Elucidation of the expression patterns and functions of the SET-domain genes ATX3 and ATX5 in *Arabidopsis thaliana.*

Napoleon Foam

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Department of Molcular Biosciences
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ABSTRACT

Covalent modifications of the DNA and histones through acetylation, phosphorylation, methylation, etc are central to gene regulation, recombination, DNA replication and many other processes in the cell. With respect to gene regulation, modifications of histones and DNA have immense influence on the expression potential of genes within the modified regions of chromatin structure, mainly on the level of DNA packaging and recruitment of the transcriptional machinery. Histone methyl transferases form a part of this complex system of genes that carry such modifications. They catalyze the methylation of the N-terminal tails of histones. This function is known to reside in an evolutionarily conserved domain, SET which is found in all the kingdoms of life. Several hundreds of the SET-domain genes have been identified in several organisms. About 30 of these SET-domain genes are thought to exist in Arabidopsis genome with relatively little or no experimental information on some of them. Many genetic analyses in several organisms show that the SET-domain genes regulate the transcription of very important genes including homeotic genes.

Two of such genes are the Arabidopsis Trithorax 3 (ATX3) and ATX5 on which data generated experimentally is too scanty. This project was therefore aimed at obtaining functional information on ATX3 and ATX5. This began with phenotypic analysis by generating 'Knockdown lines' of ATX5, using the RNA interference mechanism. Level of silencing was low. However lines where silencing is considered to have occurred exhibited phenotypic defects which gives an important hint which suggest ATX5 is involved in a number very vital processes in Arabidopsis development. Fertility and silique development, which is fertility-related, were adversely affected. The ATX5 RNAi lines also possessed abnormal phyllotaxy and enlarged inflorescence meristems. When SALK lines for T-DNA insertion for the ATX5 were later screened phenotypically, there was one isolated instance of enlarged inflorescence meristem which matched that obtained on one RNAi line.

Phenotypic analysis was followed by in *situ* hybridization and RT-PCR, both of which gave consistent results. RT-PCR results showed a relatively higher level of ATX5 mRNA in the flowers. *In situ* hybridization analysis also showed that ATX5 is expressed in very vital tissues and organs of Arabidopsis, namely the shoot apical meristems of both seedlings and embryo. ATX5 transcript was also detected in the tapetum of stamens and the gynoecia of

Arabidopsis flowers. Results from both experiments suggest that the phenotypes observed on the RNAi lines were due to down regulation of the ATX5 gene, although this was not an easy experiment to perform convincely, but does not explain the cause of the low level of silencing.

Experiments are underway to determine the interacting partners of the ATX5 protein using the glutathione Sepharose tagged (GST)- pull down assay with the interacting partners found for the structurally similar ATX4, as well as test the possibility of histone methyl transferase activity.

Preliminary results from RT-PCR analysis detected ATX3 mRNA in several tissues and organs. The mRNA level in seedlings was however higher in seedlings than in many other tissues and could be a reflection of a higher activity of ATX3 in seedlings. In situ hybridization analysis also showed that ATX3 is necessary for normal development of the pollen wall or lipid-rich exin coating, which is the function of tapetum of which the ATX3 trasncript was present. Preliminary screening of SALK lines for the ATX3 gene did not produce any obvious phenotypes.

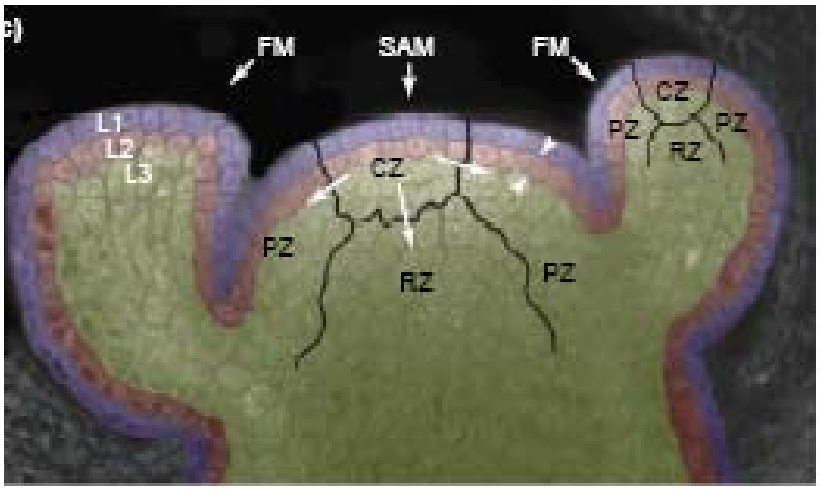
1 THEORY

1.1 Arabidopsis and its development

Arabidopsis thaliana is a small wild, annual, flowering weed that is well suited for use in classical biological research work and commonly termed wall cress or mouse-ear cress ('vårskrinneblom' in Norwegian). Though a weed it has edible cousins like cabbage, broccoli, Brussels sprouts and oilseed, all of which belong to the Brassicaceae (mustard or crucifer) family. The plant is not only small in structure, but also contains a small genome of 125 Mb that is distributed between just five chromosomes. It also has a relatively fewer gene number (26,000). Its short life cycle (6 weeks) and prolific seed production are desirable features in genetic research. Arabidopsis, unlike other organisms such as animals and bacteria, but like other plants also possesses a tremendous developmental plasticity. For example, patterning and morphogenesis in Arabidopsis and most plants occurs post embryonically. This means new plant organs are formed throughout their life by stem cells in the meristems. These and other more features have helped to make this small weed a good model organism for plants, evidenced by the sequencing of its whole genome by The Arabidopsis Genome Initiative (Thakur et al., 2003) in the year 2000.

In plants the stem cells are located in the apical meristems of the shoot (SAM) and root (RAM). Located at the shoot axis of higher plants is the shoot apical meristem (SAM) which is the site for pluripotent cells. This small pool of undifferentiated cells produces lateral organs such as leaves during vegetative development as well as floral meristems during development. Radially, the SAM is organized into zones: the central, rib and peripheral zones (CZ, RZ, PZ respectively). At the apex of SAM is the CZ which contains self-renewing cells that divide infrequently. Below the CZ is the RZ which is a site for rapidly dividing cells, whose division and elongation give rise to the stem of the plant. The PZ where the rate of cell division is relatively higher surrounds the CZ. Cells from the PZ become recruited into the organ primordia and give rise to lateral organs such as leaves, inflorescence and floral meristems in a defined and predictable pattern termed phyllotaxy. As stem cells become recruited to generate new organs at the flanks of SAM, new stem cells have to be formed to replenish and maintain the size and organization of SAM. Hence disruption of the size and dimension of the SAM in some mutants has been found to result in phyllotactic defects. In

Arabidopsis where the phyllotaxy is spiral, the regular initiation of successive lateral organ primordia occurs at a constant divergence angle approximately 137° (Richards, 1948).



(Carles, 2003)

Figure1: Arabidopsis inflorescence SAM and its adjacent floral meristems (FMs). The black outlines represent the approximate boundaries between the different meristematic zones: the peripheral zone (PZ), the rib zone (Tariq and Paszkowski) and the central zone (CZ).

After floral induction, the cells of a shoot apical meristem change in identity and form the inflorescence meristem (IM) that is characterized by a pattern of indeterminate growth and the production of flower meristems on its flanks. In Arabidopsis, these flower meristems are arranged in a spiral phyllotactic manner and are, unlike inflorescence meristems, determined. Genes that are required for vegetative shoot meristem maintenance initiation and initiation of organ primordia include the homeobox genes SHOOTMERISTEMLESS, ZWILLE, WUSCHEL, NO APICAL MERISTEM and the CLAVATA genes (Clark et al., 1996; Laux et al., 1996; Long et al., 1996; Moussian et al., 1998). Models that explain mechanisms regulating positioning of primodia to form these discrete phyllotactic patterns suggest that biochemical factors and biophysical forces in the apex of SAM are major players that determine positioning of organ primordia. By this, primordia are positioned at a point that is farthest from the apex and from existing primordia. the effects of biophysical forces for example are known to be subject to input from genetic, hormonal and environmental sources in the apex in order to determine the initiation sites of organs (Selker et al., 1992). One such hormone that triggers organ initiation and for that matter phyllotaxis is auxin, whose distribution pattern in the apex of SAM is proposed to determine organ patterning. Mutation in PIN1 a gene involved in auxin transport resulted in disturbed phyllotaxy (Reinhardt et al., 2003).

1.2 RNA interference: mechanism, components and applications

1.2.1 A conserved mechanism across species

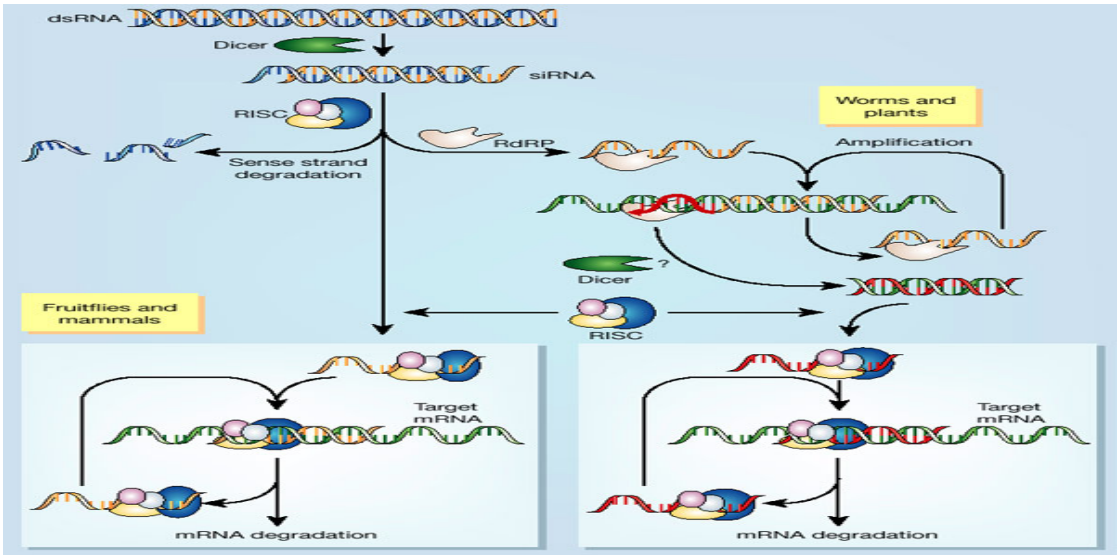
RNA interference (RNAi) or Post transcriptional gene silencing (PTGS) is an ancient and natural form of post-transcriptional gene silencing in which double-stranded RNA (dsRNA) induces interference in gene expression either through targeted degradation of homologous mRNA or through the suppression of translation. This natural form of silencing was initially discovered in plants but has since been found to be conserved across many species, and alternatively, termed RNA interference in animals, quelling in fungi, and Post transcriptional gene silencing in plants. In nature RNAi serves as a defensive mechanism against viral infection. Its is also implicated in transposon silencing as well as endogenous gene silencing (Waterhouse et al., 1998; Birchler et al., 1999; Voinnet, 2002; Zilberman et al., 2004).

1.2.2 The mechanism of RNAi/PTGS

In living cells dsRNAs do not usually occur. Therefore their accumulation during events such as viral replication in an infection, from an introduced transgene, or a foreign dsRNA evokes an antiviral response, which leads to the degradation of cognate mRNA or inhibition of translation. Figure 2 below illustrates this mechanism and the alternative pathways, depending on the organism. This response causes an RNAse III-like protein complex called DICER (Dicer-like in plants) which has nuclease activity to cleave and process the dsRNA into short interference RNA (siRNA); duplexes of 21-23 nucleotides with 2-nucleotide 3' overhangs (Zamore et al., 2000; Bernstein et al., 2001).

An RNA-induced silencing complex (RISC) which distinguishes between the different strands of the siRNA incorporates the antisense strand into the complex while the sense strand (blue) is degraded.

As part of the RISC complex, the antisense strand of siRNA is used to target genes for silencing, and has one of several fates depending upon the organism. In fruit flies and mammals the incorporated siRNA serves as guide through base-pairing to enable the RISC to cleave and degrade the target homologous mRNA (Hammond et al., 2000; Sharp and Zamore, 2000).



(Novina and Sharp, 2004)

Figure 2: The mechanism of RNAi/PTGS observed in various organisms. In mammals and fruitflies siRNA which is the processed product of the long dsRNA becomes incorporated directly into RISC where it guides RISC to homologous mRNA for degradation. However In plants and worms the antisense siRNA is used to prime, the amplification of new dsRNA.

In worms and plants, the antisense strand of the siRNA might first be used to prime the amplification of new long dsRNA using RNA dependent RNA polymerases RdRP (RdRP) (Novina and Sharp, 2004). The antisense strand achieves this by pairing up with a complementary mRNA (green) and starts the generation of new siRNAs (red), which again leads to target destruction of mRNA. In RNAi, antisense siRNA with perfect or near perfect pairing to the endogenous mRNA usually results in mRNA degradation while siRNA with partial complementarities results in microRNA (miRNA) mediated gene silencing; blocking the translation of target mRNAs into protein.

1.2.3 RNAi as a potent reverse genetic tool in molecular biology

Today RNAi is a potent genetic tool that is being greatly exploited for various goals, ranging from functional genomic studies, therapeutic studies and in diagnostics (Giladi et al., 2003; Song et al., 2003; Izquierdo, 2004; Denovan-Wright and Davidson, 2005). The embracement of this mechanism was propelled mainly by the discovery that exogenous dsRNA, when successfully delivered into living cells evoked specific and targeted silencing of endogenous genes (Brummelkamp et al., 2002; Giladi et al., 2003). This mechanism has been applied with convincing success in many organisms including *C. elegans* and *A. thaliana* and produced specific and heritable silencing of genes (Fire et al., 1998; Chuang and Meyerowitz, 2000). The search for better techniques for the efficient production and delivery of dsRNA in target

organisms is a major challenge for researchers today. For different organisms, various techniques exist for the production and efficient delivery of the dsRNA. In plants for example, stable and heritable RNAi silencing can been achieved by cloning two copies of a single DNA fragment at two insertion sites of an *Agrobacterium tumefaciens (A. tumefaciens)* compatible vector (such as pHELLSGATE8 and pKANNIBAL), but in an inverse orientation. If driven by a single promoter, transcripts from these repeats become paired and form dsRNA, due to self-complementarities between the bases on both repeats. The introduction of an intron between such repeats produces a hairpin RNA (hpRNA) which is found to contribute greatly to efficient gene silencing (Wesley et al., 2001). For delivery of these repeats for the production of dsRNA, the floral dip method of Agrobacterium-mediated transformation is by far the most efficient and commonly used technique to transfer transgenes into Arabidopsis plants (Clough and Bent, 1998). More on the techniques for generating Agrobacterium compatible vectors for RNAi and the Agrobactreium – mediated transformation is described in later section below.

Unlike other mutagenic techniques such as chemical and insertional mutagenesis, RNAi or PTGS silencing can result in complete loss of function as well as reduction of gene function with varying degree of severity in the phenotypes. This technique therefore lends itself for application on genes with lethal effects, where it is reduced possibility of generating functional information on mutants in development. RNAi also enables the researcher to target and knockdown specific genes from multiples of related gene sequences or gene families in the organism. On the other hand, gene redundancy can be eliminated or reduced by selecting RNAi fragments from conserved sequences or domains (Lawrence and Pikaard, 2003). In this way a family of genes with overlapping functions can be silenced. Additional merit in which makes RNAi or PTGS the first choice for in certain circumstances is that the resulting mutants possess variable degrees of silenced phenotypes that are reported to be heritable and stable in subsequent generations (Chuang and Meyerowitz, 2000).

1.3 Background to the Gateway® Cloning Technology

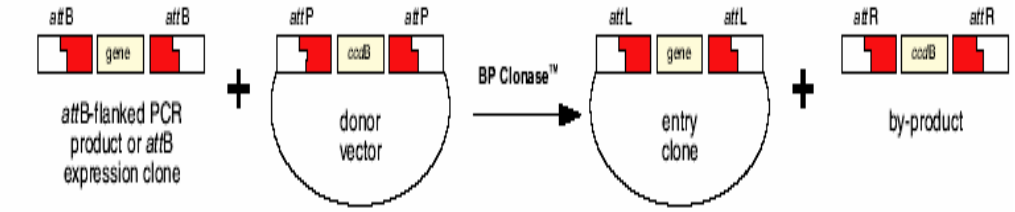
The Gateway® Cloning Technology (Invitrogen Corporation) is a universal cloning system that provides a versatile system involving two recombination reactions for transferring DNA segments between different cloning vectors. It is a rapid and highly efficient route to functional analysis, protein expression and cloning or sub-cloning of DNA segments by

replacing restriction endonucleases and ligase with site-specific recombination. It also allows cloning or subcloning from one vector backbone to another. With this technology, every subcloning reaction maintains the appropriate reading frame and orientation and multiple genes or gene fragments can be transferred to one or more vectors in a single cloning experiment.

Basically, the recombination reactions of the Gateway® Cloning Technology are *in vitro* versions of the site-specific recombination reactions of bacteriophage lambda (phage lambda) when it infects *E. coli*. Phage lambda has two alternative phases - lytic and lysogeneic phases- and can switch between them during its life cycle. The lytic phase is characterized by replication of phage lambda chromosome, production of new phage lambda which culminates in lysis of the *E. coli* cells. In the lysogenic phase however, lambda is able to excise and integrate its genome to specific sites in the *E. coli* genome with the help of proteins encoded by its genome and also from *E. coli* (Ptashne, 1992). These integrative and excisive recombination reactions of lambda form the basis for the two recombination reactions of the Gateway® Cloning Technology: the BP and LR reactions. The BP and LR recombination reactions are dependent on two major components: the DNA attachment sequences (att sites) also referred to as recombination sites and the recombination proteins that mediate the recombination reaction. The att sites flank the ends of both transgene and insertion site and ensure site recombination reactions while the proteins that mediate the reactions are called the BP and LR Clonase™ enzymes.

1.3.1 BP recombination reaction

The BP reaction (PCR fragment + Donor vector = Entry Clone) involves the transfer of PCR-generated gene or DNA fragment flanked by attB1 and attB2 sites into a donor vector which has attP1 and attP sites flanking the cloning site. The recombination reaction between the attB and attP sites yields an entry clone with attL1 and attL2 sites flanking the gene or DNA fragment. This reaction is catalyzed by the BP Clonase™ mix of the recombination proteins integrase (Int) and host integration factor (IHF). The attB and attP sites ensure site-specific recombination between only attB1 and attP1 and between attB2 and attP2 so as to maintain the orientation of the DNA fragment.

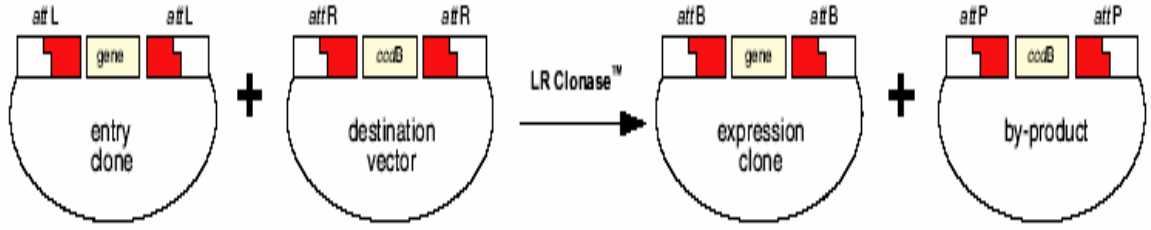


(Invitrogen Corporation)

Figure 3. A diagrammatic representation of the BP recombination reaction. The reaction produces two clones, the entry clone and a by-prodcuct which causes transformed cells to die on media, due to the *ccd*B gene.

1.3.1 The LR recombination reactions

The LR reaction (Entry Clone + Destination Vector = Expression Clone) transfers the DNA fragment in the Entry Clone to the Destination Vector with attR1 and attR2 sites. The LR reaction also involves a site specific reaction but between attL1 and attR1 and between attL2 and attR2 to create an Expression Clone. The LR Clonase™ mix mediates this reaction and conatins the Int, excisonase (Xis) and IHF recombination proteins. Cells that pick up the by-product do not survive after transformation, due to the *ccd*B gene.



(Invitrogen Corporation)

Figure 4. A diagrammatic representation of the BP recombination reaction. Only cells that take up the expression clone survive survive while those that take up the by-product donot, due to the deadly *ccd*B gene.

1.4 Gateway® cloning compatible vectors

The cloning sites in the donor and destination vectors which are flanked by the attP and attR respectively contain the *ccd*B gene, for the negative selection of right clones after transformation. The *ccd*B gene (cassette) is a toxic gene and prevents cells in which they are present from growing on growth medium, even if the cells have resistance to the antibiotic in a selective medium. It is present as a single copy in the donor vectors but two copies in the destination vectors that are designed to express hairpin RNA (hpRNA) for RNAi interference (e.g. pHELLSGATE8).

Both copies of the *ccd*B cassettes which are also the insertion sites for the RNAi fragment or foreign gene and occur in reverse repeats and separated by an intron. During the LB and LR

recombination reactions, this gene is replaced by the favorite gene fragment. As exemplified with pHELLSGATE8 vector below (Fig.5), the successful transfer of the DNA fragment from the entry vector into the destination vector during the LR reaction yields an expression clone with inverse arrangement of both copies of the RNAi fragment. The two cassettes are downstream and under the control of the cauliflower mosaic virus 35S promoter (CaMV 35S). When transferred into the Arabidopsis plant, theinverse repeats of the RNAi fragment or foreign gene become transcribed as a single unit in the host cell.

It also allows the transcript to fold back on itself due to self-complementarities. The result is a double-stranded hpRNA which is eventually spliced when it evokes the RNAi mechanism in the cell.

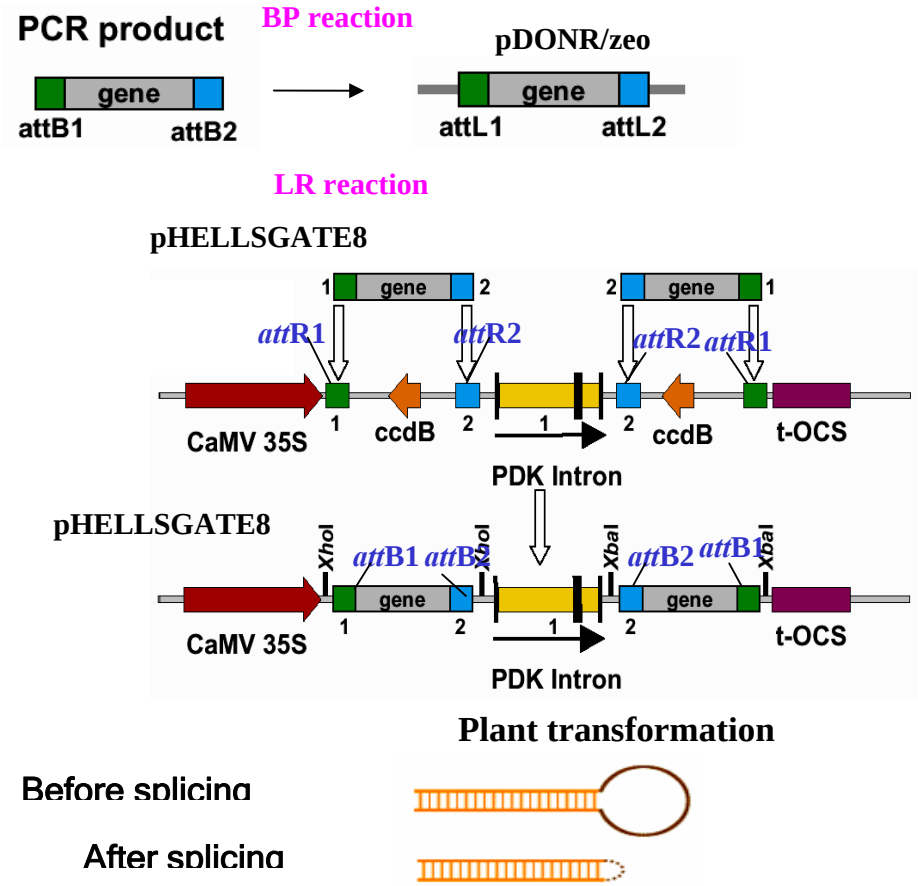


Figure 5: The pHELLSGATE8 destination vector before and after the LR reaction showing the two insertion sites occupied by the ccdB gene (orange). Both sites are separated by an intron (yello) but driven by the strong caMV 35S (plum). It also shows the hpRNA, a transcript of the inversely arranged RNAi fragments and how the spliced product.

Another feature common to the Gateway® vectors is the presence of antibiotic resistance gene for positive selection. This antibiotic resistance gene may confer resistance to commonly used antibiotics such as zeocin, ampicilin, spectinomycin, etc depending on the Gateway® compatible vector. The combination of the *ccd*B gene and antibiotic resistance genes in the Gateway® technology makes things easy by giving an extremely high yield of correct clone on the medium.

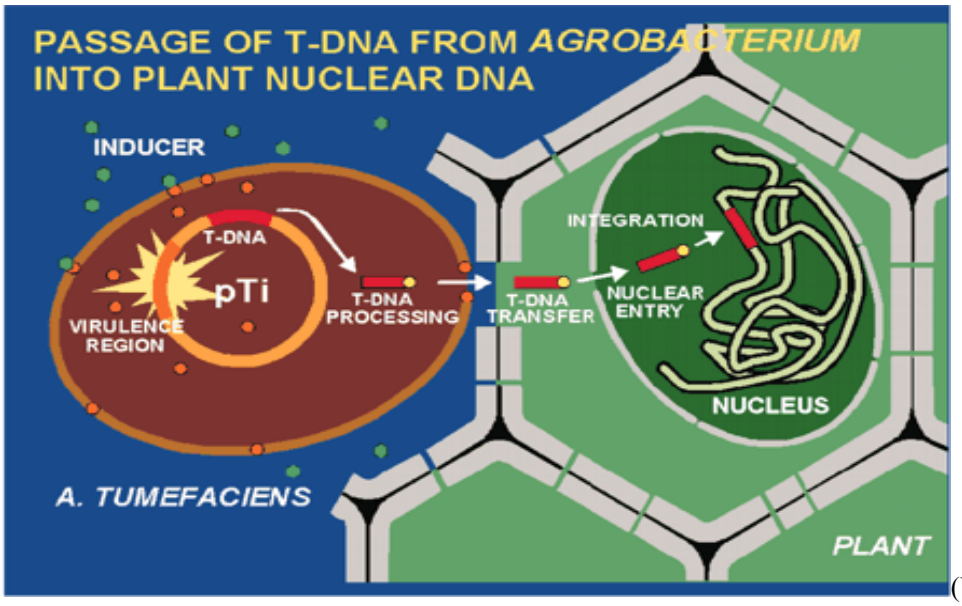
1.5 *Agrobacterium tumefaciens* transformation and T-DNA transfer

Agrobacterium tumefaciens (A. tumefaciens) is a soil bacterium, in fact a pathogen noted for inducing plant tumours (crown gall disease), by transferring a defined fragment of its DNA into the genome of dicotyledonous plants (Tzfira and Citovsky, 2002). It contains a large plasmid, the tumour inducing plasmid (Ti-plasmid) which contains the virulence genes (Vir genes) and transfer DNA (T-DNA) (Otten L, 1984; Scheiffele et al., 1995). The T-DNA which becomes transferred and integrated into the plant genome (Fig.6) is flanked by 25bp direct repeats at both the right and left borders and this ensure that any DNA between these border repeats become transferred to the plant genome. Gene products of the virulence genes enhance the plant cell division and tumor formation. The virulence (Vir) genes are in turn activated by the secretion of sugar and phenolic compounds (such as acetosyringone) from wounded plant cells. Activation of the Vir genes lead to the production of an endonuclease that excises the T-DNA as well as production of permeases necessary for the uptake of opines produced by the tumors. By a mechanism that is yet to be elucidated, the T-DNA becomes transferred to the plant where it is eventually integrated into the chromosomal DNA. Products of genes on the T-DNA include cytokines (phytohormones), indoleacetic acid, opines etc.

This capacity of Agrobacterium to carry out a natural form of genetic engineering in plants has been harnessed by researchers to transfer DNA either as mutagens or foreign gene into plant (Krysan et al., 1999).

One popular method is engineering the T-DNA to remove genes coding for the phytohormones and inserting a DNA fragment with selectable marker (usually antibiotic resistance gene) but leaving the right and left border repeats intact. The Ti plasmid is also ‘disarmed’ by deleting the genes necessary for inducing tumors so that such a plasmid is still

capable of transferring its T-DNA into the plant genome but lacks the ability to cause tumors (Schrammeijer et al., 2003). Due to the large size of the Ti plasmid, a binary vector system is developed: a self-replicating vector containing the T-DNA and the Ti-plasmid which contains the Vir genes. The Vir genes work in trans and produce the enzymes that are responsible for excising, copying and transporting the T-DNA to the plant cell though it may be located on a separate plasmid.



(Valentine, 2003)

Figure 6: Transfer of genetic material from *A. tumefaceins* to plant cell. Only the T-DNA from Ti plasmid is transferred and integerated into the plant genome.

For now the floral dip method of Agrobacterium mediated transformation has emerged as the favorite means to efficiently transform plants, though vacuum infiltration and tissue culture are alternatives. This method which was developed for Arabidopsis by Bent and Clough employs the binary vector system in Agrobacterium cells. Addition of the silvette to a culture of Agrobacterium serves as surfactant and strengthens adherence of Agrobacterium cells to inflorescences of plants after they are dipped in these cells (Clough and Bent, 1998).

1.6 SET- domain genes and their role in chromatin modification and transcription

1.6.1 SET domain genes are divided into 4 sub-groups

The SET-domain genes are conserved from yeast to animals and famous for their role in epigenetic gene regulation through remodelling of the chromatin structure into either

euchromatin or heterochromatin (Jenuwein et al., 1998). As chromatin remodelling proteins they are often involved in multimeric protein-protein interactions, to regulate the organisation of DNA and the histone octamer. Well over 300 of SET-domain genes have been identified so far, with at least 28 and over 30 found in *Caenorhabditis elegans* (*C. elegans*) and Arabidopsis respectively (Baumbusch et al., 2001; Alvarez-Venegas and Avramova, 2002; Terranova et al., 2002). According to sequence homology within their signature domain, the SET domain, these proteins are divided into four groups (Jenuwein et al., 1998; Baumbusch et al., 2001): Suppressors of position effect variegation [SU(VAR)3-9], Enhancer of zeste [E(Z)], small absent or homeotic disc1 (ASH1) and the trithorax (TRX) proteins (Jenuwein et al., 1998).

The Drosophila E (Z) protein and its homologues as well as the SU(VAR) genes establish or maintain transcriptionally active states on their target genes. They are therefore generally referred to as transcriptional repressors. This is supported by genetic evidence from experiments in among others *A. thaliana* and *S. cerevisiae* (Rastelli et al., 1993; Goodrich et al., 1997; Laible et al., 1997).

By contarst TRX genes (exemplified by the human ALL-1 (HRX), Drosophila TRX, SET1 in *C. elegans* and *S. cerevisiae*) and ASH1 genes are predominantly considered to antagonise the repressive or silencing effects of the PcG genes by maintaining transcriptionally active states of target genes (Chang et al., 1995; Rozovskaia et al., 1999; Nagy et al., 2002; Klymenko and Muller, 2004). Members of both the TRX and ASH1 subgroups belong to Trithorax-group (TrxG) a major group of genes that are required for the normal expression of homeotic genes (Shearn, 1989; Kennison, 1995).

1.6.2 Methylation histones by SET-domain proteins affect chromatin structure

The SET-domain proteins function by catalyzing mainly, the transfer of methyl groups on specific lysines or arginines of histone tails, which, depending on the specific residue, organism or level of methylation (mono-,di-, or trimethylation) can have multiple and varying effects on the chromatin structure. The enzymatic activity of these SET-domain proteins resides within the SET domain, an evolutionarily conserved 130–160 amino acids long protein sequence motif, though other conserved domains are known to be associated with these SET-domain proteins (Rea et al., 2000; Yeates, 2002).

The organisation of the eukaryotic chromatin does not only pose as a hindrance to the transcription apparatus, but also influences other processes such as DNA replication and cell division, recombination and DNA repair. The chromatin is highly packaged by proteins which reduces access of transcription factors to DNA. The basic unit of this chromatin is the nucleosomes which consists of a tetramer containing 2 each of histones H3 and H4 (H3/H4 tetramer) and 2 dimers of H2A/H2B that form a histone octamer around which a 147bp of geneomic DNA is wrapped (Arents et al., 1991). To be able to fit into the nucleus, eukaryotic nucleosomes have to be further organised into higher order structures into a 30nm chromatin fibre to compact the DNA, a function that has been attributed to linker histone H1 or H5 (Khorasanizadeh, 2004). This 30nm can as well exist in several level of packaging creating regions of heterochromatin (highly condensed and restrictive) or euchromatin (less condensed, permissive) along the chromosome.

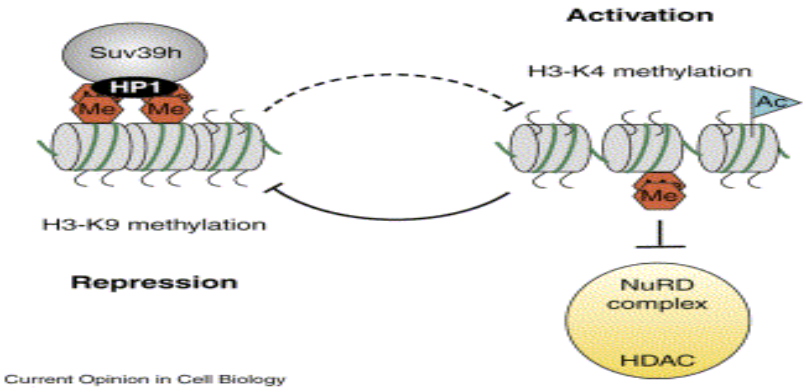
To overcome this hindrance, eukaryotic cells have developed several mechanisms to open the chromatin. One such mechanism is the covalent modification of specific amino acid residues on the N-terminal tails of histones by acetylation, methylation, phosphorylation, ubiquitylation, etc (Strahl and Allis, 2000). However, these modifications are also utilized to create heterochromatic regions in order to keep genes in a repressed state so that they are not expressed inappropriately. Chromatin can also be opened or packaged through directed nucleosome mobilization by protein complexes in an ATP-dependent process as well as by positioning of nucleosomes to create promoters with different requirement for remodelling.

2.6.3 Role of SET-domain proteins in establishing the histone code

The covalent modifications to which histone tails are subjected can occur either sequentially or in combinations on the histones. Combinatorial possibilities of these distinct modifications on histone tails represent encoded information and this extends the content of the genome beyond the DNA moiety (epigenetics) and its double helix, proposed as the histone code. This code predicts that (i) distinct modifications of the histone tails will induce interaction affinities for chromatin-associated proteins (ii) the modifications may be interdependent and generate various combinations and that (iii) local concentrations and combinations of differently modified nucleosomes determine qualities of higher order chromatin (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

Based on their specifities for residues on the histone tails, methylation by the different SET domain proteins creates or participates in establishment of epigenetic marks, which either contribute to euchromatin or heterochromatin formation (Lachner and Jenuwein, 2002).

In mammals and in plants, histone H3 methylated on K9 (H3K9) is found in constitutive heterochromatin and correlated to gene silencing. For example, the Suv39h-mediated histone methyltransferase activity on H3K9 is important for the creation of a specific binding site for the human heterochromatin protein1 (HP1). HP1 is known to be a major player in the establishment and



(Lachner and Jenuwein, 2002)

Figure 7: A model of heterochromatic self-maintenance by the SUVAR39h/HP1 complex. Methylation of lys9 by Suv39h creates recognition mark for HP1 which in turn recruits Suv39h.

maintenance of heterochromatin regions and thus is a candidate for establishing and maintaining transcriptionally repressive heterochromatin structure in human, Drosophila and *N. crasa* (Nakayama et al., 2001; Cheutin et al., 2003) (Fig. 7).

HP1 has a dual function by first recognising H3K9 methyl through its chromodomain and also in recruitment of Suv39h. In this way HP1 and Suv39h are involved in the self-maintenance and spread of heterochromatin to new regions (Bannister et al., 2001; Lachner et al., 2001). A homologue of HP1 in Arabidopsis is the *LIKE HETEROCHROMATIN PROTEIN 1(LHP1)* which has also been suggested to regulate gene expression in plants through formation of heterochromatin-like repressive complexes (Gaudin et al., 2001).

H3K4 methylation has the reverse effect of H3K9 methylation: it contributes to euchromatin formation (Bernstein et al., 2002; Santos-Rosa et al., 2002) and is synonymous to gene activation. It interferes with the substrate recognition by Suv39h and also prevents the binding

of the negatively acting nucleosome remodelling and histone deacetylation (NuRD) complex. At the same time H3K4 methylation facilitates acetylation of the H3 amino terminus, favouring the formation of more accessible nucleosome configuration that permits transcription (Lachner and Jenuwein, 2002). This form of methylation is typical of the TrG SET domain genes, counteracting the opposite and repressive effects of the Polycomb genes. For example, TrG genes like the human ALL-1, the Arabidopsis ATX1 and set1 of *Saccharomyces cerevisiae* have all been found activate their targets by methylating lysine 4 on histone 3 (Briggs et al., 2001; Nakamura et al., 2002; Alvarez-Venegas et al., 2003).

Structural differences within the SET domain as well as the SET flanking regions account for the mechanistic basis of the substrate specifities haboured by the different SET domain proteins (Marmorstein, 2003; Xiao et al., 2003). One of such regions often found flanking the N- and C-termini of the SET domain is the Cysteine-rich domain SAC (SET domain-associated cysteine-rich). It is unique for proteins containing the SET domain (Trievel et al., 2002; Landry et al., 2003). In the Arabidopsis, the SAC domain is only present in the C-terminus of the SET-domain of the trithorax sub-group (Baumbusch et al., 2001).

1.7 Arabidopsis trithorax 3 (ATX3) and ATX5 genes and their predicted functions

Both ATX3 (At3g61740) and ATX5 (At5g53430) are putative SET domain genes (Fig. 8) and belong to the Arabidopsis Trithorax (ATX) sub-group of SET-domain genes and found on chromosomes III and V respectively. The Arabidopsis genome contains at least 6 of these ATX genes (Baumbusch et al., 2001). The sequence of the ATX5 protein is predicted to be about 1044 amino acid long with a molecular weight of 119123.0 (119.123 kDa) and an isoelectric point (pI) of 8.1185. The cDNA of ATX5 is 3937 bases long while the genomic DNA is 6349 bp long. Functionally, ATX5 is predicted to methylate H3K4 and related to the Drosophila trithorax group proteins *TRITHORAX (*TRX*) and TRITHORAX-RELATED* (TRR) and the yeast gene SET1. ATX3 is predicted to have a protein sequence of 800 amino acids, a molecular weight of 91047.0 (91.047 kDa) and a pI of 7.9744. The coding sequence is 3634 bases while genomic DNA sequence of 6138bp (The Plant Chromatin Database; (www.chromdb.org). ATX5 in particular is found to share about 85% identity with ATX4 (At4g27910). With the help of the MIPS Interactive Redundancy Viewer, ATX5 and ATX4

are found be located in duplicated regions though the latter is located on chromosome IV (Baumbusch et al., 2001).

Besides the SET domain the ATX genes contain the plant homeodomain (PHD) fingers and the pro-trp –trp- pro (PWWP) motif (Fig.8). The functions of these other domains are currently unclear but thought to be important for the activity of multicomponent complexes in transcription. The PWWP domain is predicted to be involved in mediating protein - protein interactions and occurs in proteins that regulate cell growth and differentiation. Likewise the PHD is predicted to be involved in mediating protein - protein interactions. In the ATX proteins the PHD fingers are situated about midway between the PWWP motif and the SET domain, followed by the extended PHD (ePHD) (Aasland et al., 1995; Stec et al., 2000; Baumbusch et al., 2001).

Figure 8: Structures of ATX3 and ATX5 showing the various domains they contain (Baumbusch et al., 2001).

Based on sequence and structural analysis, The Institute for Genomic Research (http://www.tigr.org/) describes both ATX3 and ATX5 as putative DNA binding and nuclear proteins that are possibly involved in transcriptional regulation. Being members of the trithorax group (Trx) of genes, they are thought to be involved in activation of homeotic genes in Arabidopsis (Alvarez-Venegas et al., 2003). However the biological or molecular processes in which both ATX3 and 5 are specifically involved are not mentioned. So far the Arabidopsis Trithorax-1 (ATX1) is the closest to ATX3 and ATX5 that has been found to possess methyltransferase activity on lysine 4 of histone 3 (H3K4) and shown to have biological function. Experimental evidence show that ATX1 gene is essential for floral organ identity and development (Alvarez-Venegas et al., 2003). Hence mutation in ATX1 resulted in the down regulation of several genes including the class A floral homeotic genes, *APETALA1* (AP1) and AP2 and the class B genes *PISTILLATA* (PI) and (AP3). These findings suggest that ATX1 is required to maintain the normal expression levels of these genes. Genetic evidence also suggest that the founding member of the trxG genes, TRX in Drosophila group genes, is required in maintaining the normal expression levels of homeotic

genes such as the *BITHORAX* and *ANTENNAPEDIA COMPLEXES* (BX-C and ANT-C) which are involved in body segmentation (Shearn, 1989; Breen and Harte, 1993). Despite their similarity to other SET domain genes, coupled with predictions, there is to be produced experimental evidence which implicates ATX3 and ATX5 histone methylation, transcription and known biological processes.

1.8 Aim of the study

The vital roles played by SET domain genes in regulating development call for experimental work to elucidate their functions. This was therefore part of a larger project, The SET Project in the Arabidopsis group (University of Oslo) which seeks on a wider scope to unravel the functions of selected SET-domain genes both in Arabidopsis and Drosophila. Accordingly, this particular project had the primary goal of generating biological data that will help in unraveling the functions of Arabidopsis Trithorax 3 (ATX3) and ATX5. Current information on these genes does not go beyond bioinformatic predictions and inference which classified them as putative trithorax genes. As such the expression patterns of these genes were investigated, both *in situ*, and *in vitro*, by using the in situ hybridization method and reverse transcription PCR (RT-PCR) respectively. Information from this method reveals the tissues in which ATX3 and ATX5 are expressed and the relative amounts, and can subsequently reveal possible function.

To further reveal biological function this study also aimed at generating knockdown lines with loss or reduction of function of the ATX3 and ATX5 genes *in planta*, followed by observation of phenotypes on such lines. To this goal, RNAi was chosen as the favorite reverse genetic tool in the beginning of the study as no SALK T-DNA knock out lines were available at this time. This involved constructing RNA-interference vector which contained a fragment of the ATX5 with the help of the Gateway® cloning technology for transformation into the Arabidopsis plants. As one SALK T-DNA knock out line for ATX5 and two ATX3 SAIL T-DNA knock out lines later on were available, these were also studied at an introductory level.

Prior knowledge on other SET domain genes shows that their proteins often reside and function in multimeric protein complexes. This therefore prompted the need to find the other protein partners of these genes. Since ATX5 has strong resembland in structure with ATX4, the interacting partner for the ATX4, pulled out from a yeast two hybrid screen (Dietzel et al.

2005), could be putative interacting partner also for ATX5. The GST- (glutathione Sepharose tagged) pull down assay is one way to find out if the ATX4 interacting partners are also interacting partner for ATX5 *in vitro*. Another experiment needed to be done is Histone Methyl Transferase (HMTase) essay to find out if the catalytic site, SET, in ATX5 also has a histone methyl transferase activity. As a result a GST-fusion construct was generated to express a recombinant GST-ATX5 SAC-SET fusion protein to be further used in the above mentioned experiments. The SAC-SET domains spans the SAC (SAC (SET-associated cysteine-rich-SET) and SET domains in of the SET-domain proteins.

2 MATERIALS AND METHODS

2.1 Reverse transcription - PCR (RT-PCR) of ATX5 transcript

2.1.1 mRNA isolation and first-strand cDNA synthesis

In order to verify reduction in mRNA levels in the ATX5_RNAi_T2 lines, total RNA was isolated using the RNeasy Mini Kit (QIAGEN). The SuperScript™III Reverse transcriptase (Invitrogen, Catalog No. 18080-044) was used to synthesize first-strand cDNA from total RNA. In conjunction with PCR, the system can be used to quantify the amount of specific mRNA from a small amount of material.

Tab 1: The line and tissues from which Total RNA was isolated for RT-PCR

Plant	Tissue	Concentration	Amount (in µl) to 2µg
ATX5_RNAi_1	Cauline leaves	810 ng/µl	2.5 µl
ATX5_RNAi_20	flowers	3.1 µg/µl	0.6 µl
ATX5_RNAi_39	Rosette leaves	480 ng/µl	4.2 µl
ATX5_RNAi_74	Rosette leaves	440 ng/µl	4.5 µl
Columbia (Wt)	seedlings	1.3 µg/µl	1.5 µl
Columbia (Wt)	flowers	1.9 µg/µl	1.1 µl
Columbia (Wt)	Rosette leaves	835 ng/µl	2.4 µl
Columbia (Wt)	siliques	600 ng/µl	3.3 µl

For each sample the following components were added to a 1.5 µl nuclease-free microcentrifuge tube: 1 µl Oligo$(dT)_{12\text{-}18}$, 2 µg total (DNase I treated), 1 µl dNTP Mix (10 mM each), sdH_2O to 12 µl. The mixture was heated to 65°C for 5 min and quickly chilled on ice. The contents of the tube were collected by brief centrifugation and the following components added and mixed gently by pipetting up and down: 4 µl 5X First-Strand Buffer and 2 µl 0.1 M DTT. Each reaction was incubated at 42°C for 2 min and 1 µl (50 units) SuperScript™III RT added to each tube. The reaction was again incubated at 42°C for 50 min and finally terminated at 70°C for 15 min. The reaction was quickly chilled on ice and the contents collected by brief centrifugation. As recommended (for targets >1kb), 1 µl (2 units)

of *E. coli* RNaseH was added and incubated at 37°C for 20 min to remove RNA complementary to target cDNA.

2.1.2 PCR

To quantify ATX5 mRNA levels, target cDNA was amplified by PCR using 2 µl each of first-strand cDNA, dNTPs (2 µM), DyNAzyme II DNA Polymerase 10x buffer (10 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM MgCl2, 50 mM KCl, 0.1 % Triton® X-100), and the forward and reverse primers (2µM) and 0.5 µl DyNAzyme™ II DNA Polymerase (2 U/µl). Water was added to make a total volume of 20 µl. Three pairs of primers were used to amplify the cDNA (see appendix for primer sequence):

1. ATX5_1L_mATG and ATX5_5'Race which are specific for the ATX5 gene and use on cDNA from RNAi lines and wt Col.
2. Act2int3_sense and act2int3_antisense as control primers to quantify the level of actin in the cDNA from RNAi lines and wt Col plants.
3. Act2int2_sense and act2int2_antisense as control primers and used on both gDNA and cDNA from RNAi line to show absence of gDNA contamination on ATX5 RNAi lines.

The thermocycler conditions were 95°C for initial denaturation and 35 cycles of 95°C for 30 sec, 56°C for 30sec, 72°C for 30 sec and a final extension temperature of 72°C for 7 min.

2.2 RT-PCR to detect expression pattern of ATX3

RT-PCR was performed to detect tissues in which ATX3 is expressed as well as quantify the mRNA levels in those tissues. All tissues for this experiment were harvested from wild type Columbia plants and total mRNA isolated. The tissues include 2-week old seedlings grown in darkness and under normal conditions, rosette leaves, cauline leaves, flowers, stems, seeds, siliques and roots. The total mRNA was reverse transcribed to single-stranded cDNA and PCR carried out.

First-strand cDNA synthesis was carried out on 2µg of total RNA using SuperScript™II for RT-PCR. Protocol was same as in section **2.1.1.**

2.2.1 PCR to amplify ATX3 cDNA

The polymerase chain reaction was set as follows: 1 µl each of first-strand cDNA, 2 µl of dNTPs (2 µM), DyNAzyme II DNA Polymerase 10x buffer, and 1.5 µl each of the forward and reverse primers (2µM) (see appendix for primer sequence) and 0.5 µl DyNAzyme™ II DNA Polymerase (2 U/µl). Water was added to make a total volume of 20 µl. The primers ATX3 RT_477L and ATX3 RT_1469 reverse used (see appendix for primer sequence). In addition control primers actin 2/7L and actin 2/7R specific for the actin gene (AB016893) were used to amplify the cDNA under similar reaction conditions. The thermocycler conditions were 95°C for initial denaturation and 30 cycles of 95°C for 30 sec, 56°C for 30sec, 72°C for 1min sec and a final extension temperature of 72°C for 10 min. Two samples, each containing genomic DNA and water as templates were also included to serve as both positive and negative control samples.

2.3 In situ mRNA hybridization

Because of the abundance of RNase and the difficulty in inactivating them, RNase-free environment and samples were ensured throughout this experiment. Hand gloves were worn at all times avoiding RNase contamination from hands, bacteria, etc. Non-disposable glassware was baked at 200°C, while plastic ware (e.g eppenedorf tubes) was rinsed thoroughly in 0.1N NaOH/1mM EDTA and diethyl pyrocarbonate (DEPC)-treated water to render them RNase-free. In addition, solutions were treated with DEPC and autoclaved for 30 minutes.

2.3.1 Generation of RNA probes

In situ hybridization was used in localizing the mRNA of ATX3 and 5 within the cytoplasm, by hybridizing the sequence of interest to complementary strands of nucleotide probes. RNA probes were synthesized by *in vitro* transcription of linearised plasmid with promoters for T3, T7 or Sp6 RNA polymerases.

To generate the probes, PCR generated DNA fragments of the genes were cloned into the polylinker site of a transcription vector (pCR®4-TOPO®) which contains a promoter for SP6, T7, and T3 RNA Polymerase (Kassavetis and Geiduschek, 1982; Dunn and Studier, 1983; Studier and Dunn, 1983). The plasmid was subsequently transformed into One Shot® TOP10

Chemically Competent *E. coli* cells, using the TOPO TA Cloning® Kit for Sequencing (with pCR®4-TOPO®) from Invitrogen. The plasmid DNA was purified using the Wizard® DNA Purification system Kit (Promega).

2.3.2 Linearization and precipitation

For antisense and sense probes 2µg plasmid DNA was linearized individually with 2.5µl *NotI* and *SpeI* restriction enzymes, 10µl (10X) restriction buffer, 10µl BSA (Promega), and water to a total volume of 100µl. The restriction mixture was incubated at 37°C overnight and run on gel to verify complete linearization of plasmid.

The linearized plasmid DNA was extracted twice in 100µl RNase-free 1:1 phenol/chloroform (SIGMA®) mixture and thereafter in 100µl fresh chloroform. 10µl RNase-free 3M NaOAc and 250µl fresh 100% ethanol were added and spun for 15 min. The resulting pellet was washed in 70% ethanol (made in DEPC H2O) and spun for 5min at room temperature. The pellet was then dried and resuspended in 5µl DEPC dH2O.

2.3.3 Transcription and DNase-treatment

For ATX5, T3 and T7 polymerases (Promega) were used for generation of the antisense and sense RNA probes respectively. Likewise Sp6 and T7 RNA polymerases (Promega) were used for ATX3 antisense and sense probes respectively. The digoxygenin (DIG)-RNA labeling kit (Roche Applied Science, Indianapolis, Ind.) was used.

The reaction was set up as follows: 5 µl linearized plasmid, (ca 2 ug), 5 µl 5x transcription optimized buffer, 2µl of10x DIG Labeling mix 1 µl RNasin, 1 µl RNA polymerase T3, T7 or Sp6. DEPC-dH2O was added to a volume of 23 µl and incubated for 45 minutes at 37ºC. A second round of transcription was performed by adding 1 µl RNasin and 1 µl polymerase. The reaction was incubated for 45 minutes at 37ºC and the yield of transcript checked on agarose gel. To remove the template DNA 2µl of DNaseI was added and incubated for 10 minutes at 37ºC. These transcribed probes were precipitated by the adding of 75µl DEPC-dH2O, 1 µl tRNA [100mg/ml] (Sigma). Each sample was treated with 100 µl 4M NH_4OAc, 400 µl 100 % ethanol and precipitated overnight at -20ºC. Thereafter, it was spun 20 minutes at 4ºC and pellet washed with cold 70% ethanol, spun again for 5 minutes at 4ºC and resuspended in 50 µl DEPC-dH_2O. To estimate the yield or concentration of transcribed probes, it was run on

ethidium bromide-stained agarose gel and the relative intensity of bands compared with the Mass Ruler® as marker (Fermentas Life Sciences).

The procedure here incorporates one modified nucleotide Digoxigenin-UTP (DIG-UTP) at approximately every 20 – 25th positions in the transcripts. Digoxigenin is a steroid isolated from the blossoms and leaves digitalis plant. DIG is linked by a spacer arm containing 11 carbon atoms to the C-5 position of the uridine nucleotide and can be incorporated into the sequence during transcription.

Concentrated DIG-RNA Labeling Mix contains 10 mM each of ATP, CTP, and GTP; 6.5 mM UTP; 3.5 mM DIG -UTP pH 7.5 (20°C).

2.3.4 Preparation of tissues

2.4.4.1 Fixation of tissue

Freshly harvested tissues of different types were immediately placed in freshly made ice cold fixation solution made of 50% ethanol, 5% acetic acid, 3.7% formaldehyde. The tissues were infiltrated with vacuum at 15-20 Hg using a vacuum regulator (Bio-Rad Laboratories, Inc) for 15 minutes and thereafter incubated at room temperature for 1.5 hrs.

2.3.4.2 Dehydration, Staining and Embedding of Tissues

The tissues were dehydrated in ethanol series and stained with eosin Y and the colour washed away as follows:

50 % Ethanol	30 minutes
60 % Ethanol	30 minutes
70 % Ethanol	30 minutes
80 % Ethanol	30 minutes
*95 % Ethanol with 0,1 % Eosin Y	overnight
100 % Ethanol	30 minutes
75 % Ethanol/25 % Histoclear	30 minutes
50 % Ethanol/50 % Histoclear	30 minutes
25 % Ethanol/75 % Histoclear	30 minutes
100 % Histoclear	3 x 1 hour

To each vial with tissue about 20 paraplast chips were added and incubated at 42ºC over night. More paraplast was added and incubated until all chips melted. Vials were moved to a 55ºC oven where paraplast was changed twice daily with molten paraplast for three days. Tissues with molten paraplast were poured into plastic weigh boats and left to harden. Tissues were carefully oriented before the paraplast hardened to enhance work during sectioning.

Sections of 8µm thick were obtained with a LEICA RM 2165 microtome (Leica Microsystems) and mounted on slides (Probe on Fischer) for hybridization.

2.3.5 Slide treatment and hybridization

Mounted tissues were given pre-hybridization treatments of dewaxing, hydration, dewaxing, washing, dehydration, blocking before they were finally hybridized as outlined below:

Dewax	Histoclear	2x10 minutes
Hydrate	Ethanol series 2x100% 95% 85%/0.85% NaCl 70%/0.85% NaCl 50%/0.85% NaCl 30%/0.85% NaCl 15%/0.85% NaCl 0.85% NaCl DEPC-dH_2O	2 minutes each
Acidify	0,2 M HCl	20 minutes
Wash	DEPC-dH_2O0 PBS DEPC-dH_2O	5 minutes each
*Proteinase K	1ug/ml in TE	30 minutes at 37 °C
Wash	PBS	2 minutes
Glycine Block	2 mg/ml in PBS	2 minutes
Wash	PBS	1 minute
Refix tissue	4 % Paraformaldehyde in PBS, pH 7,0	20 minutes
Dehydrate	Ethanol series 2xDEPC-dH_2O 0.85% NaCl 15%/0.85%NaCl 30%/0.85% NaCl 50%/0.85% NaCl 70%/0.85% NaCl 85%/0.85% NaCl 95% 2x100%	2 minutes each
**Hybridization	6xSSC, 3 % SDS, 50 % Formamide, 100 ug/mltRNA]50-200 ng probe	2 days at 55 °C
Quick wash	0,2xSSC, 0,1 % SDS	
High Stringency Wash	0,2xSSC, 0,1 % SDS	2 x 10 minutes at 55 °C
Wash	2x SSC	2 minutes
RNase treatment	10 ug/ml in 2xSSC	30 minutes at 37 °C
Wash	2xSSC	2 minutes
High Stringency	0,2xSSC, 0,1 % SDS	2 x 10 minutes at 55

Wash		°C
Wash	2xSSC	2 minutes
Rinse	TBS	5 minutes
Blocking	0,5% Blocking agent (DIG-kit) in TBS	2 x 45 minutes
Rinse	TBS	1 minute
***Antibody	anti-DIG AP-coupled diluted 1:1000 in 0,5% BSA in TBS	2 hours
Rinse	TBS	3 x 5 minutes
Detection pre-incubation	10 mM Tris pH 9.5, 10 mM NaCl, 50 mM $MgCl_2$	5 minutes
Detection	****Western Blue solution with 1 mM *****Levamisol	2-6 days in the dark

*The treatment of tissues with **proteinase K** helps to remove protein that may bind to the target and hence accessibility to target RNA is enhanced.
**Slides are covered with another glass and tissues sandwiched between the two slides. The probes, (both antisense and sense strands) are applied between the glass slides in concentrations of 50, 100, 150, and 200 ng.
***__Anti-DIG AP__: an anti-digoxigenin antibody from sheep, conjugated with alkaline phosphatase (AP) which shows 100 % reactivity with digoxigenin
****__Western Blue__: staining agent
*****__Levamisol__: reduces background staining when added to western blue

2.4 Use of the Gateway® Cloning Technology and RNAi to generate knockdown lines

For functional studies of ATX5 a selected gene fragment was amplified by PCR and cloned into the pHELLSGATE8 vector as inverse repeats, using the well-advanced GATEWAY® Cloning Technology (Invitrogen, Corporation). Agrobacterium mediated transformation was employed to deliver the T-DNA with the inverse repeats downstream of the 35SL promoter into the *A. thaliana* wild type Columbia plants (Clough and Bent, 1998). This allowed for the generation of double-stranded RNA, which serves as a trigger or inducer for RNA interference (RNAi). The combination of the GATEWAY® cloning technology and its compatible vectors has been found to provide a fast and efficient route for expression of hairpin RNA (hpRNA), which has been found to be a more potent inducer of RNAi (Wesley et al., 2001).

2.4.1 Choice of and amplification of gene fragment for RNAi

By multiple alignments of the SET domain genes, a 578 bp fragment not encompassing the SET domain and other homologous regions of the ATX5 gene was selected and amplified by PCR. The aim was to avoid cross-homology silencing, since the mechanism of RNAi silencing depends on sequence homology. Since the recommended length of RNAi fragment

is 300-600bp, the selected fragment was limited to 578 bp to increase the efficiency of silencing. The attB sequences were added to the 5'-terminal of both primers since they are obligatory features of the Gateway® cloning Technology.

Polymerase chain reaction (PCR) was performed using 1µl of first–strand cDNA from seedlings, 0.5µl of *Pfu* DNA Polymerase, 2 µl of Pfu DNA Polymerase 10X Reaction Buffer with $MgSO_4$ (Promega), 2µl dNTPs (2µM of each dNTP) and 1µl each of the primers ATX5_attb1 and ATX5_attB2 (see appendix for sequence). Finally 12.5µl of water was added to a total volume of 20µl. Thermocycler conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 2 mins, and a final synthesis step at 72°C for 7 min. To generate more PCR products, 1µl of the PCR was used as template for another round of PCR, under the same conditions above. The PCR product was run in 1.0% agarose gels stained with ethidium bromide, and the DNA recovered by excising it from gel. The DNA was then purified from the gel using the Wizard® SV Gel and PCR Clean-Up System (Promega). The attB-PCR product was also sequenced to verify the sequence of the fragment before the Gateway® recombination reactions were performed.

2.4.2 The Gateway® Recombination reactions

2.1.2.1 The BP reaction

The attB-PCR product flanked by the attB1 and attB2 recombination sites was cloned into the donor vector (pDONR/zeo), by using a modified version of the manufacturer's (Invitrogen Corporation) instruction. This included the addition of the following components in a 1.5 ml microcentrifuge tube at room temperature: 1µl attB-PCR product, 2 µl pDONR™/zeo donor vector (150 ng/µl), 4 µl 5X BP Clonase™ Reaction Buffer, and TE Buffer (pH 8.0) to a total volume of 16 µl. 4 µl of the BP Clonase™ enzyme mix was added and the cloning reaction incubated at 25°C overnight. Finally, 2µl of Proteinase K solution was added to each reaction and incubated for 10 minutes at 37°C. Competent cells (DH5α) were subsequently transformed with the BP reaction mix and right transformants selected on zeocin (100mg/ml) LB medium. Colonies of transformed cells were picked and grown in selective LB liquid medium with zeocin (100mg/ml). The plasmid DNA (now entry clone) was purified using the

Wizard® Plus DNA Purification System (Promega) and used for the LR recombination reaction.

2.4.2.2 The LR reaction

A modified version of the manufacturer's (Invitrogen) standard protocol was also used to immobilize the RNAi fragment in pDONR/zeo into pHELLSGETE8 (destination vector, this time into two insertion sites. This included 3.2µl of pDONR/zeo, 1.1µl (165 ng/reaction) of pHELLSGATE8 destination vector, 3µl 5X LR Clonase™ Reaction Buffer and TE Buffer, pH 8.0 to 16µl. 3µl of LR Clonase™ enzyme mix was added and the reaction incubated at 25°C overnight. Proteinase K solution (2µl) was also added to each reaction and incubated for 10 minutes at 37°C.

The Proteinase K treatment is necessary to remove proteins from the BP clonase mix. This increases the transformation efficiency, resulting in a larger number of colonies. Proteins bind to DNA and this can interfere with the uptake of DNA by the cells during transformation.

2.4.3 Transformation of DH5α with BP and LR recombination reactions

In both transformations, 1µl of the recombination reaction (BP and LR) was added to aliquots of 50µl Library Efficiency® DH5α ™ competent cells in 1.5ml sterile eppendorf tubes and mixed gently, followed by incubation on ice for 30 minutes and heat-shocking of the cells for 30 seconds at 42°C, without shaking. The tubes were immediately transferred to ice and 450 µl of room temperature S.O.C. medium added and mixed gently by stirring with pipette tips. Tightly capped tubes were incubated with horizontal shaking (200 rpm) at 37°C for 1 hour. Amounts of 20µl and 100µl of transformations were spread on a prewarmed zeocin (100mg/ml) and spectinomycin Luria-Bertani (LB) selective plates (for BP and LR recombination reactions respectively) and incubated overnight at 37°C. Transformants from each recombination reaction were picked and grown in liquid media with appropriate selective antibiotic. Again plasmid was purified after each transformation using The Wizard® DNA Purification System Kit (Promega).

2.4.4 PCR to confirm presence of ATX5 RNAi fragment at both insertion sites in pHELLSGATE8

Before the transformation of Agrobacterium cells were with the pHELLSGATE8 vector, PCR analysis was carried out on the purified pHELLSGATE8 plasmid, by adding the following to a PCR tube: 1µl of purified plasmid DNA, 2µl of 2µM of dNTPs, 1µl each of forward and reverse primers, 2µl of 2 µM dNTPs DyNAzyme II DNA Polymerase 10x buffer and 0.5µl of DyNAzyme™ II DNA Polymerase (2 U/µl). Water was added to make a reaction volume of 20µl. Thermocycler conditions were 95°C for initial denaturation and 35 cycles of 95°C for 30 sec, 56°C for 30sec, 72°C for 30 sec and a final extension temperature of 72°C for 7 min. The primers 35SL and HU (vector specific) were each combined with AtTRX 2075 5'Race (gene specific) to form two different pairs of primers each pair to verify the presence of each fragment (See figure 13 for primers and their annealing sites).

2.4.5 Transformation of A. thaliana using the floral dip method

The *A. thaliana* wild type Columbia plants to be transformed were grown in pots until they begun to flower. To obtain more floral parts per plant, their inflorescences were pruned when most plants had formed primary buds to relieve apical dominance and encourage synchronized emergence of multiple bolts (Clough and Bent, 1998).

To mobilize the transgene from pHELLSGATE8 into the Arabidopsis cells, 40 µl of *Agrobacterium tumefaciens* (strain C58 pGV2260) cells were mixed in an eppendorf tube and transferred to cold and sterile cuvettes. The cells and DNA mixture was then electroporated with a pulse of 2.5kV at 25µF and 200Ω. The cells were resuspended in 1ml of SOC medium and incubated on a shaker for 1 hour at 28ºC. Different volumes of the transformed cells were plated on YEB medium containing 100 mg/l each of the antibiotics spectinomycin, carbenicillin and rifampicin (for pHELLSGATE8, Ti-plasmid and Agrobacterium respectively). The plates were sealed and incubated at 28°C room for 4 days as *A. tumefaciens* grows slowly. Resulting colonies from transformed cells were picked and grown at 28°C 5ml liquid YEB medium containing 100mg/ml each of spectinomycin, carbenicillin and rifampicin. To obtain active and vibrant cells for transformation, 1.5ml of each culture was added to 390ml of new YEB medium and allowed to grow to an OD of 2.0 before they were harvested by centrifugation. The concentrated cells were diluted with 5% sucrose solution to OD600=8.0. Silwet L-77 (as surfactant) was added to a concentration of 0.002% (20µl/L) in a total volume of 670ml, and mixed well.

When there were numerous unopened floral buds but few open floral buds on the previously pruned wild type Columbia plants, their above-ground parts were dipped into the transformation solution for 3 minutes. The plants were then covered and placed away from light for 3 days but brought back to normal growth conditions thereafter. This was aimed at maintaining moisture and humidity long enough for bacterial cells to attach to the plants. The Agrobacterium cells successfully transferred the T-DNA into reproductive cells and hence some of the seeds (T1). Seeds were grown on sterile MS-2 kanamycin (50mg/l) selective medium and transformants selected with the help of the kanamycin resistance gene which sits on the T-DNA.

2.5 Analysis of T-DNA Insertion Lines

ATX3 and ATX5 T-DNA insertion lines [SALK 831182(SAIL705-H05) and SALK 811973 (SAIL25S_F11) respectively] obtained from SALK Institute of Genomic Analysis Laboratory (http://signal.salk.edu/) were grown and observed for abnormal phenotypes (see appendix for plant growth conditions). At the same time, genomic DNA was isolated and PCR conducted to identify homozygous, heterozygous and wild type plants. For ATX3 the T-DNA insertion was supposedly in the promoter while it was in exon 1 of the ATX5 gene.

Genomic DNA was isolated, from tissues of the T-DNA lines using the Quantum Prep Aquapure Genomic DNA Isolation Kit (Bio-Rad Laboratories).

PCR was conducted as follows: 1.5µl of genomic DNA, 2µl (2µM) of dNTPs, 1µl each of forward and reverse primers, 2µl of 2 µM dNTPs DyNAzyme II DNA Polymerase 10x buffer and 0.5µl of DyNAzyme™ II DNA Polymerase (2 U/µl). Water was added to a total reaction volume of 20µl. Two primer sets were used for each gene in the PCR (see appendix for primer sequence).

For ATX5 the following primers were used:

Primer set 1: ATX5_SAIL_705_H05_LP + ATX5_SAIL_705_H05_RP for detection of wild type or heterozygous lines. Both primers are gene – specific and are left border and right border genomic primers with annealing sites that flank the T-DNA insert. PCR conditions allow for amplification only in wild type Arabidopsis but not in those with T-DNA, due to increased length of target DNA.

Primer set 2: ATX5_SAIL-705_H05_RP + LBb1 for detection of homozygous or heterozygous lines. LBb1 is T-DNA specific. Hence this primer pair can only amplify target DNA with T-DNA.

For ATX3 the following primers were used:
Primer set 1: ATX3_SAIL_25S_F11_LP + ATX3_SAIL_25S_F11_RP
Primer set 2: ATX3_SAIL_25S_F11_RP+LBb1

Therefore if PCR produces products in any line with both primers sets, it means that line is heterozygous for the insertion and contains T-DNA on one allele of the gene. Lines in which products are obtained with only primer set 2 are homozygous and contain T-DNA on both alleles. However, lines in which product was obtained with only primer set 1 had no T-DNA on both alleles and considered well type.

Thermocycler conditions were 4 min of 95°C for initial denaturation and 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min and a final extension temperature of 72°C for 7 min.

2.6 Generation of GST-fusion protein for Pull down Assay and histone methyl transferase (HMTase)

The ATX5 SAC-SET-domain was mobilized into an expression vector (by Gateway® cloning) and the expression of the ATX5 SAC-SET domain induced and used as bait to identify other proteins that interact physically with ATX5. Potential preys for this experiment are proteins that have been shown to interact with the ATX4 protein. ATX4 shares 85% identity with ATX5. The recombinant ATX5 SAC-SET protein will also be used to to test for histone methyl transferase activity of ATX5.

2.6.1 Amplification of ATX5 SAC-SET domain by PCR

Template for PCR was full-length cDNA purchased from RIKEN Plant Science Center (http://www.psc.riken.go.jp). PCR was carried out as follows: 1µl of template DNA, 5 µl of 2 µM dNTPs, 2.5 µl each of 2µM forward and reverse primers, 5µl of *Pfu* DNA Polymerase 10X Reaction Buffer with $MgSO_4$, and 0.5µl of Pfu DNA polymerase (Promega) 33.5 µl of dH2O was added to give a total reaction volume of 50 µl.

Thermocycler conditions were as follows: 95°C of initial denaturation for 3 min, and 35 cycles of 95°C for 0.5 min, 56°C for 0.5min, 72°C for 4 min, and a final extension temperature of 72°C for 5 min. Forward primer: ATX5_SAC-SET_attB1. Reverse primer: ATX5 SAC-SET_attB2 mstop (see appendix for primer sequence). NB! The Gateway ® Technology was selected to clone the SAC-SET fragment into the expression vector and therefore the att sites were added to the 5' end of each primer.

2.6.2 Generation of GST-fusion construct using the Gateway® cloning technology

Modified versions of the BP and LR reactions protocols of Invitrogen were used. With the BP reaction, 2.5 µl of the PCR-generated SAC-SET fragment was cloned into the pDONR/zeo plasmid vector (donor vector). The resulting clones were used to transform DH5α cells and the correct clones (now entry clones) selected on zeocin (100mg/L) selective medium. Entry clones were purified by the Wizard® DNA Purification system Kit (Promega) and used in the LR reaction to transfer the ATX5 SAC-SET domain into pGEX-GAW, an expression clone. Resulting clones were also used to transform DH5α cells and the right clones selected on ampicilin (100mg/L) selection medium. These right clones were also purified as above.

In addition to selection on selective medium, PCR analyses were conducted on the purified plasmids after both the BP and LR reactions to confirm the presence of the SAC-SET domain in both vectors.

2.6.3 Transformation of BL21 cells with the GST-Fusion construct

Transformation by electroporation was chosen for this purpose. The BL21 cells (25µl) were thawed gently on ice in a 1.5 ml eppendorf tube. Sterile cuvette was also placed on ice prior to electroporation. After thawing, 2µl of the purified pGEX-GAW/ATX5 SAC-SET construct was added to the 25µl BL21 cells**.** This was mixed gently and placed on ice for 1min.The cells were then transferred to the cold cuvette and electroporated for 5 milliseconds under the following conditions: 25 mF, 200W and 1.3kV. The cells in the cuvette were resuspended with 1ml of liquid SOC medium immediately using a pipette. The cells were again transferred to a new and sterile 1.5 ml eppendorf tube for incubation at 37ºC for 60min. Cells were then plated out on LB medium containing 100mg/L ampicilin.

2.6.4 Induction of the ATX5 SAC-SET domain in BL21 cells

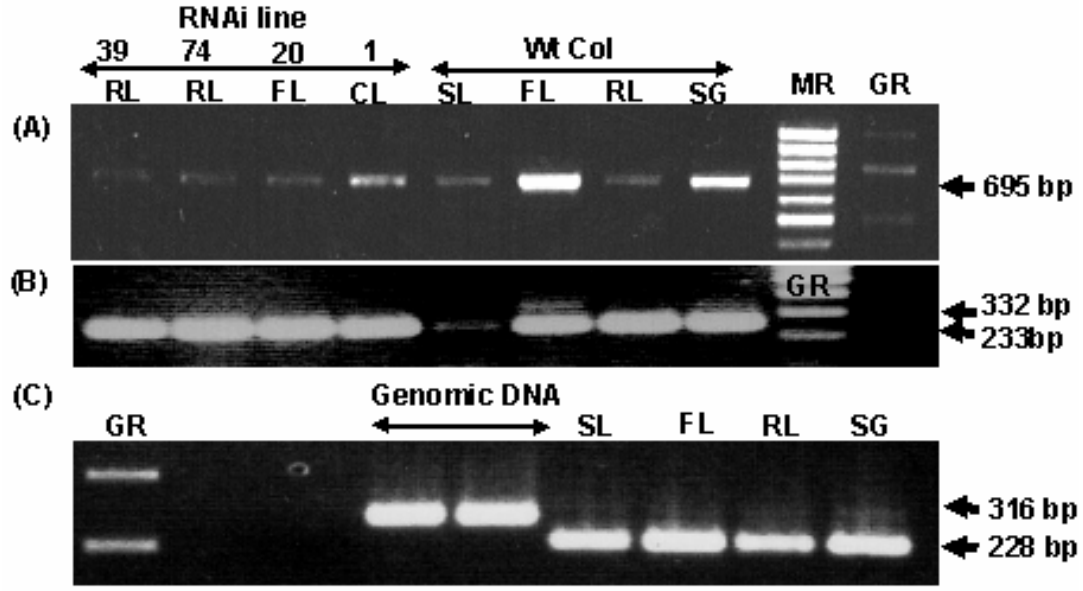
This was a test experiment to determine if the ATX5 protein will be expressed and in the right size before a larger volume of the BL21 culture could be set for expression and purification of the protein. Four (4) colonies from the ampicilin LB plates were picked and grown in 5 mL liquid LB medium containing 100mg/L ampicilin at 37ºC for 5hrs with shaking. Each culture was divided into equal halves, giving two batches (2.5 ml each). From each of the 2.5mL culture one batch was induced with 2.5µl 100mM IPTG (isopropyl-β-D-thiogalactopyranoside). Both batches were then incubated at 30ºC for 30 min with shaking to express the protein. From each culture (both batches) 20µl of cells were mixed with 5mL 4X SDS-loading buffer and denatured at 95ºC for 5min. The denatured cells were spinned down briefly and loaded in SDS-PAGE gel (running and stacking gels). The gel was run at 200V until loading buffer completely ran out of gel into the running buffer.

The gel was stained by immersing it in 0.1% Coomassie Blue with shaking for 20 min for the detection of protein bands. The gel was rinsed in dH_2O and visualised with UV light.

3 RESULTS

3.1 RT-PCR to verify reduction of mRNA levels in ATX5 RNAi lines

This semi-quantitative experiment was aimed first and foremost to verify the reduction of ATX5 mRNA in some selected T2 RNAi plants. Due to the smaller size of the RNAi lines, it was difficult to collect tissues from them for this experiment. Hence RT-PCR was performed to compare mRNA levels of the ATX5 in both RNAi plants and wild type plants (Fig.9A). In addition mRNA levels in wild type plants also threw some light on the expression pattern of ATX5. Transcript of ATX5 was detected in all the tissues that were selected from the RNAi plants. Rosette leaves from 2 selected RNAi plants showed a small reduction in the mRNA levels as compared to the levels in wild type rosette leaves.



Figures 9: RT-PCR to quantify mRNA levels in selected ATX5_RNAi lines as well as in wt Columbia tissues. (A) Contains RT-PCR products for from cDNA of both ATX5_RNAi_lines_39, 74, 20 and 1 and wt Columbia cDNA with ATX5 specific primers. (B) Contains products from amplification of cDNA of RNAi lines and wt Col plants with the control primers act2int3_sense and act2int3_antisense which are specific for the actin gene AB016893. (C) Contains products from amplification of both cDNA of RNAi lines and genomic DNA (with intron) using control primers act2int2_sense and act2int2_antisense for the actin gene. Larger band in genomic DNA verifies the absence of genomic DNA contamination in cDNA. (RL=Rosette leaves, FL=Flowers, CL=cauline leaves, SL=siliques, SG=seedlings, MR=Mass Ruler, GR=Gene Ruler).

However a significant reduction of ATX5 transcript was noticed in flowers from one RNAi mutant plant relative to level in wild type flowers. A very weak band can be seen for gDNA in the actin control. However there was no gDNA band for ATX5 observed which was expected to be 2045 bp.

Considering the expression pattern of ATX5 in only wild type plants, there was a significantly higher level of its mRNA in flowers than in siliques, rosette leaves, and seedlings. ATX5 mRNA levels in both siliques and rosette leaves were comparable, but higher in seedlings. When control primers for the actin gene AB016893 (gene bank) were used on genomic DNA from wild type plants and cDNA of RNAi plants (Fig.9C) larger product was obtained with the genomic DNA which had an intron between exons 2 and 3. This confirmed the absence of genomic DNA contamination in the cDNA.

3.2 ATX3 expression pattern revealed by RT-PCR

The RT-PCR detected ATX3 transcript in 2 week old seedlings (grown under normal conditions), flowers, rosette leaves, stems, seeds roots and siliques of wild type Arabidopsis plants. There was no transcript detected in 2 week old seedlings grown in darkness, as well as in cauline leaves and floral buds.

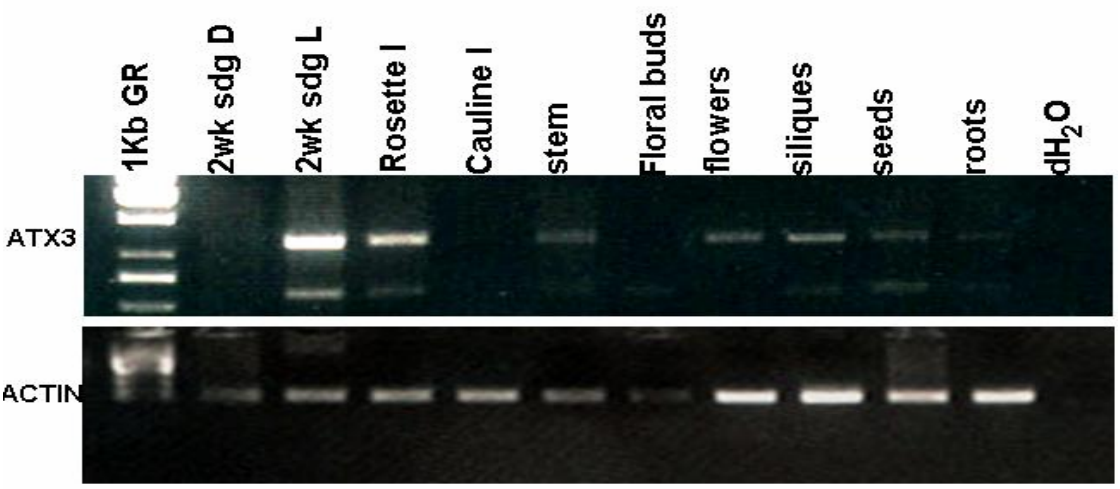


Figure 10: ATX3 mRNA detected in various tissues by RT-PCR. RT-PCR products visualized by agarose gel electrophoresis on 1% agarose gel stained with ethidium bromide. 1kb GR = 1kb Gene Ruler, 2wk sdg D/L = 2wk seedlings grown in darkness and in light respectively.

The amount of transcript varied in the various tissues in which ATX3 was detected. There was a relatively higher amount of ATX3 transcript in tissues of 2 week old seedlings grown under normal conditions than in all other tissues. There was also a significant amount of mRNA in the rosette leaves and siliques, but very low levels in stems and comparable to levels in seeds and flowers. The mRNA level in the roots was barely detectable.

3.3 Expression pattern of ATX3 and ATX5 using in situ hybridization

Knowledge of the expression pattern of a gene throws is very crucial in unraveling the role a gene plays and as such, *in situ* hybridization was performed to determine the specific tissues in which ATX3 and ATX5 is expressed.

3.3.1 Production of the DIG -non-radioactively labeled RNA probe

PCR-generated fragments meant to serve as probes for hybridization were successfully cloned into the pCR4 TOPO plasmid vector and confirmed with restriction analysis, using *Eco*RI restriction enzyme. *Eco*RI sites flank both ends of the polylinker and the resulting fragments were approximately 992bp and 695bp for ATX3 and ATX5 respectively, same as the probe lengths. The restriction enzymes *Spec*I and *Not*I were used to completely linearize 20µg of the plasmid DNA. Since *Spec*I and *Not*I restriction sites precede the transcription start sites (TSS) for T3, T7, and Sp6 RNA polymerases, *in vitro* transcription with T3 and T7 RNA polymerases produced both sense and antisense RNA probes respectively for ATX5 (Fig.11) whiles Sp6 and T7 transcribed the sense and antisense probes respectively, for ATX3 (data not shown). As expected, the transcripts appeared as smears when ran on 1% agarose gel stained with ethibium bromide, indicating a successful transcription of the probes.

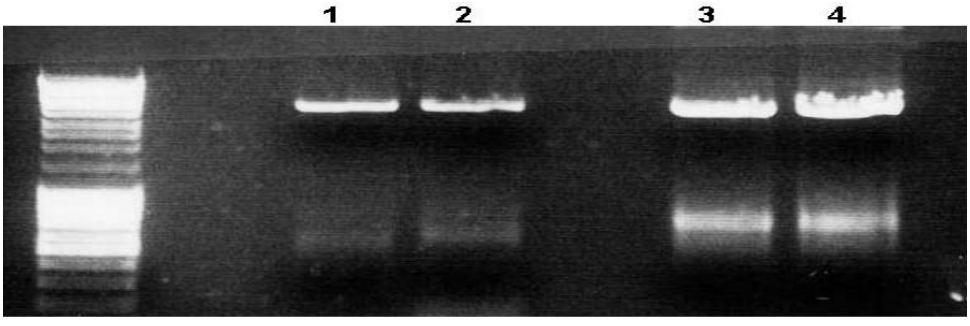


Figure 11: *in vitro* transcription of RNA sense (1and 2) and antisense (3 and 4) probes for ATX5 visualised by electrophoresis on 1% agarose gel stained with ethidium bromide.

3.3.2 Hybridization of RNA probe with ATX5 mRNA in wild type Columbia tissues

The antisense and sense strands of each gene were hybridized individually to mRNA from wild type Columbia tissues, *in situ*. The sense RNA probe which will not usually bind to mRNA served as control and helped in the identification of background signals that may result from non-specific binding. The antisense RNA probes however, which hybridize specifically to mRNA due to base complementarities helped in determining tissues in which the both ATX3 and ATX5 gene were expressed.

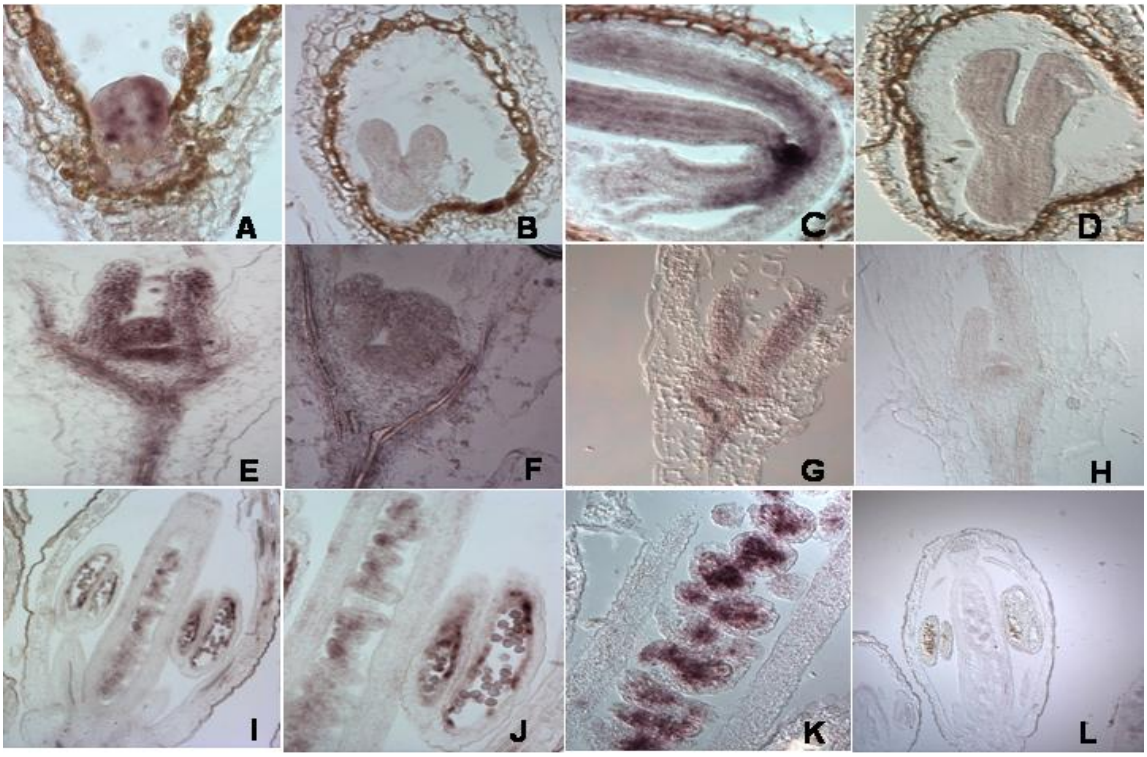


Figure 12: in situ hybridization showing expression pattern of ATX5 in wild type Columbia embryo (A-C), seedlings (E-G), in stamens and ovules of fruits (I-K) with antisense probe. D, E, and L show no detection of mRNA with sense probes.

Two rounds of hybridization carried out by Barbro Sæther (courtesy of Norwegian Arabidopsis Research Council, NARC) on two different occasions localized ATX5 transcript in the shoot apical meristem (SAM) of embryos, seedlings as well as in the male and female floral organs.

ATX5 transcript was also found to localize in the root apical meristem (RAM). In the SAM of seedlings, ATX5 was expressed mainly in the central zone (CZ) and peripheral zone (PZ) and within some regions of the rib zone (RB) (Fig.12 E-G).

ATX5 was found to be expressed as early as the triangular and globular stages of embryogenesis, though at a relatively lower level (Fig.12A).

However ATX5 was barely detectable at the heart stage (Fig.12C), yet found to be specifically expressed later in embryogenesis; in the SAM of torpedo and mature stage embryos (Fig.12B). ATX5 transcript was also localized in the root apical meristem of the torpedo and mature stage embryos. The ovules, pollen grain and tapetum of the anthers of flowers (Fig.12I-K) were also sites at which hybridization signals for ATX5 were also detected.

3.3.3 Hybridization of RNA probe with ATX3 mRNA in wild type Columbia tissues

A signficant ATX3 expression was observed in the tapetum layer in the anthers of the wild type flower from early stage until the tapetum layer was degraded (figure 10).

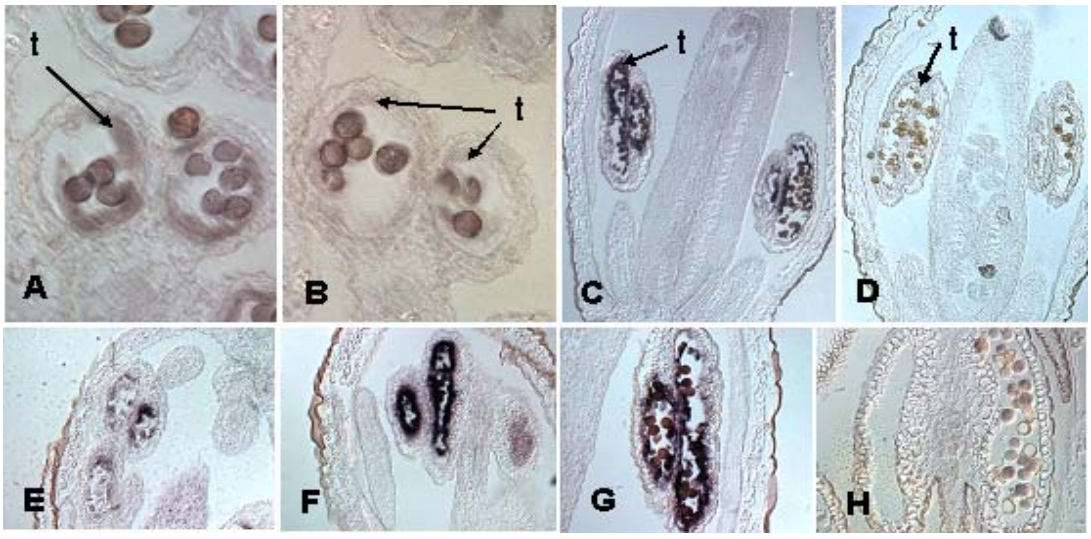


Figure 10: In situ hybridization showing expression pattern of ATX3 mRNA in wild type Columbia tapetum (t) at early stage A and E, at later stage C, E and F. No detection of mRNA is shown at later stage when tapetum is degraed. B and D show no detection of mRNA with sense probes.

3. 4 Generation of ATX5 RNAi lines

3. 4. 1 Cloning of ATX5 attB RNAi fragment into Gateway vectors

With the Gateway® cloning technology the selected gene fragment was cloned into the entry vector (using BP reaction) and subsequently transferred into the destination vector (using LR reaction). The Zeocin™ resistance and the *ccd*B genes in pDONR™/zeo allowed for the selection of the right *E. coli* transformants as large and white colonies after the BP reaction.

Negative selection by the *ccd*B ensured that no *E. coli* cell survived if even it picked relegate vectors. The purified plasmid DNA was subjected to both restriction and sequencing analysis to further confirm the presence and correct orientation of the ATX5 gene insert. With the location XhoI restriction site flanking the attachment (att) sites, digestion of the pDONR™/zeo with *Xho*I gave rise to expected fragments size (data not shown).

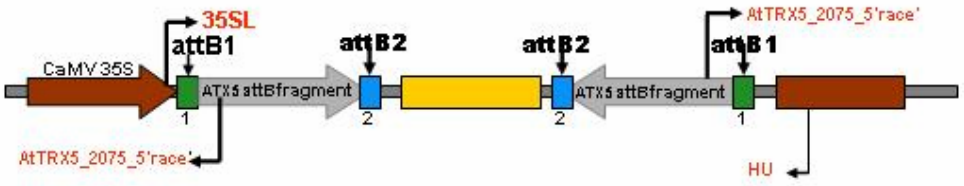


Figure 13: The pHELLSGATE8 vector with ATX5 RNAi fragment showing annealing sites of primers (red) and other features.

PCR analysis confirmed the presence of the attB gene fragments (Fig. 14) in inverse repeat at both insertion sites in the pHELLSGATE8 vector; an indication of successful LR reaction. The primers 35SL, HU (vector specific primers) were each combined with AtTRX 2075 5'Race (gene-specific primers) for each insert. Their annealing sites are indicated in figure 13. A combination of 35SL and AtTRX 2075 5'Race produced a PCR product of 215 while HU and AtTRX 2075 5'Race gave a product of 673bp.

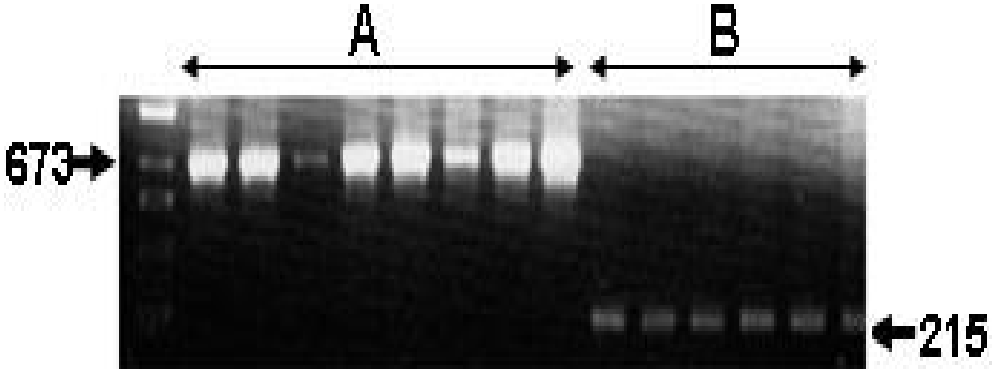


Figure 14: Agarose gel electrophoresis of PCR product to verify presence of the ATX5 RNAi fragment in pHELLSGATE8 vector. Bands in A are products of primer pair 35SL and AtTRX5_2075_5'race whiles bands in B are from products of primer set HU and AtTRX5_2075_5'race.

3.4.2 Agrobacterium transformation and selection of transformed seedlings

Before the *A. thaliana* ecotype Columbia wild type (wt) were transformed using the floral dip-method, the Agrobacterium cells were grown on both solid and liquid YEB medium (which contained carbenicillin, rifampicin and spectinomycin). The antibiotic selection marker genes

for carbenicillin, rifampicin and spectinomycin reside on the Ti-plasmid vector, Agrobacterium and the pHELLSGATE8 vector respectively. The ability of some of the Agrobacterium cells to grow on both solid and liquid YEB media, confirmed that the Agrobacterium cells had indeed taken up the correct vectors. Therefore, such cells were used for transformation of Columbia wt plants. About 77 transformants were recovered from about a thousand 1000 seeds of 20 transformed individual plants. The neomycin phosphotransferase II (NPT II) selection marker gene on the pHELLSGATE8 vector (confers kanamycin resistance) enabled transformed seedlings to be identified. These transformants were selected at the 6-leaf-stage as those with green leaves and well-established roots within the selective medium.

3.4.3 Phenotypes of RNAi 'knockdown' lines

These transformants were transplanted into plastic pots and observed for abnormal phenotypes until maturity. All T1 transformants showed a trend towards late flowering (data not shown). However, 77 of the transformants representing about 88% displayed no other distinguishable phenotypes from wild type plants. The rest of the plants, 9 in all, had a plethora of abnormal phenotypes with varying degrees of severity and considered to be lines in which there was efficient silencing of the ATX5 gene (by the RNAi mechanism). These included vegetative defects such as abnormal phyllotaxy, stunted growth and smaller plant structure. Reproductive defects included late flowering, unopened floral buds, shorter stamen filaments. In addition fertility was adversly affected, resulting in infertile 'knock down' as well as those reduced of fertility and irregularly-shaped siliques.

Unlike wild type Arabidopsis plants with spiral phyllotaxy, some ATX5 RNAi lines had 2 -3 branches or flowers in opposite arrangement or decussate phyllotaxy at some nodes along the stem. Their flowers or siliques were also irregularly spaced along the main stem and auxiliary branches (data not shown). In addition, the mutants of these silenced lines were relatively smaller in stature; shorter in height and having less branches and smaller leaves (Fig.15A-D).

Detailed observation of all the ATX5 RNAi lines also revealed that they had shorter stamen filaments on flowers of the silenced lines. This limited the ability of the stamens to deposit pollen on the stigmatic papillae of the gynoecia (Fig.15E and G). This in turn resulted in a marked reduction of fertility as well as instances of complete loss of fertility in two of the ATX5RNAi mutants. The siliques of those T1 mutants that produced seeds were also smaller

in size as compared to wild type Columbia plants. They were also bent and distorted in form (Fig.15 I).

Table2: summary of phenotypes observed in T1 and T2 generations of ATX5 RNAi lines.

Line	T1 phenotype	T2 phenotype
ATX5_RNAi_11	-smaller structure -unopened flowers -shorter filaments - infertile: seedless siliques	- *
ATX5_RNAi_14	-smaller plant structure -infertile: seedless siliques	- *
ATX5_RNAi_20	-smaller plant structure -shorter filaments and unopened flowers	-smaller siliques but generally normal
ATX5_RNAi_28	-smaller plant structure -shorter filaments -shorter filaments	-smaller plant structure
ATX5_RNAi_39	-smaller plant structure -numerous rosette leaves at base of stem -clustered flowers -fewer siliques and seeds -phyllotactic defects	-*normal
ATX5_RNAi_41	-smaller plant structure -reduced fertility - smaller leaves, late flowering	-smaller plant structure
ATX5_RNAi_46	-smaller plant structure -reduced fertility	-smaller plant structure
ATX5_RNAi_61	-smaller plant structure -reduced fertility	-phyllotactic defects
ATX5_RNAi_74	-small plant structure -reduced fertility	-normal

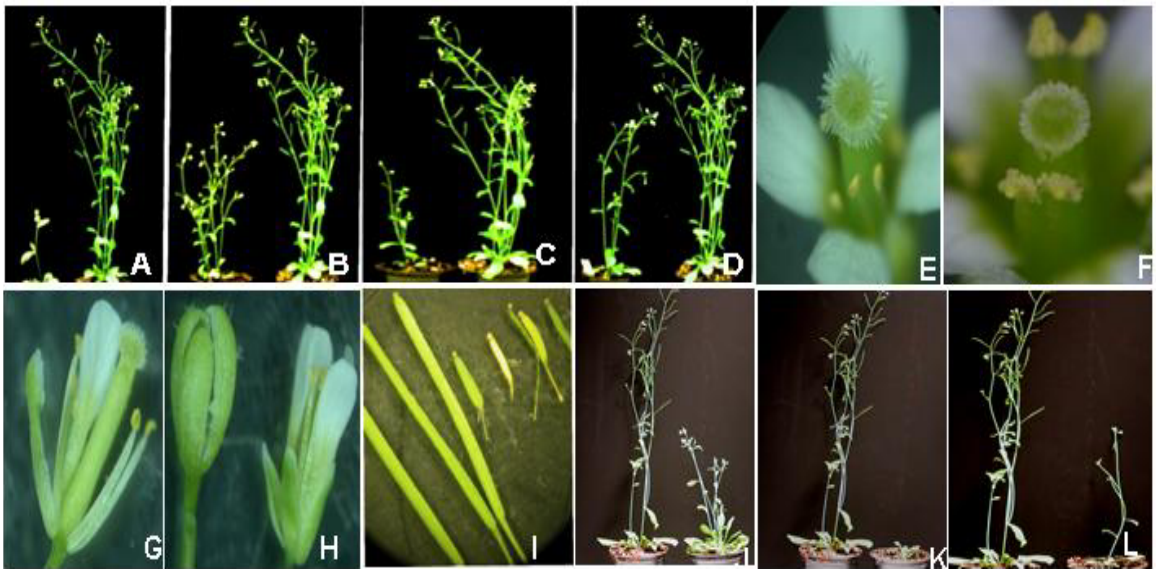


Figure 15: Defects observed ATX5 RNAi lines at the vegetative and reproductive phases. A-D shows RNAi lines (to left) that are relatively smaller (ATX5_ RNAi_61, 41, 28, 20) as compared to wild type Columbia (to right) of same age. E and G show flowers with shorter stamens whiles F shows a wild type flower which is opened and contains well developed stamens. H shows an unopened wt flower while I shows smaller siliques as compared to longer wild type siliques. J- L show some T2 plants of the ATX5 RNAi lines (to right) which are smaller (ATX5_RNAi_46, 11, 20) compared to Wt (to left).

In one extreme line (ATX5_RNAi_39), the plant was not only smallish is structure, but possessed other unique defects in the T1 generation as well. This line exhibited a bushy appearance due to precocious growth of rosette leaves at the base of the stem (Fig.16A). In his line, pin-like structures characterized the initiation of the reproductive phase. These structures eventually formed a cluster of floral bolts that emerged from the same point. This was followed by a later elongation of the structures that gave rise to solitary flowers.

Except for isolated instances of smaller plant and silique size (Fig.15 J-L) and phyllotactic defects on some few individual plants within certain lines there was no recurrence of the observed abnormal phenotypes in ATX5 T1 lines on the progeny (T2) of all the RNAi lines. Thus they were to a large extent indistinguishable from wild type Columbia plants and in strong contrast to other experiments which showed stable and heritable silencing with RNAi using similar constructs (Chuang and Meyerowitz, 2000).

Meanwhile the survival rate for 9 of the 10 T2 lines varied between 75% and 100%. Such high survival rates indirectly indicate the T1 lines carried more than a single copy of the

RNAi fragment, but does that suggest their genotypes. Southern analysis was therefore carried out to reveal the copy number of the RNAi fragment in each RNAi line but failed, mainly due to technical reasons.

Table3: Survival rate for seedlings of ATX5 RNAi lines on MS-2 selective medium containing kanamycin.

RNAi Line	**Number sown**	**Dead(white+brown)**	**No. survived (green on kanamycin MS- 2 medium**	**Survival (%)**
1	73	18	55	75.3
10	61	13	48	78.7
22	114	14	96	84.2
28	91	2	89	97.8
61	7	none	7	100
39	17	3	14	82.3
41	14	10	4	28.6
46	90	1	89	98.9
40	87	none	89	100
74	89	none	89	100

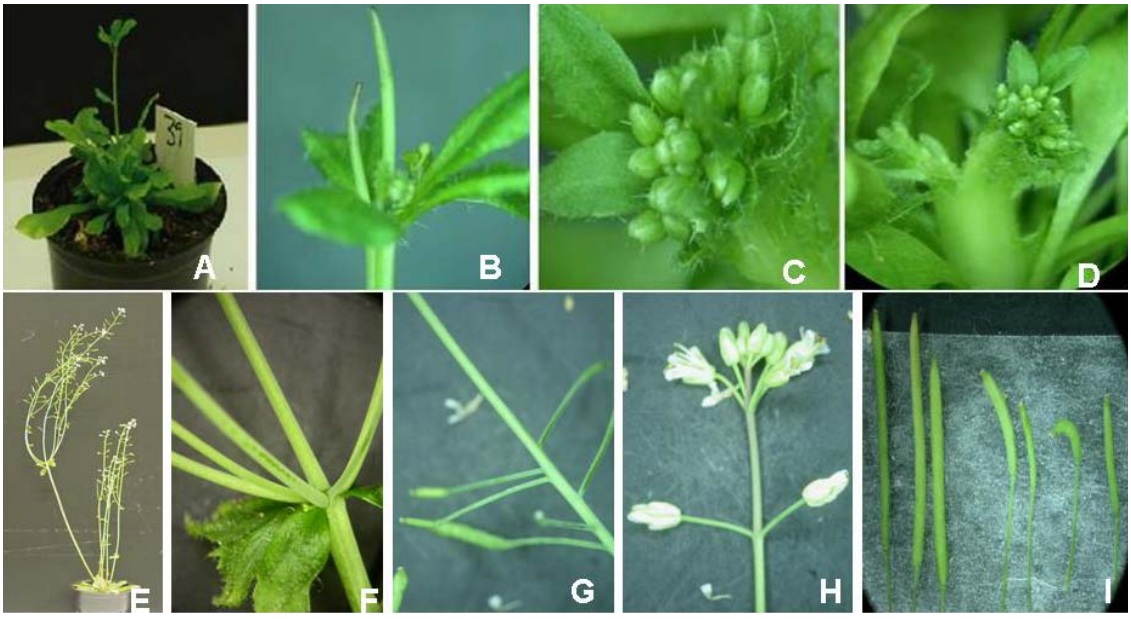


Figure 16: Abnormal phenotypes on ATX5_RNAi_39 (A-D) and hybrid plants (E-H). Line ATX5_RNAi_39 showing enhanced leaf production at the base (A) and pin-like structures that eventually produced enlarged floral meristems (B-D). Hybrid of ATX5_RNAi_11 and Columbia with aerial rosette leaves in addition to the basal rosette leaves, enhanced branching (E and F), irregular arrangement of flowers and siliques on branches (G and H), as well as smaller and bent siliques (I).

The infertile lines were able to produce seeds when pollen grains from wild type Columbia was used to pollinate them (ATX5_RNAi_11 and 14), indicating that the infertility observed in those plants resulted from the male organs. This suggests a role of ATX5 in male fertility. All hybrid (6 in number) progeny had irregular phyllotaxy; 2-3 flowers or siliques from same place (Fig.16G and H). Such siliques were also unusually smaller and sickle-shaped (Fig.16I). A dramatic change in the morphology was also observed on one of the hybrid plants; it had unusually aerial rosette leaves in addition to the rosette leaves that usually occur at the base of the main leaves.

There was also enhanced branching both at the node that gave rise to these aerial rosette leaves and the base of the stem (Fig.16E and F).

3.5 Genetic and phenotypic analyses of ATX5 SALK T-DNA insertion lines

Genotyping of the SALK lines showed 20 out of the 30 plants contained T-DNA while 10 had no insertions at all. Out of these 20 lines 13 lines were heterozygous and 6 homozygous for the insertion. All but one line had phenotypes resembling wild type Columbia plants i.e. there was no observable phenotypic defect on those lines. However, one line was observed to possess smaller plant structure (fig. 17A) throughout development, similar to those observed in the ATX5 RNAi lines (fig.15A-D). Floral bolts also began to form 4 days after wild type Columbia plants. At the reproductive phase, an unusually large number of flowers formed a relatively larger cluster of flowers (fig. 17B and C) from the inflorescence meristem, similar to that observed in line ATX5_RNAi_39_T1 (fig. 16C).

Figure17. Phenotypes an ATX5 T-DNA insertion line. Plant was smaller in size as compared to wild type (A). There was also an unusually large cluster of flowers at the inflorescence meristem (B and C) relative to wild type inflorescence meristem (D).

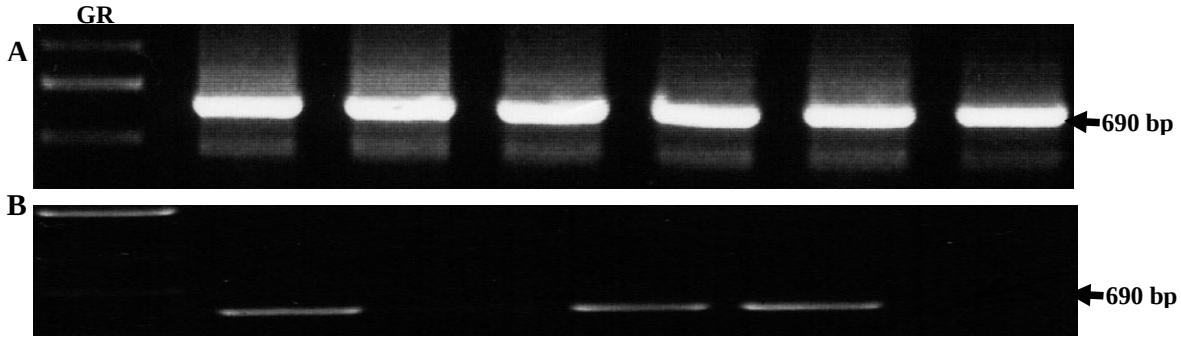
3.6 Phenotypic analysis of ATX3 SALK T-DNA lines

In all 24 T-DNA were screened phenotypically. Generally the plants the T-DNA lines did not show any significant phenotypic defects. Six (6) lines were appeared smaller early in development, possibly due to stunted growth. These lines were also about 4 days late in flowering as compared to Arabidopsis wild type Columbia (data not shown). They however assumed normal growth and appeared normal during the reproductive phase, just as the other lines. All lines were genotyped but the results are inconclusive and rquire a repetition of the experiment

3.7 Cloning of SAC-SET domains for GST-pull down Assay and HMTase

3.7.1 Verification of SAC-SET domains in both entry and expression vectors

The BP and LR recombination reactions were each followed transformation into DH5α cells and selection of correct clones on solid and liquid selective media. The growth of cells indicated successful recombination since only cells that took the right clones could survive on selective media. However PCR analysis of purified plasmid DNA was also carried out and the SAC-SET domains were found present in both the donor and expression vectors respectively. Below are pictures of PCR products visualised by agarose gel electrophoresis. The agarose gel was stained with ethidium bromide.



Figures 18 A and B: Agarose gel electrophoresis of PCR products amplified from purified pDONR/zeo vector containing ATX5 SAC-SET domain (699 bp) in A. PCR also confirmed the presence of the motif in pGEX-GAW, the expression, in B.

By using the ATX5_SAC-SET _attB1 (gene specific) and attL2 (vector specific and sits 250 bp inside pDONR/zeo) a product of 699 bp was obtained by PCR (Fig.18A). This

consolidated the results from selection on selective medium with zeocin, which previously confirmed the presence of the ATX5 SAC-SET domain in pDONR/zeo (see appendix for sequence and annealing site for attL2 in vector map).

DH5α cells were transformed with the product of the LR reaction and selected on ampicilin selective medium. Theoretically, cells that picked up pGEX-GAW containing the ATX5 SAC-SET domain were the only cells capable of growing on the medium. Therefore colonies from such cells were picked and grown in ampilicin liquid culture and the pGEX-GAW plasmid, thought to contain the ATX5 SAC-SET purified thereof, using the Wizard® DNA Purification system Kit (Promega). Both PCR (Fig.18B) and sequencing analysis also confirmed the presence of this vector as well its orientation and the absence of misincorporated nucleotides. For the PCR, the gene specific primer ATX5_SAC-SET_attB1 and the vector specific primer pGEX 3' were used (See appendix for the annealing site for pGEX 3' in vector). Now the pGEX-GAW which contains ATX5 (GST-Fusion construct) was ready for transformation into cells for expression of the recombinant GST-fusion ATX5 SAC-SET protein.

3.7.2 Induction of the ATX5 recombinant protein in DH5α cells

The denatured cells of cells induced with IPTG as well as those that were not induced were run on 10% SDS-PAGE gel and visualized with UV light.

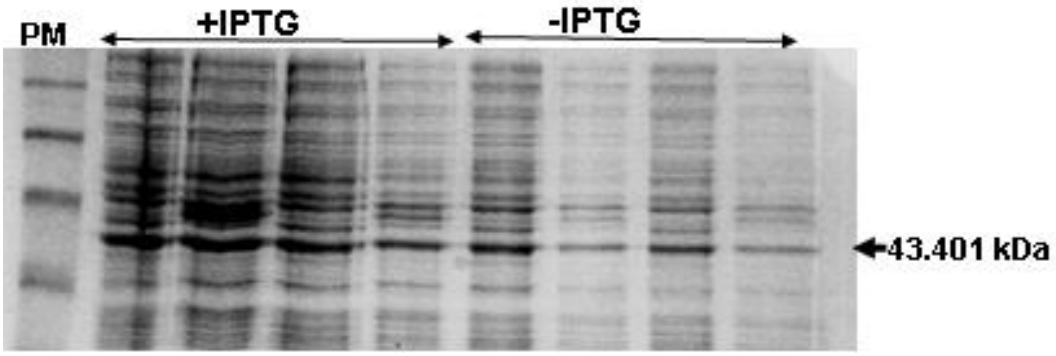


Figure 19: Induction of the recombinant GST-ATX5 SAC SET fusion protein in BL21 cells. Protein was run on 10 % SDS-PAGE gel and stained with 0.1% Coomassie Blue stain. PM: protein marker.

The bands on the gel show that the induction of the recombinant ATX5 SAC SET protein was successful. The ATX5 SAC-SET protein was expressed fused to a lac gene as both are under the control of the same promoter (lac tac promoter). The bands correspond to a size of 43.401 kDa appear stronger in samples induced with IPTG than in those without IPTG.

4 DISCUSSION

4.1 Expression pattern of ATX3 indicate role in various part of plant development including fertilization

Based on the results of the RT-PCR, it can be considered that ATX3 is expressed in several tissues, both at the vegetative and reproductive phases. The relatively higher amount of transcript detected in seedlings and rosette leaves suggest that ATX3 is highly expressed in those tissues and may be essential for the vegetative development of Arabidopsis plants and/or the transition to flowering. At the reproductive phase ATX3 is detected in the flowers, though its level is lower than in the seedlings. This also suggests that this gene is further involved in reproduction or related processes. A highly specific ATX3 in situ hybridisation pattern in tapetum indicate these genes involvement in pollen wall formation, or lipid-rich exin coating, the last being important for successful development of the pollen on the stigma surface (for review see Scott et al 2004). The lack of phenotypes on the ATX3 SAIL T-DNA lines and RNAi knock down lines hinder further speculation about the function of ATX3. The strong and highly specific expression pattern in the tapetum shown by *in situ* hybridization does not necessarily contradict the relative low RT-PCR expression pattern in flowers, as the level of transcript in the tapetum is restricted to only a few number of cells, the expression level looking at the flower as a whole will relatively also be low.

4.2 Levels of silencing and heritable phenotypes in ATX5 RNAi mutants were very low

Unexpectedly the various phenotypes observed in ATX5 RNAi lines in the T1 generation were not observed in the T2 generation, except phyllotactic defects and stunted growth in some few individual plants. This was in strong contrast to other experiments which gave heritable, stable and efficient silencing in Arabidopsis, Drosophila, *C. elegans*, etc, with similar vector constructs that express hairpin dsRNA (Chuang and Meyerowitz, 2000; Kennerdell and Carthew, 2000; Piccin et al., 2001; Wesley et al., 2001; Johnson et al., 2005). For example, there was no observable reduction in fertility in the T2 generation of some RNAi lines that had drastic reduction or loss of fertility in the T1 generation. In addition, only 9 out of 77 T1 RNAi lines contained observable phenotypic defects. This goes a long way to

add to already building list of pitfalls associated with the RNAi silencing mechanism (Frantz, 2003; Wang et al., 2005). Though much remains to be learned about the phenomenon of RNAi/PTGS, a number of parameters have already been known to account for the short-comings in RNAi. These parameters to a large extend, though not exclusively are capable of accounting for the the inconsistent and non-heritable silencing effects observed in the ATX5 RNAi lines. For example it is known that higher transcriptional activity is directly related to efficiency of RNAi silencing. Genes that are found to be expressed at relatively higher levels in wild type *C. elegans* and Arabidopsis were strongly silenced with RNAi than those expressed at moderate or low levels (Cutter et al., 2003). The mRNA of ATX5 is barely detectable with Northern blotting analysis in many tissues suggesting it is expressed at low level and that RNAi silencing may be relatively low with it (see chromdb.org).

Another determinant in RNAi efficiency is the copy number of the transgene in the genome. This is however inversely related to the efficiency of silencing by RNAi. Evidence from the works of McGinnis and other groups reveal that transgenes become subjected to some degree of transcriptional silencing when present in multiple copies (mc), consequently reducing the effectiveness of RNAi the RNAi mechanism in mc lines than in single copy (sc) lines (Kerschen et al., 2004; McGinnis et al., 2005). Positional effects of particular foreign DNA insertion events by Chuang et al as factor that can cause variation in severity of phenotypes (Chuang and Meyerowitz, 2000). Due to technical problems we could not identify mc and sc lines among the ATX5 RNAi line. Sequence composition, spatial and temporal expression pattern, the strength of the driving promoter and normal mRNA turnover rate of the target gene are also known to be major parameters that determine the efficiency of the RNAi mechanism.

At the same time, questions are also been raised as to whether there could be redundancy in gene function between ATX5 (At5g53430) and its closest relatives, especially ATX4 (At4g27910) which shares about 85% identity with the latter. Though both are found on two different chromosomes (IV and V), the extensive sequence similarity between them and their presence in high repetitive regions suggest either of them is a duplicated product of the other and may therefore have similar function. This therefore requires more genetic and phenotypic analyses for example, generation of double mutants alongside single mutants of these two genes. Taken that the ATX5 protein, like many other transcription factors is part of a multi-complex of proteins, more work has to be done to determine how essential it is for the

functioning of such a complex. Sequence comparison analysis of the PHD and PWWP domains associated with the SET-domain of ATX genes have led some scientists into speculating that these domains are involved in multimeric protein-protein interactions. If its absence is not a limiting factor to the functioning of such complexes, the unstable and low level heritability of the phenotypes observed in the RNAi lines reflects its role. If it is however the opposite, other genetic tools or a better experiment design for RNAi will have to be done once again.

4.3 Meristematic disturbance observed in ATX5 SALK T-DNA knock out line

A total of 29 ATX5 T-DNA lines were screened for phenotypic defects. Out of these, only one line was observed to possess visible defects. This line had an enlarged SAM and produced a larger cluster of flowers than in wild type plants. When all these lines were genotyped, the line with phenotype was found to be among 6 lines that were homozygous for the T-DNA insertion while 13 lines were heterozygous. The rest of the plants (10) had no T-DNA insertion. The results seemingly suggest that silencing of ATX5 results in homozygous T-DNA lines but does not account for the absence of phenotypic defects in the other 5 homozygotes. The low level of phenotype associated with this genotype is therefore inconclusive until more analyses - both genetic and phenotypic - are carried out. For example, when the wild type primers are not optimal during the PCR, false bands or no bands can occur, which might give higher number of heterozygotes or homozygotes. In addition the observed phenotype can be due to an insertion of the T-DNA within another gene than in ATX5. It is also possible that phenotypic defects due to silencing of the ATX5 gene are conditional, thus becoming visible only under specific growth conditions.

4.4 ATX5 functions both in the vegetative and reproductive phases

In general terms, the SET-domain genes are considered to catalyze the transfer of methyl groups onto specific amino acid residues of histone tails and thereby create epigenetic marks that serve to regulate the function of homeotic genes or to control the formation of heterochromatin (Jenuwein et al., 1998). Based on the observed specificities to various SET-domain genes, genes of the trithorax subgroup are proposed to maintain the activated states of target genes. This has been confirmed in many experiments including those on the ATX1

gene in Arabidopsis and trithorax (TRX) gene of Drosophila (Sedkov et al., 1994; Alvarez-Venegas et al., 2003). As such, phenotypes such as phyllotactic defects, stunted growth, shorter filaments, reduced and loss of fertility, etc observed in the silenced ATX5 RNAi lines suggest nothing short of the involvement of ATX5 in the regulation of genes controlling plant development.

All the 11 ATX5 RNAi lines in which abnormal phenotypes were observed showed varying degrees of severity with consideration to the phenotypic defects. Though all these plants were smaller in size they were by no means phenotypically identical, as some were observed to be larger in size and bore more vegetative parts than others. In addition, complete loss of fertility was observed in two of these lines whiles drastic and moderate reduction in fertility in the rest of these lines gave rise to relatively fewer seeds per plant. Such variation in degree of silencing is typical of RNAi silencing and has been reported in other laboratories (Chuang and Meyerowitz, 2000; Levin et al., 2000; Stoutjesdijk et al., 2002). This unique form of silencing by RNAi, unlike other reverse genetic tools, increases the number and type of genes on which functional studies can be carried out, even on essential genes.

Having mentioned the parameters that might have caused these unstable and non-hertable phenotypes in the RNAi lines, our attention has to be drawn to the observed phenotypes. The observed phenotypes are consistent to the expression pattern of ATX5 as shown by RT-PCR and *in situ hybridization*, given that these phenotypes are due to the down regulation of the ATX5 gene.

4.5 Reduced fertility and smaller siliques in RNAi lines links ATX5 to a role in fertility

The localization of ATX5 transcript in the anthers and gynoecium of wild type Columbia flowers implicates it in fertility and fertility-related processes. This is consistent with the ATX5 RNAi lines where reduction and complete loss of fertility were registered. The stamen filaments of the RNAi lines were relatively shorter and this greatly hindered the deposition of pollen grains onto the stigma of the carpel. This apparently led to a reduction in fertilization in some silenced lines and more extremely, a complete loss of fertility in two lines; ATX5_RNAi_11 and ATX5_RNAi_14. It is however worth noting that there was pollen production by the flowers of these lines. In addition observation of some individual flowers

on these lines revealed that not all flowers possessed abnormal stamen. The possibility of whether the pollen contained defective pollen grains or male gametophytes, especially when *in situ* hybridization localized ATX5 transcript in the tapetum of stamen was considered though it was an after-thought. It is therefore worth setting up an appropriate experiment to investigate this. The phenotypes here link ATX5 to a role in promoting fertilization but do not pinpoint the mechanism in which ATX5 is involved. This is mainly because fertility is under the control of several mechanisms and genes. But this information serves as a reference point for further work to unravel more about this link. This observation of shorter stamen filaments is not an isolated incident as mutants of the ATX-1 and ASK1 (for -*Arabidopsis skp1-like1-1*) genes have also been found to possess shorter stamen filaments (Zhao et al., 1999; Alvarez-Venegas et al., 2003). In the mutants of these genes, fertility was drastically reduced as well. In the experiment involving ASK1 the unusually shorter stamen filament was considered as a weaker form of loss of B function in floral organ, based on the ABC model of floral organ identity. Interestingly their follow-up experiments confirmed this, first by revealing genetic interaction between the proteins of ASK1 and UNUSUAL FLORAL ORGAN (UFO), a gene that controls floral meristem development and B function which is responsible for the identity of petals and stamen of flowers (Zhao et al., 1999). Not surprisingly phenotypic analysis by Zhao et al also revealed a disruption of petal and stamen identities in double and triple mutants involving ASK1, UFO and LEAFY. These phenotypes were therefore characterized as strong mutants of loss of B function genes (Zhao et al., 2001). Both UFO and LEAFY are known for their roles in regulating the B function genes APETALA3 (AP3) and PISTILLATA (PI) (Levin and Meyerowitz, 1995). Does the observation of shorter stamen filaments in ATX5 RNAi lines also suggest a weaker loss of B function?

Phytohormonal pathways such as the gibrellin-signalling pathway are also among the means by which normal development of floral organs, such as petals and stamens are regulated. When components of the giberellin-signalling pathway are mutated development of these organs become impaired. For example, the phytohormone gibberellin (GA) is known to regulate the development and fertility of *Arabidopsis* flowers by promoting the elongation of cells of stamen filament (Hynes et al., 2003). Since pollen from wild type Columbia Arabidopsis restored fertility in the sterile lines, ATX5 might be important in male fertility though its direct role(s) in female fertility and at post-fertilization cannot be underestimated or precluded.

Even in those RNAi lines where fertilization was reduced, the resulting siliques were smaller and distorted. Reason(s) for the smaller size and irregular shapes of the siliques from these mutants is not clearly known but normal silique development is at least known to be dependent on pollination and double fertilization (Gillaspy et al., 1993). The inability of the shorter stamens to appropriately deposit pollen grain on the stigmatic papillae of gynoecium in ATX5 RNAi plants caused reduced fertilization, leading to a reduction in thr number of ovules or seed per silique. Pollination and subsequent fertilization events in the ovule initiate a sequence of events including embryo development and those that lead to the senescence of floral organs and the initiation of fruit and seed development. These events involve unknown pollen-borne signal(s) which lead to inter-organ communication. In orchid this communication between the male and female gametophyte is very crucial and that ovary and gametophyte development are coordinately regulated by auxin and ethylene (Zhang and O'Neill, 1993). For example, double fertilization in the ovule is followed by division and growth of the different cell layers of the ovary until the silique matures or reaches its full length and thickness (Vivian-Smith and Koltunow, 1999; Vivian-Smith et al., 2001). Without fertilization of both the egg and central cells of the embryo silique development does not proceed as the cells degenerates during senescence of the flower. This seems to account for the total loss of fertility (no seed production) in two of the ATX5 RNAi lines and a reliable indication of the dependence of embryo and silique development on fertilization (Koltunow and Grossniklaus, 2003). Though the embryo contains other accessory cells only the egg cell and central cell fuses with the two sperm cells of the pollen tube to give rise to the embryo and endosperm respectively (Higashiyama et al., 2001; Higashiyama, 2002). The smaller siliques in those lines with reduced fertility (few seeds per silique or plant) also suggest that there were fewer sperm cells that fertilised the egg and central cells in those siliques. In
The presence of ATX5 transcript in both the tapetum of stamen and gynoecium shown by *in situ* hybridization might therefore indicate the participation of ATX5 in signalling pathway that signals for the normal growth and development of Arabidopsis embryo and siliques. Therefore one of the roles played by ATX5 is proposed to be in the determination of the proper length of stamen, probably through elongation of the cells in the stamen filaments. This assertion seems to be further consolidated strongly by the detection of a higher amount of the ATX5 transcript in flowers than in any other tissues tested with RT-PCR.

ATX5 might therefore be active both during fertilization and at post fertilization as it is not uncommon to observe the activity of genes at many stages of Arabidopsis lifecycle. For

example the Arabidopsis AINTEGUMENTA (ANT) and SUPERMAN (SUP) genes that are functional during floral meristem determination and flower organogenesis are also found to participate in ovule and seed development (Sakai et al., 1995; Elliott et al., 1996). However, the function of such genes can be masked by other genes because of redundancy. It is therefore not surprising that the mRNA of ATX5 was detected was detected during embryo development (at the globular, heart and mature stages) and seedlings of wt Col, by *in situ* hybridization.

4.6 Large cluster of flowers and phyllotactic defects suggest ATX5 is very vital in the SAM/IM

The ATX5 RNAi lines and hybrids of ATX5 RNAi _11_and wild type Columbia plants exhibited disturbed phyllotaxy, a feature of plants known for long to be regulated in the SAM by genes that maintain its identity as well as by auxin and not the least, some environmental factors like day length (Laibach, 1953; Kuhlemeier and Reinhardt, 2001; Reinhardt et al., 2003). On one of the ATX5 RNAi lines, a r cluster of flowers large than in wild type flowers formed. Such a larger cluster of flowers is observed when an enlarged an enlarged SAM develops, usually due to a relatively higher population of stem cells in the SAM. Both phenotypes seem to be under the control of two biological processes, namely one that regulate the formation of new organ primordial and another which determines the population of stem cells in the SAM. How linked these two processes can be is however unknown. This left little doubt when *In situ* hybridization detected ATX5 transcript in the central, peripheral and rib zones (CZ, PZ and RZ) of SAM, both in the embryo and seedlings of wild type Arabidopsis.

In arabidopsis, lateral organs occur in a spiral pyllotactic pattern, with organs occuring at a constant divergence angel of 137.5º. This was however not the case in the ATX5 RNAi knockdown lines, especially, their hybrids. In these lines it was common to observe two flowers diverging either by 90º (alternate phyllotaxy), or by 180º where two flowers formed at opposites positions of a node. Irregular spacing of flowers along the branches was also observed on these plants. In plants with spiral phyllotaxy an unknown gradient of negative influence is postulated to be established by existing primordia. This negative gradient which reduces with distance prevents formation of new organ primordium in the vicinity of existing primordia. As a result, new organs can only form at positions farther from existing primodium, where the negative influence is lower than the threshold (Reinhardt, 2005). The

phenotypes of these ATX5 RNAi lines therefore indicate a malfunction in this suggested negative influence. The presence of ATX5 in the SAM of wild type Columbia coupled with the abnormal phyllotaxy in ATX5 RNAi lines links ATX5 to mechanisms or pathways that maintain the integrity of the SAM. As a result ATX5 is likely to be involved in the regulation of genes that dictate the formation of organ primordia and therefore the appropriate body plan (architecture) in Arabidopsis. It is suggested that the absence or reduction of ATX5 mRNA in SAM is accountable for the observed phyllotactic defects both in the RNAi lines and the hybrid plants. Mutations in other genes that are essential for the normal functioning of SAM or their transcriptional regulators (repressors or enhancers) have in many cases resulted in abnormal phyllotaxy. Evidence for this can be derived from Arabidopsis mutants *fasciata1* (*fas1*) and *fasciata2* (*fas2*), that possess phyllotactic defects due to irregular organization of their SAM (Kaya, 2001). In mutational analysis of the BELLRINGER gene, a repressor of AGAMOUS the mutants developed phyllotactic defects in Arabidopsis plants, though no appreciable disturbance in the size of SAM was detected (Byrne et al., 2003; Bao et al., 2004). AGAMOUS is a floral homeotic gene which functions in determining the identity of floral organs as well as in the determinacy of floral meristems. There was therefore no link found between phyllotaxy and the size of SAM.

The larger cluster of flowers on one RNAi line (ATX5_RNAi_39_T1) relative to wild type inflorescence can also be attributed to a malfunction in the inflorescence meristem (IM). When SALK T-DNA lines were screened one line also possessed an unusually larger cluster of flowers. This matched the phenotype observed on ATX5_RNAi_39_T1. Such a phenotype results when the inflorescence meristem becomes enlarged than normal, as observed in mutants of the CLAVATA genes (Fletcher et al., 1999). This further consolidates the suggestion that ATX5 is involved in biological pathways that maintain the integrity of the inflorescence meristem (IM) and that its loss or reduction is responsible for the larger cluster of flowers in the IM. Proliferation of cells in the meristem must be balanced with differentiation and recruitment of those cells to the flanks of the IM to form organs. Therefore an enlarged IM results when the rate of stem cell proliferation is not balanced by differentiation, leading to accumulation of more cells in the IM. Hence mutation of genes that enhance cell differentiation in the SAM or IM results in masses of undifferentiated cells at the shoot and floral meristems than usual. The CLAVATA (CLV) genes CLV1, CLV2 and CLV3 in coordination with the WUSCEL (WUS) gene form a regulatory feedback loop that maintain the balance between cell proliferation and differentiation and for that matter the

population of cells in the SAM/IM (Schoof et al., 2000). Both the CLV1 and CLV2 genes encode leucine-rich receptor kinases for the CLV1/CLV2 receptor kinase complex, a receptor to the protein product of CLV3. The product of CLV3 is a signalling ligand, which together with CLV2 and CLV2 restricts the expression of WUSCHEL, a gene which encodes a homeodomain protein(Fletcher et al., 1999). The WUS gene promotes the formation and maintenance of SAM through cell differentiation that specifies the identity of the stem cells as well as the expression of CLV3. The CLV genes on the other hand enhance stem cell differentiation and timely division as mutations in any of those genes produce enlarged meristems (Schoof et al., 2000). In the regulatory loop that regulates the integrity of the SAM, an increase or decrease in the number of stem cells produces a corresponding level of CLV3 expression and adjustment of WUS level, in a negative feedback loop (Brand et al., 2002). For example, and increase in stem cell population results in reduced level of CLV3, leading to a reduction in the repressive effect of CLV3 on WUS. As a result, WUS is able to enhance the differentiation of the stem cells and in turn increase the expression of CLV3. In this way, the size of the SAM or IM is maintained (Sharma et al., 2003).

Other phenotypes that suggested a malfunction of the SAM was promiscuous growth of rosette leaves at the base of ATX5_RNAi_39_T1 and the presence of aerial rosette leaves and enhanced branching at the second node of a hybrid plant from ATX5_RNAi_11_T1 and wild type plants. All these phenotypes are reliable indicators of the vital role played by ATX5, considering the importance of SAM in plant development: the site for establishment of plant architecture and organogenesis (e.g. leaves, flowers, branches etc).

It is therefore not out of place to suggest that ATX5 like other trithorax proteins participates in maintaining the appropriate activation of at least one of the homeotic genes that control the integrity of the SAM/IM/FM as well as in establishment of normal phyllotaxy. A similar role is being played by TRITHORAX (TRX), the founder of the TrG genes in Drosophila. TRX regulates the expression of genes belonging to the Antennapedia and bithorax Complexes (ANT-C and BX-C) which are important in patterning or segmentation of the Drosophila body (Ingham, 1988; Breen and Harte, 1993). This finding is undoubtedly a step forward in the search for the specific and or general target genes of ATX5 as well as the biochemical process (s) in which it is involved.

CONCLUSION

Though the RNAi mechanism has been hailed as effective and reliable reverse genetic tool, it failed to produce effective and stable silencing in ATX5 RNAi lines. As a result, the phenotypic defects observed in the ATX5 RNAi lines are preliminary and require further analysis, using carefully designed experiments.

Having said this, it has to be mentioned that the phenotypic defects were observed in some of the RNAi lines matched the results of *in situ* hybridization and RT-PCR. These results implicate ATX5 in several biological processes vital for reproduction, establishment of body plan and maintenance of the shoot apical or inflorescence meristem in Arabidopsis. With respect to reproduction, ATX5 is considered to be necessary for the development of stamen filament long enough to deposit pollen grains on the gynoecia for fertilization. In addition, ATX5 is also thought to be involved in post-fertilization events necessary for normal silique development. The other phenotype of larger cluster of flowers which was apparently due to an enlarged inflorescence meristem also implicate ATX5 in maintenance of the size of the inflorescence meristem. Another potential role for ATX5 is the establishment of proper phyllotaxy. This suggests that mechanism specifying the formation of organs at the appropriate sites was malfunctional in those ATX5. Again this regulatory mechanism is thought to function in the shoot apical or inflorescence meristem.

The results obtained from ATX3 are inconclusive. RT-PCR revealed that ATX3 may be involved in several processes in development, with possibly higher activity in at the seedling stage. In situ hybridization also revealed tapetum expression, and knowing that tapetum is necessary for pollen wall formation or lipid-rich exin coating, ATX3 may have such a funcion. Phenotypic analysis of the SAIL T-DNA knock out lines for ATX3 did not show phenotypic defects except for late flowering and smaller plant structures on isolated lines.

Future perspective

The above results are preliminary and provide only evidence of suggested roles of ATX3 and ATX5. In addition they do not tell the exact or specific roles they are involved in. Hence more work is needed to pursue these goals.
One of such experiments is the GST-Pull down Assay with ATX4 interacting partners which is on-going for ATX5, and requires completion to see if one can 'fish out' prospective interactive protein partners among these. If these have functions that already are known, this will throw more light on the possible molecular processes ATX5 is involved in

Without an experiment to test histone methyl transferase (HMTase) activity in the protein products of these genes, a role assigned to both ATX3 and ATX5 based on other reported SET domain proteins, will be nothing but speculative. As a result well defined designed *in vitro* experiments are required to test if these genes have any HMTase activity, and the specific histones or residues, if such an activity exists. For ATX5 the recombinant SAC-SET-domain will also be used to test for HMTase activity of the ATX5 protein.

As a matter of fact a new round of phenotypic analysis using the RNAi mechanism will also be necessary to verify if the phenotypic defects in the ATX5 RNAi lines. Unlike in the previous experiment, generating RNAi lines with different constructs carrying ATX5 RNAi fragments from different regions of the gene is recommended. Phenotypic analysis of a next generation of the SALK T-DNA can also be carried out once again for comparison.

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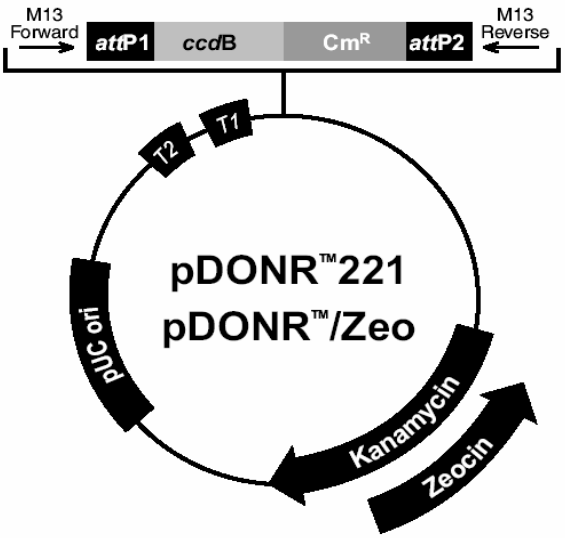
APPENDIX

Name of primers and sequence

Primer name	Sequence (read 5'-3')
ATX5_ATTB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCAACTGATGTGGAGACACTGTGG
ATX5_ATTB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCTGCGGATGGATGATGACG
ACT2INT2_ANTISENSE	CCGCAAGATCAAGACGAAGGATAGC
ACT2INT2_SENSE	CCCTGAGGAGCACCCAGTTCTACTC
act2int3_sense	TCAGGAAGGATCTCTATGGAAAC
act2int3_antisense	TTCCTGTGAACAATCGATGG
ATTL1	TCGCGTTAACGCTAGCATGGATCTC
ATTL2	GTAACATCAGAGATTTTGAGACAC
ATX5_1LM/ATG	ATGATAATCAAACGAAAGTTGAAAACTT
ATX5_SAIL_705_H05_RP	TGCTTCCTTCACGGTTTAATG
ATX5_SAIL_705_H05_LP	AAACCCCAAGCAAAGAGAAAC
LBb1	GCGTGGACCGCTTGCAAC
ATX3RT 477L	TCAAATTTCCTACGACGATGC
ATX3RT 1469R	GTCGTCCACTTAGCCCTGAC
Actin 2/7L	GCTGGTTTTGCTGGTGATGATG
Actin 2/7R	TAGAACTGGGT**CC**T**CC**TCAGGG
ATX5_SAC-SET_attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCACTTGCAGAGGACAGAAAT
ATX5 SAC-SET_attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTCATTCATGAATTTCCTGCAGT
ATX3_SAIL_255F11_LP	TCACCCTTTTTTCCTCCCATAC
ATX3_SAIL_255F11_RP	AAAGGATCATCTTCCTCCATTG

List of vectors

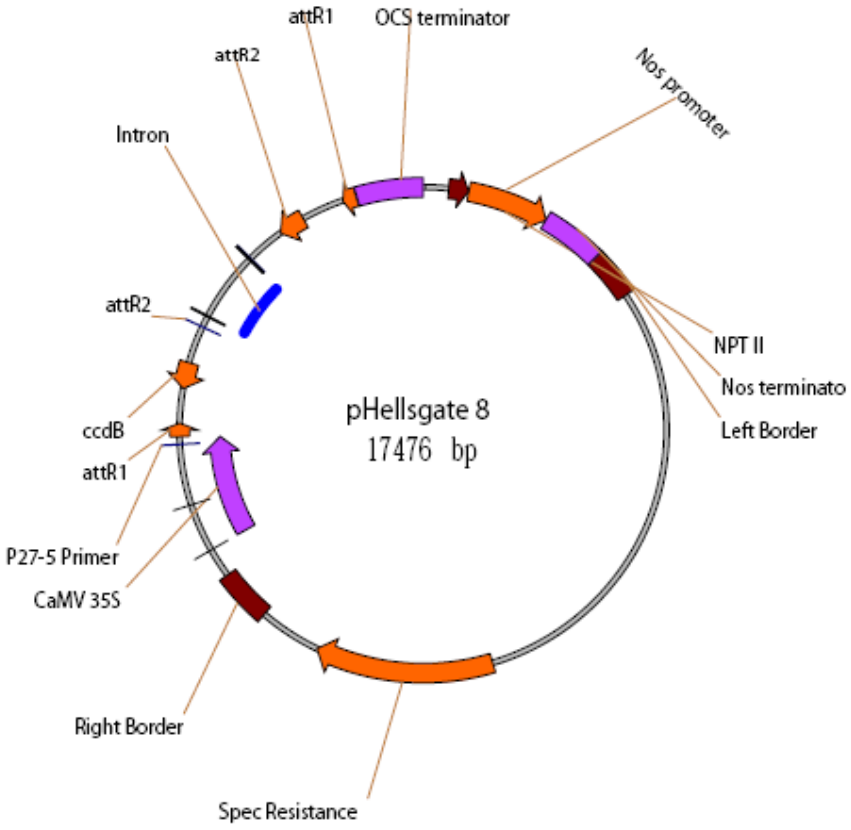
pDONR™/zeo plasmid vector used as the donor vector during the BP recombination reaction to clone the attB-ATX5 fragment



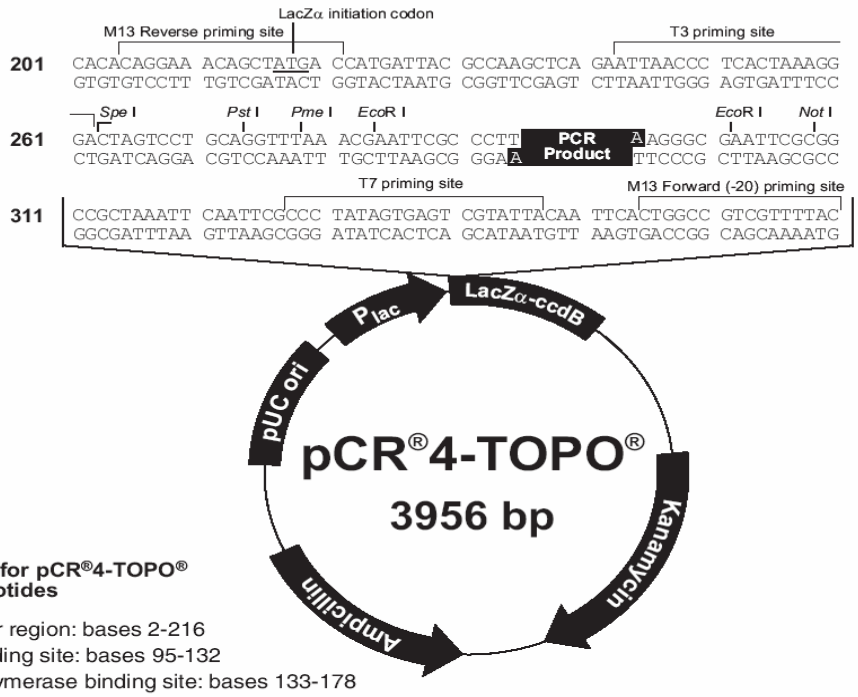
Comments for:	pDONR™221 4762 nucleotides	pDONR™/Zeo 4291 nucleotides
*rrn*B T2 transcription termination sequence (c):	268-295	268-295
rrnB T1 transcription termination sequence (c):	427-470	427-470
M13 Forward (-20) priming site:	537-552	537-552
*att*P1:	570-801	570-801
*ccd*B gene (c):	1197-1502	1197-1502
Chloramphenicol resistance gene (c):	1847-2506	1847-2506
*att*P2 (c):	2754-2985	2754-2985
T7 Promoter/priming site (c):	3000-3019	3003-3022
M13 Reverse priming site:	3027-3043	3027-3043
Kanamycin resistance gene:	3156-3965	---
EM7 promoter (c):	---	3486-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4086-4759	3615-4288

invitrogen™

The pHELLSGATE8 vector used in the LR recombination reaction for expression of double stranded RNA for RNAi



The pCR®4-TOPO® plasmid vector <u>before</u> the cloning reaction for transcription of probes for use in *in situ* hybridization



Comments for pCR®4-TOPO®
3956 nucleotides

lac promoter region: bases 2-216
- CAP binding site: bases 95-132
- RNA polymerase binding site: bases 133-178
- Lac repressor binding site: bases 179-199

Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
- LacZα portion of fusion: bases 217-497
- *ccd*B portion of fusion: bases 508-810

T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
Ampicillin (*bla*) promoter: bases 3064-3160 (c)
pUC origin: bases 3161-3834
(c) = complementary strand

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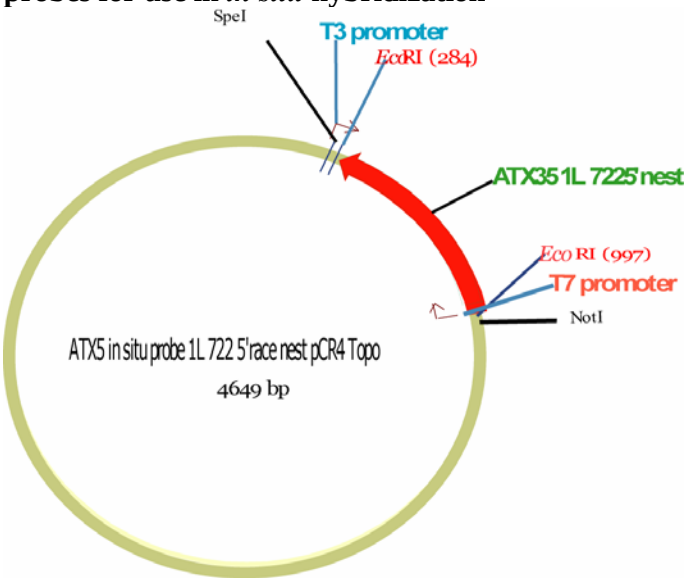
The pCR®4-TOPO® plasmid vector <u>after</u> the cloning reaction for transcription of probes for use in *in situ* hybridization



pGEX-GAW plasmid vector used to clone ATX5 SAC-SET fragment during The LR recombination reaction