Identification and analysis of the SET-domain gene ATX4 and its interacting proteins in *Arabidopsis thaliana*.

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**Thesis for the Degree of Master of Science.**

Programme for Molecular Genetics, Department of Molecular Biosciences, University of Oslo, December 2005
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Abstract.

Chromatin modulating proteins are thought to be involved in multimeric protein-protein interactions and contain characteristic amino-acid motifs like the SET domain. The SET domain proteins are believed to regulate gene expression and chromatin structure via lysine histone methyltransferase (HMT) activity. SET domain proteins can generally be divided into activators (H3K4 methyltransferases) and repressors (H3K9 methyltransferases). These proteins often reside and function in protein complexes.

The *Arabidopsis thaliana* protein ATX4 belongs to the trithorax subgroup of SET-domain proteins. ATX4 shows sequence similarity with other H3K4 methyltransferases and is probably a positive regulator of homeotic genes during plant development.

This thesis comprises expressional and functional analysis of the actively transcribed SET-domain encoding gene *ATX4*.

Expression pattern was analysed by promoter::GUS fusion constructs and bioinformatics. Promoter::GUS analysis indicates expression in vascular tissue, pollen and the papilla of the stigma of *Arabidopsis thaliana*.

Yeast two-hybrid screens provided new putative interacting partners for ATX4. In addition deletion mapping shows that the interacting partners bind specific parts of the ATX4 protein. GST pull-down has been performed to confirm one of these reactions.

T-DNA insertion lines for ATX4 show an early flowering phenotype. *ATX4* mutant plants shoots earlier and grow faster than wild type plants. This may indicate a regulatory role for ATX4 on flowering time as a putative activator of Flowering Locus C (FLC).
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACF1</td>
<td>ATP-utilizing chromatin assembly and remodelling factor 1</td>
</tr>
<tr>
<td>ADE1</td>
<td>Adenine gene 1</td>
</tr>
<tr>
<td>AF10</td>
<td>ALL1 fused gene from chromosome 10</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>API</td>
<td>Apetala 1</td>
</tr>
<tr>
<td>ASH1</td>
<td>Absent small or homeotic 1</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine trisphosphate</td>
</tr>
<tr>
<td>ATX</td>
<td>Arabidopsis Trithorax</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BRM</td>
<td>Brahma</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>ccdB</td>
<td>Controller of cell division or death B</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Chromodomain</td>
<td>Chromatin organization modifier domain.</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CO</td>
<td>Constans</td>
</tr>
<tr>
<td>C-SAC</td>
<td>C terminal SET Associated Cysteine rich region</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EFS</td>
<td>Early flowering in short days</td>
</tr>
<tr>
<td>ELF</td>
<td>Early flowering</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FLC</td>
<td>Flowering locus C</td>
</tr>
<tr>
<td>FLD</td>
<td>Flowering locus D</td>
</tr>
<tr>
<td>FLK</td>
<td>Flowering locus K</td>
</tr>
<tr>
<td>FRI</td>
<td>Frigida</td>
</tr>
<tr>
<td>GA</td>
<td>Gibberllin acid</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HIS3</td>
<td>Histidine gene 3</td>
</tr>
<tr>
<td>HKMT</td>
<td>Histone lysine (K) methyltransferase</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Km</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>KYP</td>
<td>Kryptonite</td>
</tr>
<tr>
<td>lacZ</td>
<td>Gene encoding β-galactosidase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LD</td>
<td>Luminidependens</td>
</tr>
<tr>
<td>LiAc</td>
<td>Lithium acetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LSD1</td>
<td>Lysine specific demethylase 1</td>
</tr>
<tr>
<td>MADS</td>
<td>Mcm1, Agamous, Deficiens, Srf</td>
</tr>
<tr>
<td>MEL1</td>
<td>Gene encoding α-galactosidase</td>
</tr>
<tr>
<td>nptII</td>
<td>Neomycin phosphotransferase II</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb Group</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCR1</td>
<td>Polycomb repressive complex 1</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEV</td>
<td>Position Effect Variegation</td>
</tr>
<tr>
<td>PHD</td>
<td>Plant Homeodomain</td>
</tr>
<tr>
<td>PIE1</td>
<td>Photoperiod independent early flowering 1</td>
</tr>
<tr>
<td>PRMT</td>
<td>Protein arginine (R) methyltransferase</td>
</tr>
<tr>
<td>PWWP</td>
<td>Proline (P) – tryptophan (W) – tryptophan (W) – proline (P)</td>
</tr>
<tr>
<td>QDO</td>
<td>Quadruple dropout</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio immuno precipitation assay</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>SD</td>
<td>Synthetic dropout</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SET</td>
<td>Suppressor of variegation, Enhancer of zeste, Trithorax</td>
</tr>
<tr>
<td>SOC (medium)</td>
<td>Derived from SOB (Super Optimal Broth); the “B” in SOB is changed to “C”, for catabolite repression, reflective of added glucose.</td>
</tr>
<tr>
<td>SOC1</td>
<td>Suppressor of overexpression of constans 1</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>Switch/ Sucrose non-fermenter</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer-DNA</td>
</tr>
<tr>
<td>TE</td>
<td>Tracheary elements</td>
</tr>
<tr>
<td>trxG</td>
<td>Trithorax</td>
</tr>
<tr>
<td>VIN</td>
<td>Vernalization insensitive</td>
</tr>
<tr>
<td>VIP</td>
<td>Vernalization independent</td>
</tr>
<tr>
<td>VRN</td>
<td>Vernalization</td>
</tr>
<tr>
<td>XCP2</td>
<td>Xylem specific cysteine protease-2</td>
</tr>
</tbody>
</table>
# Table of contents

1. INTRODUCTION........................................................................................................................................1

1.1 THE MODEL PLANT ARABIDOPSIS THALIANA........................................................................................1

1.2 CHROMATIN AND HISTONE MODIFICATIONS. ..................................................................................2
1.2.1 CHROMATIN STRUCTURE. .............................................................................................................2
1.2.2 HISTONE MODIFICATIONS. ...........................................................................................................3
1.2.3 THE HISTONE CODE HYPOTHESIS. .............................................................................................5

1.3 SET-DOMAIN PROTEINS.....................................................................................................................7
1.3.1 THE SET DOMAIN. ............................................................................................................................7
1.3.2 SET-DOMAIN PROTEINS IN ARABIDOPSIS THALIANA. ...............................................................7
1.3.3 OTHER DOMAINS IN THE ATX PROTEINS. ................................................................................8

1.4 FLOWERING AND FLC. .....................................................................................................................10
1.4.1 FLOWERING......................................................................................................................................10
1.4.2 FLOWERING LOCUS C (FLC). .......................................................................................................11

1.5 AIM OF THIS PROJECT....................................................................................................................13

2. MATERIALS AND METHODS.............................................................................................................15

2.1 DNA AND RNA METHODS..............................................................................................................15
2.1.1 POLYMERASE CHAIN REACTION (PCR). ......................................................................................15
2.1.1.1 PCR GENOTYPING OF THE ATX4 INSERTION LINE SALK_060156. ........................................15
2.1.2 RESTRICTION CUTTING OF PCR PRODUCTS WITH ENDONUCLEASES. ....................................16
2.1.3 AGAROSE GEL ELECTROPHORESIS. ............................................................................................16
2.1.4 PURIFICATION OF DNA-FRAGMENTS. ............................................................................................17
2.1.5 CLONING OF PCR PRODUCTS USING THE GATEWAY CLONING TECHNOLOGY. ..................17
2.1.5.1 ATX4 DELETION CONSTRUCTS ............................................................................................19
2.1.6 ISOLATION OF DNA FROM BACTERIA CULTURE. ........................................................................19
2.1.7 ISOLATION OF DNA FROM YEAST CULTURE. ...........................................................................20
2.1.8 ISOLATION OF GENOMIC DNA FROM ARABIDOPSIS THALIANA. ...........................................20
2.1.9 ISOLATION OF mRNA FROM ARABIDOPSIS THALIANA. ............................................................20
2.1.10 SOUTHERN AND NORTHERN BLOTTING. ................................................................................21
2.1.11 PROBE LABELLING. .....................................................................................................................22
2.1.12 SOUTHERN AND NORTHERN HYBRIDIZATION. .........................................................................22
2.1.13 QUANTIFICATION OF DNA..........................................................................................................23

2.2 PROTEIN METHODS..........................................................................................................................24
2.2.1 ISOLATION OF RECOMBINANT PROTEINS. ..................................................................................24
2.2.2 IN VITRO TRANSLATION OF PROTEINS. ....................................................................................24
2.2.3 GST PULL-DOWN. ..........................................................................................................................25
2.2.4 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS, SDS-PAGE. ...27

2.3 BACTERIAL METHODS (E.COLI). ....................................................................................................29
2.3.1 GROWTH OF BACTERIA. .............................................................................................................29
2.3.2 TRANSFORMATION OF BACTERIA. ..............................................................................................29
2.3.2.1 HEAT SHOCK. ............................................................................................................................29
2.3.2.2 ELECTRO SHOCK. .....................................................................................................................29
2.3.3 SCREENING OF TRANSFORMANTS. ............................................................................................30
2.3.4 SEQUENCING. ...............................................................................................................................30

2.4 YEAST METHODS (S. CEREVISIAE). ..................................................................................................31
# Table of contents

2.4.1 GROWTH OF YEAST. ................................................................. 31  
2.4.2 TRANSFORMATION OF YEAST. ................................................. 31  
2.4.3 SELECTION AND SCREENING OF TRANSFORMANTS. ................. 31  
2.4.4 MATING OF YEAST. ................................................................. 32  
2.4.5 YEAST TWO-HYBRID. .............................................................. 32  
2.5 PLANT METHODS (ARABIDOPSIS THALIANA). ............................... 34  
2.5.1 SEED STERILIZATION AND GROWTH. ....................................... 34  
2.5.2 TRANSFORMATION OF ARABIDOPSIS THALIANA. ....................... 34  
2.5.3 SELECTION OF TRANSFORMANTS. ........................................... 34  
2.5.4 HISTOCHEMICAL GUS ANALYSIS. ............................................ 34  
2.5.5 T-DNA KNOCK-OUT LINES. ..................................................... 36  
2.6 BIOINFORMATICS. ................................................................. 37  
2.6.1 SEQUENCE ALIGNMENTS......................................................... 37  
2.6.2 BLAST SEARCHES. ................................................................. 37  
2.6.3 PAIR WISE ALIGNMENT........................................................... 37  
2.6.4 EXPRESSIONAL ANALYSIS ...................................................... 37  
2.7 STATISTICS.................................................................................. 38  
2.7.1 STUDENT’S T-TEST................................................................. 38  

3. RESULTS. .................................................................................... 39  
3.1 PART A: EXPRESSION ANALYSIS. .............................................. 39  
3.1.1 ATX4::GUS EXPRESSION. ....................................................... 39  
3.1.2 BIOINFORMATICAL EXPRESSION ANALYSIS. ......................... 41  
3.2 PART B: FUNCTIONAL ANALYSIS............................................... 43  
3.2.1 YEAST TWO-HYBRID SCREEN FOR POSSIBLE INTERACTING PARTNERS FOR THE ATX4 PROTEIN. ................................................................. 43  
3.2.2 SECOND YEAST TWO-HYBRID SCREEN FOR POSSIBLE INTERACTING PARTNERS FOR THE ATX4 PROTEIN. ......................................................... 44  
3.2.3 YEAST TWO-HYBRID DELETION MAPPING .................................. 46  
3.2.4 GST PULL-DOWN TO CONFIRM YEAST TWO-HYBRID INTERACTIONS. ......................................................................................................................... 48  
3.2.5 INVESTIGATION OF PHENOTYPE FOR ATX4 KNOCKOUT LINES. ......................................................................................................................... 50  
3.2.5.1 SOUTHERN BLOT................................................................. 51  
3.2.5.2 PCR GENOTYPING. ............................................................. 51  
3.2.5.3 EARLY FLOWERING HYPOTHESIS ........................................ 52  
3.2.5.4 DOCUMENTATION OF FIRST SHOOTING DAY. ......................... 52  
3.2.5.5 DOCUMENTATION OF STEM LENGTH .................................... 53  

4. DISCUSSION................................................................. 55  
4.1 THE SET-DOMAIN PROTEIN ATX4 IS A PUTATIVE HISTONE METHYLTRANSFERASE......55  
4.2 PROMOTER-GUS EXPRESSION PATTERN. ...................................... 56  
4.3 INTERACTING PARTNERS FOR ATX4. .......................................... 58  
4.4 ATX4 MUTANT PLANTS SHOW AN EARLY FLOWERING PHENOTYPE. ...................... 61  
4.5 CONCLUSION AND FUTURE ASPECTS. ........................................... 62  

5. REFERENCES. ................................................................. 65
Table of contents

6. APPENDIX. ........................................................................................................................................69
1. Introduction.

1.1 The Model Plant *Arabidopsis thaliana*.

*Arabidopsis thaliana* is a small flowering plant in the mustard (*Brassicaceae*) family. For nearly a century this dicotyledonous plant has been used as a model organism for the study of genetics and molecular biology. There are many advantages of working with *Arabidopsis* as a model organism: *Arabidopsis* is small of size, which facilitates growth of vast numbers of the plant, it has a short generation time; a new generation is obtained in only five to six weeks, and the plant produces a large quantity of offspring.

The genome size of *Arabidopsis* is small compared to other higher plants. The 125Mb genome is completely sequenced and is organized into five chromosomes, containing about 25,500 genes that are categorized into a vast number of gene families (The *Arabidopsis* Genome Initiative, 2000). Access to the genome sequence provides a better foundation for studying the functionality of *Arabidopsis* genes, and it facilitates the discovery and analysis of new genes and gene families.

The ability to make transgenic plants by introduction of foreign DNA is of great importance for gene analysis. This is obtained in *Arabidopsis* by means of the bacteria *Agrobacterium tumefaciens* (Barghchi, 1995).

*Arabidopsis thaliana* was used as a model organism in the experimental work of this thesis.
1.2 Chromatin and histone modifications.

1.2.1 Chromatin structure.

The cells of eukaryotic organisms contain substantial amounts of DNA. A major challenge for these organisms is to fit their large genome into a small nucleus. To facilitate this packaging the genomic DNA is assembled into chromatin; a complex of DNA, specialized proteins called histones, and non-histone proteins (Figure 1.1).

![Figure 1.1 Multiple levels of chromatin folding.](image)

The basic repeat unit of chromatin is the nucleosome. These are arranged in a “beads on a string” fashion (10 nm fibre), which is further packaged into a structure called the 30 nm fibre. The figure is modified from (Horn and Peterson, 2002).

Nucleosomes are the fundamental units of chromatin. They constitute a helix of ~147bp of DNA wrapped 1.7 times around an octamer of core histones (Luger et al., 1997). A nucleosome is composed of two dimers of the core histones H2A and H2B, and a tetramer of the core histones H3 and H4. A linker histone called H1 associates with 10-60bp of DNA between the nucleosomes and stimulates folding from the 10nm fibre to the 30 nm fibre. The condensation degree of the 30 nm fibre determines the formation of
either heterochromatin or euchromatin. The more condensed heterochromatin is generally transcriptionally repressive, while the open euchromatin is transcriptionally active. Chromatin is a highly flexible structure that may undergo dynamic changes between repressive and permissive chromatin structures during different genetic processes. These include structural reorganizations that occur during DNA replication and during the cell cycle, coordinated gene expression, as well as DNA repair and recombinations.

Heterochromatin can be further differentiated into a constitutive and a facultative state. Constitutive heterochromatin is permanently compacted and is essentially found at centromeric and telomeric regions of chromosomes, and in regions containing transposable elements. In contrast facultative heterochromatin represents transiently condensed and silenced euchromatin. The best known examples of facultative heterochromatin are the inactivated X chromosome in female mammals, and position-effect variegation (PEV).

### 1.2.2 Histone modifications.

Each histone has a charged NH$_2$ tail that protrudes outwards from the nucleosome core. Amino acid residues on these tails are highly accessible to covalent post-translational modifications such as acetylation of lysines (K), phosphorylation of serines (S) and threonines (T), methylation of lysines (K) and arginines (R), ADP-ribosylation, ubiquitination (Figure 1.2) and more. The first three modifications have been studied quite thoroughly, but little is known about the other modifications. To elucidate the complete functions of all these modifications, further investigations need to be done (Margueron et al., 2005).
Acetylation is a well-known histone modification. Histone acetyltransferases (HATs) catalyze acetylation of amino acid residues on histone tails. This type of modification is generally linked to transcriptional activation. Acetylation of histones decreases inter-nucleosome interactions and interactions between histone tails and linker DNA, thereby allowing greater accessibility for the transcription apparatus (Khorasanizadeh, 2004). Acetylation also plays a role in the histone code. Histone deacetylases (HDACs) catalyze the removal of the acetyl group. HDACs generally function as repressors. The combined actions of these enzymes on histone tails serve as a rapid mean of switch between active and repressive states of DNA transcription (Vermaak et al., 2003).

Phosphorylation of histones is catalyzed by histone kinases. This modification of histones plays a part in cellular processes as diverse as DNA repair, activation and repression of transcription, mitosis, meiosis and apoptosis (Peterson and Laniel, 2004). De-phosphorylation is catalyzed by phosphatases.
Histone methylation is catalyzed by histone methyltransferases (HMTs). The HMTs can be categorized into two families; protein arginine methyltransferases (PRMTs) and histone lysine methyltransferases (HKMTs). Another layer of complexity is added to the methyl modification of histone tails by the fact that lysine residues can be mono-, di-, or trimethylated. Histone lysine methylation is considered to be a long-term epigenetic mark of maintaining chromatin states (Jenuwein and Allis, 2001). Even though the histone lysine demethylase LSD1 was recently discovered (Shi et al., 2004), and there may be future discoveries of other demethylases, this will not change the hypothesis that histone lysine methylation has a role in epigenetic regulation of genes (Kubicek and Jenuwein, 2004).

1.2.3 The histone code hypothesis.

The variations among histones, provided by amino acid residue modifications, extend the information coded by the genome beyond the genetic code. These modifications of histone tails may represent a form of language; thus came the rise of the histone code hypothesis (Strahl and Allis, 2000). This hypothesis proposes that the language specified by variations of histone modifications can dictate the regulatory features of a gene and that this language is read by an assortment of proteins that deciphers the code into a distinct chromatin state; either repressed or active.

The term “code” may be a misnomer concerning the histone code hypothesis, as “code” implies that a particular combination of histone marks will always direct the same biological function. In contrast, the nuclear genetic code is always the same no matter which gene is analyzed, in any cell type or tissue. In the case of histone modifications there are clear exceptions; a particular mark or set of marks can have different or even opposite biological consequences (Peterson and Laniel, 2004).

Chromatin binding enzymes contain the chromodomain or bromodomain protein modules that enable them to bind modified histone tails, these protein modules recognize methyl-
lysine and acetyl-lysine respectively. Other domains also bind modified histones, such as the PHD-finger, and probably additional yet to be discovered domains as well. Experimental data published in recent years supports the idea that the functional effect (repression or activation) of deciphering the histone code will depend (a) on the combinations of histone marks produced by the enzymes recruited to the gene (by a transcription factor or bromodomain/chromodomain interactions), and (b) also on the chromatin architecture of each gene (de la Cruz et al., 2005). The pattern of histone modifications can be differentially interpreted by cellular factors depending on the gene being studied and the cellular context (Peterson and Laniel, 2004).
1.3 SET-domain proteins.

1.3.1 The SET domain.

The SET domain is a 130-160 amino acid evolutionary conserved protein domain. Genes encoding proteins containing this domain were first found in the Polycomb Group (PcG) and in the trithorax Group (trxG) of *Drosophila melanogaster*, and they are named after their *Drosophila* members *Su (var)* (Suppressor of variegation), *E (z)* (Enhancer of zeste) and *trx* (trithorax) (Alvarez-Venegas and Avramova, 2002). The fact that the Polycomb group proteins maintain repression of homeotic genes during development, and the trithorax group preserves the activity of homeotic genes was first observed in *Drosophila*. These proteins often reside and function in protein complexes that can bind to specific chromosomal response elements. Examples of such complexes are the PcG complex PCR1 that inhibits nucleosome remodelling *in vitro* by blocking SWI/SNF activity, and the opposing trxG complex BRM that is highly related to SWI/SNF and catalyzes ATP-dependent alterations in nucleosome organization *in vitro* (Simon and Tamkun, 2002).

A wealth of publications during the last 4-5 years have demonstrated that the SET-domain proteins are able to regulate gene expression and chromatin structure by methylation of lysine residues on histone tails. The SET-domain proteins can generally be divided into activators and repressors depending on the specific lysine that is methylated and the number of methyl groups that are transferred. For example, SET-domain proteins methylating histone H3 lysine 4 are generally shown to function as activators whereas SET-domain proteins methylating histone H3 lysine 9 are generally shown to act as repressors.

1.3.2 SET-domain proteins in *Arabidopsis thaliana*.

The *Arabidopsis* genome contains at least 29 expressed genes encoding SET-domain proteins which can be divided into four classes exemplified by their *Drosophila* members SU (VAR)3-9, E(Z), TRX and ASH1. Functional histone methyltransferases have been identified in *Arabidopsis*; examples are the *Arabidopsis thaliana* KRYPTONITE (KYP) protein that has been shown to be a histone H3K9 specific methyltransferase that is
required for maintenance of DNA methylation (Jackson et al., 2002), and the ATX1 protein (Alvarez-Venegas et al., 2003). The *ARABIDOPSIS* TRITHORAX (ATX) subgroup of SET-domain proteins contains 5 genes (*ATX1-ATX5*) (Alvarez-Venegas and Avramova, 2001; Baumbusch et al., 2001). The ATX1 protein has been shown to be responsible for only a fraction of the H3K4 methylations in *Arabidopsis*, suggesting that other plant methyltransferases are involved in genome-wide H3K4 methylation (Alvarez-Venegas and Avramova, 2005). ATX1 is the only ATX protein described in detail so far, and the only ATX protein with known HMTase activity.

1.3.3 Other domains in the ATX proteins.

In addition to the SET-domain the ATX proteins also contain the PWWP domain, PHD finger, extended PHD finger and the C-terminal SET-Associated Cysteine rich (C-SAC) domain. In contrast to the rest of the SET-domain proteins, the ATX protein does not contain an N-terminal SAC domain. ATX1 and ATX2 also contain the DAST-domain (Alvarez-Venegas and Avramova, 2001).

The PWWP domain spans 70-80 amino acids and is present in proteins of nuclear origin. The designation of the domain is based on its central ‘proline-tryptophan-tryptophan-proline’ core. The PWWP domain is found in a wide variety of proteins playing a role in cell division, growth and differentiation, and it is hypothesized to function as a site for protein-protein interaction, influencing chromatin remodelling and thereby facilitating fine tuning of transcriptional processes (Stec et al., 2000; Slater et al., 2003). The PWWP domains of DNA methyltransferases Dnmt3a and Dnmt3b are capable of binding DNA, minimal and non-specific binding respectively, but it’s likely the domain has another property than that of DNA binding (Chen et al., 2004)

The PHD finger is a Cys(4)-His-Cys(3) zinc finger that is found in many regulatory proteins from plants or animals which are frequently associated with chromatin-mediated transcriptional regulation. The PHD finger of *Drosophila* protein ACF1 have been shown to bind to central domains of core histones (Eberharter et al., 2004)
The function of the extended PHD finger (ePHD) is unclear, but the presence of the ePHD in proteins involved in chromatin-mediated gene expression makes it plausible that this domain plays an important role in chromatin remodelling/histone modifications. The ePHD finger of AF10 has been shown to be a protein-protein interaction domain that mediates oligomerization (Linder et al., 2000).

The C-SAC domain is a group of three C-terminal cysteines in the pattern CXC(X)_4C after the SET domain (Baumbusch et al., 2001). The SET-domain may require adjacent cysteine-rich regions to confer histone methyltransferase activity (Rea et al., 2000).
1.4 Flowering and FLC.

1.4.1 Flowering.

For plants to gain maximal reproductive success they have evolved intricate mechanisms to ensure that flowering occurs under favourable conditions. Many genes coordinate flowering time with environmental variables and with the developmental state of the plant. Typical environmental variables are temperature, nutrient availability, day length, etc. The timing of floral initiation is critical for reproductive success, and many plant species have evolved multiple pathways to regulate flowering time. These pathways include the photoperiod pathway and the vernalization pathway, which depend on environmental cues, an the autonomous pathway and the gibberllin (GA) pathway which are relatively independent of environmental changes (Michaels and Amasino, 1999; Ratcliffe et al., 2001; Noh and Amasino, 2003).

In the photoperiod pathway flowering is promoted in response to day length, long days promote the transition of the shoot apical meristem from vegetative growth to flowering in Arabidopsis. Short days delay this transition (Noh and Amasino, 2003).

The exposure of a germinating seed to an enduring period of low temperature is known as vernalization. Through a vernalization response, Arabidopsis ecotypes from northern latitudes are adapted to flower in the spring following exposure to cold winter conditions. This inhibits flowering in the late summer when seed maturation might be short-lived by the onset of fall and winter conditions. When these ecotypes are grown in the laboratory they flower late, but they flower much earlier if subjected to a period of low temperature for 4 to 8 weeks while the seed is germinating (Ratcliffe et al., 2001; Rouse et al., 2002).

The autonomous pathway may coordinate flowering in respect to the developmental state of the plant, and the GA pathway mediate flowering in response to gibberllin (Noh and Amasino, 2003).
1.4.2 Flowering Locus C (FLC).

The FLC gene encodes a MADS-box transcription factor which acts as an inhibitor of flowering and is a convergence point for numerous pathways that regulate flowering time in Arabidopsis. The MADS domain-containing proteins comprise a large family of transcription factors in plants. In Arabidopsis more than 28 family members have been identified, and they seem to play several roles in development of the plant (Michaels and Amasino, 1999, 2001).

The late flowering phenotype of winter-annual strains of Arabidopsis is created by the interaction of the two genes FLC and FRIGIDA (FRI). The FLC gene is only expressed to high levels in the presence of FRI, which encodes a plant-specific gene with unknown biochemical activity. Vernalization promotes flowering in winter-annual strains of Arabidopsis by causing a down-regulation of FLC (Michaels et al., 2003). Methylation of histone tails on nucleosomes associated with the FLC gene enables the plant to “remember” that it has been exposed to a cold-period. FLC regulation is a convergence point for the control of flowering by vernalization, FRI and the autonomous pathway (He and Amasino, 2005).
Introduction

Figure 1.3 Pathways regulating flowering time in Arabidopsis.

Vernalization and the autonomous pathway repress FLC expression. The PAF1 complex mediates H3K4 methylation that activates FLC expression. The figure is modified from (He and Amasino, 2005).

Vernalization and the proteins of the autonomous pathway repress the expression of FLC. Upregulation of FLC is done by FRI and FRL1. FLC blocks floral transition in part by repressing the expression of SOC1 and FT. In contrast the photoperiod pathway promotes expression of these flowering-time integrators. Expression of SOC1 and FT leads to expression of LEAFY and AP1 respectively which induces flowering (Figure 1.3).

A plant model of how chromatin-modifying systems have developed as substantial components in the control of a major developmental switch, such as the transition to flowering, is contributed by the regulation of the FLC locus. Recent studies of FLC regulation provide perhaps the best-studied example in plants of how changes in the chromatin environment are used to modify a developmental program (He and Amasino, 2005).
1.5 Aim of this project.

This study is part of a bigger project with the goal to identify genes encoding putative chromatin modifying SET-domain proteins in the *Arabidopsis* genome, and to do functional analysis of these proteins. The complete sequencing of the *Arabidopsis* genome made it possible to do a whole genome search for genes encoding proteins showing similarity to SET-domain proteins with known functions.

The aim of this project was:

- to identify new putative interacting partners for the ATX4 protein.
- to investigate what domains of ATX4 interact with already identified interacting proteins.
- to investigate the expression pattern of *ATX4*.
- to investigate analyze the *in vivo* function of ATX4 by investigating *Arabidopsis* lines homozygous for a T-DNA insertion in the *atx4* gene.
2. Materials and methods.

2.1 DNA and RNA methods.

2.1.1 Polymerase Chain Reaction (PCR).

(Mullins, 1987; Sambrook, 2001)

PCR was applied for amplification of desired DNA fragments for cloning, screening of bacterial colonies after transformation and genotyping of T-DNA mutants. The standard set-up for PCR was 1 X reaction buffer, 200 µM dNTP, 0.2 µM primers, 0.5-1 U DNA polymerase plus a desired amount of template.

Dynazyme (Finnzymes) is a standard thermo stable DNA polymerase for screening and genotyping. For cloning the use of Advantage® 2 polymerase mix (BD), proofreading enzyme Pfu (Fermentas) and Platinum Taq HiFi was applied. The Advantage mix contains Taq DNA polymerase, TaqStart™ Antibody and small amount of proofreading enzymes. These properties make it suitable for PCR-amplification for cloning because of high accuracy of amplification. Pfu DNA polymerase possesses 3’→5’ exonuclease proofreading activity that enables the polymerase to correct nucleotide-misincorporation errors. The Platinum Taq HiFi polymerase was used for amplification of long fragments. Both Dynazyme and Advantage polymerases give a 3’dATP overhang. Pfu-generated PCR fragments are blunt-ended.

Reactions were run in accordance with manufacturer’s recommendations and PCR products were analyzed by agarose gel electrophoresis.

2.1.1.1 PCR genotyping of the ATX4 insertion line SALK_060156.

Offspring from the T-DNA insertion line SALK_060156 that contains an insertion at position 2167 in the ATX4 gene were PCR genotyped to be sure that they were homozygous (Figure 2.1).
Materials and methods

Figure 2.1 PCR genotyping of the ATX4 T-DNA insertion line 060156

The T-DNA inserted in the ATX4 gene is 5kb long. Primers shown are the primers used for genotyping. In case of a homozygous line reaction 1 will give no results, and reaction 2 will give a product of 235bp. If the line is heterozygous reaction 1 will give a product of 284bp, and reaction 2 will give a product of 235bp. If there is no insert reaction 1 will give a product of 284bp, and reaction two will give no product.

In reaction 1 primers used were left primer 060156L, and right primer 060156R. For reaction 2 primers used were left primer 060156L, and right primer LBa1. The PCR reactions were run with a synthesis step of 30 seconds, which is not enough time for the enzyme (Dynazyme) to synthesize the entire 5kb insert. The PCR reactions were run on an agarose gel electrophoresis (Figure 3.8).

2.1.2 Restriction cutting of PCR products with endonucleases.

Restriction of PCR products was done with various restriction endonucleases (BamHI, XbaI, Alu1) and was performed in accordance with manufacturer’s recommendations.

2.1.3 Agarose gel electrophoresis.

Agarose gel electrophoresis was used to separate, identify and purify DNA fragments according to size. For analysis of fragments post PCR or cutting, gels of 0.8%-1% agarose (NuSieve® 3:1 Agarose, Cambrex BioScience) were used. Samples were loaded with 1/6 volume of loading buffer (FBX) and run at 100 V in 1X TAE buffer (40 mM}
Materials and methods

Tris-acetate, 1 mM EDTA) for 30-60 minutes. For visualization of DNA fragments the gel was added 0.6µg/ml Ethidium bromide (EtBr). EtBr intercalates with DNA and emits fluorescent light when exposed to UV radiation (Sharp et al., 1973) The GeneRuler 1kb ladder (Fermentas) was used as a molecular marker to determine fragment sizes.

2.1.4 Purification of DNA-fragments.

DNA fragments were separated on agarose gels and purified using the Wizard SV GEL and PCR Clean-Up System. Gel slices containing DNA were melted and applied on columns containing a DNA binding silica membrane. After washing, the purified DNA was eluted in nuclease free water.

2.1.5 Cloning of PCR products using the Gateway Cloning Technology.

The Gateway Technology (Invitrogen) is a universal cloning method based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989). It provides a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression (Hartley et al., 2000) Lambda recombination occurs between site-specific attachment (att) sites; attB and attP on the \textit{E.coli} chromosome and on the lambda chromosome respectively. The att sites serve as binding sites for recombination proteins. The recombination occurs between the attB and attP sites to give rise to attL and attR sites. Most Gateway vectors contain two att sites flanking a cassette containing the ccdB gene for negative selection, and a Chloramphenicol resistance gene for counter-selection (Figure 2.1). The CcdB protein inhibits growth of \textit{E.coli} strains such as DH5\(\alpha\). During the BP or the LR reaction this cassette is replaced by the gene of interest to create the respective entry clone or expression clone. Cells that take up un-recombined vectors carrying the ccdB gene will fail to grow.
The fragment of interest was amplified by PCR with primers that contain modified attB site sequences. The attB sites contain mutations to ensure specificity of the reaction, elimination of stop codons and minimization of secondary structures when single stranded. The attB flanked PCR product was purified and recombined into a donor vector to give rise to an entry clone. In a BP reaction the donor vector contains the attP site found in bacteriophage lambda, with the same modifications as the attB sites. This defines the direction of the cloning (attB₁ only reacts with attP₁, and attB₂ only reacts with attP₂). 150 ng donor vector was mixed with about 100 fmol PCR product, 1X BP reaction buffer, and BP Clonase enzyme mix (containing Integrase (INT) encoded by bacteriophage lambda, and Integration Host Factor (IHF) encoded by _E.coli_.)

The resulting entry clone was recombined into a destination vector. 150 ng of entry clone was mixed with 150 ng of destination vector, 1 X LR reaction buffer, LR Clonase
enzyme mix containing INT and Exitionase (Xis) encoded by bacteriophage lambda, and IHF.

All recombinations were done at 25°C for at least one hour. For deactivation of the Clonase enzymes Proteinase K was added, and the sample was incubated at 37°C for 15 minutes. After each recombination step 2 µl of the recombination mix was used for transformation into a gyrA+ E.coli strain (i.e. DH5α). Transformants were selected on media containing the appropriate antibiotics. Cells that do not contain the donor vector will die as a result of the antibiotic in the plate medium; cells containing an unrecombined donor vector will die because the ccdB gene is still present in the donor vector.

2.1.5.1 ATX4 deletion constructs

Three deletion constructs of ATX4 were made using the Gateway Cloning Technology to investigate which parts of the ATX4 protein that interacts with already known interactants. The three fragments of ATX4 were made by running PCRs with the following primers: ATX4new GAW cdsL and AX4 GW 1387R for ATX4-1387, ATX4new GAW cdsL and AX4 GW 2395R for ATX4-2395, and ATX4SACSETGAW-L and ATXRSACSET4GAW-R for ATX4-SACSET. These fragments were recombined into the pDONR/Zeo vector. The pDONR/Zeo-ATX4-1387 and the pDONR/Zeo-ATX4-2395 consist of 1387 bp and 2395 bp of the 5´ of the ATX4 cds respectively. The pDONR207-ATX4-SACSET constitutes ~1100 bp of the ATX4 3´ end. All the constructs were recombined into the vector pGBK7-GW creating the bait vectors pGBK7/-ATX4-1387, pGBK7/-ATX4-2395 and pGBK7/-ATX4-SACSET. The constructs were sequenced using BD primers.

2.1.6 Isolation of DNA from bacteria culture.

Plasmid DNA purification was done in accordance with the Wizard® Plus SV Minipreps DNA Purification System (Promega). Cells from bacteria cultures were harvested by aliquoting 1.5 ml of culture in an Eppendorf tube followed by centrifugation at max speed for 20 seconds. This step was done two times. After neutralization, the lysate containing the plasmids was transferred to a column containing a DNA binding silica membrane.
Materials and methods

After washing, the plasmid was eluted in nuclease free H$_2$O. The DNA was stored at $\div$20°C.

2.1.7 Isolation of DNA from yeast culture.

Cells from yeast cultures were harvested by aliquoting 1.5 ml of culture in an Eppendorf tube followed by centrifugation at max speed for 20 seconds. The cells were then resuspended in 500µl lysis buffer [50 mM tris-HCL pH 7.5; 10 mM EDTA; 0.5% β-mercaptoethanol; 50 U/ml Lyticase; 80 µg/ml RNase A] and incubated at 37°C for 45 minutes. After incubation the cells were added 55 µl 10% SDS and 3 µl 10 mg/ml Proteinase K and incubated at 56°C for one hour. After lysis the plasmids were extracted by adding 600 µl of Phenol: Chloroform: Isoamylalcohol (25:24:1) and vortexed. The tubes were then centrifuged at maximum speed for 1 minute. The resulting supernatant was transferred to a new Eppendorf tube. 3M NaAc (pH 5.2) was then added in an amount of 1/10 of the volume of the supernatant. 1mL cold 96% ethanol was also added. The tube was incubated at $\div$80°C for 15 minutes. After incubation the tube was centrifuged at 13000 rpm for 10 minutes, the supernatant was removed and the precipitate was washed in 500 µl of 70% ethanol. After washing the DNA was dried at room temperature for 10 minutes, and resuspended in nuclease free H$_2$O. The DNA was stored at $\div$20°C.

2.1.8 Isolation of genomic DNA from Arabidopsis thaliana.

200 mg of rosette leaves was harvested and frozen in liquid N$_2$. The DNA isolation was done by using the BIO-RAD AquaPure Genomic DNA Isolation Kit (Biorad), scaled for 200 mg of plant tissue. The procedure was done in accordance with the Quantum Prep AquaPure Genomic DNA Isolation Kit (Biorad). After hydration the DNA stored at $\div$20°C.

2.1.9 Isolation of mRNA from Arabidopsis thaliana.

500 mg of plant tissue (seedlings, rosette leaves and flowers) was harvested and frozen in liquid N$_2$. The mRNA isolation was done by using the RNeasy® Plant Mini Kit (Qiagen) (for seedlings and flowers) and the RNeasy® Midi Kit (Qiagen) (for rosette leaves). The
Materials and methods

procedure was done in accordance with manufacturers recommendations with emphasis on working in an RNase-free environment.

2.1.10 Southern and Northern Blotting.

(Southern, 1975)
Southern blotting was used to estimate the number of T-DNA insertions the *Arabidopsis thaliana* T-DNA insertion line SALK_060156. Northern Blotting was used to confirm knock out of the ATX4 gene, and to check for a possible reduced FLC level, in the mentioned T-DNA insertion line.

For Southern blotting genomic DNA was isolated, digested with restriction enzyme HindIII and size-fractionated on a normal agarose gel (1% agarose, 1X TAE Buffer) and run at 80V for 3-4 hours. After electrophoresis the gel was soaked in denaturation solution (0.5M NaOH; 0.5M NaCl) with gentle shaking for 2 x 20 minutes, rinsed in sdH2O, soaked in neutralization solution (0.5M Tris pH7.5; 1M NaCl) with gentle shaking for 2x 20 minutes and rinsed in 10X SSC.

A 50 ml Northern gel was made by mixing 0.85 g agarose, 10 ml 5X RB (Running Buffer) and 32 ml of DEPC-H2O. This mix was boiled until all the agarose was completely dissolved. After the gel mix had cooled to approximately 70°C, 8 ml of 37 % formaldehyde was added. Prior to loading the samples were heated to 65°C for 5 minutes, and cooled on ice. The gel was run for approximately 4 hours at 80 V in Northern gel buffer (50 ml %X RB; 40 ml 37 % formaldehyde; 60 ml DEPC-H2O). After the size separation of total RNA gel was turned upside down on a glass plate that had been rinsed in 70 % EtOH. Liquid was removed from the gel using a glass rod.

Capillary transfer of the DNA or RNA to a Hybond N+ nylon membrane (Amersham Bioscience) was done over night, followed by baking of the blot at 60°C for 15 minutes and UV cross-linking of 700 kJ (Hoefer® Scientific Instruments) for covalent binding of the DNA or RNA to the membrane.
2.1.11 Probe labelling.

*NptII (neomycin phosphotransferase II)* probe template was generated by PCR on 2 ng of the pKOH (PROH) vector with primers nptII 3’ and nptII 5’, run on an agarose gel, isolated and used as a probe for Southern Blot. *ATX4* probe template was generated by PCR on 5 ng of the pGEM-T-ATX4-full vector with the primers HR and ATX41054 5’RACE, digested with restriction enzyme HindIII, run on an agarose gel. The resulting 820 bp band was isolated and used as a probe for Northern Blot. FLC probe template was generated by PCR on 500 ng of the pCR2.1-TOPO vector with FLC cDNA insert, with FLC-primers FLC-3’ and FLC-5’, run on an agarose gel, isolated and used as a probe on a Northern Blot to check for a possible reduced FLC-level in the *Arabidopsis thaliana* T-DNA insertion line SALK_060156. The probes were labelled according to the Rediprime II Random Priming Labelling System protocol (Amersham BioScience). The probe was radioactively labelled with [α-32P-dCTP].

2.1.12 Southern and Northern Hybridization.

(Galu and Hughes, 1986)

The Hybond N+ nylon membrane from the blotting was wetted in wetting solution (0.7 X SSC; 1 X SPEP) for 30 minutes. The membrane was then prehybridized for 90 minutes in prehybridization solution (0.7 X SSC; 1 X SPEP; 5 X Denhardts; 100 µg/ml heterologous DNA). The probe was added to the hybridization solution (0.7 X SSC; 1 X SPEP; 1 X Denhardts; 10% Dextran sulphate; 100 µg/ml heterologous DNA), boiled for 10 minutes, and then added to the membrane for hybridization over night. Prehybridization, hybridization and washing were done at 68°C. After hybridization the membrane was washed for 30 minutes in pre-warmed wash solution (0.7 X SSC; 1 X SPEP; 1 X Denhardts).

After wash the membrane was wrapped in plastic and exposed to autoradiographic film (Kodak) at ±80°C for an amount of time depending on the intensity of the signal. An intensifying screen was placed behind the film to increase efficiency.
For removal of the probe the membrane was soaked in heated stripping solution (0.1% SDS; 1 mM EDTA pH 8.0) and left on a shaker for 30 minutes. This step was repeated up to four times until the signal disappeared. After stripping the membrane was stored in plastic film at 4°C.

2.1.13 Quantification of DNA.

Quantification of DNA samples was done with Hoefer® DyNAquant 200 fluorometer (Pharmacia Biotech) as described by the manufacturer.

Quantification of DNA was also done on agarose gel containing EtBr. EtBr intercalates with DNA and fluorescence in UV-light proportional to the total amount of DNA. The amount of DNA in a band on the gel can be calculated by comparing the intensity of the band with a standard of known amount and intensity.
2.2 Protein methods.

2.2.1 Isolation of recombinant proteins.

BL. 21 cells (Amersham) were used for expression of GST-ATX4 fusion proteins. These cells were transformed with IPTG (isopropyl-beta-D-thiogalactopyranoside) inducible pGEX-AB-GAW expression clones containing in-frame fusions between GST and different parts of ATX4 (see section 2.1.5 for description of ATX4 deletion constructs). Cells were transformed with the appropriate pGEX clone and selected on LA-amp plates. Colonies were cultured over night in 50 ml LB-amp. The next day 25 ml of the cultures were diluted in 125 ml LB-amp and IPTG was added to a concentration of 125 μM. The cultures were induced at 150 rpm for 1 h 45 min at 30°C. After being induced the cultures where centrifuged at 5100 rpm for 25 min in a Beckman Coulter™ TJ-25 centrifuge. The supernatant was removed and the pellet was washed in 10 ml 50 mM Tris. After washing the cells were resuspended in 1 X PBS with lysozyme (0.25 mg/ml) and one Complete EDTA-free Protease inhibitor cocktail tablet (Roche Diagnostics), and incubated at RT for 30 minutes. After incubation the cells were frozen in liquid N₂ and thawed in running tap water. The cultures were then sonicated for 3 X 10 seconds (50 %; 0.5 cycles). After sonication the denatured cells were transferred to autoclaved centrifugation tubes and centrifuged at 14.000 rpm for 25 min. The supernatant containing the proteins was transferred to new 15 ml tubes and stored at - 80°C for later use. All centrifugations were done at 4°C.

2.2.2 In vitro translation of proteins.

Prey construct from the yeast two-hybrid screen (section 2.4.5) was expressed and labelled with 35S methionine using The Rabbit TnT® System (Promega). This in vitro system combines both transcription and translation in one single tube. The procedure was done in accordance with the manufacturer’s recommendations.
2.2.3 GST pull-down.

The Glutathione S-Transferase (GST) gene fusion system is a versatile system for expression, purification and detection of fusion proteins produced in *E. coli*. In this system the ability of GST to bind glutathione is used to isolate recombinant proteins fused to GST. GST fused to the protein of interest works as a tag that is used to isolate the fusion proteins. GST fusion proteins have to be expressed in BL. 21 (Amersham) cells that are optimized for protein expression. GST pull-down is a method for testing protein interactions *in vitro*. To analyse ATX4 interactions GST-ATX4 fusions were attached to glutathione coated sepharose beads and incubated with a mixture containing radioactively labelled *in vitro* translated protein (Figure 2.3). Interacting protein will bind to the GST-fused ATX4 protein. ATX4 with bound protein is eluted from the sepharose beads and loaded on a SDS-PAGE-gel.
Materials and methods

Figure 2.3 Purification of protein complexes using a GST tagged fusion protein.
GST fusion proteins, generated by standard recombinant DNA techniques, can be bound to sepharose beads coated with glutathione. To look for proteins that bind to ATX4 these beads with GST-fusion proteins bound were mixed with precleared protein lysate. The beads with bound fusion protein and interacting proteins were washed and run on an SDS-PAGE. The figure is modified from The Molecular Biology of The Cell 4th Edition.

Before pull-down the TnT translated prey clone proteins had to be precleared, and the GST-fusion proteins had to be blocked. To reduce unspecific binding 12.5 µl of undiluted TnT translation reaction was used per pull-down and precleared by adding 50 µl of 1 X binding buffer (20 mM Hepes-KOH pH 7.6; 200 mM KCl; 2.5 mM MgCl₂; 10% glycerol; 0.05% NP-40; 0.1 mM PMSF; 1mM DTT) and 12.5 µl of 50% GST slurry (50% GST sepharose; 50% PBS) and incubated on a rotating wheel at 4°C for 1 h. After pre-clearing the tubes were centrifuged at 2000 rpm for 2 minutes in a swing out centrifuge. 6 µl (¹/₁₀) of supernatant was saved for use as input control and 60 µl of supernatant from each mix
was used for incubation with GST-fusion proteins, or GST control, and added 350 μl of 1 X binding buffer.

For blocking of GST-fusion proteins beads representing 5 μg of proteins were aliquoted into 1.5 ml Eppendorf tubes, and all samples were normalized to the same volume with 50 % GST-slurry. The beads were first washed in 500 μl 1 X binding buffer, and then blocked with 1% BSA in 500 μl 1 X binding buffer on a rotating wheel for 20 minutes. After blocking the tubes were centrifuged at 2000 rpm for 2 minutes, the supernatant was discarded and the tubes were added 400 μl precleared translation mix.

For binding the tubes were put on rotating wheel at 4°C for 2.5 hours. After binding the beads were washed two times in 1 ml 1 X binding buffer, succeeded by three washing steps with different concentrations (200 mM, 400 mM, 600 mM) of 1 ml RIPA and put on rotating wheel for 5 minutes at 4°C for each step. After wash the tubes were centrifuged and the supernatant was discarded. 30 μl 200 mM RIPA and 10 μl 4 X SDS loading buffer was added to each tube. The tubes were heated on 95°C, centrifuged and 20 μl were loaded on an SDS-PAGE gel.

2.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE.
SDS-PAGE was used to separate proteins in coherence with the GST pull-down. SDS-PAGE uses a highly cross-linked gel of polyacrylamide. The proteins are in a solution that includes the negatively charged detergent; sodium dodecyl sulphate. SDS binds to hydrophobic regions of the proteins causing them to unfold and render them freely soluble in the detergent solution. Binding of SDS causes a net negative charge for the proteins. The amount of SDS bound to the protein is almost proportional to the molecular weight of the protein and is independent of sequence. The complex will migrate through the polyacrylamide gel because of the negative charge and protein-SDS-complexes will be separated according to size. A known molecular weight marker makes it possible to estimate the molecular weight of the polypeptide chain in question. The separating gel was made by mixing 1.75 ml dH2O; 1.25 ml 1.5 M Tris-HCl pH 8.8; 50 μl 10% SDS; 2 ml acrylamide/Bis stock; 25 μl 10% APS and 2.5 μl TEMED. The stacking gel was made
by mixing 3.05 ml dH₂O; 1.25 ml 0.5 M Tris-HCl pH 6.8; 50 µl 10% SDS; 650 µl acrylamide/Bis stock; 25 µl 10% APS and 5 µl TEMED. The gel was run at 200V in 1 X Running buffer (1.8 g Tris base; 8.64 g glycine; 0.6 g SDS and dH₂O added to a total volume of 600 ml). After the electrophoresis the gel was dried and left in an exposing cassette with a phosphoimager screen for screening ON. Eventually the phosphoimager screen was scanned in a Typhoon phosphoimager.
2.3 Bacterial methods (*E.coli*).

2.3.1 Growth of bacteria.

Cultures of *E.coli* were grown over night in LB media (10 g/l Peptone; 5 g/l yeast extract; 0.17 M NaCl) with proper antibiotic selection marker on a shaker at 37°C. For selection of single colonies, bacteria was plated on LA plates (LB + 5 g/l agar) and incubated at 37°C over night.

For storage, bacteria culture was added an equal volume of 50% glycerol for a final concentration of 25%, and stored at −80°C.

2.3.2 Transformation of bacteria.

*E.coli* DH5-α (Invitrogen) and BL21 (Amersham) cells were transformed by heat shock or by electro shock (DH5-α). BL21 cells are suitable for protein expression.

2.3.2.1 Heat Shock.

50 µl cells were thawed on ice. 2 µl of recombination mix was added and the sample was incubated on ice for 10 minutes. The sample was then heat shocked for 45-50 seconds at 42°C followed by additional incubation on ice for 2 minutes. 500 µl of SOC medium (2% peptone; 0.5% yeast extract; 100 mM NaCl; 2.5 mM KCl; 10 mM MgCl₂; 10 mM MgSO₄; 20 mM glucose) was added and the sample was incubated on a shaker for one hour at 37°C. 100 µl of the sample was eventually plated on plates containing selective LA-media with appropriate antibiotic.

2.3.2.2 Electro shock.

1 µl DNA was mixed with 50 µl DH5-α cells. This mix was then pipetted into electro cuvettes followed by an electro shock in a Gene Pulser™ (Bio-Rad) with 25 µF capacitance; 2.4 kV and Pulse Controller (Bio-Rad) with resistance of 200 Ω. 1 ml of SOC medium was added and the sample was incubated on a shaker for 45 minutes at 37°C. 100 µl of the sample was then plated on plates containing selective LA-media with appropriate antibiotic.
2.3.3 Screening of transformants.

Screening of transformants was done by PCR directly on colonies, or on over-night culture. The PCR was done preferably with one vector specific primer, and one gene specific primer.

2.3.4 Sequencing.

Sequencing was done at the ABI sequencing lab facility at the Department of Molecular Biosciences, University of Oslo.
2.4 Yeast methods (*S. cerevisiae*).

### 2.4.1 Growth of yeast.

*S. cerevisiae* was grown overnight in 2X YPDA media (10 g/l yeast extract; 20 g/l peptone; 50 ml/l 40% glucose; 7.5 ml/l 0.2% adenine sulphate) on a shaker at 30°C, or plated on YPDA or various Minimal Medium plates containing SD (Synthetic Dropout) and incubated at 30°C over for 3-5 days.

Yeast cultures in YPDA media were stored at −80°C after adding an equal volume of 25% glycerol for a final concentration of 12.5%.

### 2.4.2 Transformation of yeast.

Yeast transformation was done in accordance with the MATCHMAKER Library Construction & Screening Kit User Manual (Clontech). Yeast colonies were dissolved in 1.1X TE/LiAc (TE Buffer/ lithium acetate) prior to transformation. 200 ng DNA; 5 µl Herring DNA; 50 µl yeast culture and 500 µl PEG/LiAc solution (polyethylene glycol 4000/lithium acetate) was mixed in an Eppendorf tube and vortexed for 2-3 seconds. After vortexing the tube was put on a shaker (150 rpm) at 30°C for 30 minutes. After incubation 50 µl Dimethyl Sulfoxide (DMSO, Sigma) was added and the sample was heat shocked at 42°C for 15 minutes (with mixing every 5 minutes). After heat shock the sample was left on ice for 2 minutes followed by a 10 second centrifugation at max speed. The supernatant was removed and the cells were resuspended in 1X TE.

### 2.4.3 Selection and screening of transformants.

To check if the correct construct had been transformed into the yeast cells a PCR was run directly on the yeast colony, or on DNA isolated from over-night culture.

SD medium was used for selection. SD medium lacks all amino acids except the one you want to select for. To avoid colonies with more than one construct the colonies were streaked on SD/+Leu/+Trp/X-α-Gal 2-3 times to isolate colonies with only one plasmid. A mix of white and blue colonies indicates that more than one plasmid are present; blue
Materials and methods

Colonies were chosen for further selection. If these blue colonies grow on QDO/X-α-Gal they have the correct phenotype.

DNA was isolated from over-night cultures of putative positive colonies. A PCR screen with vector specific primers for pGADT7 were run, and the PCR products were digested with the restriction enzyme AluI to find unique clones among the different colonies.

Plasmid DNA from unique clones was transformed into *E.coli* (section 2.3.2) and plated on selective medium for the library plasmid (ampicillin resistance). Plasmid DNA was isolated from the transformed *E.coli* cells and sequenced.

For retesting of the positive interaction the AH109 yeast stock was transformed with the appropriate library clones, followed by a new mating and selection. QDO selects for all four of the reporter genes, the control mating was performed to eliminate proteins with unspecific binding and proteins that interact with DNA BD.

2.4.4 Mating of yeast.

The two different yeast mating types a and α can undergo sexual propagation. E.g. an a-cell will secrete an a-factor that binds to α-cells, connecting the cells together and making diploid yeast cells. 3-4 big yeast colonies, of both mating types were each dissolved in 250 µl 2 X YPDA. 20 µl of each mating type was then mixed with 160 µl 2 X YPDA in a well on a 96-well micro titration plate. The plate was incubated on a shaker at 85 rpm at 30ºC over night. The next day 120 µl was plated on SD/-Leu/-Trp plates for selection of diploid colonies.

2.4.5 Yeast Two-Hybrid.

Yeast Two-Hybrid Screens were used to identify new putative interacting partners for the ATX4 protein and to check which parts of the ATX4 protein that interacts with already identified interactants. For Yeast Two-Hybrid screening the haploid yeast strains Y187 (mating type α) and AH109 (mating type a) were used.
In MATCHMAKER Systems (Clontech), a bait gene is expressed as a fusion to the GAL4 DNA-binding domain (DNA-BD), while another gene or cDNA is expressed as a fusion to the GAL4 activation domain (AD) (Fields and Song, 1989; Chien, 1991). When bait and library fusion proteins interact in a yeast reporter strain, the DNA-BD and AD are brought together and specifically activate transcription of four reporter genes (MEL1, lacZ, ADE2, HIS3) (Figure 2). DNA-BD and AD fusions are created by cloning cDNAs into pGBK7T7 and pGADT7-Rec respectively.

![Figure 2.4 The Two-Hybrid Principle.](image)

DNA-BD, amino acids 1-147 of the yeast GAL4 protein, binds to the GAL-UAS upstream of the reporter genes. AD, amino acids 768-881 of the GAL4 protein, functions as a transcriptional activator. Figure is modified from the MATCHMAKER Library Construction & Screening Kit manual (Clontech).

To identify new interacting proteins full length BD-ATX4 was screened against an AD-fusion library constructed from flower cDNA. After mating the cultures were plated on media selecting for diploid interactants.
2.5 Plant methods (*Arabidopsis thaliana*).

Ecotype Colombia was used for all work with *Arabidopsis thaliana*.

2.5.1 Seed sterilization and growth.

For surface sterilisation seeds were incubated for 5 minutes in 70% EtOH, 5 minutes in Bleach solution (20% Klorix; 0.1% Tween20; sdH$_2$O) and finally 5 minutes in Wash Solution (0.001% Tween20; sdH$_2$O).

Seeds were resuspended in 1.5 ml 0.1% agar and pipetted on MS-2 plates (0.5 g/l MES; 4.43 g/l Murashige & Skoog salts; 20 g/l sucrose; 5 g/l bacto agar) (Murashige and Skoog, 1962). The plates were sealed with surgical tape and stored at 4ºC for 3 days to reset the seeds. After incubation the plates were moved to the growth room. After 2-3 weeks the seedlings were transferred to soil for further growth under the following conditions; 22ºC; 16 hour day (light intensity of 100µE/m$^2$) and 8 hour night. Humidity was 60%. Seeds and plants were grown under the same conditions.

2.5.2 Transformation of *Arabidopsis thaliana*.

Transformation was done by the means of *Agrobacterium tumefaciens*. All transformations were performed by Tage Thorstensen.

2.5.3 Selection of transformants.

Selection of transgenic *Arabidopsis* was done using antibiotics. The *Arabidopsis thaliana* T-DNA insertion line SALK_060156 we used contains the *nptII* gene which gives kanamycin resistance. Addition of kanamycin (50 µg/ml) to the MS-2 plates will cause the wild type to die at the dicotyledonous stage while the transformants will continue to grow and develop.

2.5.4 Histochemical GUS analysis.

(Jefferson, 1989)

Histochemical assay of GUS activity was used to analyze gene expression by fusing the promoter region of the *ATX4* gene to the *udiA* gene encoding β-glucoronidase (GUS). The GUS enzyme hydrolyzes GlcA to a water-soluble indoxyl intermediate that is further
dimerized into a dichlorodibromo-indigo blue precipitate by an oxidative reaction. In this case the \textit{ATX4} promoter was cloned in front of a promoter less \textit{udiA} gene, the gene was then regulated by the \textit{ATX4} promoter and the GUS protein was expressed according to the expression pattern of \textit{ATX4}.

The promoter region of \textit{ATX4} was amplified by PCR using ATX4-complement-GAW L and ATX4-23 GAWpromR primers on wild-type genomic DNA as template, and recombined into the vector pPZP211G GAW using Gateway Cloning Technology (Figure 2.5).

\textbf{Figure 2.5 ATX4 promoter-GUS fusion construct.}
The promoter region of \textit{ATX4} was cloned in front of a promoter less GUS-gene, and a \textit{nos} terminator in the pPZ211G GAW vector. The vector is a Ti vector containing right and left border sequences, and the \textit{nptII}-gene for selection of transformants.
Materials and methods

The construct was transformed into *Agrobacterium* strain C58 pCV2260, and wild-type *Arabidopsis* were transformed by floral dipping in the *Agrobacterium* culture. This was done by Tage Thorstensen.

50 independent lines containing the *ATX4*:GUS constructs were screened for GUS activity. The GUS assay was performed in accordance with a modified protocol from (Grini et al., 2002).

Tissue was collected and added an X-Gluc mixture [500 mM NaPO₄; 100 mM potassium-ferrocyanide (K₄Fe(CN)₆ 3H₂O); 100 mM potassium-ferricyanide (K₃Fe(CN)₆); 10% Triton X-100; 100 mM X-Gluc (dissolved in DMF)] and incubated for 3 – 12 hours at 37°C in the dark. Following a graded EtOH dehydration series from 15% to 50% EtOH, the material was post-fixed in FAA (10:7:2:1 EtOH: distilled water acetic acid: formaldehyde (37%)) on ice for 30 min and put back on 50% EtOH. Eventually the material was mounted on microscope slides in a clearing solution of 8:2:1 (w/v/v) chloral hydrate: water: glycerol (ClH). The GUS expression was documented by taking pictures with a digital camera connected to a microscope.

2.5.5 T-DNA Knock-Out Lines.

The homozygous, single copy T-DNA insertion line SALK_060156 contains an insertion at position 2167 in the *ATX4* gene. This line was investigated for a possible early flowering phenotype. First shooting day, and the number of rosette leaves were documented for the two sister lines B5, B7 and for Colombia. The data was then run through a Student’s T Test to confirm its significance.
2.6 Bioinformatics.

2.6.1 Sequence alignments.

Sequence alignments were performed with Vector NTI Advance™ Sequence Analysis and Data Management Software (http://www.invitrogen.com).

2.6.2 Blast Searches.

Blast searches were performed against the NCBI database (http://www.ncbi.nlm.nih.gov/blast).

2.6.3 Pair wise Alignment.

Genomic ATX4 sequence was aligned with the ATX4 cDNA sequence using the EMBOSS Pair wise Alignment Algorithm (http://www.ebi.ac.uk/emboss/align/).

2.6.4 Expressional Analysis

For bioinformatical analysis of the expression pattern for ATX4 two online services was used: The Arabidopsis Gene Family Profiler with a database using 353 Affymetrix ATH1 arrays. The arrays cover various organs and tissues at several developmental stages (http://arabidopsisGFP.ueb.cas.cz.), and the Arabidopsis thaliana microarray database and analysis toolbox Genevestigator which uses a large database of Arabidopsis Affymetrix GeneChip® data. The data is generated by users or retrieved from public repositories and stored in a MySQL database (Zimmermann et al., 2004). With the Gene Family Profiler the AtGenExpress function was used with MAS5 normalization and the AGI code for ATX4 (At4g27910). With the Genevestigator the Gene Atlas function was applied with wild type ATH1:22k array, the ATX4 AGI code and linear scale type.
2.7 Statistics.

2.7.1 Student’s t-test.

(Student, 1908)

Student’s t-test is a statistical method that deals with the problems associated with inference based on "small" samples: the calculated mean, $X_{\text{avg}}$, and standard deviation may by chance deviate from the "real" mean and standard deviation (i.e., what you would measure if you had many more data items: a large sample).

The Student’s t-test was performed with the online software at http://www.physics.csbsju.edu/stats/t-test_bulk_form.html
3. Results.

3.1 Part A: Expression analysis.

3.1.1 ATX4::GUS expression.

To investigate the temporal and spatial expression pattern for the ATX4 gene, wild type plants were transformed with a construct containing ~1800 bp of the ATX4 promoter cloned in front of a promoterless gene encoding β-glucoronidase (GUS) (see section 2.5.4). The screening for transgene plants on selective media containing kanamycin identified 50 independent ATX4::GUS transformants. Seeds from these lines were further selected on kanamycin containing media to identify possible one-copy lines. We selected 6 possible one-copy lines, showing 3:1 (resistant:sensitive) distribution, for further analysis. These lines were analyzed by histochemical GUS assay for expression in the seedling stage, and for expression in various tissues of mature plants. GUS expression was observed in the vascular tissue of seedlings including the root, and in the vascular tissue of rosette leaves, sepals and petals. GUS expression was also observed in stamen filaments and in pollen (Figure 3.1).
A transient GUS expression was observed in flowers (figure 3.1.D, E, F and G). No GUS expression was observed in the first closed bud (i). In the second closed bud (ii) GUS expression was observed in pollen. In the third closed bud (iii) the GUS expression in pollen was gone; however dissection of (iii) revealed GUS expression in the vascular tissue of the stamen filaments (figure 3.1.F). This also applied for closed bud four (iv) which in addition shows GUS expression in the vascular tissue of sepals. This is better documented in figure 3.1.G where one clearly sees expression in stamens, sepals, petals and the papilla of the stigma. In the first open flower (v) GUS expression was observed in the vascular tissue of petals. The second open flower (iv) showed a total loss of GUS
expression. This expression pattern was observed in all the one-copy lines we investigated. All of the flower-states in Figure 3.1 D were harvested from the same plant, at the same time.

Before X-Gluc incubation the organs were separated from the plant by using a scissor and tweezers, this separation generated cut zones in which we observed GUS expression after incubation in X-Gluc. We suspected this to be a possible stress induced expression of \textit{ATX4}. To test this hypothesis rosette leaves were sliced with a scalpel before incubation in X-Gluc mixture. No significant amount of GUS expression was visible in the cut zones (Figure 3.1 B). This suggests that slicing of rosette leaves with a scalpel does not induce expression of \textit{ATX4} in the cut zones.

\textbf{3.1.2 Bioinformatical Expression Analysis.}

Results from the \textit{Arabidopsis} Gene Family Profiler (http://arabidopsisGFP.ueb.cas.cz/) and the Genevestigator (Zimmermann et al., 2004) support the \textit{ATX4}::GUS expression results. The Gene Family Profiler shows \textit{ATX4} expression in all tissue, except mature pollen, it also shows the same transient \textit{ATX4} expression in flowers (Figure 3.2). The Genevestigator also shows \textit{ATX4} expression in all tissue, with the strongest expression being in pollen, stamens and petals (data not shown).
Results

Figure 3.2 ATX4 expression pattern.
The Arabidopsis Gene Family Profiler shows ATX4 expression in all tissue except mature pollen. The strongest expression is in young flowers. The figure is modified from the Arabidopsis Gene Family Profiler (http://arabidopsisGFP.ueb.cas.cz).
3.2 Part B: Functional analysis.

3.2.1 Yeast two-hybrid screen for possible interacting partners for the ATX4 protein.

Yeast two-hybrid is a powerful method for finding interacting proteins for your protein of interest, to confirm suspected interactions, and define interacting domains.

A yeast two-hybrid screen was done by Tage Thorstensen to identify putative interacting partners for the ATX4 protein. This was performed by mating yeast clones expressing the full-length ATX4 gene fused to the GAL4 DNA binding domain in the pGBK7 vector (section 2.4.5), against a library of GAL4 activation domain fusions constructed from flower cDNA.

The screen gave us 80 possible interacting prey clones growing on QDO plates. PCR screening and restriction cutting showed that 50% of the prey clones were unique, 15 of these were chosen for control mating. The control mating was performed as described in section 2.4.3. Table 3.1 shows the result of the control mating.

<table>
<thead>
<tr>
<th>Bait: pGBK7-ATX4-C</th>
<th>Selection media and type of growth</th>
<th>Selection media and type of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SD /-Leu/-Trp</td>
<td>QDO + Xgal</td>
</tr>
<tr>
<td>pGADT7-prey clones</td>
<td>BD / Lam ATX-C</td>
<td>BD / Lam ATX-C</td>
</tr>
<tr>
<td>I</td>
<td>DW+ / DW+ / DW+</td>
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<tr>
<td>V</td>
<td>DW+ / DW+ / DW+</td>
<td>+ / + / B+</td>
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<tr>
<td>VI</td>
<td>DW+ / DW+ / DW+</td>
<td>+ / + / B+</td>
</tr>
</tbody>
</table>

Table 3.1 Positive ATX4 prey clones.
Control mating of the prey clones was done to reduce the number of false positives, and it gave rise to six positives. D = Diploid colonies, W = White colonies, B = Blue colonies, + = growth, + = no growth.

After the control mating six prey clones, that were only able to grow when mated with the clone expressing the full length ATX4 protein, were chosen for further analysis. These clones were sequenced, and a BLAST search against GenBank was performed to identify the corresponding cDNA for the putative ATX4 interacting clones (Table 3.2).
Table 3.2 Positive ATX4 prey clones.
The clones were sequenced to confirm that they were in frame with the GAL4 DNA-BD domain. The sequences were BLASTed against GenBank to identify the corresponding genes. Unsatisfactory sequence data delivered from the ABI facility have resulted in some prey clones being denoted “not sequenced”.

3.2.2 Second yeast two-hybrid screen for possible interacting partners for the ATX4 protein.
Because of uncertainty with respect to the ATX4 SET-domains effect on the yeast reporter genes, a second yeast two-hybrid screen was done with a bait clone encoding a fragment of the ATX4 protein that does not contain the SET-domain. This fragment, denoted ATX4-2395, constitutes the N-terminal part of the ATX4 protein encompassing the PWWP domain and the PHD finger. This screen gave 30 possible interacting prey clones that were able to grow on interaction selective QDO media. PCR screening and restriction cutting showed that 26 of the interacting prey clones were unique. These clones were control mated (Table 3.3), and sequenced (Table 3.4).
## Results

### Table 3.3 Positive ATX4 prey clones.

Control mating of the prey clones was done to reduce the number of false positives. **D** = Diploid colonies, **W** = White colonies, **R** = Red colonies, + = growth, ÷ = no growth, * = sporadic/ poor growth.

<table>
<thead>
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<th>Lam</th>
<th>ATX4-C</th>
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<th>Lam</th>
<th>ATX4-C</th>
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Results

Table 3.4 Positive ATX4 prey clones.
The clones were sequenced to confirm that they were in frame with the GAL4 DNA-BD domain. The sequences were BLASTed against GenBank to identify the corresponding genes. Unsatisfactory sequence data delivered from the ABI facility have resulted in some prey clones being denoted “not sequenced”. * = Prey clones showing 100% positive growth pattern.

3.2.3 Yeast two-hybrid deletion mapping.
Bioinformatical analysis has shown that the ATX4 protein contains the following domains; PWWP, PHD finger, extended PHD finger, SET domain and C-SAC (Baumbusch et al., 2001). Yeast two-hybrid deletion mapping was performed to investigate which domains of the ATX4 protein that interacts with the six identified interactants from the first yeast two-hybrid screen.

The three deletion constructs of ATX4, and a full length ATX4 construct were in made in collaboration with Tage Thorstensen as described in section 2.1.5, the resulting destination vectors were used as bait in the two-hybrid mating. The ATX4-1387 fragment contains the PWWP domain, the ATX4-2395 fragment contains the PWWP domain and the PHD finger and the ATX4-SACSET fragment contains the SET domain and the C-SAC domain (Figure 3.3)
The six positive interacting prey clones from the first screen were mated against the bait clones expressing one of the three ATX4 deletion constructs 1387, 2395 and SACSET. As expected all the six prey clones grow when mated with full length ATX4 suggesting that they encode proteins that bind the ATX4 protein. However, when mated with the clones containing the ATX4 deletion constructs, prey clones II, III and V only showed growth when mated with bait clones containing the 1387 fragment of ATX4, suggesting interaction with the N-terminal or the PWWP domain of the protein. Prey clone IV on the other hand showed growth only when mated with colonies containing the ATX4-SACSET fragment and seems to interact with the SET-domain part of ATX4. Clones I and VI were able to grow when mated with both the 1387 and the SACSET bait clones proposing two interaction points, one in the N-terminal and the other in the C-terminal of ATX4 (Figure 3.4).
Results

3.4 Yeast two-hybrid deletion mapping with positive interacting clones.
Bait clones containing ATX4 deletions were mated with six prey clones encoding proteins that specifically interacted with full length ATX4. The mated yeast cultures containing the bait and the prey clones were plated on SD\QDO media for selection of interacting proteins.

3.2.4 GST pull-down to confirm yeast two-hybrid interactions.
The Glutathione S-Transferase (GST) gene fusion system is a versatile system for expression, purification and detection of fusion proteins produced in E.coli. For a GST-Pull down experiment the GST fusion proteins have to be expressed in cells that are optimized for protein expression. A GST pull-down was performed to confirm interaction from the yeast two-hybrid deletion mapping. As an initial approach to confirm the yeast two-hybrid data, ATX4 fragments 1387 and SACSET were fused to a GST tag and expressed and purified in E.coli. A gel was run to check that we had sufficient amounts of GST control, GST-ATX4-1387 and GST-ATX4-SACSET. In the pull-down reaction 5 μg of GST fusion protein was incubated with in vitro translated prey clone IV from the first yeast two-hybrid screen. After binding, the GST-fusion proteins were washed, denatured and run on an SDS-PAGE gel (Figure 3.5).
Results

Figure 3.5 GST pull-down.
GST pull-down experiment for prey clone 4. The SDS-PAGE gel on the left shows a severe loss of protein for the ATX4-SACSET fragment compared to the ATX4-1387 fragment. The gel on the right is the SDS-PAGE gel after autoradiography detection, it shows that clone IV binds the SACSET part of ATX4 stronger than it binds the 1387 part of the ATX4 protein.

Quantification of the 1387-band and the SACSET-band on the SDS-PAGE gel using the Image Quant TL program (Amersham) showed that the strength of the SACSET-band is only $\frac{1}{5}$ compared to the strength of the 1387-band. This shows that there has been a severe loss of protein for the ATX4-SACSET fragment. However, quantification of the bands in the same lanes after autoradiography showed that the strength of the SACSET-band now is the strongest, being 2.4 times stronger than the 1387-band. These results give a difference of $5 \times 2.4$ which equals 12. The prey clone denoted IV binds to the SACSET-part of ATX4 12 times stronger than it binds to the 1387-part of ATX4. This coincides with the yeast two-hybrid results (Figure 3.4). But because of the severe loss of ATX4-SACSET protein the pull-down must be repeated.
3.2.5 Investigation of phenotype for ATX4 knockout lines.

An effective way to elucidate the function of a gene is to create a homozygous knock-out mutant plant. Any possible phenotypic effects may indicate what processes the gene is involved in, and whether the gene is essential or redundant. A BLAST search against the SALK T-DNA-database (http://signal.salk.edu/) with the ATX4 gene sequence identified the SALK line SALK_060156, containing a possible T-DNA insertion in the ATX4 gene at position 2167 (Figure 3.6).

![Figure 3.6 ATX4 T-DNA insertion line.](Image)

The SALK insertion line 060156 has a T-DNA inserted at position 2167 in the ATX4 gene. Blue boxes indicate exons and the lines between them indicate introns. The exons were identified by aligning atx4 cDNA with the ATX4 gene sequence. cDNA was amplified by RT-PCR and cloned into the pGMT-Easy vector. The amplification and cloning was done by Tage Thorstensen.

Of the seeds from the SALK_060156 line delivered from the SALK database only 3 were able to grow beyond the seedling stage, these were denoted SALK_060156 A, B and C. PCR genotyping showed that all 3 plants contained the T-DNA insertion at the predicted position, and all were heterozygous for this insertion.
3.2.5.1 Southern Blot.

To check the number of T-DNA insertions in the plants SALK_060156A, B and C Southern hybridization using a probe against the selection marker nptII was performed on DNA isolated from the plants; this was done by Tage Thorstensen (Figure 3.7). The hybridization data show that plant 060156B contains one T-DNA insertion and the progeny of this line was thus chosen for use in further experiments. Data for plant line SALK_060156C is not shown.

![Figure 3.7 Southern blot showing the number of T-DNA insertions in SALK_060156 plants.](image)

A southern blot was performed to check the copy number of the T-DNA insertion. In the figure A is the T-DNA insertion line SALK_060156A, and B is the T-DNA insertion line SALK_060156B. 060156B shows one single hybridization band of specific size demonstrating the presence of one copy of nptII gene, and hence one copy of the T-DNA insertion.

3.2.5.2 PCR Genotyping.

Offspring from the heterozygous one-copy line 060156B were genotyped by PCR to identify homozygous plants (data not shown). The two SALK sister lines B5 and B7 were found to be homozygous and were used for further investigations. To be sure that the B5 and B7 plants produced homozygous offspring, 14 plants of each were PCR genotyped (section 2.1.1.1) and found to be homozygous (Figure 3.8).
Results

3.2.5.3 Early flowering hypothesis.

Compared to Colombia wild type, plants of the SALK_060156 lines seemed to grow faster and develop flowers at an earlier stage than the wild type plants. These plants also seemed to have fewer rosette leaves than the wild type plants. We hypothesized an early flowering phenotype for the SALK_060156 line. To investigate this we registered the first shooting day and stem length for Colombia wild type versus SALK_060156.

3.2.5.4 Documentation of first shooting day.

First shooting day was documented for the two sister lines SALK_060156B5, SALK_060156B7 and for Colombia. The plants were observed for two weeks after they had been transferred to soil. It was registered for each plant which day it shot (Figure 3.9).
3.2.5.5 Documentation of stem length.

Stem length was measured for the SALK_060156B5, SALK 060156B7 sister lines and for Colombia. The plants were observed for two weeks after they had been transferred to soil. Each day the stem length of each plant was measured to the closest 0.5 cm. These data show that offspring from the SALK_060156B line grew faster and were taller than the Colombia control line at the same stage (Figure 3.10).

Figure 3.9 First shooting day for ATX4 knockout lines vs first shooting day for Colombia.

ATX4 knockouts shoot in average one day earlier than Colombia wild type. The data was run through a Students T test to confirm its significance. The probability of the results, assuming the null hypothesis, is 0.000. A probability of 0.05 or less is highly significant and the null hypothesis can be rejected; the wild type plants and the SALK_060156 plants can be distinguished by first shooting day.
Figure 3.10 Stem length for ATX4 SALK line vs stem length for Colombia WT.
The ATX4 SALK plants grow faster than Colombia wilt type. The data was run through a Student’s T test to confirm its significance. The probability of the results is 0.001 or less. A probability of 0.05 or less is highly significant and the null hypothesis can be rejected; the wild type plants and the SALK_060156 plants can be distinguished by stem length.
4. Discussion.

4.1 The SET-domain protein ATX4 is a putative histone methyltransferase.

ATX4 is member of the ARABIDOPSIS TRITHORAX class of SET-domain proteins and contains the following domains: PWWP, PHD, ePHD, SET and C-SAC. Bioinformatics reveal that ATX4 show sequence similarity with histone H3K4 methyltransferases (Baumbusch et al., 2001), indicating that the ATX4 protein is a histone methyltransferase with H3K4 activity. Methylation of H3K4 is generally associated with gene activation.

The ARABIDOPSIS TRITHORAX gene ATX1 is the only ATX gene with known histone methyltransferase activity. ATX1 is involved in methylating histone H3K4 of only a fraction of Arabidopsis nucleosomes. This suggests that other plant methyltransferases are involved in genome-wide H3K4 methylation, and the presence of multiple Trithorax genes in the Arabidopsis genome supports this idea (Baumbusch et al., 2001; Alvarez-Venegas and Avramova, 2005).
4.2 Promoter-GUS expression pattern.

As an early approach to elucidate the function of the ATX4 gene we investigated its expression pattern by introducing into the Arabidopsis genome a promoter::GUS construct. This gave us detailed information on the temporal and spatial expression pattern of ATX4. The ATX4 promoter directs GUS expression as described in section 3.1.1, showing GUS expression in the entire plant in the seedling stage, except roots in young seedlings, and expression in rosette leaves and flowers of the adult plant. In young seedlings strong GUS expression is observed in the hypocotyl and in the vascular tissue of cotyledons. Adult plants show expression in the vascular tissue of rosette leaves and a transient GUS expression in buds and flowers. The expression of ATX4 in seedlings indicates a role for ATX4 early in plant development. The ATX4 expression observed in vascular tissue of various organs suggests that the ATX4 protein may regulate genes that have roles in transport of water or solutes; genes expressed in vascular tissue are suggested to be candidates for transport of peptides (Dietrich et al., 2004), ATX4 may regulate such a gene. Our results do not indicate whether the vascular expression is in the xylem or in the phloem. To be sure that this GUS expression is not unspecific the results should be confirmed by in situ hybridization.

In addition to the vascular expression in seedlings and rosette leaves, the ATX4 promoter also directs GUS expression in flowers, particularly an interesting transient expression from small closed buds to fully grown open flowers (Figure 3.1.D). This expression is impermanent both temporally and spatially. The transient temporal expression shown in anthers in Figure 3.1.D. ii) and in 3.1.E is similar to the expression pattern for the AMS gene, for which the suggested function is direct or indirect regulation of pollen mitosis I (Sorensen et al., 2003). The transient expression in flowers may indicate a role for ATX4 in developing pollen, stamens and petals; when the pollen is mature ATX4 expression is turned off in pollen and turned on in stamens.
A bioinformatics approach to investigate the \textit{ATX4} expression pattern was also done. The two online services the \textit{Arabidopsis} Gene Family Profiler (http://arabidopsisGFP.ueb.cas.cz) and the Genevestigator (Zimmermann et al., 2004) were used. These show \textit{ATX4} expression of various degree in all tissue, but most notably both show strong expression in flowers. The Gene Family Profiler shows no expression in mature pollen, whereas the Genevestigator shows general pollen expression. These are not necessarily opposing results, as our \textit{ATX4}::GUS result show an early expression in pollen, but no pollen expression in later floral stages. An early pollen expression will show on a general pollen expression scale, but not on a scale for mature pollen. Genes expressed specifically in pollen will show high expression in "stamen", but also in the categories "flower" and "inflorescence" in such programs with results based on microarray data.
4.3 Interacting partners for ATX4.

Yeast two hybrid screening is a powerful method for identification of interacting partners for your protein of interest. To find new putative interacting partners for the ATX4 protein two yeast two-hybrid screens were done as described in sections 3.2.1 and 3.2.2. These screens gave us numerous putative interacting partners (Table 3.2 and Table 3.4). All six prey clones in Table 3.2 show positive growth pattern. Among the results in Table 3.4 are five definitive false positives that either interacts with GAL BD and Laminin, or they do not interact with the ATX4 full length protein. These false positives are prey clones 2, 5, 14, 19 and 23. The prey clones 6 and 8 are also deemed false positives since the show some sporadic growth when mated with the GAL BD alone, and when mated with the full length ATX4 protein, the latter which should give rich growth in case of a positive interaction. Prey clones 3 and 7 show some growth when mated with the GAL BD, and prey clones 9, 10, 11 and 15 show poor growth when mated with the full length ATX4 protein; these may also be false positives and control mating should be repeated. However the 11 remaining putative interacting proteins from the two-hybrid screen suggest that ATX4 may be part of one or several protein complexes. Amino acid alignments place ATX4 in the trithorax class of SET-domain protein, and such proteins often reside in protein complexes (Mahmoudi and Verrijzer, 2001). Among the putative interacting partners found for ATX4 were a putative bifunctional nuclease, a cysteine endopeptidase, papain-type (XCP2) and a putative SET-domain transcriptional regulator, denoted ASHR2 (Baumbusch et al., 2001).

Bifunctional nucleases are capable of degrading both RNA and DNA (LeBrasseur et al., 2002). Single-strand specific nucleases are active during plant growth and developmental processes such as germination and xylem differentiation (Perez-Amador et al., 2000). However since this protein showed some interaction with the GAL4 BD it may be a false positive.

Proteases/peptidases are enzymes that degrade proteins by hydrolyzing some of their peptide-bonds. Papain-type endopeptidases are involved in many aspects of plant growth
and development. In the root hypocotyl of *Arabidopsis* transcripts of the papain-type cysteine endopeptidase XCP2 (Xylem specific Cysteine Protease-2) is found almost exclusively in xylem (Zhao et al., 2000). XCP2 is detected generally in stems and flowers where cells are growing and differentiating, and not in mature tissue. This suggests that XCP2 has specialized function related to growth and/or differentiation. XCP2 has been shown to localize within the vacuoles of TE (tracheary elements, the water conducting cells of the xylem), which may mean a possible role for this enzyme as a peptidase that is an effector of autolysis; a critical final process in TE differentiation (Funk et al., 2002).

The putative SET-domain transcriptional regulator ASHR2 is related to the ASH homologues of *Arabidopsis* SET-domain proteins. These proteins have their SET-domain not in their C-terminal part, but rather placed in their central region. ASHR2s only known domain is the SET-domain (Baumbusch et al., 2001). ASHR2 is suggested to have a role in vegetative development and to be an activator of the photoperiod flowering pathway by maintaining the normal expression of FT and SOC1 (Lin et al., 2005).

XCP2 being active in xylem differentiation may coincide with the results showing *ATX4* expression in vascular tissue of rosette leaves and petals. XCP2 is also associated with plant growth and development. Putative interacting partners may bind to ATX4 and other components, and together constitute a protein complex that modifies chromatin and/or regulate genes.

ATX4 does not contain any known nuclear localization signal (NLS), and transient expression assay in onion epidermis cells have been unsuccessful (Tage Thorstensen, unpublished data). It is therefore possible that ATX4, although most probably being a chromatin modifying HMTase, may be functioning outside the nucleus by methylating other proteins than histones. Several of the translational apparatus proteins are methylated in all kinds of organisms (Toledo et al., 1988). XCP2 is localized to vacuoles, and probably interacts with ATX4, this supports the hypothesis that ATX4 may be functioning outside the nucleus.
The yeast two hybrid deletion mapping (Figure 3.4) show that the analyzed prey clones specifically interact with different parts of ATX4. The results indicate that prey clones II, III and V (*Arabidopsis thaliana* expressed proteins) interact with the 1387 bp N-terminal fragment containing the PWWP domain, and prey clone IV, *Arabidopsis thaliana* hsp70 like protein interacts with the ~ 1100 bp C-terminal fragment containing the SET-domain. Proteins I and VI seem to interact with both the N-terminal and the C-terminal part of the protein, this may be explained if the folding of the full-length ATX4 protein brings domains in both the N- and C-terminal in close proximity to each other, so that proteins encoded by clones I and VI potentially may bind both domains. A GST pull-down was performed to confirm the interaction, and the result coincided with the yeast two-hybrid deletion screening (section 3.2.4). Due to lack of time GST pull-down was not performed for the remaining prey clones, this should however be done to confirm their interactions with ATX4.
4.4 atx4 mutant plants show an early flowering phenotype.

PCR genotyping and Southern Blotting show that the T-DNA insertion line SALK_060156 is homozygous, single copy for its T-DNA insertion in the ATX4 gene. Quantitative measurements such as registration of first shooting day and stem length were used to compare Colombia wild type plants with plants from the SALK_060156 line. The atx4 mutants showed earlier shooting and longer stems than wild type plants, demonstrating that atx4 mutants show an early flowering phenotype.

However to confirm that this phenotype is due to a mutation in the ATX4 gene, a complementation test has to be done; the mutant line has to be transformed with the wild type ATX4 gene to see whether this restores the wild type phenotype, and in the putative complement line new measurements has to be done.

One hypothesis that may explain the early flowering phenotype seen in the SALK_060156 line is that ATX4 may be an activator of the flowering repressor FLC. Histone H3K4 trimethylation is associated with active FLC expression, and a histone methyltransferase that methylates FLC has been identified. This histone H3K4 methyltransferase, denoted EFS/ASH2 (Soppe et al., 1999; Baumbusch et al., 2001; Kim et al., 2005), is required for elevated FLC expression and thus the vernalization responsive delayed flowering seen in winter-annual Arabidopsis ecotypes. It is possible that EFS/ASH2 is recruited by the PAF1 complex to confer methylation of FLC (Figure 1.3). Additional histone methyltransferases may be involved in methylation of FLC chromatin during seedling early stages of development (Kim et al., 2005). ATX4 may activate FLC in wild type Arabidopsis leading to a high level of FLC, which again leads to normal flowering time, however in the absence of ATX4 the FLC level will be reduced leading to early flowering. This suggests a putative role for ATX4 in methylation of FLC.
4.5 Conclusion and future aspects.

The fact that ATX4 contains a SET-domain and shows sequence similarity to histone H3K4 methyltransferases is a strong indication of ATX4 being a histone methyltransferase. However to confirm this, a histone methyltransferase assay has to be performed with the ATX4 protein on histones. On the other hand if ATX4 is to be confirmed as a methyltransferase specific for other proteins than histones, a methyltransferase assay has to be performed with the ATX4 protein on interacting proteins.

ATX4 shows strong expression in seedling hypocotyl and in vascular tissue of cotyledons, rosette leaves and flowers, and yeast two hybrid screens indicate that ATX4 interacts with proteins with roles in development and vascular differentiation. This suggests a role for ATX4 in development of the vascular tissue. The interacting partners are active in the xylem, so further investigations are needed to check if ATX4 is expressed in xylem of phloem. The GUS results should also be confirmed by in situ hybridization.

The transient ATX4 gene expression in buds and flowers may indicate a role of the ATX4 protein in pollen and stamen development. ATX4 is early expressed in pollen, and then turned off, succeeded by expression in the stamens, an expression that also vanishes. The pollen expression is similar with the expression pattern for AMS which is suggested to be involved in pollen mitosis. ATX4 is also expressed in the vascular tissue of petals and sepals, further supporting the notion of ATX4 having a role in vascular development. Mature flowers show a total loss of expression.

The discovery of many putative interacting partners for ATX4 supports the hypothesis that it may be part of one or several protein complexes. However all the yeast two-hybrid results should be further confirmed in vitro by GST pull-down, followed by in vivo confirmation by co-immuno precipitation.
Considering that \textit{atx4} mutants show an early flowering phenotype, is a putative histone H3K4 methyltransferase and FLC has shown to be trimethylated at lysine 4 on histone 3, it is tempting to speculate that ATX4 is an activator of FLC. HMTase activity is, as mentioned, yet to be confirmed. The early flowering phenotype in the \textit{atx4} mutant line has to be complemented. Interaction studies to see whether ATX4 binds any of the proteins in the PAF1 complex would further support the hypothesis that ATX4 is an activator of FLC.

Interestingly, yeast two-hybrid screening show that another SET-domain protein called ASHR2 interacts with ATX4. However, ASHR2 is suggested to be an activator flowering, in contrast to ATX4 which we propose to be involved in repression of flowering. All though speculative, these preliminary results could be explained if methylation of ATX4 by ASHR2 leads to inactivation of the ATX4 protein, or vice versa, leading to activation or repression of flowering respectively.
5. References.


Michaels, S.D., and Amasino, R.M. (2001). Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of FRIGIDA and
autonomous pathway mutations but not responsiveness to vernalization.
Plant Cell 13, 935-941.

FLOWERING LOCUS C activity as a mechanism for the evolution of
summer-annual flowering behavior in Arabidopsis. Proc Natl Acad Sci U S A
100, 10102-10107.


Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and
bioassays with tobacco tissue cultures. Plant Physiology 15, 473-497.

Noh, Y.S., and Amasino, R.M. (2003). PIE1, an ISWI family gene, is required for
FLC activation and floral repression in Arabidopsis. Plant Cell 15, 1671-
1682.

Perez-Amador, M.A., Abler, M.L., De Rocher, E.J., Thompson, D.M., van Hoof, A.,
bifunctional nuclease induced during leaf and stem senescence in

Biol 14, R546-551.

of flowering in Arabidopsis by an FLC homologue. Plant Physiol 126, 122-
132.

Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil,
Regulation of chromatin structure by site-specific histone H3

FLC, a repressor of flowering, is regulated by genes in different inductive
pathways. Plant J 29, 183-191.

edition. (Cold Spring Harbor Laboratory Press.).

endonuclease activities in Haemophilus parainfluenzae using analytical

and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine

chromatin: mechanisms of Polycomb and trithorax group complexes. Curr
Opin Genet Dev 12, 210-218.


efs is involved in the autonomous promotion pathway of Arabidopsis
thaliana. Development 126, 4763-4770.


6. Appendix.

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