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Regulation of the *FT2* gene in European aspen

Master's thesis in Biotechnology

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DEPARTMENT OF BIOLOGY AND BIOLOGICAL ENGINEERING

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MASTER'S THESIS

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Cover:
A gene edited European aspen plant with a predicted enhancer region of *PtFT2* deleted.

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Abstract

A genomic region containing the *PtFT2* gene has previously been shown to be involved in the adaptation of *Populus tremula* (European aspen) to different daylengths. In this study regulatory elements of the gene, which is responsible for growth maintenance and inhibition of bud set, was investigated. The phenotypic and genetic effects of deleting predicted enhancer regions (PER)s, located in the *PtFT2* genomic region and hypothesized to be involved in regulation of *PtFT2*, were analyzed. Plants with one PER upstream of the *PtFT2* deleted and plants with one PER downstream of the gene deleted were grown in long day conditions followed by short day conditions. Expression of *PtFT2* and traits related to growth cessation and bud set were measured. Indications of a down regulated gene expression and changes in growth cessation and other phenotypic traits were observed.

Furthermore, a bioinformatic analysis of the PERs, to find conserved binding sites for potential homologous TFs present in *Arabidopsis thaliana*, was carried out. Homologous binding sites were found and the TFs binding to them in *A. thaliana* were involved in flowering, flower development and phase transitions.

Finally, an experiment investigating gibberellins (GA)s effect on growth in *Populus tremula x tremuloides* (hybrid aspen) was performed as it can affect growth cessation. GA treatment counteracted growth cessation, slowed growth and bud set in hybrid aspen plants with *PtFT2* deleted. This implies that gibberellin can affect growth cessation independently of *PtFT2*.

Keywords: *Populus tremula*, *FT2*, growth cessation, bud set, transcription factors, gibberellin

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List of abbreviations

Umeå Plant Science Centre	UPSC
Predicted enhancer region	PER
Transcription factor	TF
Gibberellin	GA
FLOWERING LOCUS T	*FT*
Populus tremula FT	*PtFT*
Long day	LD
Short day	SD
Single-nucleotide polymorphism	SNP
The Swedish aspen collection	SwAsp
Wild type	WT
Forward	F
Reserve	R

1. Introduction

This master's thesis project was performed at Umeå Plant Science Centre (UPSC) in the Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, and presented, defended and examined at Chalmers University of Technology. The study topic and aim were chosen as flowering and growth cessation in trees are essential biological processes for tree breeding and for understanding trees adaptation ability to different growth conditions, which are increasingly crucial as a result of the ongoing and upcoming climate changes.

UPSC is a world leading scientific research center for experimental plant biology and tree related research. Their work is mainly implemented with the model organisms *Arabidopsis thaliana* and *Populus tremula x tremuloides* (hybrid aspen), though they are also working with *Picea abies* (Norway spruce), *Pinus sylvestris* (Scots pine), *Populus tremula* (European aspen) and a few other plant species.

This study investigates regulatory elements of *PtFT2*, a gene which is responsible for growth maintenance and inhibition of bud set. It was performed by investigating the phenotypic and genetic effects of deleting predicted enhancer regions (PER)s, of *PtFT2*, in European aspen. The PERs were believed to be enhancer region for the regulation of the gene. They were proposed to contain binding sites for transcription factors (TF)s which could regulate the expression of *PtFT2*. Therefore, a bioinformatic analysis to find potential homologous TFs present in *A. thaliana* was performed. Finally, an experiment investigating gibberellins (GA)s effect on growth in hybrid aspen was preformed, to determine whether or not GA works independently of *PtFT2*, as it has previously been shown that GA also affects growth cessation.

The choice to implementing the main part of the study in European aspen was based on previous work of studying the species local adaptation in Sweden [1].

1.1 Background

Species in the poplar genus *Populus* spp. (poplar, aspen, cottonwood) are fast growing deciduous trees from the Northern Hemisphere [2]. They are commonly used as model organisms for trees, not only because of their fast growth habit but also their easiness of being vegetatively propagated [3]. Recent developments that further make aspen great model organisms include availability of sequenced genomes and transformation protocols for them [3]. In 2006 *Populus trichocarpa* was the first tree to be completely sequenced. This facilitated gene functions studies for traits connected to perennial growth habit. [4]

Even though the availability of genetic information facilitates tree breeding, there are still major obstacles to overcome. The main issue is the long generation time of trees. It generally takes many years before trees leave their juvenile phase and start to flower, develop seeds and reproduce. [5][6] It is highly desirable to create earlier flowering trees to be able to improve fruits and wood in a reasonable time span. Most commercially grown trees lack a complete domestication, in comparison with herbaceous crops, and have only been selected for a few generations [5]. A shortening of trees juvenile phase would therefore not only speed up the breeding process, but also make it easier to improve desirable traits.

To be able to successfully breed trees with earlier flowering it is essential to understand the underlying genetics. So far, most research have been implemented with the annual plant model organism, *Arabidopsis thaliana* [4]. It is an excellent model organism in some respects, such as that it is small, has a short generation time and a small genome. Nevertheless, it lacks traits that are important for perennial plants like trees. These include wood formation and growth connected to seasons. [4] Thus the underlying genetics behind these traits are difficult to study in *A. thaliana*.

The *FLOWERING LOCUS T* (*FT*) gene is responsible for flowering time regulation in *A. thaliana* through photoperiodic responses to daylength [6][7]. A genomic region called block C is believed to be important for regulation of the *FT* gene in *A. thaliana* [8][9]. This is an enhancer region, which can increase the transcription of genes when it is present. Proteins called transcription factors (TF)s regulate the expression of genes by binding to these regions. [10] TFs are involved in several types of biological processes such as stress response, growth and flowering [11][12]. Several TFs are involved in flowering in *A. thaliana* by binding to block C, these include SPL, CDF, TEM, NF-Y, SVP, AGL, and FLC. [13]

It has been shown that there is a high level of genetic conservation of traits among plant species [14]. Homolog *FT* genes are for example also found in aspen tree species. These genes are paralogs, arising from a genome duplication, and have distinctive functions. [7][9] *PtFT1* is speculated to have the conserved function of initiating flowering and reproduction, though this is not yet determined. *PtFT2* regulates growth cessation, maintenance of vegetative growth and inhibition of bud set, the formation of buds. [15][1]

Aspen plants grow during the long days (LD) in summer and initiate growth cessation during short days (SD) when the day length is below a critical length [16]. Before going into dormancy buds are formed as a protection from frost damage [17]. By measuring night length aspen plants can decide when, in autumn, to initiate growth cessation. The procedure is crucial for their survival during winter. [16] This biological process is controlled by *PtFT2* when information about the night length is transmitted from a gene called LATE ELONGATED HYPOCOTYL 2 (*LHY2*) [15].

Besides, it has been shown that the growth cessation initiated by *PtFT2* can be counteracted by gibberellin (GA). An increase of GA production in aspen delays the growth cessation, the bud set, and the growth stop. [18] Aspen plants with an increase of GA are insensitive to the *PtFT2*s growth cessation signals. GA and *PtFT2* are therefore believed to work in parallel to control similar processes. [18] However it is not known if the GAs effect on growth cessation is dependent or independent of *PtFT2*.

The *PtFT2* gene has its highest expression in the end of the day in LD conditions. During SD conditions the gene expression is downregulated compared to the expression in LD. [15] Recently it has been shown that there exist a third homolog of *FT* in aspen. It is a copy of *PtFT2* called *PtFT2β*. The gene previously called *PtFT2* will therefore be renamed as *PtFT2α*. *PtFT2β* is located 20 000 base pairs upstream of *PtFT2α*. [1] The discovery of *PtFT2β* is quite new, thus there is not much information about its function and expression in different situations [1]. When mentioning *PtFT2* it is for simplicity generally referring to both paralogs hereafter, if nothing else is stated.

It has also been shown that *PtFT2* plays a major role in the adaptation of European aspen to the different daylengths across Sweden [1]. Photoperiodic signals are crucial for the trees ability to determine the optimal time for growth cessation and thus bud set [1]. In a study single-nucleotide polymorphisms (SNP)s, present in the *PtFT2* genomic region, among the Swedish aspen collection (SwAsp) [19], believed to be responsible for the local adaption, were analyzed [1]. Intron 3 of the *PtFT2* genetic region is shown to contain the phenotype-associated SNP with the strongest signal of local adaptation. 65 % of the observed genetic difference in timing of bud set in European aspen is explained by this SNP. [1] The northern trees have obtained an allele with a nucleotide change from the T nucleotide found in the southern trees to a G nucleotide, as an adaptation to the shorter days in northern Sweden [1]. Northern trees show a significant difference in growth cessation and initiation of bud set in a shift from LD conditions (23 hours day) to SD (19 hours day) and a down regulation of *PtFT2* compared to southern trees which continue to grow without setting their buds at the SD conditions. The northern trees have a down regulated expression of *PtFT2* compared to southern trees under both SD and LD conditions, that is when they are initiating their growth cessation and when they are actively growing. [1]

To further understand the underlying genetics of European aspens adaptation to different day lengths it is of interest to study the regulation of *PtFT2*. However, it is likely that the regulation resembles the procedure in *A. thaliana* [6][7]. TFs homologous to TFs, that bind to enhancer regions and are involved in flowering, in *A. thaliana* are probably involved in the regulation of *PtFT2*. Furthermore, flowering is a crucial biological process in all flowering plants and similar TF homologs exists in several other species. [20][12]

Locating enhancer regions is however a challenging task as they are located and operating in a lot of different ways. Enhancer regions are located all over the genome, their location can be both upstream and downstream of the gene they regulate, there can be other genes between the enhancer region and the target promotor and finally some enhancer regions can regulate more than one gene. [21][22] Thus there might be several different potential enhancer regions controlling the *PtFT2* gene. Annotating the *PtFT2* genetic region to known bindings sites for TFs in *A. thaliana* can be useful for identifying binding sites for TFs and thus enhancer regions of it. A functional approach to characterize potential enhancer regions is by CRISPR/Cas9 deletions and study the effects of it [23][24]. CRISPR deletions can be both homozygous and heterozygous, which might influence expression of the gene of interest. [25][26] Thus it is important to detect the zygosity. [27] After confirmation of the deletions through genotyping, the gene edited plants should be grown in different conditions, phenotyped based on how trait of interest change, and their gene expression examined to gather as much data as possible for analyzing. It is necessary to analyze both phenotypic and gene expression data as the different type of data might reveal differences that cannot be seen by one of them alone.

It might be nonintuitive to study growth cessation for improvements of tree breeding, however adaption to different day lengths is useful to understand. Trees generally initiate growth cessation way before it gets cold and start to grow again after the risk of frost occurring is long gone. They do this to be sure that they will not be exposed to temperatures they cannot cope with in the active growing state. [6] If the mechanism behind growth cessation is better understood the growth season could be extended, to a reasonable length, by shortening the dormancy period, probably without risk for damages [19][28]. This will be especially useful when the climate changes lead to longer periods of summer. Eventually the trees will probably adapt to the new conditions but as the day lengths are fixed and trees have a long generation time this will take very long time. It is also useful to being able to change the dormancy period when moving trees from one latitude to another as the day length response is genetically controlled and therefore preserved, even if the new environment has another day length [6].

1.2 Aim

The aim of this study is to clarify how the *PtFT2* gene in European aspen is regulated and it can be divided into three different but related parts. Firstly, it is to investigate the phenotypic and genetic effects of deleting the predicted enhancer regions (PER)s. Another aim is to find potentially conserved transcription factors (TF)s. The last part is to analyze GAs effect on growth in aspen plants.

1.3 Specification of issue under investigation

Hypothesizes that were going to be verified or rejected during the project:

Gene expression and growth habit differs between wild type (WT) aspen and aspen with PERs of the *PtFT2* gene knocked out.

Southern aspen plants with the PERs of *PtFT2* deleted will behave as northern aspen plants, which means that they will have lower gene expression, slower growth and earlier growth cessation and bud set compared to the WT southern plant.

Homologous *A. thaliana* binding sites for TFs exist in the PERs of *PtFT2*.

Gibberellin (GA) affects growth cessation and early bud set in aspen independently of *PtFT2* and counteracts it.

1.4 Demarcations

This thesis project will only be able to analyze plants generated with two different constructs due to the amount of time it takes for aspen plants to be generated and grown. It will focus on plants with one upstream target and plants with one downstream target. The data analysis of TFs will in addition to the upstream and downstream predicted enhancer regions also include analysis of intron 3 in the *PtFT2* genome sequence. No other part of the gene sequence will be analyzed.

2. Prior works of importance to the study

Before the start of this master's thesis project necessary preparation work was done. A European aspen plant, SwAsp15, from the Swedish aspen collection [19] was used for the preparation work as well as the main study. A *FT* enhancer region, block C, of *A. thaliana* was aligned to the genomic sequence of European aspens *PtFT2* gene. With the highest score it aligned to one upstream region of the *PtFT2* gene transcription start and one region downstream of the transcription stop. Hereafter these regions are called predicted enhancer regions (PER)s.

It was also shown that most of the phenotype-associated SNPs found in a study about local adaptation of European aspen [1] were located in the PERs. However, the strongest associated SNP found in that study was not located within any of the two PERs. Constructs for CRISPR/Cas9 deletion of the PERs were created. Clones of the SwAsp15 plants was then transformed. One genotype, 966, with the upstream PER deleted and one genotype, 971, with the downstream PER deleted were obtained in time for the start of this master's thesis project and was therefore used in it. Multiply versions, lines, of the two genotypes were created from independent transformation events. A schematic figure of the *PtFT2* genetic region can be seen in Figure 1.

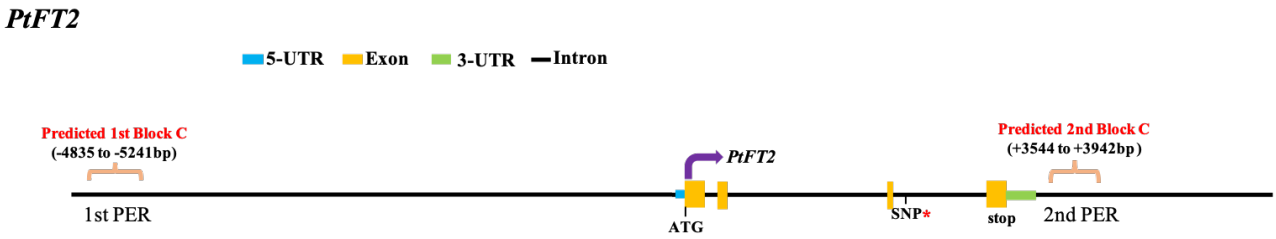

Figure 1. Schematic figure of the *PtFT2* genetic region. The curly brackets indicate the predicted enhancer regions and block C. ATG indicates the start of the *PtFT2* gene. SNP indicates were the strongest SNP is located.

3. Methodology

This chapter covers the methodology part of the project and is divided into four different parts. They cover the methodology of genotyping, phenotyping, gene expression analysis, transcription factor study and gibberellin treatment.

3.1 Genotyping of CRISPR edited aspen plants for confirmation of genetic deletion

In order to determine which of the CRISPR edited lines, created from the independent transformation events, that had the predicted enhancer regions (PER)s deleted, a genotype analysis was performed. For this the Phire Plant Direct PCR kit and gel electrophoresis were used. Lines with confirmed deletion were chosen for further phenotype and gene expression analysis.

Small leaf samples were taken from each obtained line and crushed into 200 µl PCR tubes, containing 40 µl dilution buffer, until the solution turned light green. The samples were collected with a pipette filter tip that was dipped into liquid nitrogen to dry the samples and make the collection easier. Only a little bit of the sample was crushed, the rest of the sample was discarded together with the tube. A new tube was used for each new sample. Wild type (WT) samples were also taken for use as control. 0.5 µl of the obtained solution was then mixed with 3.9 µl distilled water, 5.5 µl 2x Phire Master Mix, 0.55 µL Primer1 10 µM, 0.55 µl Primer2 10 µM and 0.5 µL gDNA. A control mix was also used. This contained 4.78 µL distilled water, 5.5 µL 2x Phire Master Mix, 0.22 Control primer mix 25 µM and 0.5 µL gDNA. The DNA samples were then amplified using the following PCR program: 1. 98 ° C 5 min, 2. 98 ° C 5 s, 3. 62 ° C 5 s, 4. 72 ° C 20 s, 5. 72 ° C 1 min, 4 ° C hold, run step 2-4 for 40 cycles.

The samples were loaded on a gel and the resulting bands, after using gel electrophoresis, were investigated. This was done with a forward primer (F) and a reserve primer (R) and again with F and a second reserve primer (R2) for both genotype 966 and genotype 971. Lines, created from independent transformation events, that containing evidence of gene deletion were randomly selected to grow further. Five lines from genotype 966 and six lines from genotype 971 were selected. To further confirm the deletion in the selected lines, DNA was extracted from the gel, using the NucleoSpin® Gel and PCR Clean-up kit, and sent to a sequencing company. A slice of the gel, surrounding the sample of interest, was cut out with a scalpel, weighted and placed in a tube and 200 µL Buffer NTI per 100 mg gel was added to the tube to suspend the gel. To bind the DNA a gel sample was added to a NucleoSpin® Gel and PCR Clean-up column placed into a 2 mL collection tube, that was then centrifuge for 30 s at 11,000 x g. The flow-through was discarded and the column was placed back into the collection tube again.

To wash the silica membrane 700 µL Buffer NT3 was added to the NucleoSpin® Gel and PCR Clean-up column and centrifuged for 30 s at 11,000 x g. The flow-through was once again discarded and the column was placed back into the collection tube. This washing step was repeated one time. To dry the silica membrane the collection tube with the column was centrifuge for 1 min at 11,000 x g to remove the Buffer NT3. The NucleoSpin® Gel and PCR Clean-up column was placed into a new 1.5 mL microcentrifuge tube and 30 µL Buffer NE was added. Then the sample was incubated at room temperature for 1 min and centrifuge for 1 min at 11,000 x g. This was done to dilute the DNA and then the obtained sample was sent to the sequence company for sequencing. The procedure was repeated for all samples from lines chosen for further study.

3.2 Phenotyping of aspen plants for observation of growth-related traits

To interpret the effect of a genetic deletion in plants it is necessary to grow the plants of interest in different conditions and observe the phenotype and how important traits change in response to them. In this study traits related to growth and growth cessation, height, leaf and branch number and bud formation, were of interest.

The plants that were selected to grow further after confirmation of deletion of the PERs were replicated to nine clones and later planted in pots and covered with plastic bags to keep a moist environment. 17 WT, non gene edited clones of SwAsp15, plants were used as control for the 966 genotype and 13 WT plants for the 971 genotype. The plants were grown in growth chambers were the growth conditions were customized. After one week the corners of the plastic bags were removed to let some air in and after two weeks the bags were removed. The growing conditions were long day (LD), 18 hour day and 6 hour night, with light switched on 03:00 and of 21:00, a day temperature of 20 ° C, a night temperature of 15 ° C and a humidity of 60 %. 46 days after potting the first phenotype measurements were performed by measuring the plants height and leaf and branch number.

49 days after potting for genotype 966 and 55 days after potting for genotype 971, that is when the growing conditions were changed to short day (SD), 14 hour day and 10 hour night, with light switched on 03:00 and of 17:00, a day temperature at 20 ° C and a night temperature at 18 ° C and a humidity of 75 %. Thereafter the height was measured, and the buds evaluated and scored for different stages of bud set, two times a week, until the buds were completely formed, which took 62 days for line 966 and 66 days for line 97, approximately four weeks. The buds were scored by following a method used in a previous study [1]. Four different stages were used for the scoring, active growth (3), growth cessation (2), bud formation (1) and bud set (0).

3.3 Gene expression analysis of aspen plants for detection of expression differences

To get a further understanding of the effect of a genetic knock out it is necessary to analyze the gene expression of the gene of interest, in addition to analyzing the phenotype. A gene expression analysis might reveal differences that cannot be seen by observing the plants only. Therefore a gene expression analysis of the two paralog versions of the *PtFT2* gene, *PtFT2α* and *PtFT2β*, were performed.

Leaf samples from three plants of each line were collected 42 days after potting at 20:00, in the end of the day period, when the expression of the *PtFT2* gene is the strongest. Each sample consisted of one large leaf per plant. As soon as a leaf was removed, it was folded into aluminum foil, putted into liquid nitrogen and then in a fridge with minus 80 ° C until the samples were analyzed.

For analyzing the samples, a modified version of the CTAB method for RNA extraction and iScript™ cDNA Synthesis kit for cDNA conversion and reverse transcription quantitative PCR (RT-qPCR) amplification were used. Gene expression of a gene coding for ubiquitin was used as reference.

The frozen leaf samples were homogenized with a mortar and pestle and the resulting powder was moved to a tube with 0.8 mL 65 ° C extraction buffer and then 16 µL β-ME was added. After that the samples were vortexed and then kept in 65 ° C for 1 min. Extraction was performed with 0.8 mL chloroform-isoamylalcohol (24:1) that was added to the tubes. The samples were mixed moderately by inverting the tubes for 20-30 s and then they were centrifuged at 13,000 rpm for 10 min in room temperature.

The light green clear upper phase was removed and added to new 2 mL tubes and the dark green lower phase was left in the old tubes. This extraction procedure was repeated, and the upper phase was added to new 1.5 mL tubes. LiCl (8M) was then added until 1/3 of the volume in the tubes was filled and the tubes were putted on ice at 4 ° C for 1 hour in a fridge.

The samples were then centrifuged at 13,000 rpm for 20 min in 4 ° C. Then the pellets were dissolved carefully in 400 µL RLT buffer, without β-ME, and 200 µL ethanol was added to the clear lysate. This was immediately mixed, by pipetting up and down, and loaded onto a RNeasy Mini column. The samples were centrifuged for 15 s at 10,000 rpm and the supernatant was discarded. 350 µL buffer RW1 was loaded onto the column and centrifuged for 15 s at 10,000 rpm and the flow-thru was discarded.

10 µL DNAse I stock solution and 60 µL buffer RDD were mixed, loaded onto the column and kept at room temperature for 15 min. Then 350 µL of buffer RW1 was loaded onto the column, spun and the flow-thru was discarded. This procedure was repeated but with 480 µL buffer RPE instead and then this step was repeated. The column was then dried by centrifugation at max speed for 2 min. Then the RNeasy Mini column was placed in a new 1.5 mL collection tube from the RNeasy Mini kit and 35 µL RNase-free buffer EB was directly added to the spin column membrane. After 1 min the samples were centrifuged for 1 min at 10,000 rpm to eluate RNA. The obtained solution was then eluted again.

The RNA concentration of the final samples was measured using NanoDrop. It gave the concentrations in ng/µL and to obtain the volume of the samples that were going to be used next, the weight of the samples, 1000 ng, were divided with the given sample concentrations. The calculated volume for each RNA samples was added into PCR tubes, together with 4 µL 5x iScript Reaction Mix, 1 µL iScript Reverse Transcriptase and nuclease free water. As the total volume in the tubes was supposed to be 20 µL, the water volume used in each tube was calculated by taking 15 minus the volume of the RNA sample. Then the samples were incubated in a thermal cycler using the following program: 1. Priming 5 min at 25 ° C, 2. Reverse transcription 20 min at 46 ° C, 3. RT inactivation 1 min at 95 ° C, 4. Optional step Hold at 4 ° C.

The gene expression was calculated from the resulting data using the ΔΔCt-method [29]. Later the gene expressions were compared to the observed phenotypes to see if plants with a phenotype that differed from the WT also had a different gene expression. This information taken together was then used to draw conclusions about the effects of the deletion of the PERs.

3.4 Identification of potentially conserved transcription factors for flowering in aspen

To better understand how the PERs are involved in regulation of the *PtFT2* gene, a bioinformatic analysis with the purpose of finding potential binding sites for transcription factors (TF)s in the *PtFT2* genetic region was performed. This was implemented by annotating the genomic sequences of the upstream PER, the downstream PER and intron 3, which contain the strongest associated SNP related to local adaptation of European aspen, to binding sites of known TFs in *Arabidopsis thaliana*. The annotation was performed using PlantPAN 3.0 [30]. *PtFT2* is known for being responsible for maintenance of vegetative growth and inhibition of bud set. It is hypothesized that this is regulated by TFs that bind to the PERs and enhance the expression of the gene. Deleting these regions would, as proposed, therefore lead to a down regulation of the gene expression. In *A. thaliana* the homologous *FT* gene regulates flowering through TFs that bind to enhancer regions of this gene.

There are however no known *A. thaliana* TFs connected to vegetative growth maintenance and inhibition of bud set, that binds to these regions of the *FT* gene. TFs involved in regulation of flowering were therefore identified instead. The gene ID of each TF was used to search for information about them on the Arabidopsis Information Resource (TAIR) [31] and to some extent Uniprot [32]. Identified TFs that were involved in regulation of flowering and a few other related traits were proposed to be potential homologs to TFs in European aspen.

3.5 Analyzing the effects of gibberellin treatments on growth in hybrid aspen plants

A small study of gibberellin (GA) treatments effect in *Populus tremula x tremuloides* (hybrid aspen) was also performed to further investigate how the *PtFT2* gene is regulated as it has been showed to be involved in growth cessation [6].

Three different types of plants, WT, a type with *PtFT2* deleted and a type with an over-expresser of a known *FT* repressor, the SVP protein, were grown for three weeks in growth chambers. The growing conditions were LD, 18 hour day and 6 hour night, with light switched on 03:00 and of 21:00, a day temperature of 20 ° C, a night temperature of 18 ° C and a humidity of 75 %. After the three weeks the growing conditions were changed to SD, 14 hour day and 10 hour night, with light switched on 03:00 and of 17:00, a day temperature of 20 ° C, a night temperature of 18 ° C and a humidity of 75 % and a GA treatment was initiated. Two times a week for five weeks, three plants of each type were watered with 50 mL of a GA solution and the rest of the plants were watered with 50 mL pure water as control. Once a week the height and the number of the leaves were measured. The buds were also evaluated and scored for different stages of bud set by using the same method as previously mentioned.

4. Results

This chapter covers the results part of the project and is divided into four different parts. They cover the results of genotyping, phenotyping, gene expression analysis, transcription factor study and gibberellin treatment.

4.1 CRISPR edited plants had heterozygous deletion

To find out if there was a deletion and in that case which lines of each genotype that had deletion of the predicted enhancer regions (PER)s, leaf samples of gene edited SwAsp15 plants with the upstream (genotype 966) and the downstream (genotype 971) PER knocked out were analyzed using PCR and gel electrophoresis. Lines with the PER knocked out are expected to have short bands on the gel, with PCR samples containing the forward (F) and the first reserve (R) primers. To determine the zygosity of the lines a second gel, with PCR samples containing the F and the second reserve (R2) primer was made. Lines with a heterozygous deletion of the PERs will have a band on this gel. A schematic figure of the *PtFT2* genetic region can be seen in Figure 2A for genotype 966 and Figure 2C for genotype 971. The resulting gels can be seen in Figure 2B for genotype 966 and Figure 2D for genotype 971. Lines showing signs of deletion were chosen to continue to grow for further phenotypic and gene expression study.

Most of the investigated lines, from independent transformation events, showed two bands on the type of gel where the F and the first R primers were used in the PCR. This can be seen in Figure 2. Two bands on the gel indicates that the CRISPR deletion was heterozygous as most samples have both shorter, non-edited, bands and longer, wild type (WT), bands on the gel. A heterozygous deletion means that the genetic region within the binding sites for the primers, the PERs, were deleted on only one of the two chromosome copies of the genome. Thus, resulting in two different bands.

The heterozygosity is further confirmed with the type of gel were the F and the R2 primer were used in the PCR. All samples on this gel clearly has bands, as shown in Figure 2. If the deletion was homozygous, deletion on both chromosome copies, no band would be present on the second gel as the biding site for the R2 is within the region surrounded by the bindings sites for F and R. Taken together the results from both type of gels indicate that there was indeed a CRISPR deletion of the PERs, but only on one of the two chromosome copies. This was true for both genotype 966 and genotype 971, which can be seen in Figure 2. Five lines of genotype 966 and six lines of genotype 971 were randomly selected, among the lines that showed evidence of deletion, to grow further. Growth habit and gene expression of these plants were later analyzed.

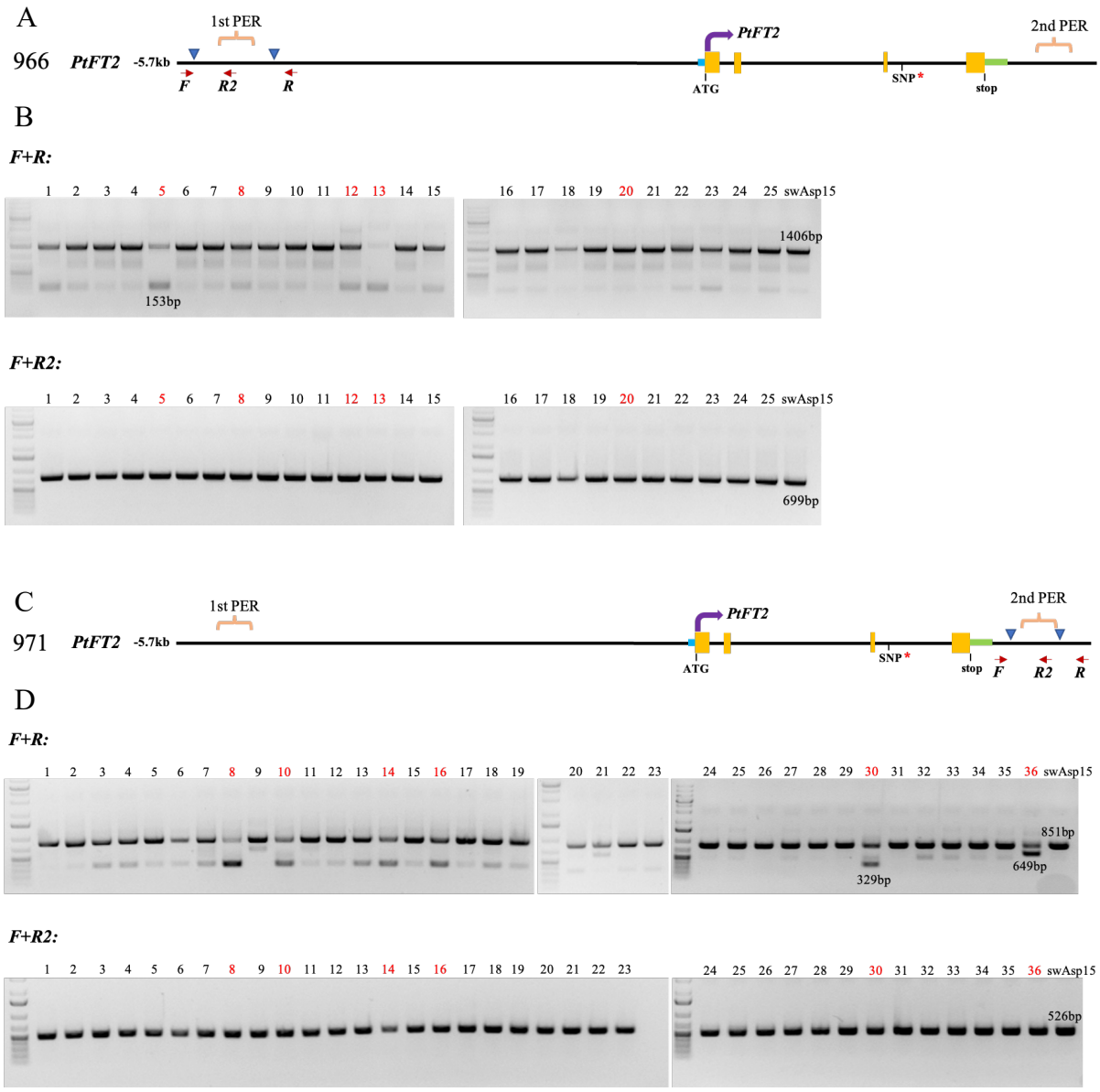

Figure 2. A and **C** Schematic figure of the *PtFT2* genetic region. The blue arrows indicate were the CRISPR/Cas9 attached and deleted the enclosed sequence. The red arrows indicate forward (F), reserve (R) and second reserve (R2) primer for the PCR amplified regions. The curly brackets indicate the predicted enhancer regions. ATG indicates the start of the *PtFT2* gene. SNP indicates were the strongest SNP is located. -5.7 kb indicates number of bases away from the gene start. A Genotype 966. C Genotype 971. **B** and **D** Amplified and gel electrophoresis analyzed DNA sequences from CRISPR edited SwAsp15 aspen plants with the PERs deleted. The red marked lines were chosen, among the lines with two bands showing, for further study. Although no lines with homozygous deletion could be found. The numbers on the gel followed by bp indicates the length of the adjacently bands in number of base pairs. B Genotype 966. D Genotype 971.

4.2 Abnormal phenotypes were seen in gene edited aspen plants

To see whether deleting the PERs had an impact on vegetative growth, different phenotypic traits of aspen plants were measured. Growth cessation and bud set were of special interest. Lines with signs of deletion, detected in the genotype analysis, were selected to growth further in growth chambers. Five lines of genotype 966 and six lines of genotype 971 were selected. WT, non-edited, SwAsp15 plants were also grown as controls. Nine clones of each line were used as replicates. 17 WT plants were used as control for genotype 966 and 13 WT plants for genotype 971. Lines lacking the PERs were expected to show signs of slowed growth and earlier growth cessation and bud set.

46 days after potting all the plants were phenotyped by measuring leaf number, branch number and height, to detect potential difference in these traits prior to the light condition change to short days (SD). Since potting the plants had been grown in long day (LD) conditions, 18 hours light and 6 hours dark. Indications of slowed growth were expected to be seen among the plants lacking the PERs. The mean values of leaf and branch number of each replica can be seen in Figure 3A and Figure 3B for genotype 966 respectively genotype 971. Height can be seen as the first datapoint in Figure 5. and Figure 6. for respective genotype. Representative replicates from different lines were also photographed, which can be seen in Figure 4.

There were indications of lower leaf number compared to WT. Though to confirm a potential difference, a statistical test would have needed to be performed. This was not done as leaf number was only measured once and determined to not have a major impact on the results. Some of the lines had significantly more branches, although the variation within each line were apparent. A distinctively lower height could be seen in line 966-8, 966-20 and 971-14. General abnormality in morphological traits, such as increased number of branches, thinner stem and smaller and wrinkled leaves, were common among the gene edited plants. This can be seen in Figure 4. The first phenotypic measurements indicated deviating morphological traits and to some extent slowed growth in the gene edited lines.

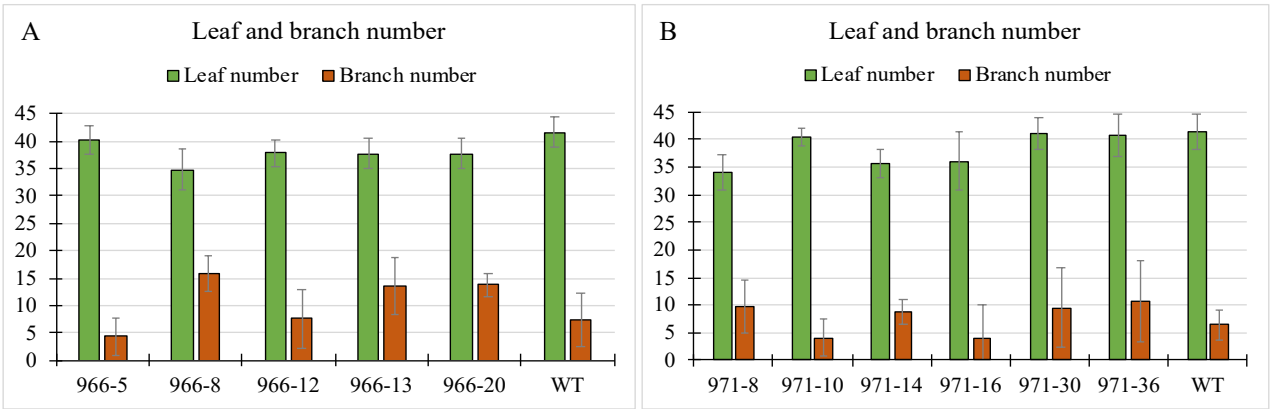

Figure 3. Mean values of leaf and branch number of 46 days old aspen plants grown in LD conditions (18/6 h). The line numbers represent the mean values for the replicates within each line. The error bars show the standard deviation of the leaf and the branch number for the replicates. **A** Genotype 966. **B** Genotype 971.

Figure 4. 46 days old aspen plants grown in LD (18/6 h) and then SD (14/10 h) conditions. The colored dots indicate the colors given to each line at the potting day. Black indicates WT. Each plant is a representative replicate from the different lines. **A** Plants of genotype 966 placed in order of observed phenotype difference. Starting with the most different compared to WT and ending with WT, from left to right. **B** The most different version of genotype 971, 971-14, compared to WT and a WT plant. The other versions of genotype 971 did not look significantly different from WT, and are therefore not shown.

The phenotypic measurements were continued in order to determine the timing of growth cessation, when the light conditions changed to SD, and the long-term differences. Gene edited plants were expected to have earlier growth cessation, earlier bud set and slowed growth. At day 49 for genotype 966 and day 55 for genotype 971, the same day as the light conditions were changed from LD to SD conditions, 14 hours light and 10 hours dark, phenotyping in short conditions was initiated. The light conditions were changed to initiate growth cessation of the plants. Height and bud score were measured two times a week, until the buds were completely formed, at day 36 for genotype 966 and day 34 for genotype 971 after the light conditions were changed. Height measurements for genotype 966 can be seen in Figure 5A and in Figure 6A for genotype 971. Bud score measurements can be seen in Figure 5B for genotype 966 and in Figure 6B for genotype 971. Score 3 equals active growth and 0 equals fully formed bud. Line 966-8, 966-20 and 971-14 had slowed growth and indications of earlier growth cessation. No lines showed significant difference in bud set.

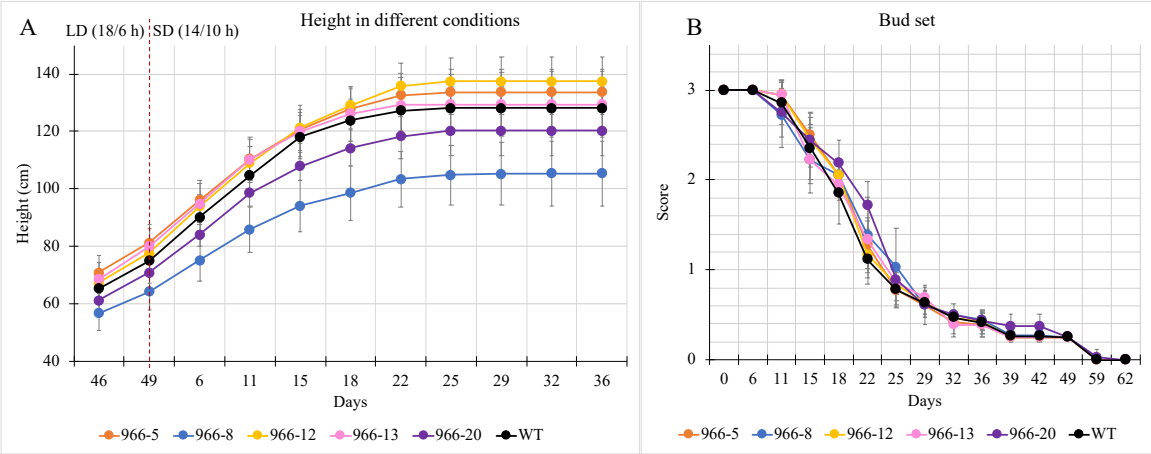

Figure 5. A Height mean values of aspen plants of genotype 966, measured over 39 days. The line numbers represent the mean values for the replicates within each line. The first two measurements were taken in LD conditions (18/6 h) and the rest were taken in SD conditions (14/10 h). The red line indicates the shift from LD to SD. The error bars show the standard deviation of the height for the replicates. **B** Bud score mean value of aspen plants of genotype 966, measured over 62 days. The line numbers represent the mean values for the replicates within each line. The measurements were initiated at the day of the shift from LD (18/6 h) to SD (14/10 h). The error bars show the standard deviation of the bud score for the replicates.

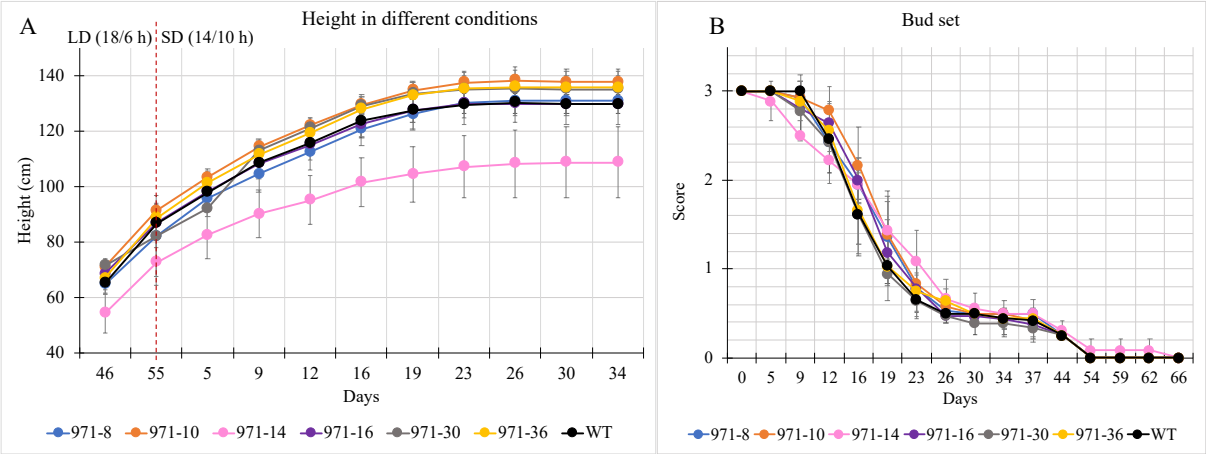

Figure 6. A Height mean values of aspen plants of genotype 971, measured over 43 days. The line numbers represent the mean values for the replicates within each line. The first two measurements were taken in LD conditions (18/6 h) and the rest were taken in SD conditions (14/10 h). The red line indicates the shift from LD to SD. The error bars show the standard deviation of the height for the replicates. **B** Bud score mean value of aspen plants of genotype 971, measured over 66 days. The line numbers represent the mean values for the replicates within each line. The measurements were initiated at the day of the shift from LD (18/6 h) to SD (14/10 h). The error bars show the standard deviation of the bud score for the replicates.

4.3 Deletion of predicted enhancer regions led to gene expression differences

Leaf samples from three of the replicates in each line, were collected 42 days after potting, when the plants were still growing in LD conditions. The samples were then analyzed to see if the expression of *PtFT2* in the gene edited lines differed and especially if it was downregulated compared to the WT plants expression of the gene. Both paralogs of *PtFT2*, *PtFT2α* and *PtFT2β*, were analyzed. It was expected to see a downregulated gene expression of these genes in the lines lacking the PERs. The results of the analysis can be seen in Figure 7A and 7C, for genotype 966, and Figure 7B and 7D, for genotype 971. Gene expression is shown as $\log_2$ of the ΔΔCt-values. All the different versions of genotype 966 had a downregulation expression of *PtFT2α* and *PtFT2β* in comparison to the WTs expression of these genes. Four of six lines of genotype 971 had downregulation of both paralog genes. The two remaining lines had upregulation of the genes compared to WT. In all lines, except line 971-14, *PtFT2β* was expressed more than *PtFT2α*.

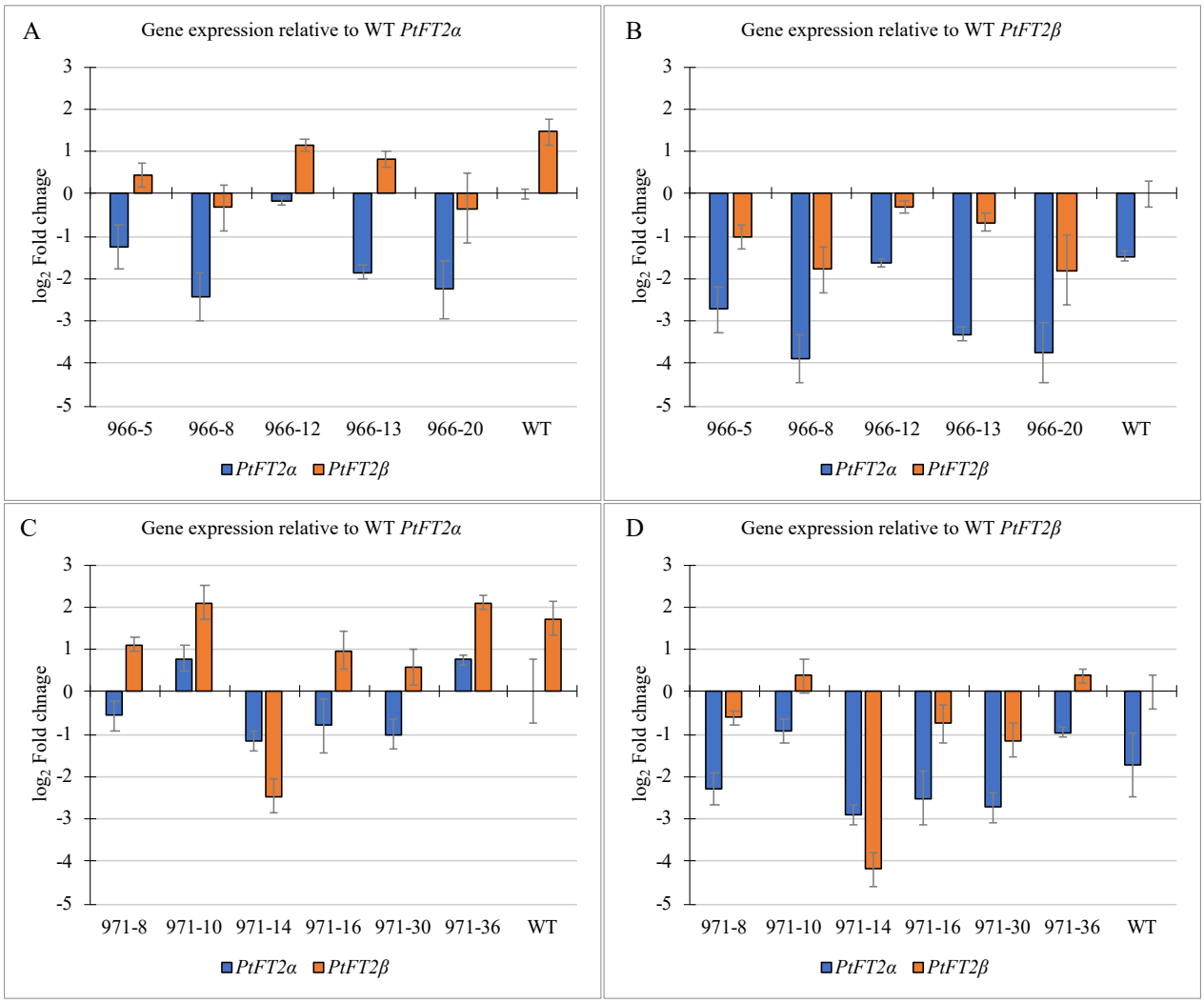

Figure 7. Gene expression of 42 days old aspen plants from genotype 966 and 971. The line numbers represent the mean values of replicates within each line. The error bars show the standard error of the gene expression. **A** and **C** The gene expression of the plants relative to the WTs expression of *PtFT2α*. **B** and **D** The gene expression of the plants relative to the WTs expression of *PtFT2β*.

4.4 Homolog binding sites for transcription factors were found

Transcription factors (TF) bind to enhancer regions which can increase the transcription of genes. The PERs of *PtFT2* are not located in the gene, thus the regulation of the gene, by them, must be through TFs binding to these regions. To determine if the PERs contain potential binding sites for TFs, in order to better understand the regulation of *PtFT2*, a bioinformatic analysis was performed. The upstream PER, the downstream PER and the intron 3 sequences were annotated to binding sites for known TFs involved in flowering in *Arabidopsis thaliana*. It was hypothesized that the PERs would contain potential homologous binding sites for TFs involved in flowering in *A. thaliana*.

The identified TFs, related to flowering, of the upstream PER can be seen in Figure 8, the TFs of the downstream PER in Figure 10 and the TFs of intron 3 in Figure 12. Position weight matrixes, of the corresponding sequences to the annotated binding places for the potential homologous TFs related to flowering, can be seen in Figure 9 for the TFs of the upstream PER, in Figure 11 for the TFs of the downstream PER and in Figure 13 for the TFs of intron 3. The full list of annotated TFs can be seen in Table 1 for the upstream PER, in Table 2 for the downstream PER and in Table 3 for intron 3.

Potential homologous binding sites for TFs were found and several TFs bound to them. Fifteen unique TFs involved in regulation of flowering were found and four of them were present in both the upstream PER, the downstream PER and intron 3.

The upstream predicted enhancer region

```
AATCTAAGCGTGTTGTTGTATGTTTATTTTTTATCGCTTTCTGAATGCAAAGTGAATATTATCCAAAGAAATGTT     75
TTAGATTCGCACAACAACATACAAATAAAAAAATAGCGAAAGACTTACGTTTCACTTATAATAGGTTTCTTTACAA

TCGCCAGCTGCATGCGTGAAATATTTACTGGCTGTGGAGAAAAATGATTTGTAAAATTAGAAAAATAATCGGAGT    150
AGCGGTCGACGTACGCACTTTATAAATGACCGACACCTCTTTTTACTAAACATTTTAATCTTTTTATTAGCCTCA

GGAATTACAAGAACAGTGAAGGAATCATTTGGCAGTGGTAATGATGATCTGCATGTATTAGGCAGAGATTGATGT    225
CCTTAATGTTCTTGTCACTTCCTTAGTAAACCGTCACCATTACTACTAGACGTACATAATCCGTCTCTAACTACA

TGCCTATCGCAGCAGACAGGGCTAAGAGATTGGACTGACATCATAGGAGTACTGGAAGATACCTTCCCGACATCT    300
ACGGATAGCGTCGTCTGTCCCGATTCTCTAACCTGACTGTAGTATCCTCATGACCTTCTATGGAAGGGCTGTAGA

GAGTTTGCTTCGTATACATCAACTTCTGTGATTCAGTTATGTCATGATGCATCCACGTTTGCATTGAATACAAGC    375
CTCAAACGAAGCATATGTAGTTGAAGACACTAAGTCAATACAGTACTACGTAGGTGCAAACGTAACTTATGTTCG

TGCGAAGTGGCGTTATTCAACTGATTAGATTTAAAGTTTAGTTTTTAAAGATTGCTAGAAATATTGTAATGAATT    450
ACGCTTCACCGCAATAAGTTGACTAATCTAAATTTCAAATCAAAAATTTCTAACGATCTTTATAACATTACTTAA

TTAGGACTATTAAAACATACATCATTATTAATTTTAATTAATTTTAAAAATTTACATAAACTTTTATTTTTTTAG    525
AATCCTGATAATTTTGTATGTAGTAATAATTAAAATTAATTAAAATTTTTAAATGTATTGAAAATAAAAAAATC

AAAAAAATAATTGTCAAAACAATATTTTTTTTATTATCTTTTA                                     295
TTTTTTTATTAACAGTTTTGTTATAAAAAAAATAATAGAAAAT
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SPL CDF NF-YC3 TEM1 FLC NTL8 DDF HB16

Figure 8. The upstream predicted enhancer region and annotated binding places for potential transcription factors related to flowering within this region. Each color represents a transcription factor. The numbers to the right represent number of base pairs on each row.

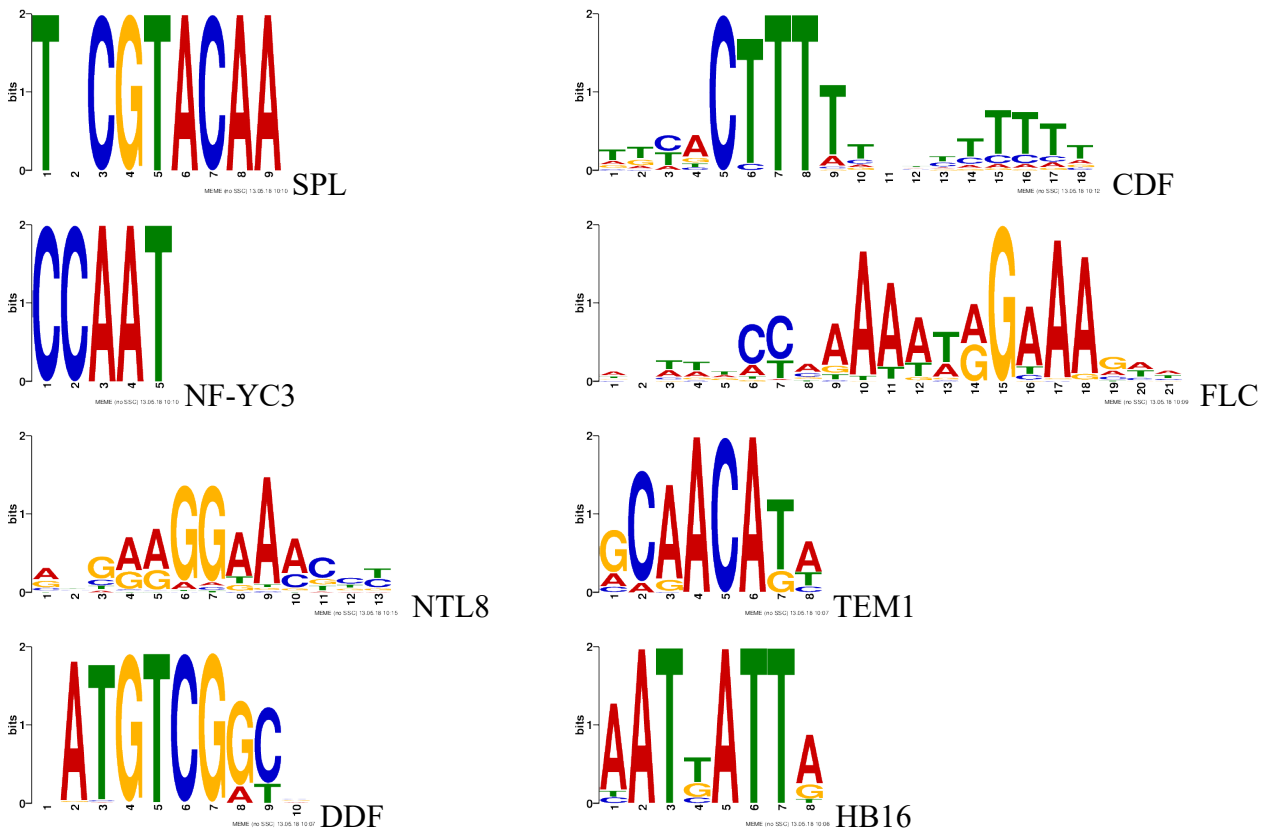

Figure 9. Position weight matrixes of the corresponding sequences to the annotated binding places for potential transcription factors related to flowering within the upstream predicted enhancer region. The matrixes represent the sequences with the highest similarity scores.

Table 1. Annotated potential transcription factors, within the upstream predicted enhancer region, related to flowering and other functions of interest for the regulation of the *PtFT2* gene.

TF ID	Gene name	TF Family	Function	Position	Strand	Score
AT5G43270	SPL2	SBP	Phase transition	309, 16	+/-	0.75, 0.75
AT2G33810	SPL3	SBP	Flowering	309, 16	+/-	0.75, 0.75
AT1G53160	SPL4	SBP	Flowering	309, 16	+/-	0.75, 0.75
AT3G15270	SPL5	SBP	Flowering	309, 16	+/-	0.75, 0.75
AT2G42200	SPL9	SBP	Phase transition	309, 16	+/-	0.75, 0.75
AT1G27370	SPL10	SBP	Phase transition	309, 16	+/-	0.75, 0.75
AT1G27360	SPL11	SBP	Phase transition	309, 16	+/-	0.75, 0.75
AT3G57920	SPL15	SBP	Phase transition	309, 16	+/-	0.75, 0.75
AT5G39660	CDF2	Dof	Flowering	Various	+/-	1.00,1.00
AT3G47500	CDF3	Dof	Flowering	507	+	0.95
AT1G69570	CDF5	Dof	Flowering	505, 509	+/-	0.87, 0.92
AT5G10140	FLC	MADS box	Flowering	121	+	0.89
AT1G12610	DDF1	AP2;ERF	Flowering	282, 290	+/-	0.86, 0.93
AT1G63030	DDF2	AP2; ERF	Phase transition	284, 284	+/-	0.91, 0.92
AT1G25560	TEM1, EDF1	AP2; RAV	Flowering	221	-	0.97
AT4G40060	HB16	Homeodomain	Flowering	530	-	0.95
AT1G54830	NF-YC3	NF-YC	Flowering	Various	+/-	0.80, 1.00
AT2G27300	NTL8	NAC	Flowering	166	+	0.91
AT5G37020	ARF8	B3	Flower development	291	+	1.00
AT4G35390	AHL25	AT-Hook	Phase transition	545, 485	+/-	0.93, 1.00

The downstream predicted enhancer region

```
TTTTAAATTAAATGAGTAAATACAGATTTAATCAGGTCATAATATAAAATTAAGTATGATCTCAAATAAGAATTA      75
AAAATTTAATTTACTCATTTATGTCTAAATTAGTCCAGTATTATATTTTAATTCATACTAGAGTTTATTCTTAAT

TTAAACATTAGGTGGTCCCAATAAATTAAACTTTTAGATAACTTAAGATTAACTAATTTGGCATGCTACTGAATT     150
AATTTGTAATCCACCAGGGTTATTTAATTTGAAAATCTATTGAATTCTAATTGATTAAACCGTACGATGACTTAA

GGCTTTTATGTACACATGTTGAATTAAAACTTTTCATCATATAGTTAATAACTTTTTATCTTTATTGACTAATGT     225
CCGAAAATACATGTGTACAACTTAATTTTGAAAAGTAGTATATCAATTATTGAAAAATAGAAATAACTGATTACA

ATTGTCCGCATCAACTTTCACATTATTCCTTGTAATTAATTTTGATAAAATCTTTGGATGAACATATTAGAGAAT     300
TAACAGGCGTAGTTGAAAGTGTAATAAGGAACATTAATTAAAACTATTTTAGAAACCTACTTGTATAATCTCTTA

TACATGAATAAATTCAACATTACAATTAAATGATGTTTTAAGGTAAAGTCTATTATAAGTGTGTGATCACTCAAT     375
ATGTACTTATTTAAGTTGTAATGTTAATTTACTACAAAATTCCATTTCAGATAATATTCACACACTAGTGAGTTA

GTCCTTACCTTTTGTTTAATTTATATTGTGCTTACAAGAAATTCGTTGTTGAGGACTTGTACATCCTCAGAA       447
CAGGAATGGAAAACAAATTAAATATAACACGAATGTTCTTTAAGCAACAACTCCTGAACATGTAGGAGTCTT
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SPL CDF NF-YC3 TEM1 AGL U2AF35B HB16 KHZ1

Figure 10. The downstream predicted enhancer region and annotated binding places for potential transcription factors related to flowering within this region. Each color represents a transcription factor. The numbers to the right represent number of base pairs on each row.

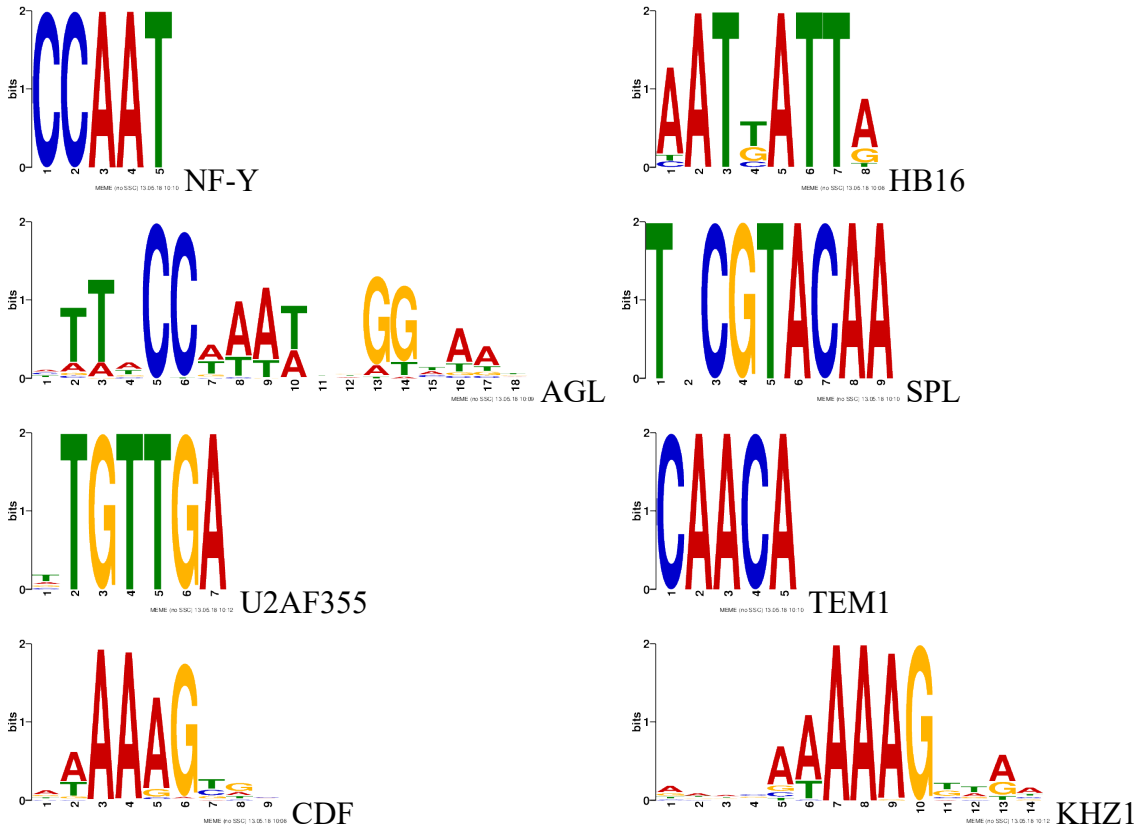

Figure 11. Position weight matrixes of the corresponding sequences to the annotated binding places for potential transcription factors related to flowering within the downstream predicted enhancer region. The matrixes represent the sequences with the highest similarity scores.

Table 2. Annotated potential transcription factors, within the downstream predicted enhancer region, related to flowering and other functions of interest for the regulation of the *PtFT2* gene.

TF ID	Gene name	TF Family	Function	Position	Strand	Score
AT5G43270	SPL2	SBP	Phase transition	Various	+/-	0.88, 0.88
AT2G33810	SPL3	SBP	Flowering	Various	+/-	0.88, 0.88
AT1G53160	SPL4	SBP	Flowering	Various	+/-	0.88, 0.88
AT3G15270	SPL5	SBP	Flowering	Various	+/-	0.88, 0.88
AT2G42200	SPL9	SBP	Phase transition	Various	+/-	0.88, 0.88
AT1G27370	SPL10	SBP	Phase transition	Various	+/-	0.88, 0.88
AT1G27360	SPL11	SBP	Phase transition	Various	+/-	0.88, 0.88
AT3G57920	SPL15	SBP	Phase transition	Various	+/-	0.88, 0.88
AT5G39660	CDF2	Dof	Flowering	199	-	0.99
AT3G47500	CDF3	Dof	Flowering	198, 196	+/-	0.95, 0.93
AT1G69570	CDF5	Dof	Flowering	378, 196	+/-	0.84, 0.93
AT2G45650	AGL6	MADS box	Flower development	123	-	0.87
AT5G60910	AGL8	MADS box	Flower development	123	-	0.87
AT1G26310	AGL10	MADS box	Flower development	123	-	0.87
AT4G11880	AGL14	MADS box	Phase transition	123	-	0.87
AT3G57230	AGL16	MADS box	Flowering	123	-	0.87
AT3G57390	AGL18	MADS box	Flowering	123	-	0.87
AT4G22950	AGL19	MADS box	Phase transition	123	-	0.87
AT5G62165	AGL42	MADS box	Flower senescence	123	-	0.87
AT5G51870	AGL71	MADS box	Flowering	123	-	0.87
AT3G30260	AGL79	MADS box	Phase transition	123	-	0.87
AT1G25560	TEM1, EDF1	AP2; RAV	Flowering	315, 167	+/-	1.00, 1.00
AT1G13260	RAV1, EDF4	AP2; RAV	Flower development	315, 167	+/-	1.00, 1.00
AT4G40060	HB16	Homeodomain	Flowering	71	+	1.00
AT5G03790	HB51	Homeodomain	Phase transition	70	+	0.94
AT1G28420	RLT1	Homeodomain	Phase transition	282	+	1.00
AT5G44180	RLT2	Homeodomain	Phase transition	282	+	1.00
AT3G12130	KHZ1	Zinc finger	Flowering	198	-	0.99
AT5G42820	U2AF35B	Zinc finger	Flowering	166, 421	+/-	1.00, 1.00
AT1G54830	NF-YC3	NF-YC	Flowering	Various	+/-	1.00, 1.00
AT1G69490	NAP	NAC	Leaf senescence	71	+	0.98
AT4G01500	NGA4	B3	Flower development	27	+	0.94
AT1G67260	TCP13	TCP	Flower development	87	-	1.00
AT1G68640	PAN	bZIP	Flower development	68	+	1.00

Intron 3 (with the strongest SNP)

Figure 12. Intron 3, containing the strongest SNP, and annotated binding places for potential transcription factors related to flowering within this region. Each color represents a transcription factor except the single T, indicated with a white letter on black background, which represents the strongest SNP. The numbers to the right represent number of base pairs on each row.

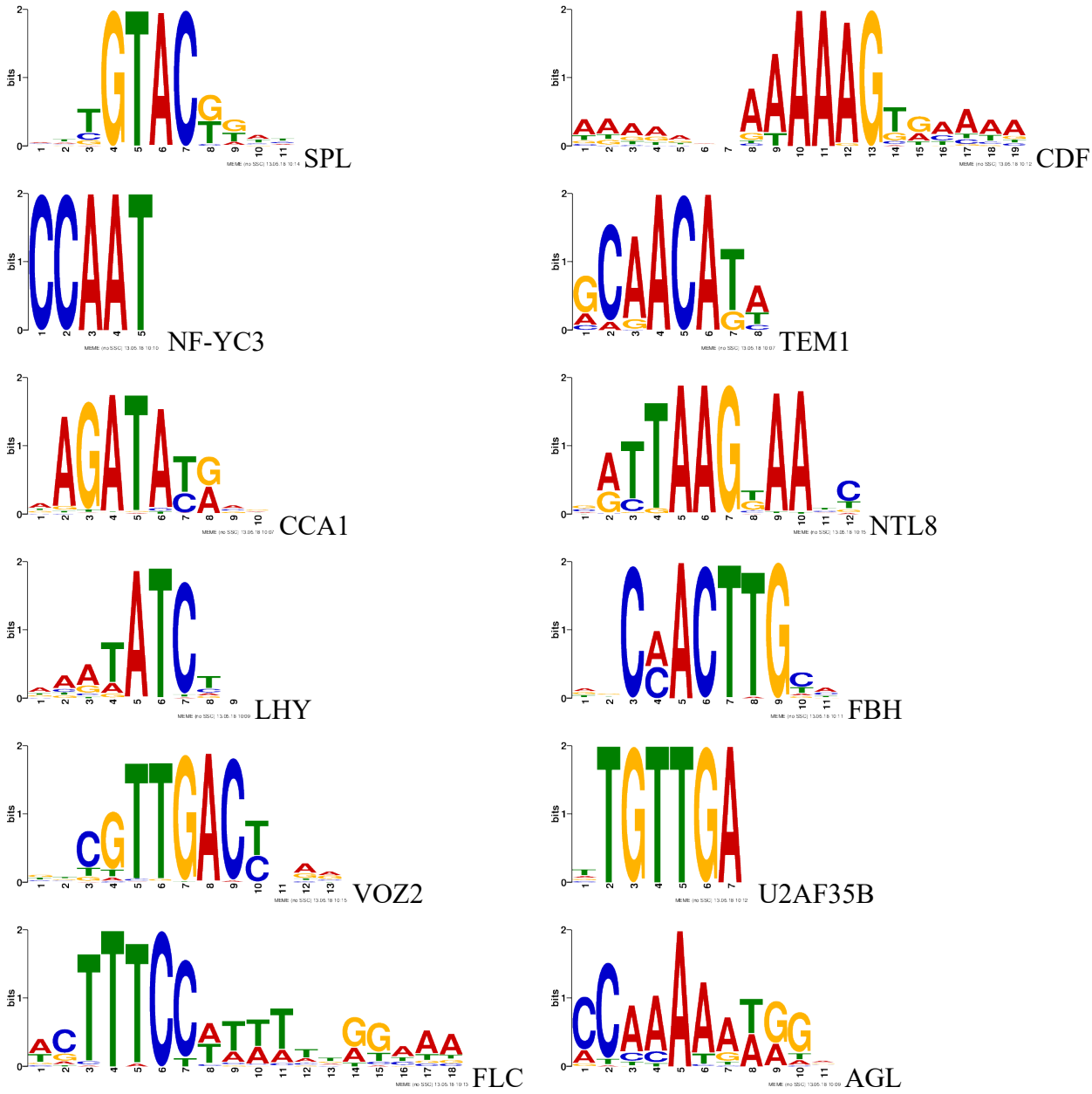

Figure 13. Position weight matrixes of the corresponding sequences to the annotated binding places for potential transcription factors related to flowering within intron 3. The matrixes represent the sequences with the highest similarity scores.

Table 3. Annotated potential transcription factors, within intron 3, related to flowering and other functions of interest for the regulation of the *PtFT2* gene.

TF ID	Gene name	TF Family	Function	Position	Strand	Score
AT5G43270	SPL2	SBP	Phase transition	Various	+/-	0.99, 0.96
AT2G33810	SPL3	SBP	Flowering	Various	+/-	0.99, 0.96
AT1G53160	SPL4	SBP	Flowering	Various	+/-	0.99, 0.96
AT3G15270	SPL5	SBP	Flowering	Various	+/-	0.99, 0.96
AT1G02065	SPL8	SBP	Flowering	Various	+/-	0.99, 0.96
AT2G42200	SPL9	SBP	Phase transition	Various	+/-	0.99, 0.96
AT1G27370	SPL10	SBP	Phase transition	Various	+/-	0.99, 0.96
AT1G27360	SPL11	SBP	Phase transition	Various	+/-	0.99, 0.96
AT3G57920	SPL15	SBP	Phase transition	Various	+/-	0.99, 0.96
AT5G62430	CDF1	Dof	Flowering	Various	+/-	1.00, 1.00
AT5G39660	CDF2	Dof	Flowering	Various	+/-	1.00, 1.00
AT3G47500	CDF3	Dof	Flowering	258, 848	+/-	0.90, 0.91
AT2G34140	CDF4	Dof	Flowering	Various	+/-	1.00, 1.00
AT1G69570	CDF5	Dof	Flowering	848, 852	+/-	0.86, 0.91
AT1G26790	CDF6	Dof	Flowering	Various	+/-	1.00, 1.00
AT2G45650	AGL6	MADS box	Flower development	853	-	0.90
AT5G60910	AGL8	MADS box	Flower development	853	-	0.90
AT1G26310	AGL10	MADS box	Flower development	853	-	0.90
AT4G11880	AGL14	MADS box	Phase transition	853	-	0.90
AT3G57230	AGL16	MADS box	Flowering	853	-	0.90
AT3G57390	AGL18	MADS box	Flowering	853	-	0.90
AT4G22950	AGL19	MADS box	Phase transition	853	-	0.90
AT4G24540	AGL24	MADS box	Flowering	853	-	0.90
AT5G62165	AGL42	MADS box	Flower senescence	853	-	0.90
AT5G51860	AGL72	MADS box	Flowering	853	-	0.90
AT3G30260	AGL79	MADS box	Phase transition	853	-	0.90
AT1G24260	SEP3	MADS box	Flower development	853	-	0.90
AT5G10140	FLC	MADS box	Flowering	783	-	0.88
AT1G25560	TEM1, EDF1	AP2; RAV	Flowering	287	+	0.94
AT1G13260	RAV1, EDF4	AP2; RAV	Flower development	Various	+/-	1.00, 1.00
AT5G42820	U2AF35B	Zinc finger	Flowering	784	+	1.00
AT1G54830	NF-YC3	NF-YC	Flowering	Various	+/-	0.80, 1.00
AT1G69490	NAP	NAC	Leaf senescence	604	+	1.00
AT3G15170	NAC1	NAC	Flower development	622	-	0.97
AT5G39610	NAC6	NAC	Flower senescence	621	+/-	0.85, 0.87
AT2G27300	NTL8	NAC	Flowering	314	-	0.92
AT1G67260	TCP1	TCP	Flower development	240	-	0.94
AT1G68640	PAN	bZIP	Flower development	158	-	1.00
AT2G46830	CCA1	MYB	Flowering	354, 278	+/-	0.87, 0.96
AT1G01060	LHY	MYB	Flowering	355, 355	+/-	0.98
AT5G37260	RVE2; CIR1	MYB	Flower development	355, 355	+/-	0.98
AT1G06180	MYB13	MYB	Flower development	74, 71	+/-	0.91, 0.91
AT1G35460	FBH1	bHLH	Flower development	567, 567	+/-	0.94, 0.93
AT1G51140	FBH3	bHLH	Flowering	566	-	0.96
AT2G42280	FBH4	bHLH	Flowering	567	-	0.96
AT2G42400	VOZ2	VOZ	Flowering	603, 602	+/-	0.87, 0.91
AT4G01250	WRKY22	WRKY	Leaf senescence	602	+	0.84

4.5 Gibberellin treatment counteract growth cessation and early bud set

To determine if gibberellin (GA) can counteract early bud set in aspen independently of *PtFT2*, hybrid aspen plants, without the gene were grown and treated with GA. It was expected that GA would either counteract growth cessation or not do that in the gene edited plants. Replicates of three different types of hybrid aspen plants were grown in a growth chamber for 8 weeks. The different types were WT (T89), a type with an over-expresser (OX) of a known *FT* repressor, the SVP protein, and one with the *PtFT2* gene deleted with CRISPR. After the first three weeks some of the replicates were treated with a GA solution and the rest were treated with pure water, two times a week. The height, new leaf number and bud score were measured once a week, until the buds were completely formed. This data can be seen in Figure 14. It was seen that GA counteracted growth cessation.

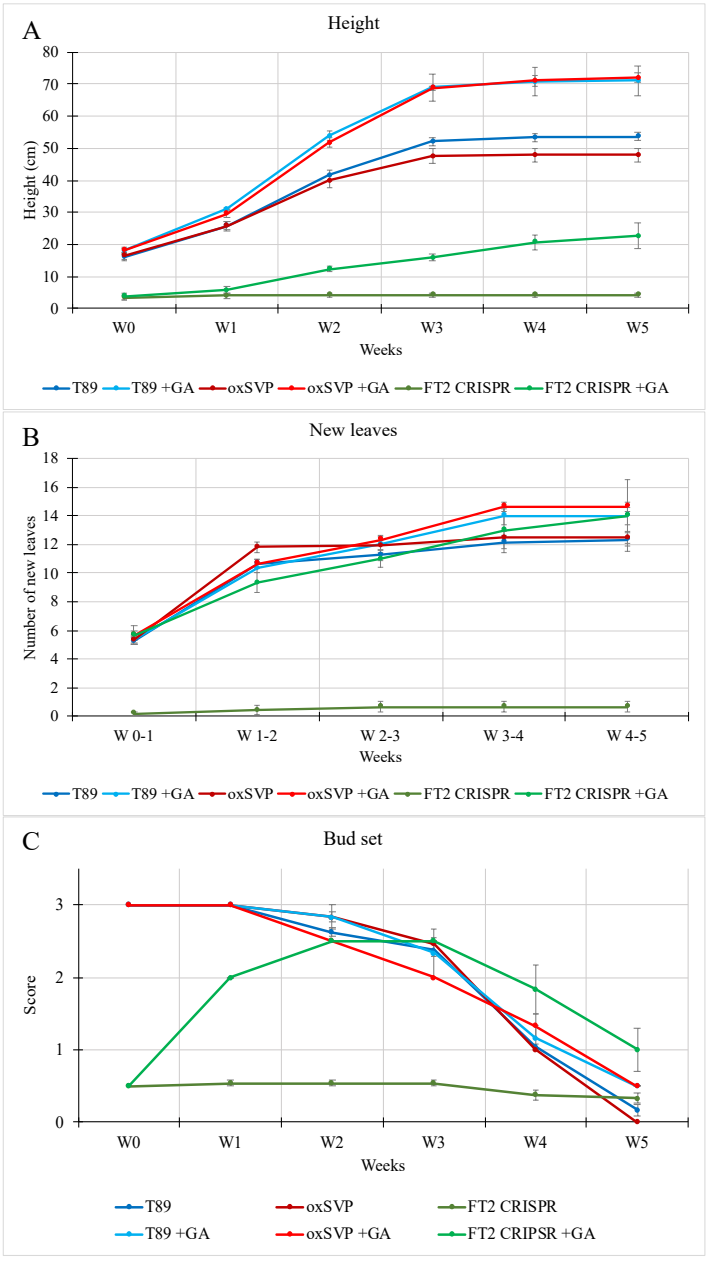

Figure 14. Phenotype measurements of hybrid aspen plants treated with a gibberellin solution and control plants treated with pure water for five weeks. **A** Mean value of height of the different plants over the five week period. **B** Mean values of new leaves of the different plants over the five week period. **C** Mean values of bud score of the different plants over the five week period.

5. Discussion

This chapter covers the discussion part of the project and is divided into four different parts. They cover discussion regarding genotyping, phenotyping, gene expression, transcription factors study, gibberellin treatment and some final remarks.

5.1 Heterozygous deletion might influence the observed effects

The genotyping preformed to confirm deletion of the PERs indicated a heterozygous deletion. It is difficult to determine the underlying reasons to why it was not homozygous, but it is most likely due to the relatively new utilization of CRISPR gene editing of European aspen. Sometimes when a CRISPR deletion is made the non-homologous end joining repair mechanism completely repairs one of the chromosomes, thus giving rise to deletion in only one of the chromosomes. [25] The mutation efficiency of CRISPR/Cas9 is depending on various factors such as target site, vector system, transformation method and plant species [25]. An optimized mutation procedure is essential for creating desirable mutations.

Even though a heterozygous deletion can have an effect on both phenotype and genotype of the gene edited plants it will most likely be different from the effect of a homozygous deletion. In a study on the resistance to a pathogenic fungus of *Vitis vinifera* (grape) it was shown that the type of knock out of the gene of interest resulted in different degree of resistance. A heterozygous deletion resulted in lower degree of resistance to the pathogen than a homozygous deletion, though the resistance was still greater than the wild types (WT)s. [26] However in order to obtain a functional knockout it is necessary to knock out both copies of the gene [27]. This is important to keep in mind when analyzing the phenotypes and gene expressions.

5.2 The phenotyping indicated changes in growth cessation

The phenotyping was expected to reveal differences in growth cessation between WT and gene edited plants. It was hypothesized that a deletion of the PERs would lead to a downregulated *PtFT2* expression as deleting an enhancer region would have a similar effect as downregulating the gene. The down regulation was expected to result in slowed growth and earlier growth cessation and bud set.

The first phenotyping performed was to measure the height and the leaf and branch number of the plants, which can be seen in Figure 3. This was done in order to see if there were any difference in these traits prior to the change of light conditions from long day (LD) to short day (SD). The bud set should not start until the days are short enough. However, it could be expected to see some difference in the growth pattern. In addition to preventing bud set, the *PtFT2* gene is also responsible for maintaining growth [4]. A deletion of one of the PERs might therefore lead to a slowed growth. The replicates of line 966-8, 966-20 and 971-14 had a mean value of height that were indeed shorter than the WT already at the first measurement, which can be seen in Figure 5A for genotype 966 and Figure 6A for genotype 971. Leaf mean value of the replicates were also lower in all 966 lines and in some 971 lines, which further indicates a slowed growth.

Furthermore, most of the replicates of line 966-8, 966-20, 966-13 and to some extent line 966-12 had a branching phenotype and thus a quite different growth habit compared to the WT plants. Representative plants from each replicate are shown in Figure 4A. The branching phenotype is unexpected as it has been shown that overexpression of *FT* homologous genes in several tree species, among them the *FT2* gene in aspen, leads to increased branching. [5][6] It is expected that the downregulated expression of *PtFT2* would lead to more branches.

The branching phenotype might instead be a stress response as most of the plants showed other signs of stress, such as red leaves, too. Some of the WT plants also had more branches than expected. Thus, strengthen the branch phenotype as a stress response. Nevertheless, most of the gene edited lines had more branches than the WT as seen in Figure 3A. If the PER is not directly responsible for the branch phenotype, the gene edited plants could still be more prone to stress due to their lack of an important enhancer region, as stress responses are often regulated by TFs that bind to enhancer regions [10].

Some of the 971 lines also had more branches than the WT, but the difference was not as obvious as with the 966 lines. Most of the 971 lines resembled WT, both in height and leaf and branch number. The only line that had a significant different growth habit was line 971-14. Its divergent phenotype can be seen in Figure 4B. There were lines with replicates with higher branch and lower leaf mean values, but the replicates of line 971-14 were more homogenous. The most significant difference where however its short height, which can be seen in Figure 6A, strange toothed leaves and general abnormality of morphological traits, such as thinner stem and smaller leaves, seen in Figure 4B.

Following the first phenotyping a continues phenotyping was initiated, right after the change from LD to SD conditions. Leaf and branch number was not counted further as it was deemed to be too labor intensive and not have a significant contribution to the overall phenotypic difference. Height was however still measured, and bud set scoring was initiated. The height of line 966-8, 966-20 and 971-14 remained low compared to WT and the height difference increased further during the measurement period. This confirms the initially indicated slow growth habit of these lines and the hypothesis that a deletion of the PERs lead to a slowed growth.

Differences in bud set between the lines were more difficult to determine. All lines set buds over a similar time span and they followed the same pattern. This did not confirm the hypothesis, as an earlier bud set of the gene edited lines were expected. A deletion of the PERs was believed to lead to a decrease in expression of the *PtFT2* gene. Apart from being important for growth it is also responsible for inhibition of bud set and it was therefore suggested that the lower expression would reduce the inhibition and result in an earlier bud set. Earlier initiation of bud set was seen in line 971-14 and to some extent line 966-8 and line 966-20, though it was not significant.

There are two major potential reasons to why the expected observations were not seen. First, the bud scoring is based on subjective evaluations and not exact quantification. The bud scoring is therefore influenced by several factors, such as the experience of the one that evaluates the buds. Second, the deletion of the PERs were heterozygous. This means that even though the PER is deleted on one of the chromosomes it could still influence the expression from the other chromosome. Thus, leading to different expression patterns depending on the underlying type of expression mechanism. [7] The gene on the non-affected chromosome might take over the expression completely. This could explain why earlier bud set was not clearly observed even when the PERs were confirmed to be deleted. The expression could also become intermediate, as in the mentioned case with grape. Because of this it is important to known how the gene is expressed. This problem might be solved by increasing the short day to a length that would initiate growth cessation in the edited plants but not in the WT. In this case the buds would start to form earlier in the edited plants and the difference would be clearly seen.

5.3 Gene expression analysis revealed differences in expression

To determine if the two paralog *PtFT2* genes, *PtFT2α* and *PtFT2β*, were downregulated in the plants with the PERs deleted, gene expression of collected leaf samples were analyzed. As hypothesized, most of the lines showed a down regulation of *PtFT2*. Deleting the enhancer regions did thus reduce the expression of the gene in general. The down regulation explains the slowed growth of line 966-8, 966-20 and 971-14 as *PtFT2* is responsible for maintained growth. Without sufficient expression it is expected that the growth slows down. Another interesting observation is that the two lines of genotype 971, 971-10 and 971-36, that had upregulated expression of the *PtFT2* genes, grew more than the WT, as seen in Figure 6A. The reason for the upregulation is not known, but perhaps it led to the increased growth. However, it could just be a coincidence as the height difference was quite small. Anyhow it would be interesting to further investigate the underlying reason for these lines upregulated gene expression.

Why the lower expression did not lead to slowed growth in the other lines and why an earlier bud set was not clearly seen in any line is difficult to determine. However, it could be worth noting that the lines with no inhibited growth, compared to WT, had a less reduced gene expression compared to the lines that showed clear signs of inhibited growth. The decrease in expression was perhaps not enough to slow down the growth of those lines. Maybe this is the underlying factor for the absence of early bud set too. If the genes had been further down regulated a more obvious early bud set might have been observed. A homozygous deletion would most likely also result in an earlier bud set as none of the chromosomes then could contribute to the enhancement of the gene.

5.4 Binding sites for potentially conserved transcription factors exist in aspen

If the PERs that were deleted contain binding sites for transcription factors (TF) and what type of potential TFs that bind to them is not known. In order to understand the underlying functions of the PERs, a bioinformatic analysis of these sequences and the sequence containing the strongest SNP associated phenotype for local adaption, intron 3, was performed. The upstream PER, the downstream PER and intron 3 were annotated to binding places of known TFs in *A. thaliana* to find potential homologous TFs in European aspen. *A. thaliana* has several binding sites for TFs in the *FT* enhancer regions, corresponding to the PERs of *PtFT2*, and they are known for regulating flowering, by enhancing the *FT* expression and photoperiodically control the gene. [8][9]

In aspen the two paralogs of the *FT* gene, *PtFT1* and *PtFT2*, have separated functions. *PtFT1* is speculated to has the conserved function of initiating flowering and *PtFT2* is responsible for maintenance of vegetative growth and inhibition of bud set. There is no *FT* gene responsible for vegetative growth and inhibition of bud set in *A. thaliana* and thus no know TFs controlling it through *FT* either. Therefore, it was not possible to search for TFs controlling growth maintenance and inhibition of bud set. Instead TFs related to flowering was identified.

Fifteen unique TFs involved in regulation of flowering were found. Four of them were present in both the upstream PER, the downstream PER and intron 3. Two were in the upstream and the intron 3, but not in the downstream region and two were in the downstream and the intron 3, but not in the upstream region. One was in the upstream and downstream but not in the intron 3 region. Finally, one was unique to the upstream, one was unique to the downstream and four were unique to the intron 3 region. The TFs of the upstream PER can be seen in Figure 8, the TFs of the downstream PER in Figure 10 and the TFs of the intron 3 region in Figure 12. Many of the TFs had more than one binding site.

This was especially common among the TFs that were present in all three regions. Another interesting finding was that some of the binding sites were binding sites for more than one different type of TF. In a study of TFs in *A. thaliana* it was shown that half of the investigated TFs recognize more than one binding site, so these findings were not unexpected [10].

TFs that were common among all regions include *NF-YC3*, *TEM1* and different versions of *SPL* and *CDF*. These TFs have been shown to regulate the expression of the *FT* gene in *A. thaliana* [11]. When analyzing the position weight matrixes, of the corresponding sequences to the annotated binding places for the potential homologous TFs, it could be seen that they generally had at least four to five bases with high binding affinity and low variation. This and that the corresponding sequences in the *PtFt2* gene generally have almost the same bases, indicate that there are conserved binding sites for TFs involved in flowering in European aspen. The matrixes are shown in Figure 9 for the TFs of the upstream PER, in Figure 11 for the TFs of the downstream PER and in Figure 13 for the TFs of the intron 3.

Some of the TFs involved in regulation of flowering, among them *NF-YC3* and *CDF*, are also involved photoperiodic responses, which further indicate that they could be responsible for bud set and growth maintenance as these are photoperiodic responses, too. Taken together the collected information indeed indicates that homologs to these TFs, and probably at least some of the other TFs involved in flowering, are controlling growth maintenance and inhibition of bud set in aspen by binding to the PERs. It seems likely that the TFs in the *PtFT2* region have changed their functions, in a similar way as the genes themselves, to be involved in growth maintenance and inhibition of bud set, while they still, as speculated, regulate flowering in the *PtFT1* region. Though it would be useful to annotate enhancer regions of *PtFT1* as well, to see if they contain similar homolog binding sites for TFs involved in flowering, to strengthen this hypothesis.

In addition to the TFs that regulates flowering, TFs involved in other related biological processes, that could be of interest, were also identified. These processes were mainly flower development and phase transition, transition from a vegetative growth phase to a flowering growth phase. The full list of annotated TFs can be seen in Table 1 for the upstream PER, in Table 2 for the downstream PER and in Table 3 for intron 3.

Whether the potentially conserved TFs are responsible for regulating the *PtFT2* gene cannot be concluded from this analysis alone. The purpose of the analysis was limited to identifying potential homolog TFs. Thus, detailed information, beyond the type of biological processes they are involved in, was not collected. For further confirmation it is necessary to, inter alia, test their binding affinity to the PERs and do precise knock outs or knock downs, of the TFs proposed binding regions, and analyze the effects.

5.5 Gibberellin counteract growth cessation independently of *PtFT2*

In addition to the analysis of European aspen a small study of *Populus tremula x tremuloides* (hybrid aspen), was performed to further investigate the regulation of the *PtFT2* gene. It has previously been shown that an increase of gibberellin (GA) production in aspen delays growth cessation, the bud set and the growth stop. It was concluded that plants with increased GA production are insensitive to the *PtFT2* signals that tells the plant to stop growing and that GA and *PtFT2* therefore work in parallel to control similar processes. [12] However it is not known whether the GAs effect on bud set works through or independently of *PtFT2*. It was therefore tested in this experiment.

GAs effect on WT plants, plants with an over-expresser (OX) of a known *FT* repressor, the SVP protein, and plants with the *PtFT2* gene CRISPR deleted, compared to control plants, without GA treatment, were analyzed and can be seen in Figure 14. The height, number of new leaves and the bud score of the treated plants were different from the untreated plants. Number of new leaves is useful to measure as it is an indication of if the plants increased height is due to continued growth or just elongation. As excepted the treated plants were taller, had more leaves and a delayed bud set for all three types. Thus, again confirming the delayed growth secession through increased GA concentration. However, the number of new leaves and the bud set was not that different from the untreated plants for the WT and the OX.

The difference between the different types of plants and especially the difference between the WT and the CRISPR plants is more interesting. Their height and leaf number were significantly lower, and the bud set is significantly earlier compared to the WT. This clearly shows the effect of knocking out the *PtFT2* gene and its importance for growth maintenance. When the CRISPR plants were treated with GA their height increased, the number of new leaves reached similar levels as the other type of plants and their bud set went from a near set bud to an almost undeveloped bud and then back to a more developed bud. This significant difference in growth habit between the CRISPR plants, treated with and not treated with GA, indicates that an increased concentration of GA counteracts growth senescence independently of *PtFT2*. However, this was a small study so it would probably be necessary to investigate this further in a larger study to confirm the indications of the observations.

5.6 Final remarks, improvements and future focus

The combined results of the phenotype and the gene expression analyses indicates an effect on growth cessation in European aspen. Deleting the PERs of *PtFT2* led to down regulations of both *PtFT2* paralogs in line 966-8, 971-14 and somewhat in 966-20 and 971-30. Line 966-8 and 971-14 had slower growth and earlier growth cessation. Slightly slowed growth was also seen in line 966-20. In addition to the low height among these lines, they and most of the other lines had fewer leaves than WT, which is an indication of slow growth, though the difference was not significant.

Phenotype differences, such as an increase in branch number, were common among the gene edited plants. Though they were most distinctive in the beginning of their growth period and to some extent present in WTs. The increasing homogeneity in phenotype over time might be signs of the plant's adaption from the lab conditions to the growth chamber conditions. To avoid stressing plants, optimal growth conditions and a smooth transition between them should be adopted. This is essential, so not inaccurate conclusions are drawn.

If these observations are not stress related, they are most likely an effect of the deletion of the PERs. However, an increase in branches as an effect of lower gene expression of *PtFT2* is unexpected. Still, lines with low expression showed strange phenotypes, in addition to the expected slowed growth and growth cessation. This might be due to changes in expression of other unknown genes as enhancer regions can regulate more than one gene. Therefore, it is useful to try to figure out these connections. The downstream PER might be a good candidate for regulating more genes than *PtFT2* as line 971-14 had an unusual leaf phenotype, which is a trait not known for being related to growth cessation.

This part of the study was used as an initial analysis of the PERs effect on growth in aspen and used to screen for lines to be further investigated. Replicates of line 966-8 and 971-14 should be grown again in future experiments to confirm the observations regarding them. Line 966-20 and 971-30 might also be grown again. In addition to this, new lines with both PERs deleted could be generated to see the combined effect of deleting them. Lines with intron 3 deleted should also be generated to determine its role in the regulation of *PtFT2*. Finally, the deletions should be redone with an optimized procedure to obtain plants with homozygous deletions.

To confirm or reject the identified TFs as homologs, their binding affinity to the annotated potential homolog binding sites should be tested. If they bind to these regions, it is likely that they are conserved and regulate the expression of *PtFT2*. It would also be of interest to test if the TFs binding affinity differ between northern and southern European aspen plants. A difference would contribute to the overall expression difference between them and could explain the down regulation of the gene expression in northern plants compared to southern plants. The observation that GA counteract growth cessation independently of *PtFT2* clarifies some of the complexity behind *PtFT2*'s role in growth cessation. However further studies are needed as there are still a lot to be investigated regarding it.

Taken together the observations of this study revealed some of the issues regarding the regulation of *PtFT2* in European aspen. They can be used as a starting point for further studies of its regulation, its involvement in growth cessation and the underlying factors for the adaptation of aspen plants to different light conditions. Understanding these processes is useful for the adaptation of trees to new latitudes and longer growing seasons. Finally, findings of importance to the *PtFT2* gene might be useful for understanding flowering in trees, as the homolog gene in *A. thaliana* is involved in flowering. It is difficult to study flowering in trees but it is of major importance for tree breeding. Thus findings that can contribute to understanding flowering in trees are exceptionally valuable.

6. Conclusion

European aspen plants with PERs of *PtFT2* deleted had a deviating phenotype and gene expression. Some of the plants also grew slower and initiated growth cessation earlier, compared to WT southern plant line. Thus, they showed indications of behaviors associated with northern aspen trees. These findings were not preserved among all analyzed lines. Hence further studies need to be performed for confirmation of the observations. None of the lines had significant differences in timing of bud set. The confirmed heterozygous deletion of the PERs could be the reason for the ambiguous results.

Potential homologous binding sites, for *A. thaliana* TFs, exist in the PERs of the *PtFT2* gene. The TFs are from various TFs families and involved in flowering, flower development and phase transitions. Growth cessation of hybrid aspen plants, with the *PtFT2* deleted, was counteracted by GA treatment. This indicates that GA affects the bud set in aspen independently of *PtFT2*.

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