flower delaying effect on CO further supported by the flowering behavior of plants overexpressing miP1a in a *co* mutant background These plants are indistinguishable in their flowering phenotype from *p35S::miP1a* or simple *co* loss of function mutants, indicating that miP1a has no influence on flowering in the absence of CO (Fig 3.4 E).

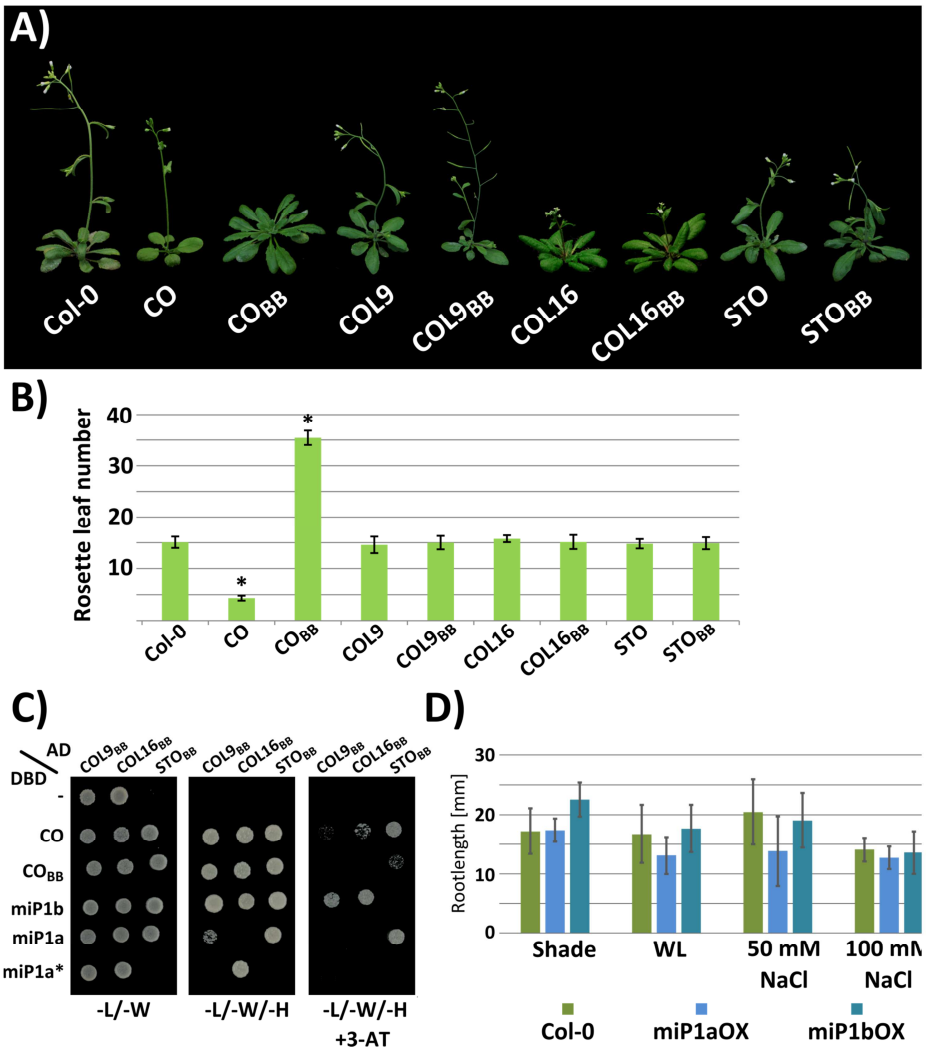


Figure 3. 5 Characterization of further B-Box proteins
A) Flowering behavior of different 4 week old plants expressing full length CO/COL9/COL16/STO or the respective B-Box domains from the viral 35S promoter; **B)** Leaf number at bolting of plants grown under long day conditions, Asterisks indicate significant differences compared to the Col-0 wild type plants;
C) Yeast-two hybrid experiment showing the growth of yeast co-transformed with CO/CO_{BB}/miP1a/miP1b/miP1a* and the B-Box of COL9/COL16/STO on media without L/W, L/W/H and L/W/H plus 10 mM 3-AT; **D)** Root growth of Col-0, *p35S::miP1a* and *p35S::miP1b* seedlings grown in shade, white light (WL) and on media containing 50 mM/100 mM NaCl

Specificity of the interaction of miP1a/b with CONSTANS

Both miP1a and miP1b proteins have a B-Box zinc finger domain allowing them to interact with CO and potentially also with the many other proteins containing a similar B-Box domain. Furthermore, overexpression of the two microProteins causes late flowering under long day conditions, similar to *co* loss of function mutant plants. Interestingly, the *CO* locus produces an alternatively spliced transcript, which could potentially produce a protein with only the B-Box domains. Overexpression of this CO

splice variant (CO_{BB}) resulted in a similar late flowering phenotype like we observed for miP1a and miP1b (Fig. 3.5 A, B).The flowering time effect we observed for the CO_{BB} splice variant of CO indicates, that flowering can be affected by the ectopic expression of B-Box proteins. To further investigate the possible use of B-Box proteins as modulators of flowering time, we overexpressed B-Box proteins of group II (COL9), group III (COL16) and group IV (STO); we also included artificial microProtein versions ($COL9_{BB}$, $COL16_{BB}$, STO_{BB}) encoding only the respective B-Box domains. The initial analysis of T1 transgenic plants revealed that none of these transgenic lines was able to significantly promote or delay the floral transition (Fig 3.5 A) and none of the T2 lines beside *p35S::CO* and *p35S::CO_{BB}* showed altered flowering behavior under long day conditions (Fig 3.5 B). A yeast-two hybrid experiment in which we tested the ability of miP1a and miP1b to interact with the B-Box of those proteins showed, that in principle all tested B-Box proteins show at least weak interactions, as the yeast cultures co-transformed with any combination of B-Box proteins were able to grow on selective media without Histidine (Fig 3.5 C). The addition of 3-AT in a concentration of 10 mM reduced the number of interactions. However, as the overexpression of STO and the STO_{BB} protein did not affect the flowering behavior under long day conditions despite the strong observed interaction between CO and STO_{BB} in the yeast-two hybrid experiment, such interactions seem insufficient to cause the observed strong flowering time effect of miP1a and miP1b overexpression.

To exclude effects of miP1a and miP1b on other members of the B-Box family, we analyzed if the known processes in which other B-Box proteins are involved are affected in our overexpressing lines. Particularly, members of the STO/STH branch of the B-Box family are involved in the developmental adaption to abiotic stresses. For example when overexpressed, STO can promote root growth in high salt conditions whereas a loss of function diminishes root elongation under such conditions (Nagaoka and Takano, 2003). BBX19 and BBX32 affect root elongation under shade conditions (Holtan et al., 2011a; Wang et al., 2015). Using the same growth conditions, we tested if miP1a/1b might have additional effects when ectopically expressed. In response to high salt concentrations neither *miP1a* nor *miP1b* had a significant effect on root elongation growth (Fig 3.5 D) supporting the idea that the major role of miP1a/b lies in flowering time control.

Analysis of plants with reduced expression of miP1b and miP1a/1b

In order to study the effect of lost *miP1a/b* activity we tested available T-DNA insertion lines and transgenic plants overexpressing artificial microRNAs. Owing to the small size of genes encoding microProteins, T-DNA insertions in microProtein genes are more infrequent compared to larger genes. We have characterized the only available T-DNA insertion line in the *miP1a* gene (GABI-KAT line *288G08*), but this line did not show a

reduction or loss of *miP1a* mRNA levels; it rather had slightly increased levels of miP1a expression and flowering time was comparable to wild type plants (Suppl. Fig. S1 A, B). Transgenic plants overexpressing artificial microRNAs targeting both *miP1a* and *miP1b* neither showed a mutant phenotype nor were *miP1a/b* mRNA levels substantially decreased (Suppl. Fig. S1 C, D).

To study the flowering behavior of plants with reduced *miP1a/b* expression levels we used the microRNA-induced gene silencing (MIGS) technology (Felippes et al., 2012) and overexpressed the sequence encoding the miP1a/b-specific carboxy terminal region of *miP1b* or a combinatorial construct against miP1a and miP1b, fused to a *miR173*-binding site. This fusion construct is recognized by *miR173,* eliciting the production of trans-acting siRNAs (*tasi-RNAs*) from the MIGS-construct, which target either *miP1a* or *miP1b* mRNA and causes their degradation.

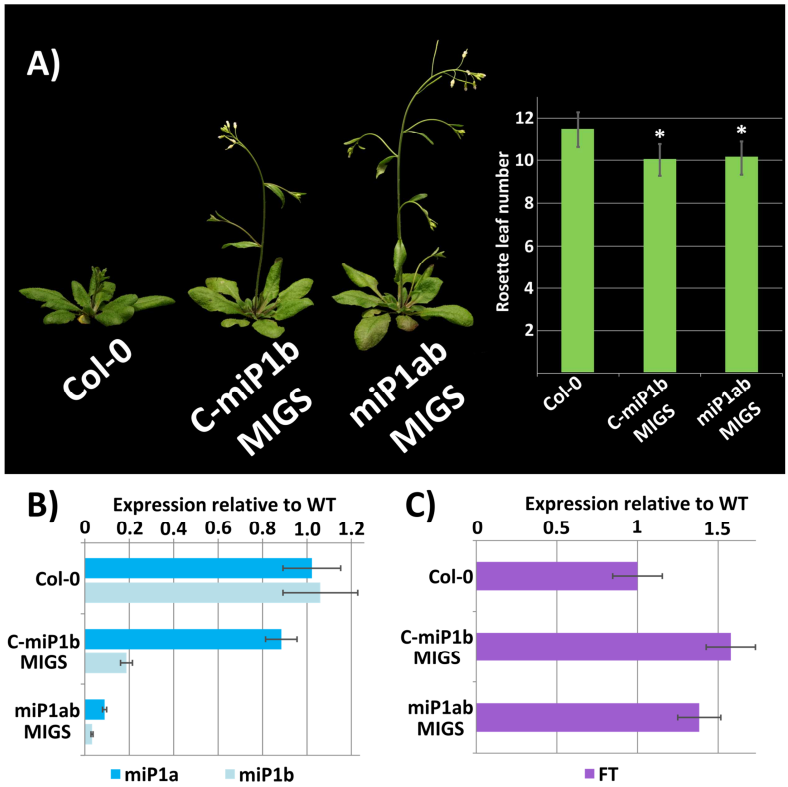


Figure 3. 6 Characterization of MIGS lines A) leaf number at bolting of Col-0, p35S::MIGS C-miP1b and miP1ab lines, Asterisks indicate significant differences compared to Col-0; **B)** miP1a and miP1b expression relative to the wild type ;**C)** FT expression relative to the wild type

From the ten T2 lines expressing a MIGS construct targeting the C-terminus of miP1b six flowered slightly but significantly earlier then wild type plants grown under the same conditions. Because this flowering time phenotype was very weak, we performed a double-blind flowering time study of progeny plants of one representative line in long day conditions. In this experiment *miP1b-MIGS* transgenic plants still flowered slightly earlier compared to Col-0 wild type plants (Fig. 3.6 A, B). From the three T2 lines expressing a MIGS construct targeting miP1a and miP1b, two flowered significantly earlier than the in parallel grown Col-0 plants.

The *miP1b* mRNA levels in the MIGS C-miP1b line and the *miP1a* and *miP1b* mRNA levels in the MIGS miP1ab line are reduced and *FT* mRNA was increased in expression compared to Col-0 (Fig. 3.6 B, C). These findings support the role of miP1a and miP1b as modulators of CO activity. It is noteworthy, that *miP1a* levels in the C-miP1b MIGS plants are comparable to wilt type, indicating a high specificity of the generated *tasi-RNAs*.

The diurnal pattern of *miP1a/b* mRNA expression partially coincides with *CO* mRNA expression peaks

CO is expressed in a diurnal manner and shows the highest level of mRNA abundance at 14 h and 21 h after dawn. Only under long day conditions is CO protein stabilized and can activate *FT* expression. Many of the B-Box proteins are known to be regulated by the circadian clock; therefore we performed a time course experiment to see when *miP1a* and *miP1b* are expressed under long and short day conditions (Fig. 3.7). Samples of Col-0, *p35S::miP1a* and *p35S::miP1b* seedlings were sawn on MS media, stratified for 4 days at 4°C and grown under long or short day conditions for 14 days. Whole seedlings were collected during a 24 h time course every three hours, starting from the onset of the light period (Friends, 2015). RNA was extracted from all samples, used in a reverse transcription to generate cDNA, which was used for qRT-PCR analysis. The results are summarized in Figure 3.7

In Col-0, CO shows the expected diurnal expression pattern under long and short days. Only under long days, the expression peak of *CO* at 14 h is followed by a peak of *FT* expression (Fig. 3.7 A, D). *MiP1a* under long day shows the highest abundance at the end of dark/begin of light period, a smaller peak between 3 h and 6 h and low levels in the afternoon, when *CO* and *FT* become induced (Fig. 3.7 G). *MiP1b* is highly abundant in the middle of the night under long days (Fig 3.7 I). Under short days both *miP1a* and *miP1b* reach high expression levels in the prolonged dark period (Fig. 3.7 H, J).

When *miP1a* is ectopically expressed at high levels, *CO* mRNA abundance remains fairly unchanged while the expression levels of *FT* typical peak at the end of the long day (Fig. 3.7 B, E). High ectopic expression of *miP1b* also caused changes to the circadian expression of *CO* mRNA and the peak towards the end of the light period was absent (Fig. 3.7 C, F). In summary, ectopic expression of *miP1a* or *miP1b* resulted in reduced *FT* expression in response to long day conditions, explaining the late flowering phenotype of the respective plants. Both miPs show diurnal expression profiles with maxima coinciding with elevated levels of *CO* mRNA. This finding supports the idea that CO protein activity is affected when miP1a/b levels are ectopically high.

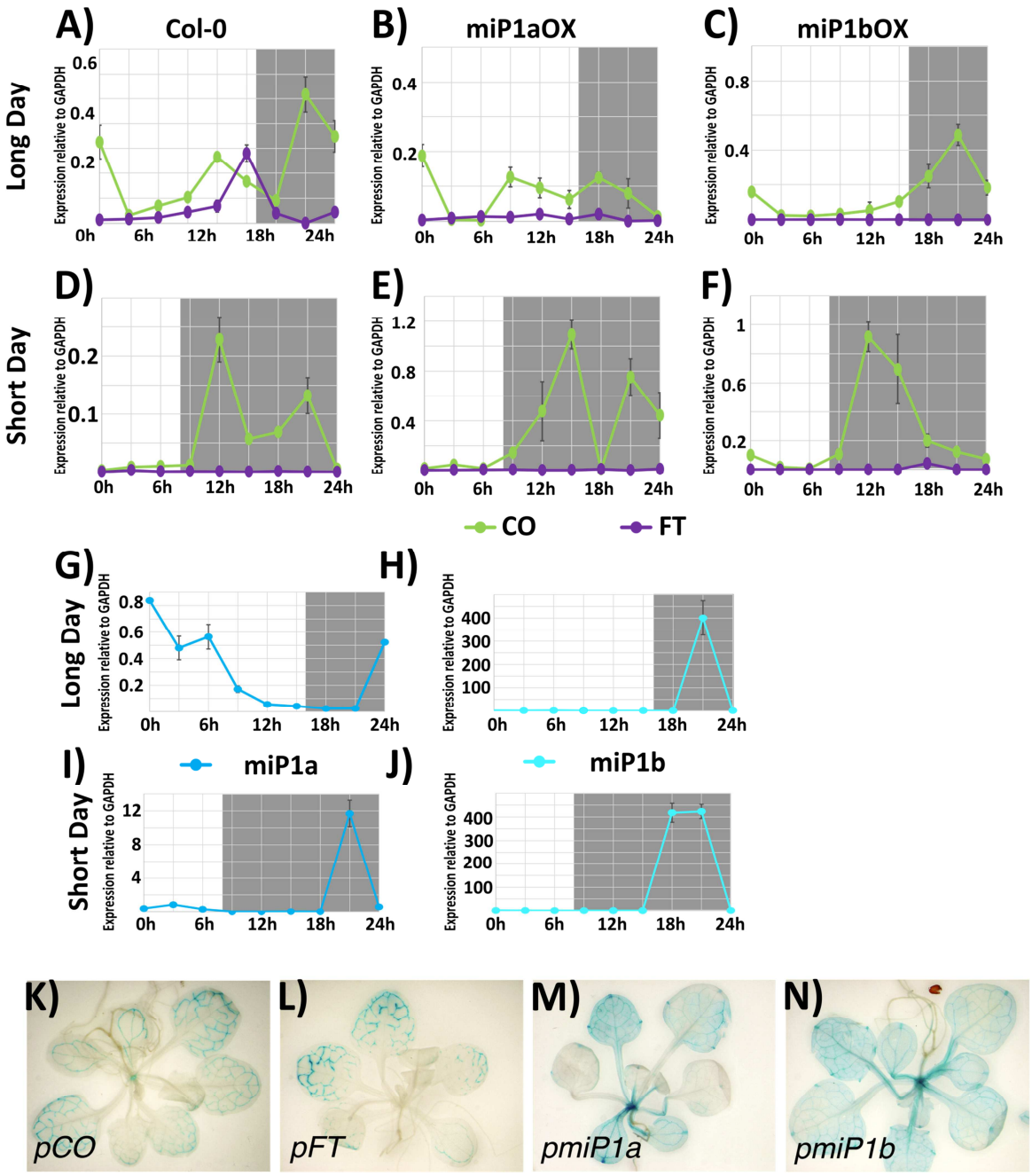


Figure 3. 7 Temporal and spatial expression of *miP1a* and *miP1b*

CO and FT expression during a 24h time course in 14 day old Col-0/*p35S::miP1a*/*p35S::miP1b* seedlings grown under long day (**A-C**) and short day (**D-F**) conditions; Expression of miP1a (**G,H**) and miP1b (**I,J**) in Col-0 seedlings grown under long and short day conditions; GUS staining of 3 week old pCO::GUS (**K**), pFT::GUS (**L**), pmiP1a::GUS(**M**) and pmiP1b::GUS (**N**) plants

The microProteins *miP1a/b* are expressed in the vasculature of leave

After the temporal analysis of *miP1a/miP1b* expression, we assessed their spatial expression pattern. *CO* and *FT* are expressed in the vasculature of leaves (Fig. 3.7 K, L), as previously reported (Takada and Goto, 2003). Expression of both genes in vascular cells is also sufficient to trigger the transition to flowering (An et al., 2004). Expression analysis of *miP1a* and *miP1b* in transgenic plants, where a genomic fragment consisting of the 1500 bp upstream region from the transcription start site of either *miP1a* or *miP1b* fused to the beta-glucuronidase gene (GUS), revealed that both microProteins have a broader and more patchy pattern of expression compared to *CO* but are also predominantly expressed in vascular tissues (Fig. 3.7 M, N) of the plant's aerial parts. Therefore they are present in the leaves, the place where CO is acting to regulate photoperiod-dependent flowering.

In addition to the expression in leaves we detected also GUS expression for both *miP1a/b* in petioles of leaves where *CO* does not seem to be expressed. It is interesting to note that both microProtein genes are highly abundant in the shoot apical meristem, where *CO* also seems to be expressed but *FT* is not induced.

The finding that *miP1a/b* are co-expressed in vascular tissue and have the ability to interact with CO, supports a regulatory role. Furthermore, when ectopically expressed in the phloem companion cells from the SUC2-promoter, miP1b can also strongly delay the floral transition indicating that it is functional in the phloem and that CO is likely its major target (Suppl. Fig. S2).

Identification of transcripts affected by CO inactivation

To further corroborate the idea that the predominant function of miP1a/b is to regulate CO protein activity, we characterized transcriptomes of two week old seedlings from Col-0 wild type, *co* mutants (*co-SAIL*) and the transgenic plants overexpressing *miP1a* and *miP1b* using RNA-Seq (Greaff et al. unpublised). The downregulated-transcriptomes of *35S::FLAG-miP1a* and *35S::FLAG-miP1b* have a 60% overlap which is quite substantial but not surprising. Interestingly, around 80% of the genes down-regulated in the *co* mutant background (relative to Col-0) are also down-regulated in the transgenic *35S::FLAG-miP1b* plants (Fig. 3.8 A) supporting the idea that CO protein activity is strongly compromised by miP1b-overexpression. To validate the observation that differentially expressed genes identified by mRNA-Seq are truly altered in expression, we performed individual qRT-PCRs to test expression of five candidate genes (Suppl. Fig. S3). These RT-PCRs confirm the RNA-Seq results. We find genes that are down-regulated compared to Col-0 in all three genotypes

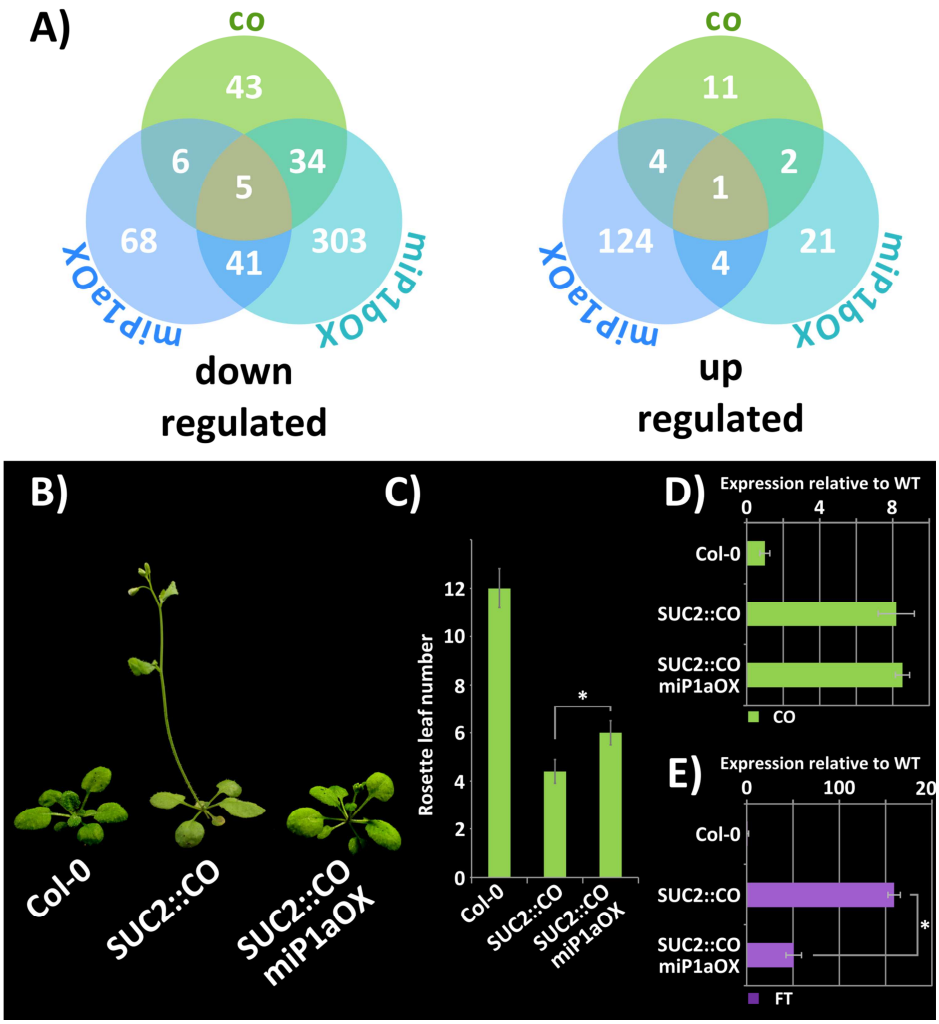


Figure 3. 8 A) summary of the mRNAseq results, showing the number of genes in *co*, *p35S::miP1a* or *p35S::miP1b* up or down regulated in their expression compared to Col-0. The analysis was performed by Daniel Straub
B) Three week old Col-0, pSUC2::CO and pSUC2::CO x p35S::miP1a plants **C)** leaf number at bolting, Asterisks indicate significant differences in the leaf number; Expression of CO (**D**) and FT (**E**) in the respective plants compared to Col-0, Asterisks indicate significant differences in expression

(e.g. *FUL* and *At3g49340*) but also genes whose expression is unchanged in *35S::FLAG-miP1a* but down-regulated in *co* mutants and *35S::FLAG-miP1b* (e.g. *ZAT7*) indicating that miP1a and miP1b might also have diverging functions. The same is true for genes up-regulated in the investigated genotypes (Suppl. Fig. S3). In all three genotypes (*co*, *35S::FLAG-miP1a*, *35S::FLAG-miP1b*), the expression levels of *FT* are among the top down-regulated genes confirming that the late flowering phenotype of *35S::FLAG-miP1a* and *35S::FLAG-miP1b*, like in *co* mutants, is due to the failure of inducing *FT* expression. Another flowering time gene found to be down-regulated in all three genotypes is *FRUITFUL* (*FUL*) which acts downstream of FT (Teper-Bamnolker and Samach, 2005), further supporting the hypothesis that miP1a/b act by inhibiting CO activity. These findings are in agreement with unchanged *CO* mRNA levels in *miP1a/b* over-expression plants, which indicate that the inhibition of CO likely occurs at the post-translational level. We also analyzed genes up-regulated in *co*, *35S::FLAG-miP1a*, *35S::FLAG-miP1b* and found *MADS AFFECTING FLOWERING5* (*MAF5*) to be up-regulated in all three genotypes relative to

Col-0 (Fig. 3.8 A). MAF5 acts as a floral repressor that is strongly controlled epigenetically (Kim and Sung, 2010; Shen et al., 2014), which is in line with the late flowering phenotype observed in *co* loss-of-function and *miP1a/b* gain-of-function plants. Whether and how elevated *MAF5* mRNA levels contribute to the late flowering phenotype of *co* mutant plants is currently unknown.

Overexpression of *miP1a* in transgenic plants ectopically expressing *CO* alters flowering time

To assess whether miP1a/b have a negative effect on CO activity, we crossed very early flowering *SUC2::CO* plants with late flowering *35S::miP1a* plants. Progeny plants carrying both transgenes show an intermediate flowering behavior when compared to wild type and *SUC2::CO* plants (Fig. 3.8 B, C). This delay in flowering is not due to an effect on the levels of *CO* expression (Fig. 3.8 D). However, *FT* levels are significantly lower in *SUC2::CO 35S::miP1a* plants compared to *SUC2::CO* plants (Fig. 3.8 E). When compared to wild type plants, the levels of *FT* expression in *SUC2::CO 35S::miP1a* plants are still strongly induced (around 50-fold), explaining the still earlier flowering of the *SUC2::CO 35S::miP1a* plants compared to the wild type. This might be due to the strong activity of the *SUC2* promoter in the phloem companion cells, causing higher CO then miP1a abundance in this tissue.

Phylogenetic analysis of miP1a/b-type microProteins in different plant genomes

To gain more information on how miP1a/b-type proteins have evolved, we used the Phytozome database (Goodstein et al., 2012). With help of this database we identified and extracted the available miP1a/b related proteins from the genomes of different plant species.

A multiple sequence alignment of all species revealed that the first B-Box and the remnants of the second B-Box are highly conserved. Surprisingly, there is a very high conservation for the last five amino acids, constituting a motif of PF(V/L)FL (Fig. 3.9 A and Suppl. Fig. S4 A). Phylogenetic analysis revealed that miP1a/b-type proteins evolved in the *Pentapetalae* family of dicotyledonous plants. Using the last five amino acids as anchor, we find that the carboxy terminal motif of the most ancient miP1a/b-type proteins in the *Fabidae* family varies significantly (Suppl. Fig. S4 B). In *Glycine max* e.g. we find one protein with the sequence LSLLL that strongly resembles the LxLxL motif, which has been shown to mediate interactions with TOPLESS-related co-repressor proteins. It is interesting to note that the PFVFL motif that is found exclusively in the *Brassicaceae*

family evolved by acquiring a single point mutation that changed the leucine in the middle position to a valine. Because of the high degree of conservation of the PF(V/L)FL motif, we can assume that it confers a biological activity to miP1a/b-type proteins. The finding that the ancestral motif strongly resembles a TOPLESS-interaction motif suggested to us that these small proteins might function by engaging with TOPLESS/TOPLESS-related co-repressor proteins. This idea is supported by the identification of miP1a as an interacting partner for the two TOPLESS-RELATED-PROTEIN 2 and 4 (Causier et al., 2012).

MiP1a/b act by recruiting TOPLESS co-repressor proteins

To test if miP1a/b type microProteins interact with TOPLESS (TPL), we performed a direct yeast-two-hybrid

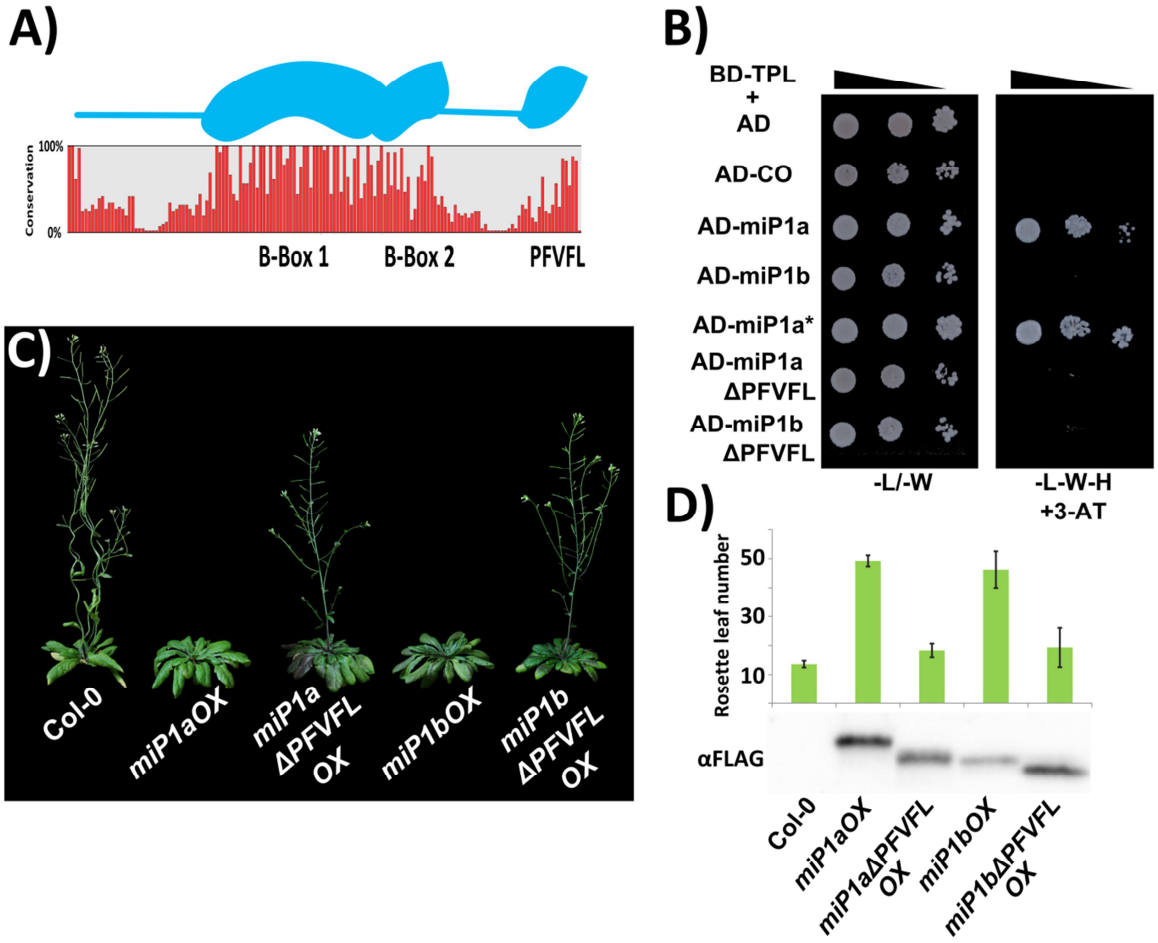


Figure 3. 9 Structural analysis and characterization of the PFVFL-motif A) Amino acid conservation of miP1a/1b orthologs and depiction of the conserved features; **B)** Yeast-two hybrid assay with DBD-TPL and AD-CO/miP1b/miP1a and different variants on L/W and L/W/H selective media; **C)** 3 week old Col-0, p35S::miP1a/1b and p35S::miP1a/1bΔPFVFL plants; **D)** leaf number of the respective grown at long day conditions plants at bolting and immunoblot signal using an αFLAG antibody, showing the abundance of proteins in the expected size

interaction test. In this assay both miP1a and miP1a*, the latter having mutations in the B-Box domain, interacted with the TPL protein (Fig. 3.9 B). CO protein did not interact with TPL in this assay and neither did miP1aΔPFVFL, a miP1a variant lacking the last five amino acids (Fig. 3.9 B).

To further explore the possibility that the PF(V/L)FL motif has an *in vivo* function, we compared transgenic plants overexpressing full-length miP1a/b proteins with protein variants lacking the last five amino acids (*35S::FLAG:miPa/bΔPFVFL*). Under inductive long day conditions, both miP1a/b over expressing plants exhibit a late flowering phenotype whereas transgenic plants overexpressing either miP1aΔPFVFL or miP1bΔPFVFL flower almost as early as the wild type (Fig 3.9 C, D). To exclude the possibility that these transgenic plants accumulate diverging amounts of miP1a/b proteins we determined protein expression levels by western blot analysis. We find that the levels of transgenic proteins are largely similar (Fig. 3.9 D) excluding the possibility that removal of the PF(V/L)FL motif affects transcript or protein stability.

Because miP1a/b-type microProteins do not harbor a DNA-binding motif it seems likely that they act as adaptors to recruit TPL/TPR co-repressor proteins to transcription factors and bridge between the transcription factor and the co-repressor complex. To investigate this hypothesis we performed a yeast-three-hybrid study and tested whether miP1a and miP1b are able to mediate interaction between CO and TPL, which showed no interaction in the yeast-two-hybrid system (Fig. 3.9 B). When co-transformed with the empty pDR plasmid AD-CO and BD-TPL were still unable to induced yeast growth on selective medium. However, in the presence of the miP1a or miP1b protein, yeast growth was strongly induced, supporting the idea that in the presence of miP1a/1b CO and TPL can interact. Without the PFVFL-motif, this interaction was abolished (Fig. 3.10 A). The idea of miP1a allowing the formation of a tripartite complex with TPL and CO is further supported by the observation that GST-miP1a is able to pull down 6xHis-TPL and MBP-CO in an simultaneous *in vitro* pull down experiment with recombinant protein. GST-miP1aΔPFVFL is only able to pull down MBP-CO but not 6xHis-TPL and GST-ZPR3 interacts with neither of the two target proteins (Fig. 3.10 B). These findings support the idea that miP1a/b-type proteins act as TPL/TPR-bridging factors for B-Box transcription factors and engage these transcription factors in transcriptional repressor complexes.

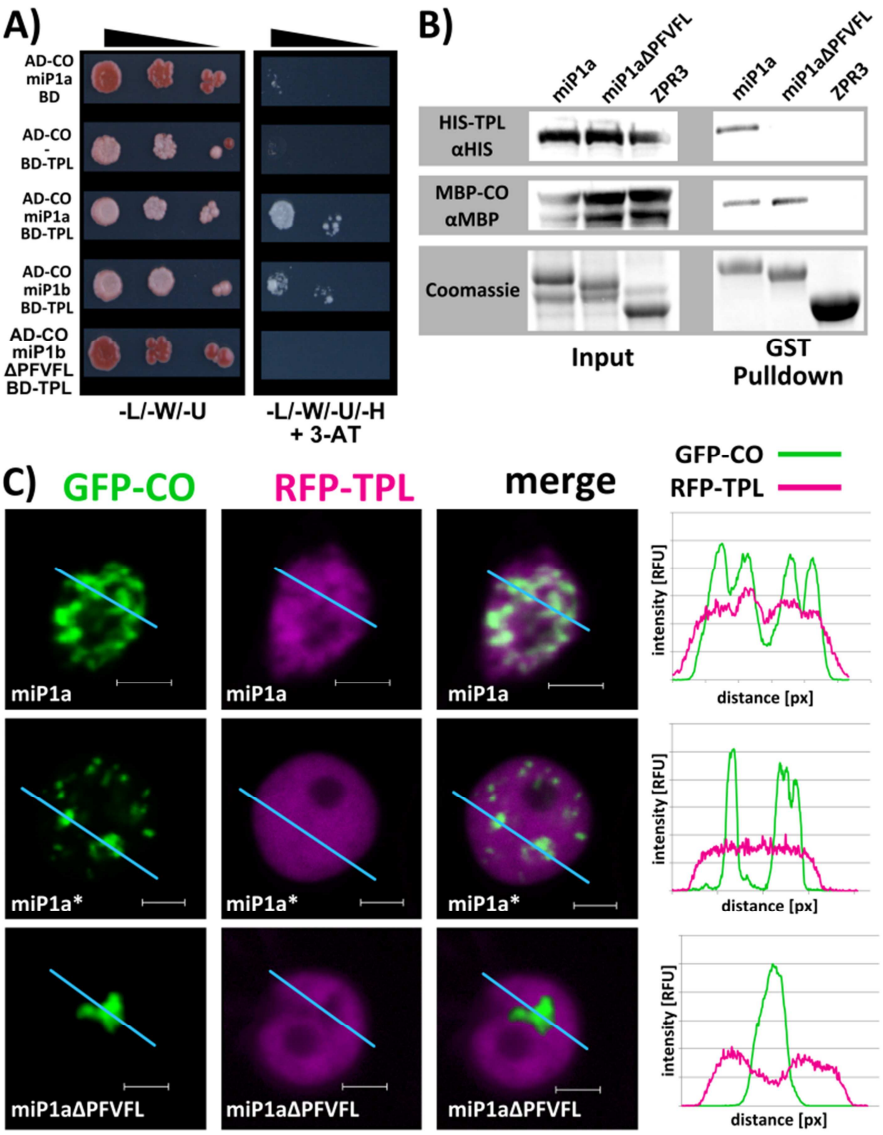


Figure 3. 10
A) Yeast three hybrid experiment with AD-CO and BD-TPL together with miP1a/1b or miP1bΔPFVFL; **B)** Immunoblot with αHIS, αMBP and Coomassie staining of input and GST-pulldown fraction of GST-miP1a/-miP1aΔPFVFL/-ZPR3 incubated with 6xHIS-TPL and MBP-CO **C)** Co-localization of GFP-CO and RFP-TPL in nuclei of transiently transformed tobacco leaf epidermis cells. Additionally the leaves were infiltrated with either p35S::miP1a, miP1a* or miP1aΔPFVFL (bar length 5 µm)

Colocalization

In order to study the interaction behavior of CO, miP1a and TPL in planta, tobacco leaves were transiently transformed with GFP-CO, RFP-TPL and different variants of miP1a, all proteins being expressed from the viral p35S-promoter. The localization of the two fluorophores was observed in the epidermis cells of tobacco plants, two and three days after infiltration (Fig. 3.10 C) .GFP-CO localizes, as previously observed (Fig. 3.3 C), to the nucleus as does RFP-TPL. RFP fluorescence can be observed in most parts of the nucleus, excluding a region assumed to be the nucleolus, whereas GFP-CO localizes in a distinct pattern that differs between the co-infiltrated miP1a variants. Co-localization analysis along a cross section of the analyzed nuclei revealed a strong co-localization of GFP-CO with miP1a and miP1aΔPFVFL. Likewise the localization of RFP-TPL, although in general more evenly dispersed in the nucleus, was different, depending on the co-infiltrated proteins. RFP signal accumulates in the same regions where GFP signal can be detected if GFP-CO and miP1a are expressed in the cells. However, RFP

distribution does not follow GFP-distribution if the miP1a* variant is present. The differences observed seem to be due to the different miP1a variants, with miP1a promoting GFP-CO and RFP-TPL colocalization, and miP1a* not influencing it. In the context of GFP-CO and miP1aΔPFVFL, where GFP-CO shows the distinct pattern displayed when interacting with miP1a; RFP signal is even excluded from the regions of GFP accumulation. In summary we see colocalization of RFP-TPL with GFP-CO and miP1a only if miP1a contains a functional B-Box and a PFVFL-motif, indicating that its ability to interact with CO and TPL *via* these regions is necessary for the observed co-localization of the proteins.

4. DISCUSSION

Several microProteins have been identified in plants in the past years (Hyun and Lee, 2006; Wenkel et al., 2007; Zhang et al., 2009b; Mara et al., 2010; Hong et al., 2011). A commonality among these proteins is the ability to sequester larger, multi-domain proteins into non-productive heterodimeric complexes. Our study reveals a number of small proteins that have a protein-protein-interaction domain and that might modulate the formation of higher order protein complexes (Suppl. Table 1).

CO activity can be regulated by the formation of different types of protein complexes

Transcription factors are often organized in gene families and the type of complexes they engage in can strongly modulate their activities. For CO it was recently shown that interaction with the BBX19 transcription factor renders CO non-functional (Wang et al., 2014a), indicating that other B-Box proteins can also influence CO activity directly. However, more recent observations suggest an additional role for BBX19 in the control of shoot elongation and PIF expression rather than flowering time regulation (Wang et al., 2015). BBX19 interacts with ELF3 and COP1, mediating the COP1 dependent ubiquitination and degradation of ELF3, thereby promoting the expression of *PIF4* and *PIF5*, affecting hypocotyl elongation in the evening positively. How this relates to the control of CO activity and stability remains unresolved. The assumption that BBX19 sequesters CO in a non-functional complex (Wang et al., 2014a) seems as likely as BBX19 interacting with CO and COP1 and promoting the COP1 dependent

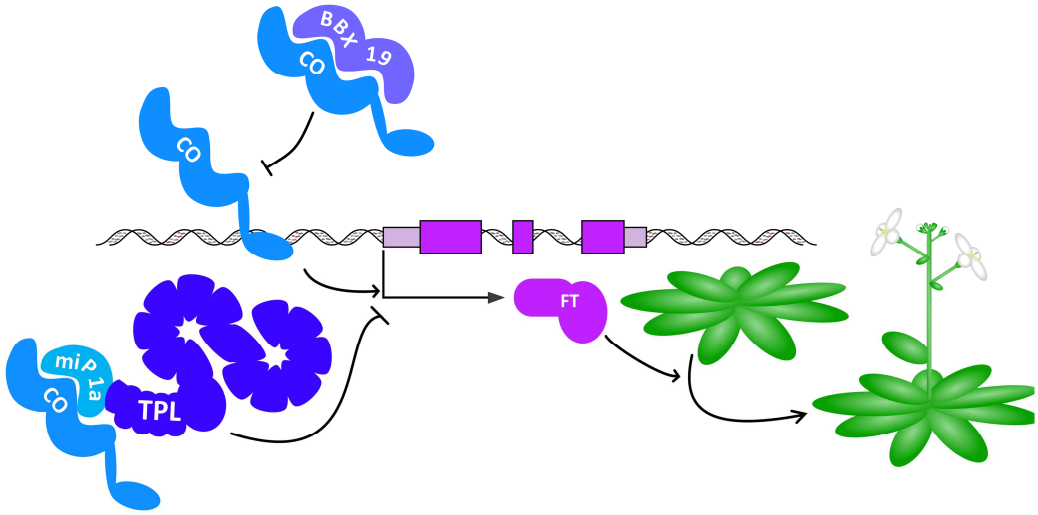


Figure 4. 1 Model of CO activity regulation by BBX19 and the miP1a/1b-TPL complex

degradation of CO in a similar manner like ELF3 or affecting CO proteins stability passively by promoting *PIF4* and *PIF5* expression (Wang et al., 2015).

We show that the transition to flowering, a trait controlled by the CO protein, can also be attenuated by overexpressing naturally occurring miP1a/b-type microProteins. MiP1a/b-type microProteins are not only binding CO and render it non-functional (Fig. 4.1): they also link CO to co-repressors of the TPL/TPR family by simultaneous interaction with these two proteins. Depletion of the TPL/TPR from the CO/miP heterodimeric complex by removing the PFVFL-motif alleviates flowering time further, suggesting that the interaction with TPL/TPR proteins is crucial for the flower repressing function of the two microProteins (Fig 3.9 c).

It is interesting to note, that an alternatively spliced product for CO exists, which would produce a truncated protein lacking the middle region and CCT-domain, largely resembling the CO_{BB} artificial microProtein. Due to the presence of a premature termination codon, it is, however, conceivable that this splice variant might be a target for nonsense-mediated mRNA decay (Isken and Maquat, 2008). This splice variant of the *CO* gene, encoding the CO_{BB} microProtein, might be expressed and stabilized under certain environmental conditions (Drechsel et al., 2013; Filichkin et al., 2015). The CO_{BB} protein could inhibit CO or buffer its activity by sequestering it. Moreover, it is also possible that miP1a/b-type proteins interact with BBX19 or miP1a/b-type proteins and thus shield CO from engaging in a non-productive complex. Such a tripartite switch was discovered in the basic helix-loop-helix (bHLH) transcription factor family for HBI1, IBH1 and the HLH-type microProtein PRE1 (Zhang et al., 2009a; Bai et al., 2012). On that account it is likely that miP1a/1b analogously engage CO and TPL/TPRs in a tripartite complex.

TPL/TPR proteins in the regulation of flowering time

The connection between the two newly identified microProteins miP1a and miP1b and TPL/TPR transcriptional co-repressors depicts a novel way of microProtein function. Co-repressors orthologue to TPL/TPR can be found in all eukaryotes and in plants they play important roles in many developmental processes like ovule and embryo development, stem cell and polarity establishment and circadian rhythm (Long et al., 2006; Smith and Long, 2010; Wang et al., 2013; Ryu et al., 2014; Wei et al., 2015). They are also essential components of Auxin, Jasmonate and Brassinosteroid signaling pathways (Szemenyei et al., 2008; Pauwels et al., 2010; Oh et al., 2014). In fact, more transcriptional repressors have been identified to interact with TPL/TPR proteins than with other co-repressors and the high degree of conservation underlines the importance of this protein family in plant development (Causier et al., 2012). TPL has been previously reported to play a role in flowering time

control, as TPL/TPRs interact with TOE1/2 and TEM1, known repressors of *FT* expression (Jung et al., 2007; Castillejo and Pelaz, 2008). The flower-delaying effect of TOE1 overexpression is abolished in tpl-1 mutant plants, indicating the importance of TPL-TOE interacting for the correct function of these proteins. However, the observed late flowering phenotype is relatively mild compared to miP1a/1b overexpression and *FT* expression is only slightly affected by TOE1ox in *tpl-1*. In general, tpl-1 plants exhibit higher *FT* expression and earlier flowering compared to *Landsberg erecta* wild type plants, indicating further TPL-dependent mechanisms affecting *FT* expression (Causier et al., 2012). Hence, the here discovered and described CO-miP1a/b-TPL/TPR complex further might present a previously unknown way of the elaborate regulation of *FT via* TPL/TPR.

PFVFL-motif as a new TPL interaction motif

TPL/TPR proteins interact with transcriptional repressors *via* a short amino-acid motif that interacts with their N-terminal TOPLESS-DOMAIN (Ke et al., 2015). The first of this motifs to be characterized was the EAR motif (for ERF1-associated amphiphilic repression), a short series of amphiphilic amino acids that are essential for the transcriptional repressive character of many repressor proteins (Ohta et al., 2001). Further motifs have been identified that differ in the amino acid composition but share the amphiphilic character (Kagale and Rozwadowski, 2011; Ke et al., 2015). MiP1a was already previously identified as a protein interacting with TPL/TPR proteins, but as it neither harbors a canonical EAR-motif nor another described TPL/TPR interacting motif, it was unknown how miP1a interacts with TPL/TPR proteins (Causier et al., 2012). The C-terminal PFVFL-motif we identified shows a high degree of conservation among orthologues and the assumed evolution of this motif (Supp. Fig. S4) indicates that these five amino acids are the TPL/TPR interaction site. We observe interaction between miP1a and TPL in yeast-two hybrid experiments, only the PFVFL-motif is present (Fig. 3.9 B). Likewise is the TPL-miP1a interaction *in vitro* dependent on this motif (Fig. 3.10 B). MiP1b seems not to interact with TPL in the yeast-two hybrid assay (Fig. 3.9 B), but the normal flowering phenotype of the miP1bΔPFVFLOX plants (Fig 3.9 C, D) and the observed interaction between CO and TPL in the presence of miP1b in yeast (Fig. 3.10 A) suggests, that CO-miP1b is able to interact with TPL/TPR. Whether the PFVFL motif (like the EAR-motif) is sufficient to render a protein into a transcriptional repressor, (Hiratsu et al., 2003) remains to be further investigated.

B-Box mediated protein interaction

We observed a strong CO antagonistic function for the CO_{BB} splice variant when overexpressing it. CO_{BB} contains both CO-B-Boxes, so it can be assumed that it interacts with the full length CO protein, sequestering it in a non-functional state. From the animal TRIM proteins (RING, B-Box, coiled-coil tripartite motif) it is known that they tend to homodimerize *via* their B-Box domains (Mrosek et al., 2008; Sugiura and Miyamoto, 2008; Tao et al., 2008; Huang et al., 2014). It is also observed that the B-Box mediated interaction between TRIM proteins is the basis of higher order complex formation (Li et al., 2011). Plant B-Box proteins which do not contain, unlike their animal counterparts, RING and coiled-coil domains, might engage in even higher order complexes. Indeed they have been shown to interact with many different kinds of proteins *via* different regions (Gangappa and Botto, 2014). The CO_{BB} protein would in this context prevent the formation of functional CO-dimers or higher order complexes.

The assumption that similar B-Boxes have a high affinity towards each other could explain why transgenic plants over-expressing the CO_{BB} protein variant are very late flowering (Fig. 3.4 A and B). Following that assumption it could be reasoned that the interaction strength between BBX19 and CO is higher than between CO and miP1a/b-type proteins. We show that the different types of B-Box proteins are able to interact in yeast (Fig. 3.5 C). However, the hypothesis that such interactions would influence flowering by sequestering CO is not supported by the observation that the overexpression of different B-Box group proteins and their B-Boxes as artificial microProteins does not cause a late flowering phenotype like it is observed for CO_{BB}, BBX19 and miP1a/1b (Fig. 3.5 A, B). Further interactions might be necessary to cause an effect such as the observed one. Here it would be interesting to characterize the interaction strength between the different B-Box proteins on the molecular level, using for example surface plasmon resonance spectroscopy or thermophoresis (Madeira et al., 2001; Wienken et al., 2010). Such measurements would also help to understand the dynamics of the interaction within the B-Box family and will, together with experiments on their affinity and stability in plants, broaden our understanding of the role of this family in plant development.

BBX19 like STO belongs to the class IV of B-Box proteins. Overexpression of STO was recently shown to promote early flowering under both short and long day conditions in a CO-independent manner (Li et al., 2014). These findings further suggest that sequences outside the B-Box might contribute to the dominant-negative function of BBX19 and its interaction with COP1 and influence on ELF3 stability (Wang et al., 2015) might additionally contribute to its ability to affect flowering. We did not observe such effects of STO

overexpression, but we also did not characterize the STO/STO_{BB} overexpressing plants in the same detail as Li et al. and might therefore have overlooked the milder effects.

Evolution of miP1a/b-type microProteins, an example for functional specialization?

Phylogenetic analysis of miP1a/b-type proteins across different genomes suggests that these proteins evolved after the separation of the monocotyledonous and dicotyledonous lineages. A remarkable difference exists in the regulation of flowering time in rice, a monocotyledonous short-day plant and *Arabidopsis*, a dicotyledonous long-day plant: The rice CO-orthologue HEADING DATE 1 (Hd1) acts as an activator of flowering time in response to short days (analogous to Arabidopsis CO in long days) but has an additional activity in long days where it acts as a repressor of flowering time (Hayama et al., 2003). The fact that miP1a/b-type proteins can only be found in dicotyledonous plants implies that they could serve as an example for functional specialization and engage CO into a transcriptional repressor complex (Fig. 4.2). Our observations suggest that in dicotyledonous plants CO also has a flower promoting or hampering effect, depending on the amount of miP1-type microProteins present.

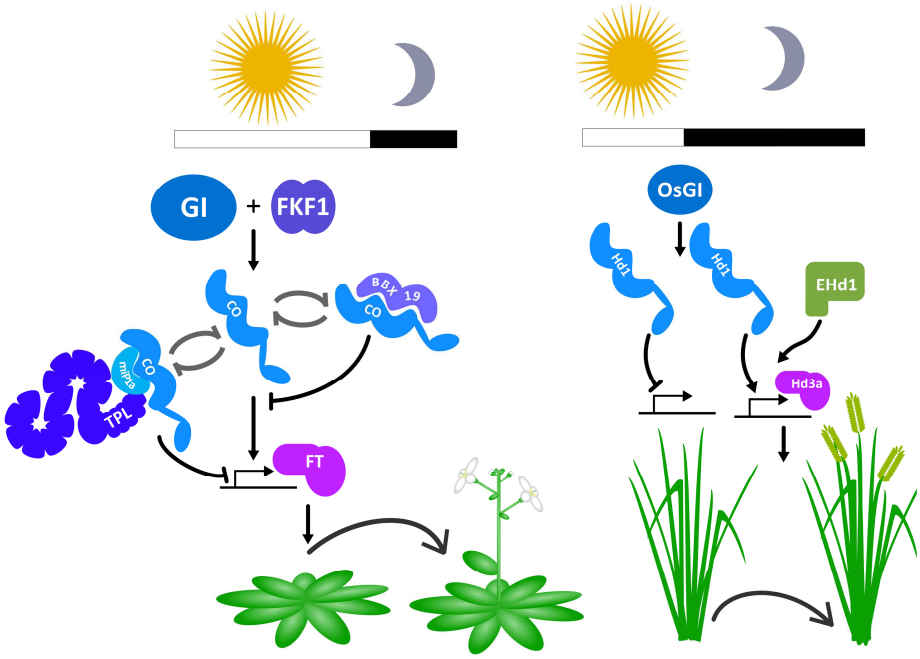


Figure 4. 2 Role of CO in flowering regulation in *Arabidopsis* and Rice
Hd1 induces flowering in short days but represses it in long days; miP1a/1b engage CO in a repressive complex in *Arabidopsis*

Analysis of the B-Box domains of miP1a/b-type proteins further revealed that they are structurally different from CO/COL proteins. CO/COL proteins have the following structure: $Cx_2Cx_8Cx_7Cx_2Cx_4Hx_8H$ whereas miP1a/b-type proteins are one residue shorter, resulting in the following structure: $Cx_2Cx_7Cx_7Cx_2Cx_4Hx_8H$ (Fig. 3.2 C) (Robson et al., 2001).

This latter type B-Box motif is only shared among five members of the group V BBX-proteins (BBX28, BB29, BBX30 (miP1a), BBX31 (miP1b) and BBX32). Compared to all group V BBX proteins, miP1a/b are much shorter, have a unique amino-terminus and the additional carboxy terminal PFVFL motif. These three features make them remarkably different from all other group V BBX proteins. Furthermore, overexpression of *BBX32* has only mild flower attenuating effect and BBX32 predominantly affects light-dependent hypocotyl elongation (Holtan et al., 2011b).

miP1a and miP1b: all different and yet the same?

Both miPa1 and miP1b harbor a complete B-Box and remnants of a second B-Box domain and contain the carboxyterminal PFVFL motif (Fig. 3.9 A). They have a 65.5% sequence identity towards each other, but miP1b is 4 amino acids shorter than miP1a (117 aa vs. 121 aa). Yet, all missing residues are found in the sequence after the second B-Box, which might not have biological activity. Our results show that both miP1a and miP1b act as genuine microProteins and possess the ability to dominantly suppress the activity of CONSTANS. Inhibition of CO results in inability to induce *FLOWERING LOCUS T* in response to long day photoperiods causing these plants to flower extremely late. Furthermore, the late flowering phenotype of both miP1a and miP1b depends on the presence of the PFVFL motif. Both *miP1a/b* microProteins are expressed in the vasculature of leaves like *CO* and *FT*. Expression of *miP1b* in the vasculature under the control of the *SUC2* promoter also delays the floral transition, indicating that miP1b is active in that tissue.

Both *miP1a* and *miP1b* genes exhibit diurnal patterns of expression. In short day conditions both genes peak in expression in the second half of the dark period. Under long days *miP1b* peaks around the same time but *miP1a* expression is high in the first half of the day and then successively decreases (Fig. 3.7 a and b). This implies that miP1a and miP1b might function as buffers to ensure the inactivity of CO in the morning and in the night. Although CO is supposed to be degraded rapidly in the dark, the high amounts of miP1a and miP1b would form a high threshold that needs to be overcome by residual CO before it can activate *FT* expression. This idea is supported by the slightly earlier flowering MIGS lines (Fig. 3.6). It would be interesting to see, how plants with reduced or higher levels of miP1a/b behave under different light regimes than the classical long or short day and if gradual changes of the flowering time can be observed.

The role of chromatin modification in the regulation of FT expression

In the last years it became clear that the transcriptional regulation of the *FT* locus is a very complex process in which many key factors of the different floral transition pathways are involved (Amasino, 2010; Song et al., 2013; Romera et al., 2014). In addition, also structural features on the DNA and chromatin level have a strong influence on these processes. Four regions at the FT locus have been found to play major roles:

1) A genomic region covering the first exon and intron of *FT* contains binding sites for several factors affecting *FT* expression, especially repressors like the FLC-SVP complex (Lee et al., 2007) .

2) The AP2 like proteins SMZ, SNZ and TOE1&2 additionally seem to bind to a region 1500 bp downstream of the *FT* coding region and affect the activity of the locus by doing so (Mathieu et al., 2009). The factors binding to those regions are involved in long term regulation of *FT* expression and known to be affected by temperature, like FLC via vernalization (Bastow et al., 2004), and the developmental age of the plant, via miR172 (Jung et al., 2007; Mathieu et al., 2009; Wu et al., 2009).

3) The region containing the 5'UTR of FT until approximately 500 bp upstream from there. Several of the factors activating or repressing *FT* are known to bind very close towards the transcription start site. GI-FKF1 as well as PIF4 bind within this region and promote *FT* expression (Fig. 1.4) (Sawa and Kay, 2011; Kumar et al., 2012). The repressor TEM1 binds to the 5'UTR coding region and might hereby hinder CO from interacting with it (Castillejo and Pelaz, 2008). CO was identified to bind within a region close to the translational start site (Song et al., 2012).

4) Two CORE elements in the proximal promoter region (251 bp and 191 bp upstream from the translational start site) were identified (Tiwari et al., 2010), although CO demonstrates only a weak affinity to those *in vitro*. However, the importance of this proximal promoter region is supported by the high degree of in comparison between different plant species (Adrian et al., 2010). Adrian et al. analyzed the conservation of the *FT* promoter between different species and additionally identified a conserved site more than 5000 bp away from the translation start site. Hap3a and Hap5a, two proteins from the nuclear factor Y family, known to interact with the C-terminus of CO (Wenkel et al., 2006), bind to the CCAAT-box elements within this region and cause the formation of a chromatin loop, most likely by interacting with CO bound towards the CORE elements (Cao et al., 2014).

The involvement of chromatin modifications on the regulation of *FT* activity adds another level of complexity. Post-translational modifications on the different histones - like methylation, acetylation, phosphorylation or ubiquitination- are known to influence the accessibility of the chromatin for the transcription machinery and influence the expression of the respective genomic regions (Kouzarides, 2007). How chromatin modifications affect flowering is better understood from their influence on *FLC*, which is repressed by chromatin modifications established during vernalization (Searle et al., 2006; Han et al., 2007; Marquardt et al., 2014); it is also important for the regulation of *FT*. Several activating or repressing chromatin marks like histone 3 Lysin 4 acetylation (H3K4ac) and histone 3 Lysin 27 trimethylation (H3K27me3) have been identified at the *FT* locus changed by activating or repressing factors binding to it (Adrian et al., 2010; Romera et al., 2014). Even cyclic changes during the course of the day for these marks have been identified (Gu et al., 2013). Such histone modifications are applied by various classes of proteins (e.g. methylatransferases, acetylases and deacetylases) and mutations in those genes have been found to alter the floral transition (Lu et al., 2010; Jeong et al., 2015). The Polycomb Repressive Complexes 1 and 2 (PRC1/2) are multi-protein complexes that mediate the deposition and maintenance of mainly H3K27me3 and other repressive chromatin marks. PRC1/2 are not only important for FLC repression (Bastow et al., 2004) but also involved in the repression of *FT* (Turck et al., 2007). FLC itself interacts with EMBRYONIC FLOWER 1 that forms a PRC1 complex with LHP1 and JMJ14 and the binding of this complex to the *FT* locus represses its expression (Wang et al., 2014b). The described formation of a high-order complex affecting *FT* expression can explain some of the observed changes, especially at the known FLC binding sites in the beginning of the *FT* coding sequence, but it is not sufficient to explain other observations like the maintenance of an LHP1 depleted region in the distal regulatory region (Adrian et al., 2010) or changes in the acetylation of chromatin in the FT promoter region (Gu et al., 2013).

Our findings on the interaction of mip1a/1b with TPL and CO, causing the formation of a tripartite complex, might explain how these sites in the *FT* promoter are modified in a CO dependent manner. TPL/TPR proteins themselves cannot bind DNA or modify histones directly but they serve as a scaffold for a variety of chromatin modifying proteins with repressive character, like histone methylases or deacetylases (Kagale and Rozwadowski, 2011; Causier et al., 2012; Krogan et al., 2012). The involvement of CO in a TPL/TPR complex by miP1a/1b would therefore change its role from an activator of the floral transition to a repressor. It is noteworthy, that CONSTANS if equipped with an additional EAR-motif – a classical TPL interaction motif found in many transcriptional repressors – develops a strong flower repressing character, likely due to direct interaction with TPL/TPR co-repressors (Takase et al., 2007). MiP1a and miP1b can engage CO in such a

complex, and evolved maybe to prevent flowering under unfavorable conditions. Further research and characterization of this interaction is necessary to understand, where and when this complex is formed, which chromatin modifications it initiates and which chromatin modifying proteins are additionally involved. Finally the binding sites at the *FT* promoter and the changes in the chromatin state at these loci can be identified in this manner. Loss of function mutants of *miP1a* and *miP1b* and crosses of those plants harboring mutations in other known components of this network will also help to elucidate the role of the two microProteins in *FT* regulation.

Conclusion

MicroProteins bind specifically to their respective target proteins and function as negative regulators. Considering the complexity and plasticity of the genetic networks regulating plant development, microProteins can fulfill an important function as simple and flexible modulators of protein activity. The identification of more than 30 potential new microProteins, demonstrates that this mechanism might be involved in the regulation of protein activity to a higher extent than previously assumed. The microProtein qualities of the two here newly characterized B-Box proteins BBX30 and BBX31, validates our approach and the underlying concept. The observed phenotype of plants ectopically expressing miP1a/1b is strongly dependent on the presence of CO, as miP1a overexpression has no additional effects on flowering in a *co* loss of function mutant. MiP1a overexpression in a context with high levels of CO strongly affects flowering.

Furthermore, we show that the two microProteins are not only trapping CO in a passive and non-functional state, but that they are also able to engage CO in a complex containing TPL/TPR transcriptional co-repressors. The idea of transcriptional repressors engaging transcription factors and co-repressors in specific and potent repressive complex is already known for hormone signaling pathways, where AUX/IAAs (Szemenyei et al., 2008) or JAZ (Pauwels et al., 2010) proteins function in this manner, and we are able to extend this idea to the process of flowering time regulation.

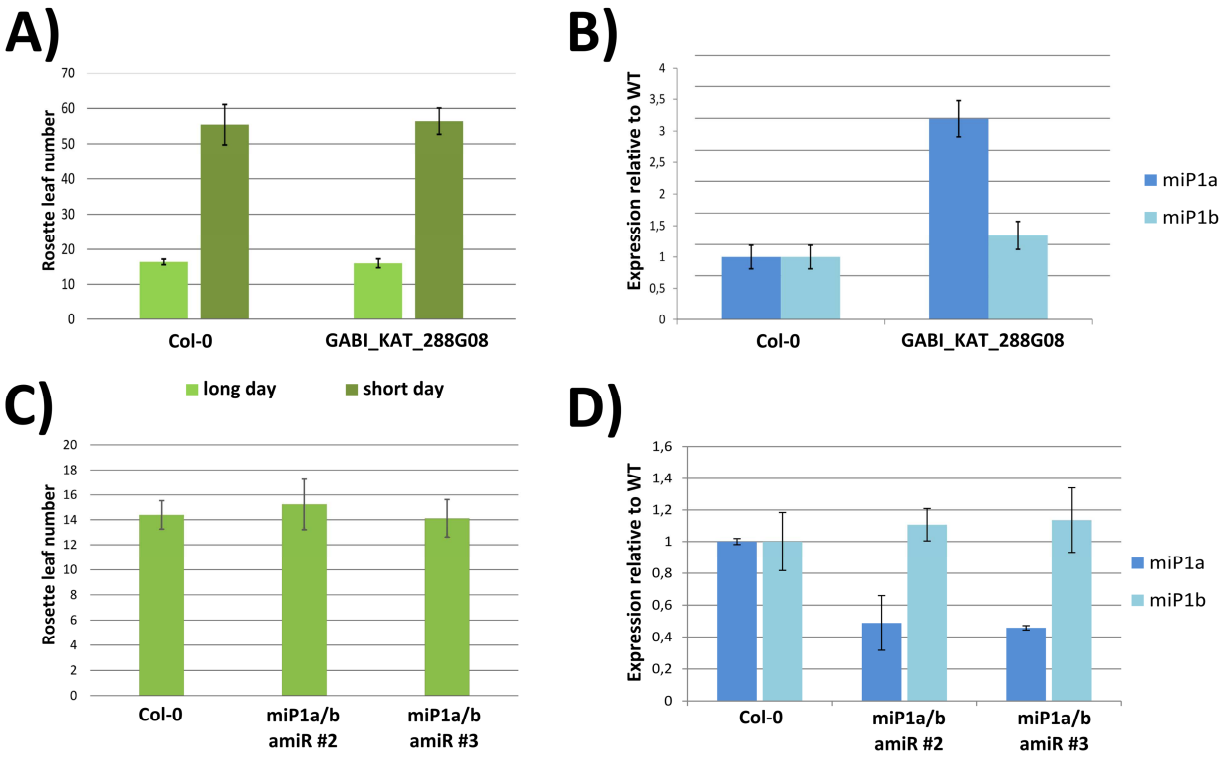
Thus, our finding unraveled a new function of CONSTANS ; that is to engage in a TPL/TPR trimeric complex, which has the potential to fine-tune the flowering response of dicotyledonous plants. The detailed analysis of how TPR/TPL affects flowering is likely complex and requires viable or conditional higher order mutant plants.

SUPPORTING INFORMATION

Supplemental table 1 MicroProteins identified in this study.

AT-number	Annotation	Pfam
AT1G02210.1	NAC (No Apical Meristem) domain	PF02365
AT1G18770.1	RING/U-box superfamily protein	PF00097
AT1G18835.1	MIF3, mini zinc finger	PF04770
AT1G24580.1	RING/U-box superfamily protein	PF00097
AT1G26945.1	KDR, basic helix-loop-helix (bHLH)	PF00010
AT1G31760.1	SWIB/MDM2 domain superfamily protein	PF02201
AT1G72070.1	Chaperone DnaJ-domain superfamily protein	PF00226
AT1G74500.1	ATBS1, BS1, TMO7	PF00010
AT1G74660.1	MIF1, mini zinc finger 1	PF04770
AT1G75390.2	AtbZIP44, bZIP44, basic leucine-zipper 44	PF00170
AT2G26320.1	AGL33, AGAMOUS-like 33	PF00319
AT2G31215.1	basic helix-loop-helix (bHLH)	PF00010
AT2G33735.1	Chaperone DnaJ-domain superfamily protein	PF00226
AT2G35605.1	SWIB/MDM2 domain superfamily protein	PF02201
AT2G35795.1	Chaperone DnaJ-domain superfamily protein	PF00226
AT2G38880.4	ATHAP3, ATNF-YB1, HAP3, HAP3A	PF00808
AT2G38880.6		PF00808
AT3G04410.1	NAC (No Apical Meristem) domain	PF02365
AT3G09700.1	Chaperone DnaJ-domain superfamily protein	PF00226
AT3G17609.3	HYH, HY5-homolog	PF00170
AT3G21890.1	B-box type zinc finger family protein	PF00643
AT3G28917.1	MIF2, mini zinc finger 2	PF04770
AT3G47710.1	BHLH161, BNQ3, BANQUO 3	PF00010
AT3G51325.1	RING/U-box superfamily protein	PF00097
AT3G56770.2	basic helix-loop-helix (bHLH)	PF00010
AT3G62190.2	Chaperone DnaJ-domain superfamily protein	PF00226
AT4G00305.1	RING/U-box superfamily protein	PF00097
AT4G04632.1	Protein kinase superfamily protein	PF00069
AT4G12190.1	RING/U-box superfamily protein	PF00097
AT4G15248.1	B-box type zinc finger family protein	PF00643
AT4G24204.1	RING/U-box superfamily protein	PF00097
AT4G26810.1	SWIB/MDM2 domain superfamily protein	PF02201
AT4G26810.2		PF02201
AT5G01070.1	RING/FYVE/PHD zinc finger superfamily protein	PF00097
AT5G03030.1	Chaperone DnaJ-domain superfamily protein	PF00226
AT5G05770.1	WOX7, WUSCHEL related homeobox 7	PF00046
AT5G15160.1	BHLH134, BNQ2, BANQUO 2	PF00010
AT5G16650.1	Chaperone DnaJ-domain superfamily protein	PF00226

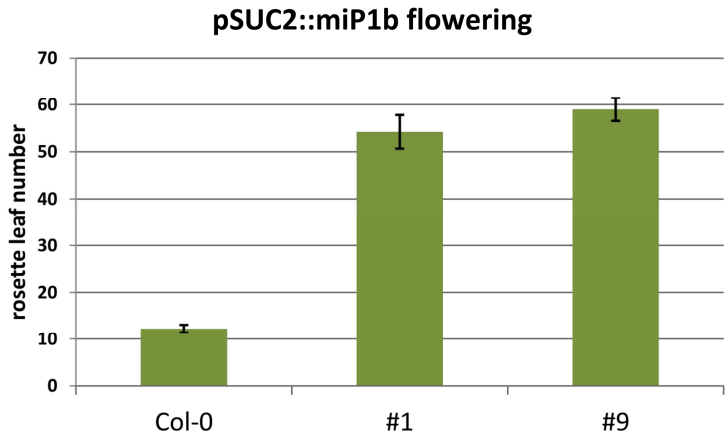
AT-number	Annotation	Pfam
AT5G18037.1	NAC (No Apical Meristem) domain	PF02365
AT5G27050.1	AGL101, AGAMOUS-like 101	PF00319
AT5G27810.1	MADS-box transcription factor family protein	PF00319
AT5G41440.1	RING/U-box superfamily protein	PF00097
AT5G46010.1	Homeodomain-like superfamily protein	PF00046
AT5G57565.2	Protein kinase superfamily protein	PF00069



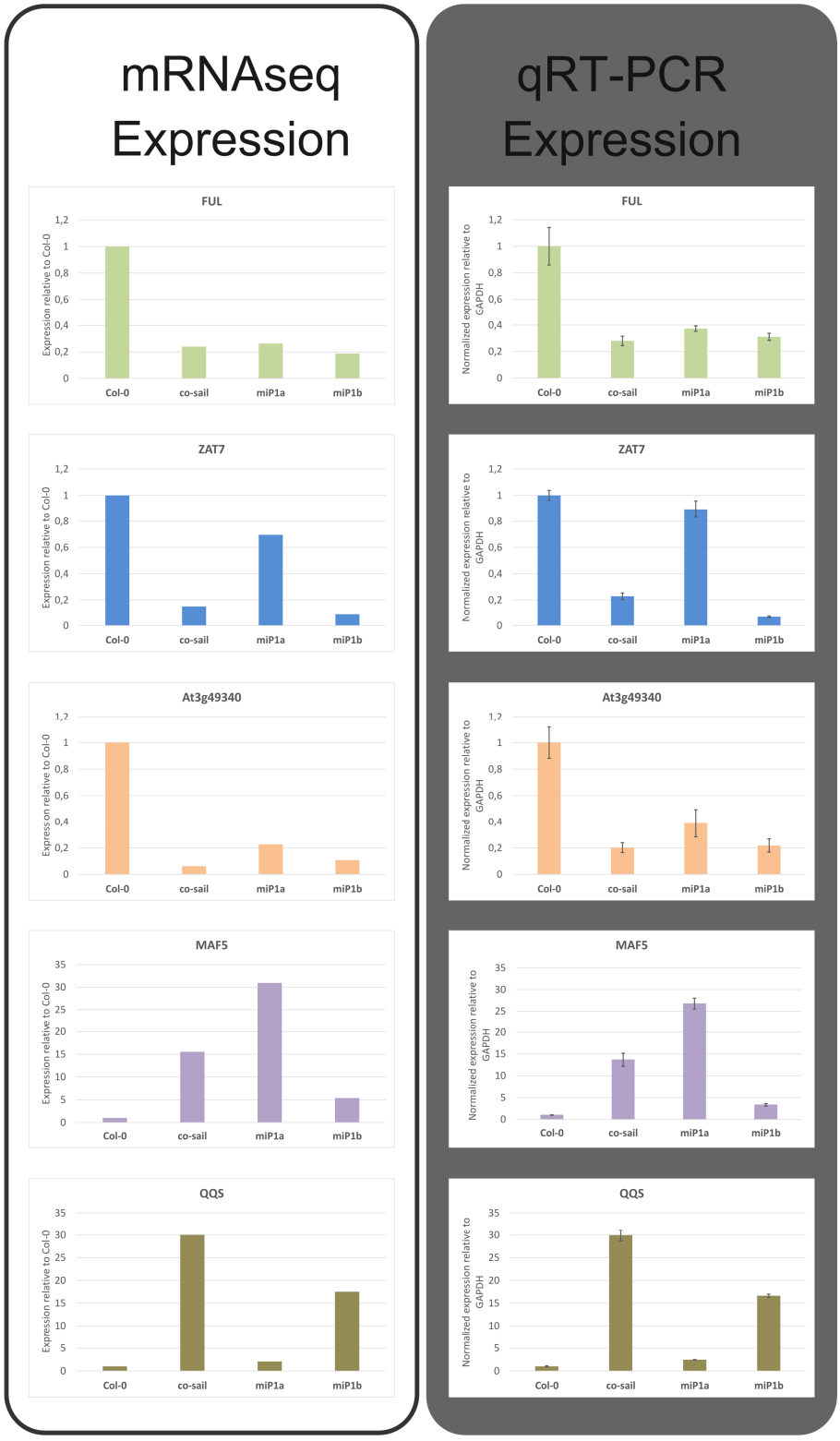
Supplemental Figure S 1 Leaf counts and miP1a/1b expression in TDNA and amiR lines

The characterized T-DNA insertion line for miP1a, GABI_KAT_288G08, does not show altered flowering behavior **(A)** or decreased miP1a expression levels **(B)** when compared to Col-0

The created amiR against miP1a/1b does not cause changes in flowering time behavior **(C)** and affects onli miP1a expression slightly **(D)**



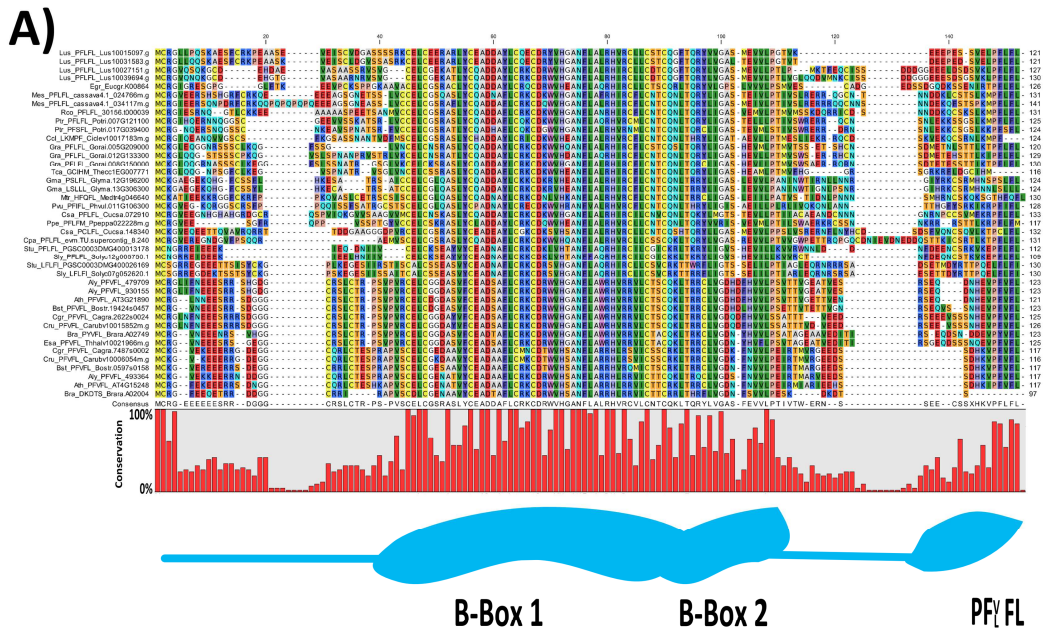
Supplemental Figure S 2 Leaf counts of *pSUC::miP1b* T2 lines

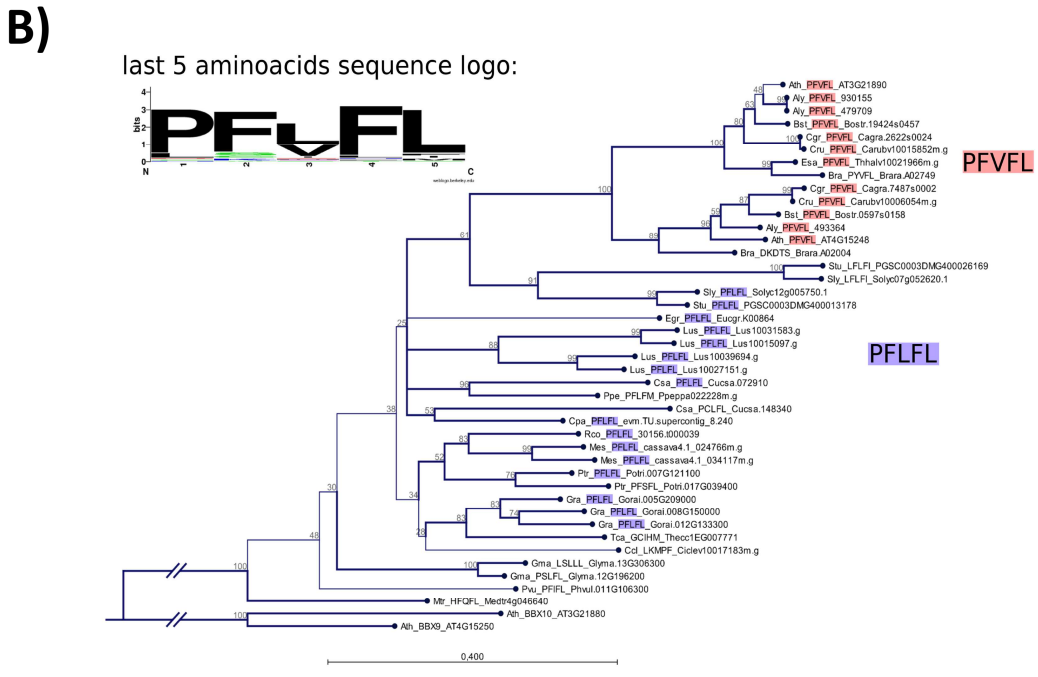


Supplemental Figure S3

Comparison of mRNAseq and qRT-PCR results

The expression of FUL, ZAT7, At3g49340, MAF5 and QQS - all showing significant differences in their expression in co-SAIL, pJAN33::miP1a or pJAN33::miP1b plants compared to Col-0 in the mRNAseq - were tested with qRT-PCR.
All tested genes showed the same expression pattern as in the mRNAseq experiment





Supplemental Figure S4

A) ClustalW alignment of miP1a/1b orthologues identified in the Phytozome database including the sequence conservation and a schematic representation of the miP1-type microProtein structure is shown.

B) Phylogenetic tree, based on the ClustalW alignment of miP1a/1b orthologues, kindly created and provided by Daniel Straub. The Neighbor-joining method with 1,000 bootstrap replications was used. Branches ≥0.25 are shown, branches >0.5 are depicted in bold

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