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Genetic Variation in the Ft1 Locus Involved in Reproductive Onset in Populus Deltoides

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Genetic variation in the *FT1* locus involved in reproductive onset in *Populus deltoides*

By

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The onset of reproduction is an important trait and is controlled by the *FT1* locus in poplar (*Populus sp*.). Sequence variation in this locus is not well-understood. This study's aim is to identify sequence variation in the *FT1* locus in a small population of *Populus deltoides* with varying reproductive onset. Gene specific primers were designed to amplify *FT* from 14 genotypes. The sequence analysis showed 12 single nucleotide polymorphisms and four insertion-deletion sites located in introns of *FT1*. No connection was observed between the identified polymorphisms and variation in reproductive onset. Further DNA sequencing of the genotypes needs to be done on the promoter region of *FT1* to conduct an association study to statistically assess the connection between polymorphisms and phenotypic variation in a larger population. This information is expected to help us understand the genetic basis of phenotypic variation in reproductive onset.

DEDICATION

I am dedicating this research to my wife (Ayfer Akgul) and my son (Salih Yigit Akgul).

ACKNOWLEDGEMENTS

I am thanking my graduate advisor Dr.Yuceer for mentorship and support during my research. I am also thankful to my committee members Drs. Diehl, Roberts, and Harkess for guidance. I extend my thanks to Dr. Chuan-Yu Hsu for help and advice during my research and to Ms. Asli Ozdilek for support. Finally, I am grateful to the Ministry of National Education of the Republic of Turkey for financial support during my Master of Science studies.

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CHAPTER I

INTRODUCTION

Reproductive onset, induction of flowering for the first time in the life cycle of a tree, is a crucial step in the development and adaptation of trees. Investigating reproductive onset at the molecular level provide us with an understanding of how trees adapt and survive as well as how to breed more efficiently for various ecological and economic traits (Brunner et al., 2004). It was found in photoperiodic tobacco that long-day plants begin reproducing when the day length is longer than a critical period, while short-day plants reproduce when the day length is shorter (Garner, 1920). Later, it was shown that photoperiod induction of reproduction is sensed by leaves (Knott, 1934). Chailakhyan hypothesized an unknown flowering compound (florigen) is produced in leaves under inductive photoperiods and moved to the shoot apex to initiate reproduction (Chailakhyan, 1936).

Recent studies have shown the protein product of the *FLOWERING LOCUS T* (*FT*) gene is part of florigen. *FT* was initially discovered to induce reproductive onset under long days in the annual model plant *Arabidopsis thaliana* (Kardailsky et al., 1999; Kobayashi et al., 1999). When a light signal was perceived by leaves of the long-day plant *Arabidopsis* and the short day plant rice (*Oryza sativa*), the CONSTANS protein activated *FT* in the leaf phloem (An et al., 2004; Ayre and Turgeon, 2004). Subsequently, FT protein appeared to be translocated to the shoot apex where it formed a protein

complex with FLOWERING LOCUS D (FD) protein. This complex activated the floral meristem identity gene *APETALA1 (AP1)* to trigger reproductive development (Abe, 2005; Wigge et al., 2005; Corbesier et al,. 2007; Mathieu et al., 2007; Tamaki et al., 2007). Other studies also provided evidence that the FT protein is a mobile floral signal transmitted from leaves to the shoot apex (Lifschitz et al., 2006; Jaeger and Wigge, 2007; Notaguchi et al., 2008) via phloem (Giavalisco et al., 2006; Lin et al., 2007; Aki et al., 2008).

A similar gene to *FT* (*FLOWERING LOCUS T1*) has been identified in poplar (Bohlenius et al., 2006; Hsu et al., 2011). Overexpression of *FT1* induced reproductive onset within several months in juvenile poplar. *FT1* was activated in response to cold in winter and determined the reproductive fate of axillary meristems on preformed shoots enclosed in vegetative buds (Hsu et al., 2011). Warming temperatures in spring rapidly suppressed *FT1* transcription, ending the onset of reproduction and marking the developmental beginning of reproductively determined meristems into flower buds. Homologs of *CONSTANS* in poplar (*CO1* and *CO2*) did not appear to be involved in reproductive onset (Hsu et al., 2012), one of the major differences between *Arabidopsis* and poplar. This is perhaps because *FT* in *Arabidopsis* is regulated by long days, whereas *FT1* in poplar is regulated by cold, suggesting different evolutionary tracks for annual and perennial species.

Reproductive onset in poplar often occurs between ages 3 and 10 years from seed. Although a large phenotypic variation in reproductive onset exists in poplar (Braatne, 1996), research has yet to be conducted to determine the underlying reasons for this variation. The reasons for this critical gap in the knowledge base include the lack of

knowledge of an appropriate gene involved in reproductive onset and a suitable poplar population with a large phenotypic variation in reproductive onset. To enable future large-scale polymorphism studies, the aim of this study was to identify sequence variation in the *FT1* locus in a small population of *P. deltoides* with varying reproductive onset. Genetic variation in transcription factors and transcription factor binding sites plays a major role in phenotypic variation within species and divergence between species (Brunner et al., 2011). Using the identified polymorphisms in the *FT1* locus, a larger population of *P. deltoides* with known phenotypes can be used to conduct an association study to statistically assess the link between polymorphism in the *FT1* locus and phenotypic variation in reproductive onset. This would represent an important step towards developing reliable genetic markers to help breeders select early or late reproducing trees at early ages.

CHAPTER II

MATERIALS AND METHODS

A step-wise systematic approach

A systematic approach to accomplish the specific aim was taken (Figure 1). In this approach, samples were collected from 14 *P. deltoides* genotypes, genomic DNAs were isolated, the *FT1* locus was amplified using polymerase chain reaction (PCR), cloned, sequenced, assembled, and aligned to identify polymorphic regions, and eventually the findings were interpreted in light of DNA polymorphism and the timing of reproductive onset.

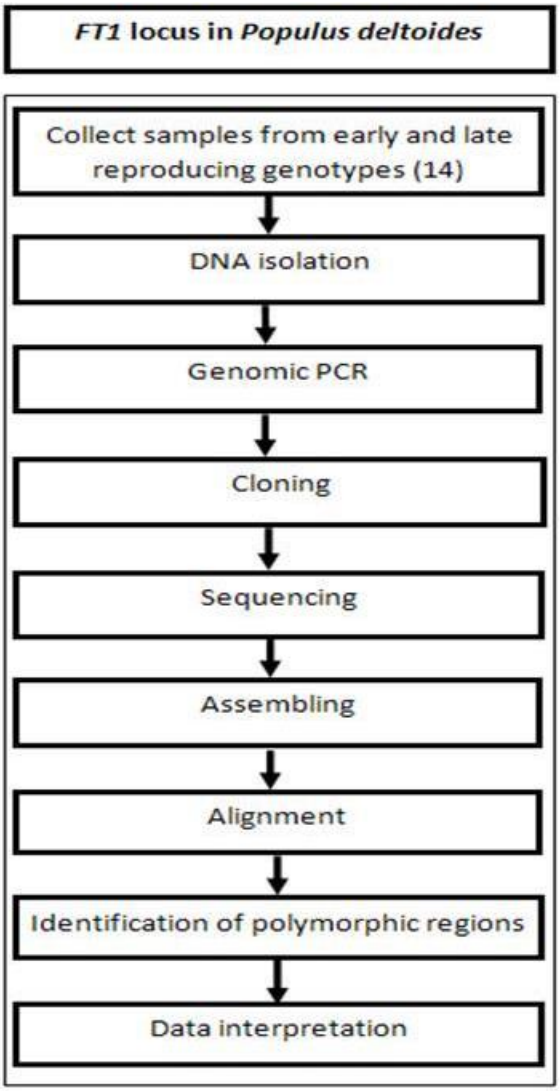


Figure 1 A step-wise systematic approach.

Includes steps of molecular analysis, and a specific genetics approach in a small population.

Plant materials

Reproductive or vegetative buds or leaves of *P. deltoides* were used as plant material. Fourteen *P. deltoides* genotypes are selected from a common garden in the Blackjack research facility of Mississippi State University. These plants were generated from dormant cuttings, except Pd-F3-OP and Pd-F2-OP, which were generated out of

seed from randomly selected open pollinated treed in the field. The dormant cuttings were obtained from a stool bed *P. deltoides* nursery maintained by Mississippi State University in Stoneville, MS. The age of material in the stool bed was not known, but they had been regularly cut back every year to maintain juvenility. All the *P. deltoides* trees in the Blackjack facility were irrigated during the summer, and the field was regularly mowed. The selected genotypes showed early or late onset of reproduction based on field observations beginning in 2008. There is no accepted definition for "early" or "late" onset of reproduction in poplar. It is arbitrarily defined "early" as reproduction by age 5 and "late" as reproduction after age 5. Tissues were collected from the selected population.

Genomic DNA isolation

Genomic DNAs were isolated from 14 genotypes using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA). 100-200 µg of tissues were ground in liquid nitrogen by pestle in a mortar that contained 400 µl AP1 buffer (cell lysis buffer) and 4 µl RNase. After vigorous vortexing, the mixtures were incubated at 65 ^{o}C for 10 min in a water bath and mixed 2 to 3 times by inverting the tubes. The samples were centrifuged for 5 min at 20,000 x g, the lysates were transferred to mini-spin tubes which were centrifuged again for 2 min at 20,000 x *g*, 130 µl of precipitation buffer (AP2) was added, and the samples were incubated 5 min on ice. Six hundred microliters of lysate cleaning buffer and 500 µl protein denaturation (AW) buffer were added onto the DNeasy Mini column and centrifuged for 1 min at 6,000 x *g*. Fifty microliters of elution (AE) buffer were added onto the DNeasy membrane, which was incubated 5 min at room temperature. The samples were centrifuged for 1 min at 6,000 x *g* to elute. After DNA isolation, the DNA

samples were quantified using a Spectronic Biomate 3 UV-Visible Spectrometer (Themo Spectronic, Rochester, NY).

Molecular analysis of *FT1* locus

PCR amplification

PCR was performed to amplify the *FT1* locus from the genomic DNA. Locus-specific forward and reverse primers were designed and used to amplify the protein coding region and introns (below). Total PCR volume per reaction was 50 µl, containing 100-200 ng of genomic DNA, 1.25 units of Ex *Taq* polymerase (Takara Mountain View, CA), 4 µl of 2.5 mM dNTPs, 1µl of 10 µM forward, 1µl of 10 µM reverse primers, and 5 µl 10X reaction buffer. PCR amplification was performed using the Eppendorf Mastercycler epgradients PCR system (Eppendorf, Westbury, NY)

Table 1 Primer sequences used for amplifying the 3' region of the *FT1* locus (exons and introns).

Regions	**Primer Sequence (5-`→3-`)**
Region 1	Forward- ATGTCAAGGGACAGAGATCCTCTGAGC Reverse- GCAAATATTCTCTTAGGCTGGGGTCAC
Region 2	Forward- CCAAGTGACCCCAGCCTAAGAGAATAT Reverse- ACCAAAGCTTGCCCCAGTTGTTGCTG
Region 3	Forward- GATATTCCAGCAACAACTGGGGCAAG Reverse- TTATCGCCTCCTACCACCAGAGCCAC

To amplify three regions of *FT1*, the following PCR parameters were used: initial denaturation of DNA at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 58 °C for 30 s, extension at 72 °C for 4 min, and final extension at 72 °C for 5 min.

Agarose gel electrophoresis

The amplified PCR products were analyzed using the agarose gel electrophoresis technique. By mixing 20 ml of 50X TAE (containing 242 g/L of Tris base, 57.1 ml/L acetic acid, 100 ml/L 0.5M EDTA) stock solution and 980 ml distilled water (ddH_2O), 1X TAE running buffer was prepared. To prepare a1%-agarose gel, 0.3 g of agarose was dissolved in 30 ml 1X TAE buffer and boiled in a microwave until a clear solution was observed. The solution was cooled, and 6 µl EtBr from 5 mg/ml stock was added into the solution. The solution was transferred to a gel apparatus and cooled at room temperature. After solidification, the comb was removed, and the gel was transferred into a tank

containing 1X TAE buffer. Then, DNA samples were mixed with 1 µl 6X loading dye and loaded into gel wells. Gel electrophoresis was run on constant voltage (65V) for 90 min. The gel was viewed under UV in a gel documentation system to visualize the DNA bands. The desired DNA fragments were isolated from the gel using the QIAEX II gel extraction kit (Qiagen, Valencia, CA)

Competent cell preparation

Competent *Escherichia coli* cells were prepared via a chemical method prior to cloning the *FT1* genomic DNA from all genotypes. First, a single colony of *E. coli* cells were inoculated from Luria-Bertani Broth (LB) plate into 2 ml LB liquid medium. After overnight culturing, it was chilled 15 min on ice and centrifuged 10 min at 4,000 rpm at 4 °C. The cell pellet was then resuspended in 25 ml cold $CaCl_2$. The final concentration was adjusted to 15%. The cell suspension was aliquoted into sterile 1.5 ml microcentrifuge tubes and stored at -70 °C.

Cloning

The pGEM®-T Easy Vector System (Promega, Madison, WI) was used for cloning the PCR product of *FT1*. For this process, 10 µl-mixure reaction was set up: 5 µl 2X rapid ligation buffer, 0.5 µl insert-ready vector (50 ng), 4 µl PCR product, and 0.5 µl T4 DNA ligase (3 Weiss units/µl). The ingredients were mixed gently and incubated at 4 °C overnight.

Competent cells were thawed on ice for 15 min and mixed carefully with 10 µl mixture of ligation reaction in the culture tube. The reaction was incubated on ice for 20 min. The cells were then heat-shocked at 42 °C for 2 min in a water bath without shaking,

and the tubes were immediately returned to ice for 2 min. 400 µl LB medium was added to tubes and mixed in the incubator for an hour at 37 °C with shaking (~150 rpm). During incubation, LB + Ampicilin plates were warmed and 40 µl of X-gal (20 mg/ml) and 200 µl IPTG (0.1M) mixture was added to the plates. Plates were left to dry, and then cells were immediately spread onto the LB Ampicillin plates. The ampicillin plates with cells were incubated upside down overnight at 37 °C.

For each *P. deltoides* genotype, four white colonies were selected to sequence. Plasmids containing the *FT1* genomic DNA were isolated using the QIAGEN Plasmid mini kit (Qiagen, Valencia, CA).

Sequences analysis

Sequencing was performed following the manufacturer's instructions using the CEQ 8000 DNA Sequencer genetic analysis system (Beckman Coulter Inc., Brea, CA). At least two colonies of each fragment were sequenced. The Dye Terminator Cycle Sequencing using the Quick Start Kit (Beckman Coulter Inc., Brea, CA) was used. T7 and SP6 primers for sequencing were provided in the sequencing kit. For internal regions of the second and third introns, other primers were designed and used (Table 2).

Table 2 Primers that were used to sequence internal regions of introns 2 and 3.

Primer name	**Primer Sequence (5-`→-3-`)**
FT1-intron2 Internal	Forward- GCTGGTAGCACTCTTGTAGAATTGTTGG
FT1-intron3 Internal	Forward- CATATGATTGGATCGAAATTTAGTTG
FT1-intron3 Internal	Reverse- TTTCTACGAGCCCGCAGGTGGAAATG
FT1-intron3 –Internal3	Reverse-2- GTTCTGATTGGTGAACTGTCTTTGTG

Identification of polymorphic regions

Using Lasergene software (DNASTAR Inc., Madison, WI), multiple sequence alignments were conducted to identify single nucleotide polymorphisms (SNPs), small insertions or deletions (indels, ≤50 bp), and structural variants (>50 bp). Assembly and alignment of sequences were performed using MegAlign program of the Lasergene software (DNASTAR, Madison, WI). If a particular SNP was found in more than three genotypes, it was called "true." The biological significance of identified polymorphic regions was inferred from the literature. The PLACE database (Higo et al., 1999) was used to screen polymorphic DNA regions to determine if a previously identified *cis*-acting regulatory element was present in such regions. The *P. deltoides* genotypes were clustered showing similar patterns of polymorphism and the relationships between the clustered genotypes and their phenotypes were examined. Phylogenetic analysis was conducted using the Clustal X method (Saitou and Nei, 1987). Pairwise and multiple alignment parameters were set independently. Gap opening and gap extension were set at 35 and 0.75, respectively, for pairwise and at 15 and 0.3, respectively, for multiple alignment. The parameter for delay divergent sequences in multiple alignments was set to 25%. TreeView software was used to visualize the phylogenetic tree. Bootstrap analysis based on 1000 replicates was conducted to show the support among nodes.

CHAPTER III

RESULTS AND DISCUSSION

Identification of early and late reproducing genotypes

Phenotypes of 14 *P. deltoides* genotypes whose timing of reproductive onset was observed on at least four ramets are shown in Table 3. Seven genotypes reproduced at age 3, three genotypes reproduced at age 5, and the other four genotypes had not yet reproduced as of spring 2013 (Table 3). Based on these observations, 10 genotypes were assigned to the "early reproducing group" and the other four were assigned to the "late reproducing group."

Table 3 Experimental population of *P.deltoides* with early or-late reproducing groups.

Genotype	**Planting date**	**Gender**	**Reproductive onset**	
Pd 10175	2008	M	2011	
Pd 10917	2008	M	2011	
ST-261	2008	M	2011	
Tx 8-1	2008	M	2011	
Tx-61	2008	F	2011	Early reproducing
Tx-74	2008	F	2011	
134442	2008	M	2011	
Pd10112	2008	M	2013	
UK100	2008	M	2013	
Pd-F3-OP	2008	M	2013	
ST-285	2008			
Pd-F2-OP	2008			Late reproducing
AC9714	2008			
AC9718	2008			

Isolation of genomic DNA

Concentration of genomic DNA (gDNA) isolated for each genotype ranged from 40 to150 ng/µl. Visual inspection of gDNAs on agarose gel showed little degradation indicating quality gDNAs (Figure 2).

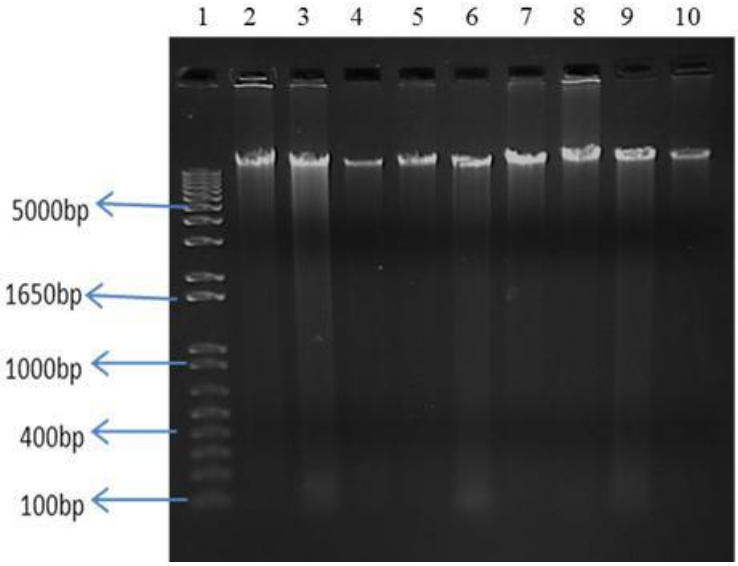


Figure 2 Analysis of genomic DNAs from some *P. deltoides* genotypes on 1% agarose gel.

Lane 1, 1 Kb plus DNA marker; Lane 2,UK-100; Lane 3, Pd10112; Lane 4, Pd10175; Lane 5, AC97-18; Lane 6, Pd10917; Lane 7, 134442; Lane 8, F2; Lane 9, F3; Lane 10, AC97-14.

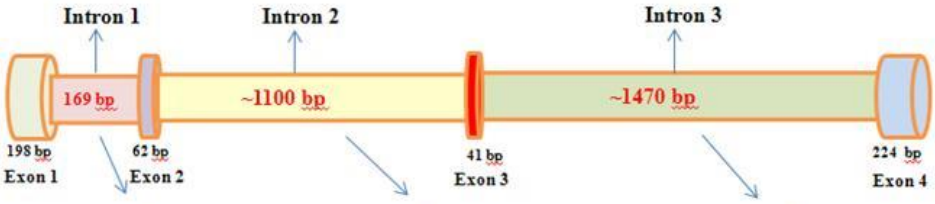
Location of polymorphisms

An approximately 3.3 kb region of *FT1,* consisting of four exons and three introns, was sequenced from the 14 *P. deltoides* genotypes (Figure 3A). Coding and intron sequences were compared among genotypes to identify SNPs. While no SNPs were located in coding regions, several SNPs were found in all three introns (Figure 3B).

Among the 12 SNPs, four led to transitions, the first transition region being in 484th nuclotide and in 5 genotypes as a change between A and G. Also, there were another three regions (655-667-906) with changes, and the changes were between C and T in 7, 4, and 4 genotypes, respectively. Six SNPs led to transversions, and in four regions (257, 2253, and 2415) the variation was between A and T in 4, 5, and 4 genotypes, respectively. In other two regions, changes were between A and C in 1122 and 2844 in 5 and 6 genotypes.

The frequency of SNP identified in this study was one per 272 bp in the population. This was calculated by dividing the average total nucleotides in the population by the total SNPs (~3260/12). This frequency was relatively low compared to other studies. For example, a frequency of one SNP for every 100 bp was found in *Zea mays* (maize) (Rafalski, 2002); one in 77 bp in *Gossypium spp*. (cotton) (Chuanfu et al., 2008), and one in 45.7 bp in *Helianthus annuus* (sunflower) (Kolkman et al., 2007). SNP frequency found in *Glycine max* (soybean) (one in 273 bp) was similar to that in this work (Zhu et al., 2003). The small sample size of 14 genotypes, and studying only a single locus, could have affected the SNP frequency observed in this study.

SNP polymorphism in the genus *Populus* shows variation among species. The rate of polymorphism was found to be one SNP per 130 bp in *P. trichocarpa* (Gilchrist et al., 2006), and one per 60 bp in *P. tremula* (Ingvarsson, 2005). In other tree genera (e.g. *Pinus, Eucalyptus, Chamaecyparis*) the SNP rate was estimated to be between 2 to 16 SNPs per 1,000 bp (Narender et al., 2005). Consequently, the SNP rate found in this study is somewhat lower than that found in other poplar studies, as well as studies of other species.



Genotypes	257	484		655	667	681	906	1122	1244		2253	2257	2415	2844
Pd10175	T	A		T	C	T	T	C	C		A	T	A	A
Pd10917	T	A		C	C	-	C	C	T		A	-	T	A
ST-261	T	A		T	C	-	T	C	C		T	-	T	C
TX8-1	A	A		T	T	-	T	C	T		A	A	A	C
TX-61	T	A		T	C	T	T	A	C		A	A	A	A
TX-74	A	G		C	T	-	T	A	T		T	-	T	C
134442	T	G		C	T	-	C	A	C		T	-	T	C
Pd10112	T	A		T	C	T	T	A	C		T	-	T	C
UK100	T	A		C	T	-	T	C	T		A	A	T	C
Pd-F3-OP	T	A		C	T	T	T	C	C		T	-	T	A
ST-285	T	G		T	T	-	T	A	T		A	A	A	A
Pd-F2-OP	T	G		T	T	-	T	A	C		T	-	T	A
AC97-14	A	A		C	T	-	C	C	T		T	-	T	A
AC97-18	A	A		C	T	-	C	C	T		T	-	T	A

Figure 3 Schematic structure of the *FT1* locus and SNP analysis in 14 *P. deltoides* genotypes.

A. The re-sequenced 3-kb *FT1* genomic region contains four exons and three introns. **B.** SNPs in intron 1, intron 2 and intron 3 were identified.

Indels were also found in introns 2 and 3. In intron 2, the first indel was between nucleotides 655 and 695, while the second indel was between1110 and 1120 (Figure 4). In intron 3, the first indel was between nucleotides 1590 and 1640 and the second indel was between nucleotides 2240 and 2280 (Figure 5).

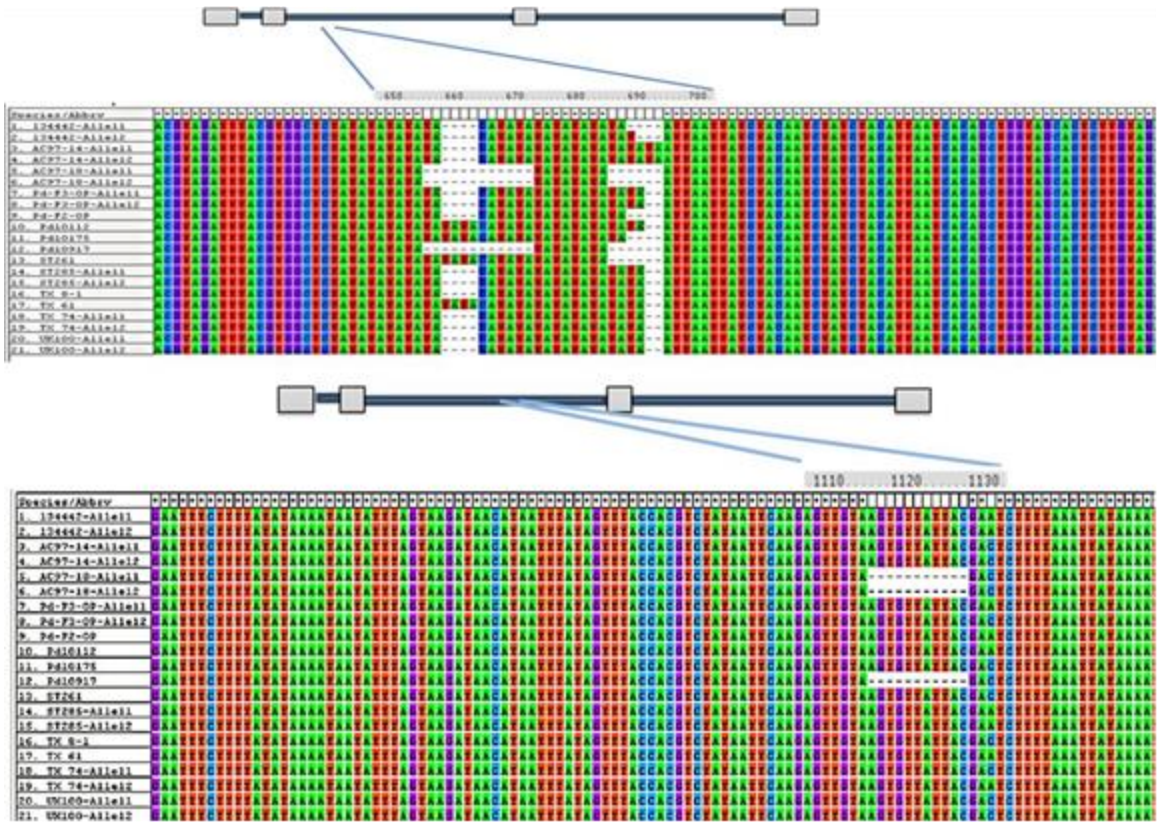


Figure 4 Indels in intron 2 with schematic structure of *FT1* gene.

Dashes show missing nucleotides.

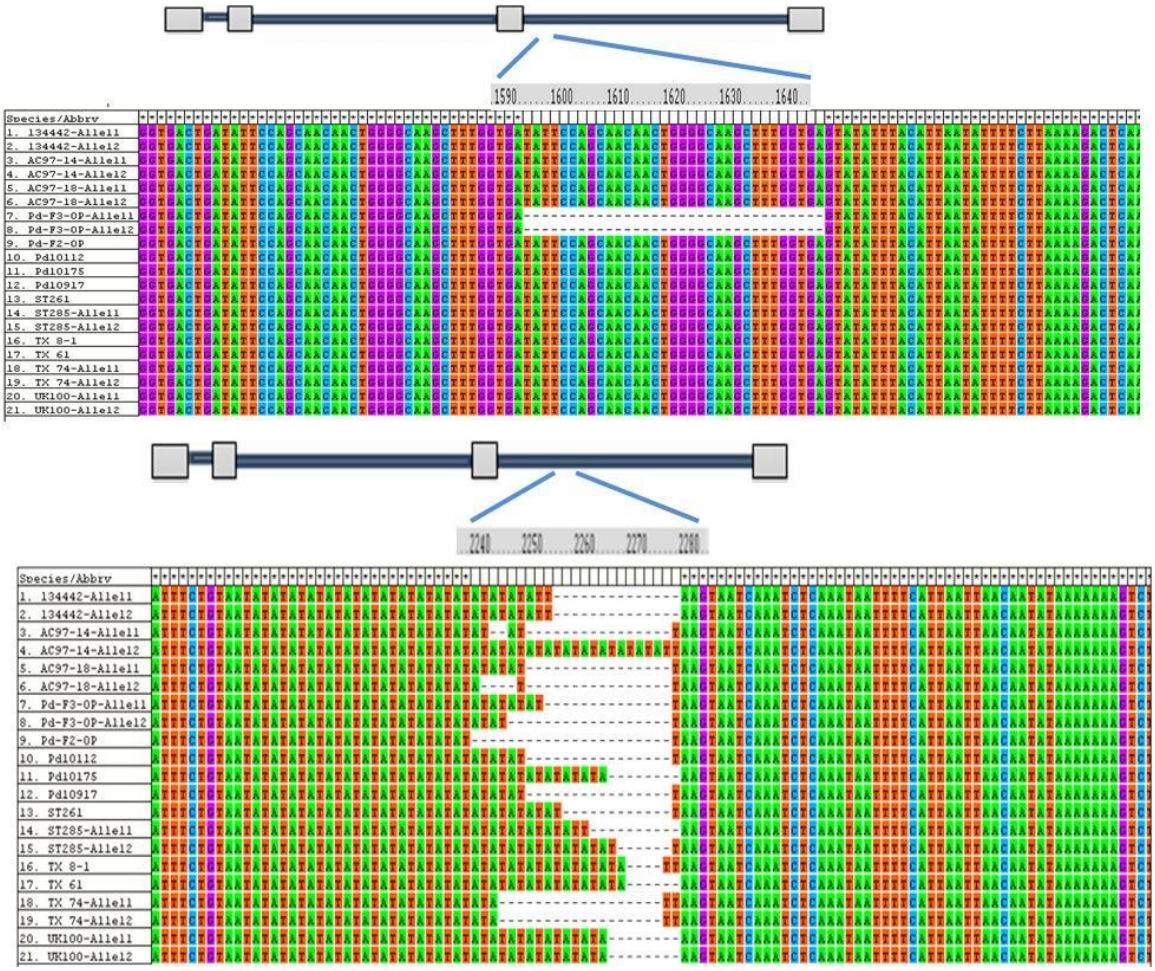


Figure 5 Indels in intron 3 with schematic structure of *FT1* gene.

Dashes show missing nucleotides.

Phylogenetic analysis

I hypothesized that there would be a clear separation of early and late reproducing genotypes based on polymorphisms in the introns of *FT1*. Although phylogenetic analysis showed three clusters, late and early reproducing genotypes were intermixed throughout the tree (Figure 6). This observation suggests the lack of relationship between SNPs/indels and phenotypes in this study. This is a primary reason the promoter region of

FT1 should also be resequenced, since the transcription factor binding sites may have polymorphisms affecting the timing of reproductive onset.

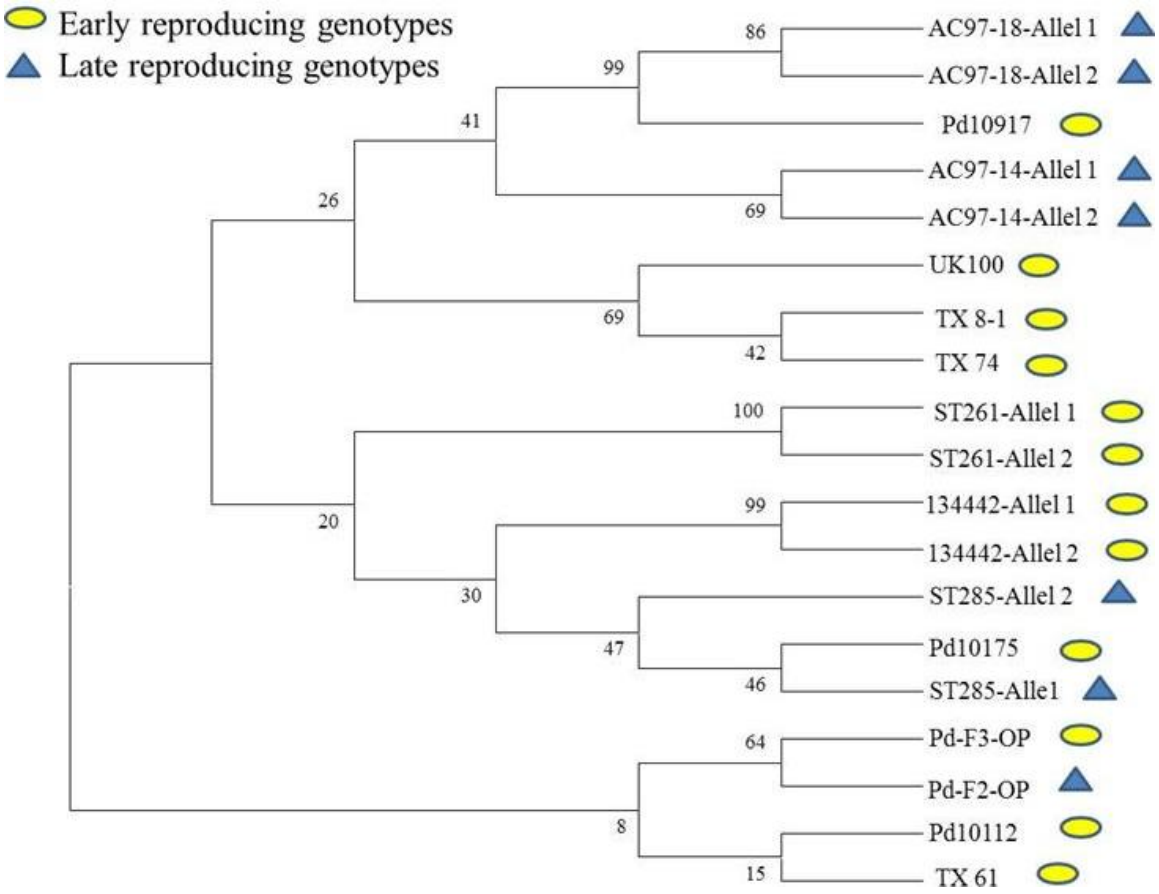


Figure 6 Clustering early and late reproducing genotypes based on polymorphisms in intron of *FT1*.

The phylogenetic tree shows three clusters of *P. deltoides* genotypes. Bootstrap percentages (n=1000) are located at nodes.

A putative *cis*-acting element located in a polymorphic region of *FT1*

A putative *cis*-acting element (CAACA) that was deleted in the *FT1*allele in intron 3 was located in the Pd-F3-OP genotype upon comparison with previous *cis*-acting element studies (Figure 7). In this genotype, there is a 40-nt deletion in this region. The

function of this element is to bind the *AP2* domain in RAV1 protein (Kagaya et al., 1999). The *AP2* domain plays an important role in the regulation of reproductive pathways (Okamuro et al., 1997).

The *AP2* domain is located in many proteins. One of the proteins is SHN, a member of the plant superfamily of *AP2* transcription factors that is involved in flower development, or a mediator in the plant responses to various environmental stresses (Okamuro et al., 1997). This element potentially plays a similar role in poplar and may show an effect on reproduction, but this needs to be studied functionally.

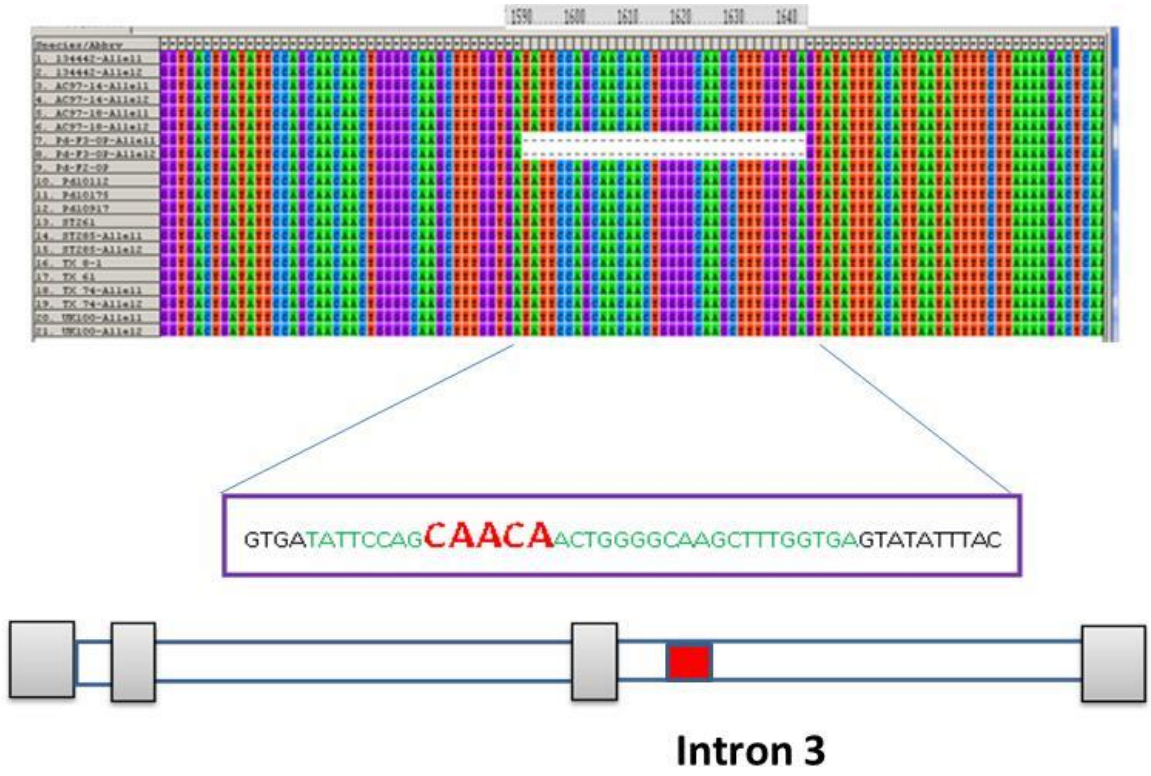


Figure 7 A putative *cis*-acting element that was deleted in the *FT1* allele in the Pd-F3-OP genotype.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Sequence variation in the *FT1* locus was identified in a small population of *P. deltoides* with varying phenotypes in reproductive onset. After the sequence analysis, 12 SNPs and 4 indels were located in non-coding regions (introns). However, phylogenetic analysis did not provide evidence for a link between the polymorphisms and the phenotypic differences with respect to reproductive onset among the 14 genotypes. Although a putative *cis*-acting element was located in an indel in one of the genotypes, its functions needs to be more fully studied to understand how it might affect reproductive onset in poplar. Consequently, further DNA sequencing of the same genotypes on the promoter region of *FT1* is suggested. Once this has been completed, using the identified polymorphisms in the *FT1* locus, a larger population of *P. deltoides* with known phenotypic differences needs to be genotyped and an association study conducted to statistically assess the connection between polymorphisms in the *FT1* locus and phenotypic variations in reproductive onset.

Adaptation is an important trait for the study of allelic variation. Genetic variation and fitness are the basis for the ability of forest tree populations to adapt to different environments (Krutovsky and Neale, 2005). Trees are always challenged by dynamic environmental conditions during their lifetime, thus, adaptive genetic variation in relevant genes and phenotypic plasticity are essential for their long-term adaptation to stressful

conditions. The timing of reproductive onset is an important adaptive trait and is controlled primarily by the *FT1* locus in poplar. Sequence variation in this locus needs to be more carefully studies in order to understand diversity in reproductive onset within populations. This study provides a beginning to the understanding of this important issue.

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