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Molecular Characterization of the *EFFECTOR OF TRANSCRIPITION* (*ET*) Gene Family in *Arabidopsis* and its Role in Plant Development

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List of abbreviations

ABA	abscisic acid
ATP	adenosine triphosphate
bp	base pair(s)
bHLH	basic Helix Loop Helix
BSA	bovine serum albumine
CAPS	cleaved amplified polymorphism sequence
cDNA	complementary DNA
Col0	*Arabidopsis* ecotype Columbia0
ELISA	enzyme linked immunosorbent assay
ET	EFFECTOR OF TRANSCRIPTION
GA	gibberellin
GFP	green fluorescent protein
GUS	β- glucoronidase
mRNA	messenger RNA
NLS	nuclear localization signal
OD	optical density
PAGE	poly acrylamide gel electrophoresis
SAM	shoot apical meristem
VCM	vascular cambial meristem
WS2	*Arabidopsis* ecotype Wassilewskija2
WT	wild type

Legend

AtET2	indicates the name of the gene
AtEt2	indicates the mRNA, or cDNA
AtET2	indicates the protein
atet2	indicates a mutant allele

Declaration

Hereby, I declare that all the work presented in this manuscript is my own, carried out solely with the help of the literature and the aid cited.

Rumen Ivanov

Gatersleben, June 2005

Introduction

The development of a mature multicellular organism from a single cell requires the concerted action of many factors. The processes leading to the formation of organisms have been extensively studied on different levels over the years, but certain aspects still remain poorly understood.

In higher plants, following fertilization, the embryo and the endosperm are coated in maternal tissue to form a structure known as seed. Seeds support the embryo ensuring it with sufficient energy for proper development. Once the seed matures, it is released from the mother plant and finding appropriate conditions, it germinates starting the formation of the tissues and organs of the sporophyte- the mature plant. This is also the time when the sexual organs are initiated. Special sets of cells undergo meiosis to form the male and female gametophyte. Following fertilization, they merge to form a zygote and endosperm, this way completing the cycle.

Though these events are well described, we are still searching for understanding on the molecular mechanisms that drive the cells into differentiation in the right time, at the right place and at the right moment.

Many key regulators of development have been isolated and characterized. Formulating new hypotheses and testing new ideas requires choosing certain model organisms. A preferred plant test model is the small cruciferous plant *Arabidopsis thaliana* because of its small size, short life cycle, prodigious seed production, availability of the whole genomic sequence and a large array of described mutants.

1. Embryogenesis

1.1 Early embryogenesis in *Arabidopsis*

Plant embryogenesis is initiated when a haploid sperm nucleus fuses with the egg cell to produce a diploid zygote. The second nucleus from the pollen grain unites with the central cell of the embryo sac giving rise to the triploid endosperm tissue that will be the nutrient source for the developing embryo.

The early embryogenesis is characterized by rapid cell divisions, pattern formation and morphogenesis. The first division of the zygote gives a terminal cell which develops into embryo, and a basal cell that will form the suspensor to provide the nutrients from the endosperm during the early phases. First clear appearance of differentiated cells in the embryo is at globular stage where an outer layer of cells, named protoderm, forms and the uppermost cell of the suspensor differentiates into so called hypophysis. The latter will participate in the formation of the root apical meristem. In the transition phase between globular and heart stage, the procambium forms and develops (Jurgens, 1994, Scheres et al., 1995, Busse and Evert, 1999). In the heart stage, the apical domain becomes quiescent and forms the shoot apical meristem (SAM) under the action of a number of genes including STM (discussed below), while division on its two sides results in formation of the cotyledons and the embryo acquires a heart- like form (Figure 1).

Once all the tissue layers have been established during the torpedo stage, the embryo expands and finally fills the seed (Goldberg et al. 1994, Meinke 1994). During this time, the endosperm is consumed and in the mature seed it remains as a single layer of cells surrounding the embryo. In other species, among which the cereals and tobacco, endosperm is preserved and acts as a storage tissue, providing nutrients during germination.

1.2 Late embryogenesis

During the expansion phase, a switch occurs from pattern formation to maturation program. The meristematic cells of the hypocotyl and the cotyledons differentiate, becoming highly specialized, and start accumulating large amounts of storage products. This way, the late embryogenesis begins, characterized by accumulation of storage compounds, acquisition of desiccation tolerance and fall into dormancy (Figure 1). The main storage products synthesized are the lipids, proteins and carbohydrates.

Lipids, consisting mainly of triacyl glycerols (TAG), accumulate in spheroid structures (oil bodies, oleosomes, spherosomes, Herman, 1995). The TAG core of an oil body is surrounded by a monolayer of phospholipids and oleosins (Huang, 1994), a special set of proteins involved in the preservation of the oleosome structure through the desiccation period. During germination, they associate with lipases to initiate its breakdown. Degradation of the lipids is the main energy source of the germinating embryo.

Accumulation of proteins is required as a reservoir of nitrogen and carbon. Along with the proteins that have structural, regulatory and metabolic role, a special set of proteins, named seed storage proteins, is synthesized in the seed to provide a store of amino acids for the germination period and the early seedling growth (Shewry et al., 1995). In *Arabidopsis* there are two sets of seed storage proteins- 12s globulins (named cruciferin) and 2s albumins (named napin). They enter the rough endoplasmatic reticulum (rER) cotranslationally, where they are folded and processed (Von Heijn, 1984). These processes require the action of molecular chaperones and are ATP dependent (Nam et al., 1997). Further, they are accumulated in special granules within the cytoplasm, named protein bodies. They can be either modified vacuoles or are derived from the endoplasmatic reticulum (Tykarska, 1987, Herman and Larkins, 1999). Expression of the storage proteins is seed specific and is strictly transcriptionally regulated. Thus, misexpression of several genes may result in ectopic appearance of storage proteins in vegetative tissues (Ogas et al., 1997, Reidt et al. 2000).

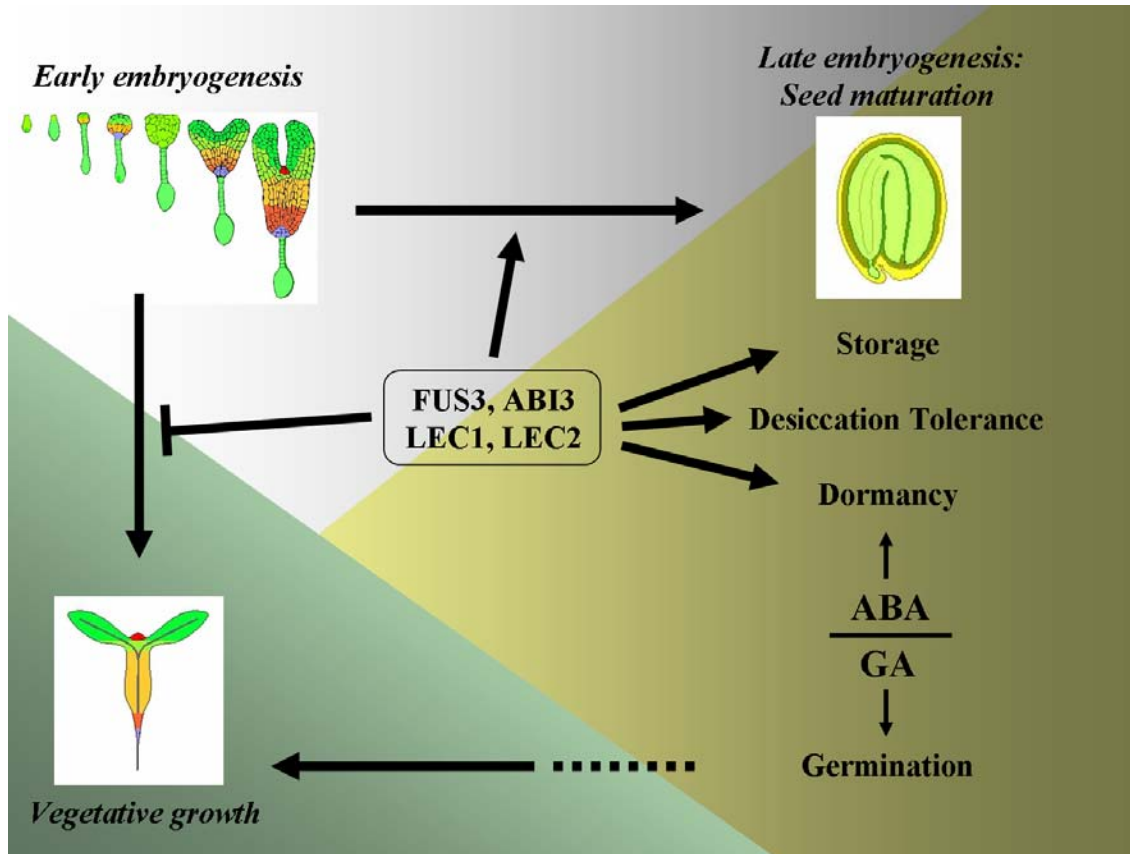

Figure 1. Scheme of *Arabidopsis* embryogenesis (see text for more details)

After early embryogenesis plant embryos are generally capable to initiate vegetative growth. In spermatophytes, this is interrupted by the processes of late embryogenesis and seed maturation. This developmental stage is characterized by the massive synthesis and accumulation of storage compounds and the acquisition of desiccation tolerance and dormancy. Later, a lowered ABA/GA ratio determines the initiation of germination. The transcription factors FUS3, ABI3, LEC1 and LEC2 are essential regulators of these processes.

In order to survive the harsh environmental conditions following its release from the mother organism, the seed needs to significantly reduce its water content. During the late stages of development, it acquires desiccation tolerance, which is associated with the expression of the Late Embryogenesis Abundant (LEA) proteins. Their role is not completely elucidated but it is considered that they are acting as osmoprotectors and chaperones (Close, 1996, Cuming 1999, Swire-Clark and Markotte, 1999). Some of them may coordinate water keeping a minimal amount of it in the seed, while others were shown to sequester ions or metabolites during desiccation. When ectopically expressed, some of them confer better osmotic stress tolerance (Xu et al., 1996). Their expression begins at the initiation of desiccation and soon their mRNA becomes the most abundant one in the seed. LEA proteins are not seed specific and they can be detected in vegetative tissues upon different forms of drought stress. Their regulation is achieved mostly on transcriptional level and is dependent on ABA and cellular water loss.

Starch is not a major storage component in *Arabidopsis*, and its accumulation is mainly studied in other species, like barley and legumes (Weber et al., 1997). During the storage phase, sucrose is imported to the seed and used for the starch synthesis. It is then hydrolyzed and the products are used either in the seed metabolism, or in starch synthesis. Several sucrose transporters have been identified in *Vicia faba* (Weber et al., 1997) and barley (Weschke et al., 2000). In *Vicia faba*, starch appears in certain regions of the embryo during the transition phase and later, during the storage phase, it fills the embryo (Weber et al., 2005). In barley, its accumulation begins even before fertilization. Later, following a strict pattern, the starch content rapidly increases (Weschke et al., 2000). A threshold level of approximately 20mM sucrose is required for the initiation of starch synthesis (Weber et al., 1998).

1.3 Control of seed maturation

The study of abscisic acid (ABA) and gibberellin (GA) mutants in *Arabidopsis* and other species have demonstrated the role of the balance between these two phytohormones for

the regulation of seed maturation and germination (Koornneef et al., 1998, White and Rivin, 2000).

ABA is a key regulator of maturation. Its levels in the seed rise in the transition to late embryogenesis, reach a peak during the maturation phase and decline in the mature seeds. Its presence is required to prevent germination immediately after the completion of morphogenesis (precocious or premature germination), but its decreasing levels do not mean that it is not active in the later stages of maturation as inhibitors of ABA (like norfluorazon) still promote germination (Debaujon and Koornneef, 2000). Mutants with defects in ABA synthesis or response can germinate precociously, fail to express maturation specific mRNAs (of storage and LEA proteins) and are intolerant to desiccation (Black, 1991).

Sugar gradients also play decisive regulatory role in the fate of the embryo development. The gradients of hexoses and sugar have important regulatory role throughout the seed development, as it is known that higher hexose concentrations promote cell division, probably by affecting D- type cyclines (Riou-Khamlichi et al., 2000). It has been suggested that through invertase activity and production of hexoses, the seed coat determines the concentration and composition of sugar supply for the embryo (Weber et al., 2005). Effects of ABA on cell division may also be mediated by the sugar metabolism as ABA has inhibitory effect on invertases and promotes cyclin dependent kinase inhibitor, both events leading to a cell cycle arrest (Weber et al., 2005).

Sucrose, along with its function as a metabolite, can itself be the cell differentiation signal (Weber et al., 2005).

Gibberellins (GA) are a group of diterpenoid compounds playing important role in regulation of processes like cell division and expansion and secondary metabolism (Olszewski et al., 2002). GAs are required for the seed germination. Upon external GA treatment, seeds are capable of overcoming the effect of ABA and germinate prematurely. It has been demonstrated that the effects between ABA and GA are ratio dependant in a wide range of hormone concentrations (Debaujon and Koornneef, 2000, White and Rivin, 2000). This way the balance between the two is the force driving the seeds into maturation or germination.

Mutant analysis has revealed several key regulators of seed development, most of which act as transcription factors. The B3 domain containing factors FUSCA3 (FUS3), LEAFY COTYLEDON2 (LEC2) and ABA INSENSITIVE3 (ABI3) regulate many aspects of seed maturation, like storage protein and lipid synthesis, and acquisition of desiccation tolerance, through transcriptional regulation on RY element in the promoter of their target genes (Figure 1). Additionally, they are involved in a very complex interaction between each other (Reidt et al., 2000, Kroj et al., 2003).

Apart from ABI3, there are four other ABA- insensitive loci in *Arabidopsis* (Koornneef, 1984, Finkelstein 1994). In addition to their action in ABA response, they have been shown to interact with other signaling pathways as light and sugar (Brocard-Gifford et al., 2003).

PKL gene encodes a CHD3 chromatin remodeling factor (Ogas et al., 1999). Homologous proteins from human yeast and *Xenopus laevis* were shown to act as a complex with histone deacetylases, suggesting a role of chromatin mediated transcriptional repression. PKL acts as a GA- mediated repressor of the embryogenic identity and plays significant role in transition to vegetative stages of plant development (Ogas et al., 1997, Rider Jr. et al., 2003)

1.4 Dormancy and premature germination

Despite being fully developed, the seed is prevented from germinating before maturation is complete. It falls dormant and this way it can survive extended periods of unfavorable conditions being in a dry state. Seed dormancy is defined as a failure of a fully viable seed to germinate in favorable conditions (Bewley, 1997). It can be caused by the mechanical restraint of the seed coat, but is also an effect of a molecular mechanism that drives the seed into a maturation phase.

Several important regulators of maturation are known in *Arabidopsis* as ABA-INSENSITIVE3 (ABI3), FUSCA3 (FUS3) and LEAFY COTYLEDON (LEC1 and LEC2). Mutants for these genes are non- dormant, show a defective maturation and fail to

acquire desiccation tolerance. Mutations in homologous genes in other organisms result even in vivipary as is the case with *VIVIPAROUS1* (*VP1*) in maize (McCarty et al., 1991).

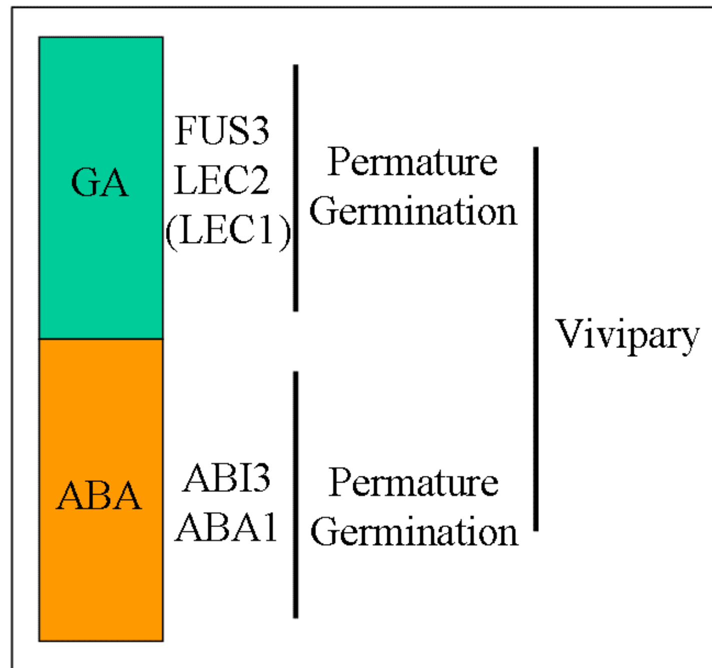

Figure 2. Factors involved in the regulation of dormancy in *Arabidopsis* (Raz et al., 2000)

At least two pathways are involved in *Arabidopsis* seed dormancy regulation. Factors like FUS3, LEC2 and possibly LEC1 act through a GA dependant regulatory mechanism, while others, like the ABA biosynthesis gene *ABA1* and the ABA response gene *ABI3* act in an ABA dependent pathway. Mutantion in these genes lead to the development of non- dormant seeds exhibiting premature germination. To create viviparous seeds, double mutants affecting both pathways are required.

Recent studies have demonstrated that these transcription factors apply their control on dormancy at different stages and by at least two different pathways (Raz et al., 2001). They were conditionally named ABA and GA controlled dormancy (Figure 2). LEC2, FUS3 and possibly LEC1 (Bentsink and Koornneef, 2002) are members of the GA pathway, and biosynthetic *ABA1* and ABA response gene *ABI3* comprise, at least

partially, the ABA pathway. GA mutants are able to germinate as early as 8- 9 days after pollination, while the ABA controlled dormancy occurs later. The effects of both pathway are additive as double mutants of members within the same pathway yield no difference in the premature germination phenotype, while crosses between the pathways result in enhancement of the phenotype and even vivipary (Raz et al., 2001, Figure 2). This way in *Arabidopsis*, suppression of vivipary is achieved by a complex regulatory network involving the phytohormones ABA and GA, and their effectors on molecular level.

2. *KNAT* genes and their role in cell differentiation

Knotted- like homeobox (*KNOX*) plant genes are a subfamily of the TALE (Three Amino Acid Loop Extension) homeodomain family. They contain a conserved 61 amino acid homeodomain for sequence specific DNA binding and an ELK domain with unknown function. Structurally they are distinguished by the MEINOX domain needed for protein-protein interaction both for plant and animal members (Bellaoui et al., 2001). A loss of function mutation in maize (*Zea mais*) led to the identification of the first member of the family in plants (Freeling and Hake, 1985, Vollbrecht et al., 1991). Due to the characteristic phenotype of the mutant, the gene was named *KNOTTED1*. Homologues are now found in many other plants like poplar, tobacco and potato. Sequencing of the *Arabidopsis* genome revealed several *KNOX* genes named collectively *KNAT* (Knotted-like of *Arabidopsis thaliana*).

KNAT genes fall into two classes, I and II, where class I have (with several exceptions) meristem- specific expression and class II are expressed more broadly (Reiser et al., 2000).

2.1 Role of *KNOX* genes in *Arabidopsis* meristem formation and function

Extensive research on the *KNOX* genes in different organisms in recent years has revealed their key role in formation and maintenance of plant meristems. Expression of *KNOX* genes is required for supporting the cells in an undifferentiated state (for a review see Hay et al., 2004). Class I genes are expresseed in the shoot apical meristem (SAM) and act in concert in the fine regulation of its function.

Early in *Arabidopsis* embryo development, at the late globular stage, the formation of the two cotyledon primordia becomes visible. The zone between them is later distinguished as SAM. Its formation is dependant on the action of SHOOTMERISTEMLESS (STM) and CUP- SHAPED COTYLEDONS (CUC) 1 and 2 (Aida et al., 1999). STM is first expressed in several cells of the globular embryo. Its expression is downregulated in the regions surrounding the central SAM. STM seems to be absolutely required for the embryonic SAM initiation as no SAM is developed in the seedlings of the *stm-1* mutant

(Long et al., 1996), whereas in the weak *stm* mutants meristems can be initiated after germination. Another *Arabidopsis* gene *KNAT1* (*BP1*), which was recently shown to be deleted in the *brevipedicellus* mutant (Venglat et al., 2002), and sharing a similar pattern of expression and regulation, might be responsible for promotion of SAM formation as KNAT1 overexpressing plants tend to form ectopic meristems on the upper leaf surface (Chuck et al., 1996).

After meristem initiation, presence of STM is essential for its maintenance whereas *KNAT1* mutant plants (*bp* phenotype) showed that meristems are sustained also in its absence (Douglas et al., 2002, Venglat et al., 2002).

The role of several other *KNAT* genes still remains to be investigated. *KNAT2* and *KNAT6*, which share a high sequence similarity, are considered to act redundantly. A knock- out mutation in *KNAT2* gene has failed to produce a notable phenotype (Byrne et al., 2002). Additionally, little is known for the role of the class II *KNAT* genes.

The DNA binding helix of the *KNOX* genes' homeodomain has the conserved amino acid sequence WFIN, which in animal MEIS homeodomain proteins recognizes the TGACAG(G/C)T sequence. STM and KNAT1 bind this sequence *in vitro* as well (Hake et al., 2004). It is currently considered that the TGAC in the 5' part of the sequence is the minimal KNOX target. This has been proven for several proteins as the tobacco NTH15 in the first exon of the ga20 oxidase (Sakamoto et al., 2001), OSH15 from rice (Nagasaki et al., 2001) and POTH1-StBEL5 complex in potato (Chen et al., 2004).

2.2 Regulation of the *KNAT* genes

Being essential for keeping the plant meristem active, the *KNAT* genes are subjected to strict regulatory control. During the last five years, a number of groups reported about interactions on transcriptional and protein level both within and outside the SAM.

KNAT genes are expressed in domains inside the SAM, but excluded from the cell specialization zones. For example, for leaf founder cells it was demonstrated that differentiation may occur through region specific transcription factor gene regulation. The MYB- like transcription factor ASYMMETRIC LEAVES 1 (AS1) and the Zn-finger/Leu zipper ASYMMETRIC LEAVES 2 (AS2) genes are negatively regulating the

expression of the class I *KNAT* genes *KNAT1*, *KNAT2* and *KNAT6*, but not *STM,* thus excluding them from the zone of young leaf differentiation (Byrne et al., 2000, Iwakawa et al., 2002). Studies using promoter-GUS reporter gene fusions demonstrated that this regulation continues as well in mature leaves with expression of *KNAT1* upregulated in both *asymmetric 1* and *asymmetric 2* mutants (Ori et al., 2000). Double mutants between *as1* or *as2* and *serrate*, a mutant with characteristic alterations in the inflorescence and leaf shape, enhance the *as* phenotype and show some phenotypical aspects of the KNAT1 overexpressing plants, such as lobed leaves and ectopic meristem initiation. In return, within the SAM STM inhibits transcription of the *AS1* gene. Such complex negative interactions between KNOX and MYB genes have been demonstrated also in other plant organisms. The *PHANTASTICA* (*PHAN*) gene from *Antirrhinum majus*, which encodes a MYB factor, is required for exclusion of AmSTM from organ primordia (Waites et al., 1998). The same is true for its maize homolog*,* ROUGH SHEATH 2, and *KNOTTED 1* (*KN1*) respectively (Tsiantis et al., 1999, Timmermans et al., 1999).

YABBY (*YAB*) genes may contribute to repression of *KNAT* in differentiation zones. *YAB* genes in *Arabidopsis* encode putative transcription factors with high mobility group (HMG) and Zn- finger domains (Eshed et al., 1999, Sawa et al., 1999, Siegfried et al., 1999). The expression of the *FILAMENTOUS FLOWER* (*FIL*, *YABBY1*, *AFO*) gene is required for normal flower development and is considered redundant with *YAB2* and *YAB3* due to their overlapping expression patterns and high sequence homology (Chen et al., 1999, Kumaran et al., 1999, Sawa et al., 1999, Siegfried et al., 1999). The single *fil* mutant, otherwise defected in floral development, lacks any vegetative phenotype. Double mutant between *fil* and *yab3*, though, enhances the flower phenotype and produces leaves with altered surface structure and vascular pattern (Siegfried et al., 1999). Stronger allele double mutants lead also to induction of ectopic meristems on the lateral organs (Kumaran et al., 2002). This comes as a result of overexpression of *KNAT1* and *KNAT2* genes and, unlike the above mentioned *as1*/*as2* effects, in the *yab* mutants there is an overexpression of the *STM* gene. This observation, along with the unaltered levels of AS1 and AS2 in the *fil yab3* plants, suggests an AS – independent mechanism, by which YABBY proteins modulate expression of the class I *KNAT* genes.

Possible mediators in the repressor- *STM* interactions are the *ZWILLE/PINHEAD* and *ARGONAUTE1* (*AGO1*) genes. They are members of a poorly characterized gene family with a possible involvement in gene silencing. This has been demonstrated for *AGO1* in addition to its function in gene methylation (Fagard et al., 2000). *ZLL/PNH* function is needed for a high expression of *STM* inside the apical meristem (Moussain et al., 1998) Double *zll/pnh ago1* mutants fail to express *STM* and are defective in shoot meristem. It has been suggested that high levels of ZLL/PNH and AGO1 are required to prevent *STM* downregulation within the SAM through silencing its negative regulators.

Additional factors that may contribute to the *KNAT* regulation, are the GA signal transduction pathway regulator PICKLE (PKL) and the C2H2 Zn finger protein SERRATE. *PKL* gene encodes a CHD3 chromatin remodeling factor (Ogas et al., 1999). Homologous proteins from human, yeast and *Xenopus laevis* were shown to act as a complex with histone deacetylases, suggesting a role in chromatin mediated transcriptional repression. PKL acts as a GA- mediated repressor of the embryogenic identity and plays significant role in transition to vegetative stages of plant development (Ogas et al., 1997, Rider Jr. et al., 2003). Likewise, SERRATE is also considered a chromatin remodeling factor (Prigge and Wagner, 2001) due to sequence homology to *Arabidopsis FIS2* (Luo et al., 1999) and Drosophila *Thiothorax- like* factor *GAF* (Farkas et al., 1994). Mutants of both genes themselves do not misexpress *KNAT* genes, but strongly enhance the *as1* and *as2* phenotypes. This suggests that regulation of the *KNAT* requires chromatin rearrangements and might be dependent on the action of phytohormones.

To fulfill their task as transcription factors, the *KNAT* genes should be able to form complexes with partner proteins, which allow an additional level of regulation. A known set of such partners are the *KNOX* genes' closest homologues from the *BELL* subfamily. These interactions were demonstrated for several plant species (Bellaoui et al., 2001, Muller et al., 2001, Smith et al., 2002, Chen et al., 2003) and occur between the MEINOX domain of the KNOX protein and the MEINOX Interacting Domain (MID) of the BELL factor. Despite the low sequence similarity between the two domains it is considered that they are derived from a common ancestral domain (Becker et al., 2002). The homeodomain does not play a role in the dimerization.

In yeast two- hybrid experiments it has been demonstrated that the interactions at least in *Arabidopsis* are specific, as BELL1 associates with STM, KNAT1 and the class II gene KNAT5, but not with KNAT3, KNAT4 and KNAT7 (Bellaoui et al., 2001). *BELL* gene encodes a protein absolutely required for proper ovule development and mutations lead to inability to initiate the formation of lateral integuments, which would develop into seed coat. Additionally, the *Arabidopsis* BELL-like gene *PENNYWISE* (*PNY*) was also demonstrated to bind STM and KNAT1, but much less efficiently KNAT2, in a far-Western blot assay (Smith and Hake, 2003). This was confirmed using a yeast two-hybrid approach (Byrne et al., 2003). The expression pattern of *PNY* overlaps with *STM* and *KNAT1* in inflorescence meristem and *STM* in the floral meristem (Byrne et al., 2003, Smith and Hake, 2003). Mutation studies showed that PNY acts together with STM to maintain the meristem identity and together with KNAT1 in internode and pedicel patterning (Smith and Hake, 2003), which is an example how in different tissues a protein shifts its partners to change the specificity and the function of the complex. In addition, the DNA recognition helix of the BELL homeodomain also contains the WFIN sequence for TGAC recognition and in this way may add to the specificity of the complex.
Along with heterodomerization, KNOX proteins may form homodimers (Muller et al., 2001, Nagasaki et al., 2001). This interaction requires the homeodomain in addition to the MEINOX one. It has been proposed that this way the excessive amounts of KNOX are inactivated. This process may also require conformational changes (Hake et al., 2004).

An interesting feature of *KNOX* gene regulation is the movement between cells demonstrated for some members. This was observed when comparisons between *Kn1* mRNA and protein localization were made (Jackson et al., 1994, Smith et al., 1992). KN1 protein (and mRNA) is translocated through the plasmodesmata and the movement was found to be dependant on two amino acids near to the homeodomain (Lucas et al., 1995). Furthermore, grafting experiments in tomato revealed a long distance movement of a homeobox mRNA in tomato (Kim et al., 2001). In *Arabidopsis*, the KNAT1 protein shows a limited mobility and partial rescue of the *stm* mutation (Kim et al 2002, 2003). In

comparison, the translocation of KN1 in *Arabidopsis* occurs with a much better efficiency. KNAT2, KNAT3, KNAT4 and KNAT6 did not show any similar capability.

Still, a largely unexplored field remains the protein stability. It has been noted that a KN1-GR fusion located in the cytoplasm was quickly degraded and undetectable, whereas upon activation the protein could be detected in leaves. In deletion experiments in rice, it was shown that overexpression of a truncated version of OSH15 results in a more severe phenotype than the full length (Nagasaki et al., 2001). In maize, truncated versions of KN1 were observed to be more stable than the wild type one. The common in these results was the lack of a certain amino acid sequence in the more stable truncated forms. It is a domain rich in Proline (**P**), Glutamate (**E**), Serine (**S**) and Threonine (**T**) (PEST). PEST domains are responsible for the targeting of a number of proteins for degradation (Rogers et al., 1986, Rechsteiner and Rogers, 1996) and might function as another way of KNOX posttranscriptional control.

3. Regulation of vascular cambial meristem maintenance and xylem differentiation

3.1 Vascular cambial meristem (VCM)

Vascular tissues are initiated during embryogenesis and distinguishable as slightly elongated cells (Jurgens, 1994, Scheres et al., 1995, Busse and Evert, 1999). In the stems of the mature *Arabidopsis* plants, they form a ring of 5- 8 bundles consisting of phloem on the outer side and xylem towards the center of the stem. In roots and the hypocotyl, vascular tissues form the central cylinder with xylem taking the central part and phloem situated on the periphery. In both cases, two- three thin layers of small cells with thin cell walls are situated between the two tissues. These cells form the vascular cambial meristem (VCM). Unlike the apical meristems, the VCM is not completely autonomous in its maintenance and function. Cultured *in vitro*, it would form undifferentiated callus, unless subjected to physical pressure, mimicking the *in vivo* situation (Brown, 1964). Cambial division provides supply of cells towards the centre or the periphery of the stem which differentiate into xylem and phloem respectively, following processes of division, expansion, secondary wall formation, lignification and cell death.
Maintenance of VCM and transition to differentiation is controlled by many factors, in first place the phytohormones auxin and gibberellins (GA).

Auxin has an inductive effect on vascular tissue initiation (Jacobs, 1952), but it has also a negative influence on vascular development (Sachs, 1966). Studies in aspen and poplar trees have shown a strict correlation between cambium location and increasing auxin concentrations (Uggla et al., 1996, Uggla et al., 1998), with concentration gradient changing according to the cambial growth rates. Several auxin- related genes have been identified. Auxin response factors (ARF) act as transcription factors and are believed to mediate auxin promoted gene expression. IAA proteins are most probably acting as regulators of the *ARF* genes (Tiwari et al., 2001). Indeed the IAA12 protein, named

BODENLOS (BDL), interacts *in vitro* with MONOPETROS (MP), also called ARF5, (Hamann et al., 2002). The similar phenotypes of *bdl* and *mp* mutants and expression pattern of the two proteins suggest that they interact *in vivo* with BDL repressing the transcriptional activation ability of *MP*.

Maintaining the meristematic tissues of the plant requires exclusion of GAs from the region, but on the other hand, GA is required for the initial steps of cell differentiation. The *GA20 oxidase* gene is excluded from the shoot apical meristem but expressed in the zones where initial differentiation occurs. Evidence for this has been shown in *Arabidopsis*, tobacco (Sakamoto et al., 2001a) and rice (Sakamoto et al., 2001b). Moreover, the penetration of bioactive GAs in the meristems from the neighboring tissues in rice, and most likely in *Arabidopsis*, is prevented by expression of the catabolitic enzyme GA2 oxidase (Sakamoto et al., 2001b). Accordingly, GA20 oxidase overexpressing plants – aspen (Ericsson et al., 2000, Israelsson et al., 2003) and tobacco (Biemelt et al., 2004) - in addition to their larger size have a significantly increased biomass accumulation and higher levels of xylem formation. The opposite effect in GA deficiency was also true for GA2 overexpressing tobacco plants (Biemelt et al., 2004), this way demonstrating that GA plays important role in cell differentiation and xylem formation.

3.2 Role of *KNAT* genes in VCM maintenance

Being key regulators of meristem identity, it is expected that *KNAT* genes would play a significant part in maintaining the undifferentiated state of the cells comprising the VCM. Indeed, studies in poplar have demonstrated a rapid disappearance of the *Kn 1*- like *Knap2* transcript outside the cambial zone (Hertzberg et al., 2001). In a more functional approach, in *Arabidopsis*, the role of KNAT1 was investigated using knock-out and overexpressing plants (Mele et al., 2003). The overexpressors had a severe lignin deficiency while the knock-out plants were over accumulating lignin compared to the corresponding wild types. This reveals KNAT1 as an important regulator of cell

differentiation. Presence of KNAT1 allows the cambium derived cells to maintain their meristematic identity and prevents them from being reprogrammed. The protein itself may be a transcriptional repressor of the several lignin biosynthetic genes suggesting a direct role in suppression of cell differentiation. This result might also indicate a great importance of the transcriptional regulation on the *KNAT1* gene as in the KNAT1 overexpressing plants presence of excessive amounts of the protein is obviously tolerated by the regulation machinery.

3.3 *GASA* gene family and GA dependent cell division

A search for GA regulated genes in tomato, led to the isolation of *GAST1* (GA-Stimulated Transcript, Shi et al., 1992), which encodes a Cysteine rich protein regulated additionally by ABA. Five sequence homologues were identified in *Arabidopsis*, named *GASA1-5*. Expression studies in the GA biosynthesis mutant *ga5* showed that transcription of two of them, *GASA1* and *GASA4*, was induced 2 to 6 fold upon external GA treatment (Herzog et al., 1996). Expression of one of them, GASA4, is blocked during embryogenesis but is induced at germination (Aubert et al., 1998). Promoter driven GUS reporter gene expression demonstrated that it was expressed in regions of active cell division, which suggests a role of GASA4 in GA dependant cell division of certain cell types. Importance of GASA4 in vascular tissue formation was demonstrated in studies on hybrid aspen where independently two groups showed a peak in the expression of its homologue in the phloem and xylem regions bordering the VCM. In the corresponding zones of differentiation, the transcript disappeared (Hertzberg et al., 2001, Israelsson et al., 2003).
Though the exact function of GASA4 remains uninvestigated for the moment, its abundance in key zones of cell division and dependence on GA make it an important factor in progression of the cells in the differentiation pathway.

4. Role of *ET* genes in plant development

4.1 Discovery of the *ET*

EFFECTOR OF TRANSCRIPTION (*ET*) genes were discovered by the use of a South-Western screen with the aim of isolating transcription factors important for embryonic gene regulation. Three independent screenings using seed specific libraries from *Vicia faba* (M. Ellerstrom, T. Wohlfarth, P. Wycliffe, L. Rask and H. Baumlein, unpublished), *Brassica napus* (Ellerstrom et al., 2005) and *Hordeum vulgare* (Raventos et al., 1998) resulted in isolation of several DNA-binding proteins sharing a conserved Cysteine-Histidine rich repeat.
The repeat, designated as ET domain, was present twice in the *V. faba* protein (VfET, EMBL/Genbank accession number X97909), four times in the *B. napus* protein (BnET, accession number AY533506) and three times in the *H. vulgare* protein (named HRT for **H**ORDEUM **R**EPRESSOR OF **T**RANSCRIPTION). Blast searches showed that members of this family occur in other dicots, monocots as well as in mosses (Figure 3), but are also unique to plants as no similarity was found to sequences outside the kingdom. The conserved structure of the ET-domain contains the common pattern $C\text{-}X_{8/9}\text{-}C\text{-}X_9\text{-}R\text{-}C\text{-}X_2\text{-}H\text{-}K$ (Figure 3b). So far, members with two, three or four domains have been identified. A factor found in *Physcomitrella patens* seems to contain only a single domain structure. Even if no functional data are available for the *Physcomitrella* protein, the presence of a similar domain also in such a distantly related phyle as mosses suggests an evolutionary conserved and therefore most likely important function in photosynthetic organisms.

4.2 Function of ET in regulation of plant development

Initial *in vitro* data on HRT showed that it is capable of binding the central sequence of the GA Response Element (GARE) and in transient assays can repress the GA induction of promoters containing this sequence. The latter holds true also for the BnET, where, though, no sequence specific binding could be observed (Ellerstrom et al., 2005).

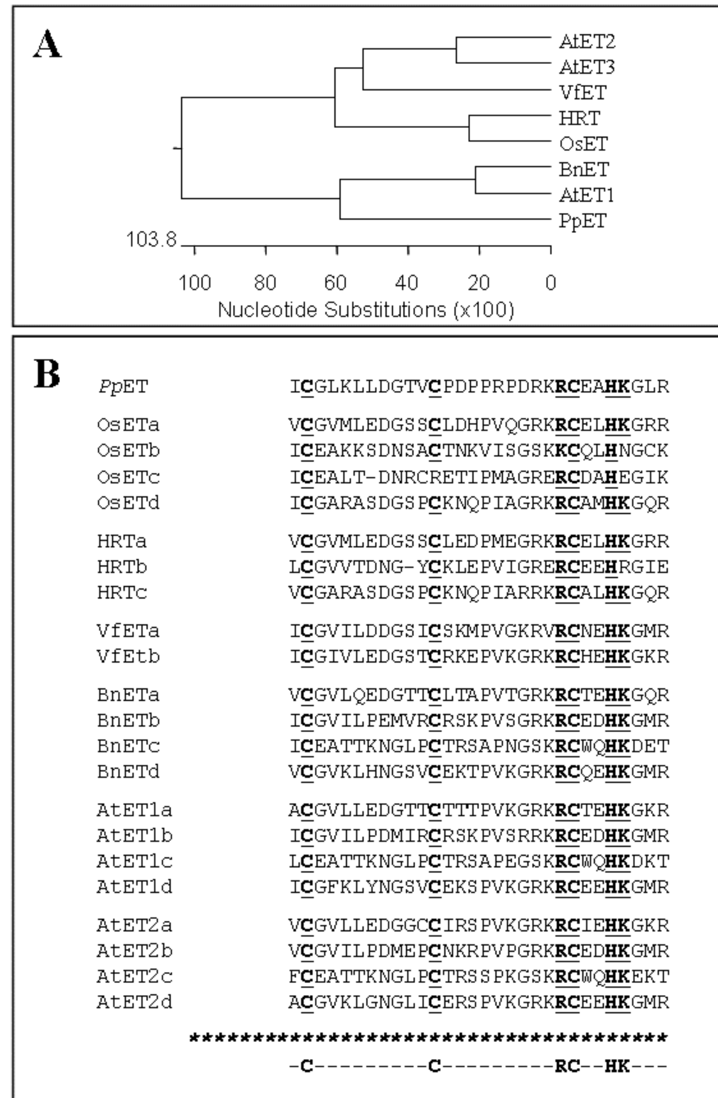

Figure 3. Structure of the ET protein family

A. Similarity tree of the known ET family proteins aligned by ClustalW algorithm

B. Alignment of all known ET repeats. Pp *Physcomitrella patens*, Os *Oryza sativa*, Vf *Vicia faba*, Bn *Brassica napus*, At *Arabidopsis thaliana*

Functional analysis of BnET overexpressing tobacco and *Arabidopsis* plants revealed heavy germination defects and confirmed the involvement of ET in regulation of certain aspects of the GA response pathway.

Additionally to seed phenotypes, the 35S promoter driven overexpression leads to dwarf growth, delayed flowering and severe lack of lignin, indicating the involvement of ET in regulation of developmental processes. ETs probably act as differentiation maintenance factors as leaf cells overexpressing BnET cannot dedifferentiate and form callus in the proper hormonal environment, unlike the wild type (Ellerstrom et al., 2005).

Aim of the project

By the use of overexpression in tobacco and *Arabidopsis*, we have demonstrated the significance of the ET proteins for the proper plant development as regulators of cell differentiation (Ellerstrom et al., 2005). This observation opens a new dimension of questions regarding the mechanism by which ET proteins, as putative transcription factors, act to control these processes, which plant regulatory mechanisms are they influencing and, the no less important question, – how are ET products regulated by the plant organism in return.

Understanding the significance of a protein or a protein family for the plant organism requires a complex characterization of mutant plant phenotypes, application of suitable model systems and *in vitro* experiments. Of special importance is the choice of the model plant. For such we chose *Arabidopsis thaliana*, due to its short life cycle, sequenced genome and large amount of data on its regulatory networks collected over the last 25 years.

We aim to investigate the role of the *Arabidopsis* ET family by T-DNA insertion mutant analysis of phenotypes in seed and vegetative development. Furthermore, we will employ gene expression screening techniques and try to correlate the plant phenotypes to the expression levels of important developmental regulators and phytohormone influence.

Materials and methods

1. Plants and plant growth

Arabidopsis thaliana plants of the ecotypes Columbia0 and Wassilewskija2 (WS2) were used as wild type. If needed, the seeds were surface sterilized by treatment with 70% ethanol and 12% NaClO, and the plants were grown in soil at 25 °C in 16h light and 8h dark conditions unless stated otherwise.

2. Molecular cloning

Molecular cloning techniques such as PCR, enzyme digestion and DNA ligation were performed according to standard protocols (Molecular Cloning Third Edition, eds. Sambrook J. and Russel D., CSH Laboratory Press).

3. Generation of transgenic lines

Full length *AtEt2* cDNA was cloned behind the constitutive CaMV 35S promoter in vector pBinAR (Roeber et al., 1996) using the KpnI and SalI restriction sites. The obtained construct was used to transform *A. tumefaciens* using a modified version of the freeze-thaw method (Hoefgen and Willmitzer, 1988). Competent *Agrobacterium* cells were mixed with 1.0 µg of plasmid DNA and successively incubated for 5 minutes on ice, liquid nitrogen and 37 °C.Before plating the cells on YEB plates, containing the appropriate antibiotic, they were diluted in 1 ml of YEB medium and shaken for 2 hours at 28°C.

Transformation of *Arabidopsis* was done according to the protocol of Bechthold *et al*. (1993). Plants of *A. thaliana* ecotype Wassilewskija were grown for three weeks under short day conditions (8 hours light, 16 hours dark) and transferred to long day (16 hours light, 8 hours dark). After three weeks, the emerging bolts were cut to induce growth of secondary bolts. Vacuum infiltration of plants with the suspension of *Agrobacterium tumefaciens*, containing the vector, was done one week after the clipping. Bacteria were grown till OD_{600}>2.0, harvested by centrifugation and resuspended in three volumes of infiltration medium supplemented with 0.01% Silwett (Sigma). Inflorescences were submerged into the *A. tumefaciens* suspension in a beaker. Vacuum was applied by an oil pump for 5 minutes and than rapidly released. Plants were removed from the beaker, placed on their side and kept at high humidity under plastic wrap. After 24 hours, they were uncovered and set upright. Seeds were harvested from dry siliques, sterilized and plated onto selection plates containing Kanamycin. After two weeks, viable plants were transferred to soil, grown up and their seeds collected. Transgenic plants were analyzed by PCR or Northern hybridization.

4. Screening for an *AtET2* T-DNA insertion mutant

The line *et2-4* was chosen from the collection of the *Arabidopsis* Knock- out Facility (AKF) at the University of Wisconsin Biotechnology center, following a pool screening for insertion in *AtET2* gene. The line was proven homozygous using a pair of PCR reactions with custom olignonucleootide primers (Metabion) amplifying the intact gene and the insertion within the gene, respectively. The primer combinations were as follows:

Intact *AtET2* gene reaction:

5’-ATGGAATTCGGCGACGGCG-3’ and

5’-GGTGATTCTCATTCCCTTATG-3’

Insertion reaction:

5'-TGGGAAAACCTGGCGTTACCCAACTTAAT-3' and

5’-TGCTCTTCACATCTCTTACGTCCTTTTAC-3’

PCR conditions:

Initial denaturation – 95 ^{o}C, 4 min

35 cycles:

95 °C, 30 s

55 ^{o}C, 30 s

72 ^{o}C, 45 s

Final extension - 72 ^{o}C

Optimal results were achieved with the use of Taq DNA Polymerase (Qiagen).

5. CAPS marker for the presence of a mutated *Atet1* allele

Genomic fragment of 1244 bp, containing the mutation site and an additional EcoRI cleavage site was amplified from the *AtET1* gene sequence. The primers used were as follows:

5’- ATGTTCAAGAGAGACGACTACA-3’ and

5’-ATCCTCGCATCGTTTTCTCC-3’, Tm= 55 °C, 35 cycles

Following PCR, the fragment was digested, without prior purification, for two hours with EcoRI (Amersham) and loaded on a 1.5% agarose gel. The intact allele would produce a major band at 1082 bp, while the frameshifted allele - one at 897 bp, due to the presence of a second EcoRI site.

6. Seed germination

Plants, including wild type, were grown under the conditions described above (see **Plants and Plant Growth**). Seeds were collected from desiccated siliques and kept for one month in a dark and dry place. After that, they were surface sterilized and spread on petri dishes over water moistured filter paper (Sartorius) supplemented with 10 µM GA_3 (Duchefa) where indicated. The dishes were cold treated for 1 to 7 days prior to incubation in a growth chamber (16 hours day, 8 hours night, 25 °C). Germination rate was determined after 7 days unless indicated otherwise.

7. Seed “rescue” experiment

Wild type and *et2-4* plants were grown under the conditions described above (see **Seed germination**). Seeds were collected from green siliques and immediately grown on plates, containing Murashige and Scoog medium (MS, Duchefa). Germination rates were counted for up to 30 days after rescuing.

8. Hypocotyl growth induction

Single plants were grown in soil in standard *Arabidopsis* pots (25 cm^2 surface, 6 cm depth). Hypocotyl growth was stimulated by continuous excision of the stem and bolts for four weeks. At the end of this period the hypocotyls were taken and 20- 50 µm fresh cross-sections were prepared using a VT1500 vibratome (Leica). Lignin autofluorescence was visualized by 325 nm UV light excitation on Axiovert135 inverted fluorescent microscope (Zeiss).

9. Submerged *Arabidopsis thaliana* cultures

Seeds were surface sterilized and grown with gentle shaking for 10 days in 100ml flasks, containing 50ml ½ MS medium (Duchefa) with added 10 g/l sucrose (Sigma) and 0.5 g/l MES buffer (Duchefa), pH 5.6. The resulting seedlings were used for phenolic compounds measurement and RNA isolation for RT-PCR and array hybridization.

10. Array hybridization

mRNA was isolated with Dynabeads mRNA Direct Kit (Dynal Biotech) according to manufacturer's instructions. First strand cDNA was synthesized directly on the beads using AMV Reverse Transcriptase (Promega). The probes were labeled with ^{33}P-dCTP by random priming using Klenow fragment (Amersham).
The membranes were prehybridized for 2 hours at 65 °C in Church buffer (7% SDS, 0.5 M $NaPO_4$, 1% Albumine fraction (Roche), pH 7.2) supplemented with 40 µg/ml Herring Sperm DNA (Stratagene). After an overnight hybridization, washing was done as follows: 2x15 minutes 2x SSC, 0.1% SDS, 2x15 minutes 1x SSC, 0.1% SDS and 2x 0.5 SSC, 0.1% SDS; all at 65 °C.
The filers were exposed to Phosphoimager screen (Fuji) and scanned.

11. RT-PCR

Total plant RNA was isolated from the indicated organs using the Total RNA Isolation Reagent (Biomol) following the manufacturer's instructions. The obtained RNA was treated with DnaseI (Fermentas) for 15 minutes at 37 °C. The enzyme was inactivated by the addition of 2M EDTA followed by heat treatment at 70 °C. Single stranded cDNA was synthesized using First strand cDNA Synthesis Kit (Fermentas) and was subsequently used as a PCR template. The annealing temperature and number of cycles for each primer pair were optimized in order to ensure quantitative measurement. The

PCR reactions were run on 1.5% Agarose gels, capillary transferred on Hybond+ membranes (Amersham) and hybridized. The labeling of the cDNA probe was carried out with [^{32}P]dCTP by the means of Rediprime^{TM} II Random Prime Labeling Kit (Amersham). The radioactive membranes were washed and exposed to Phosphoimager (Fujifilm). The primer pairs and their respective PCR conditions are as follows:

AtEt1: 5'-ATGTTCAAGAGAGACGACTACATTGC-3'
5'-AAGATGTCATTCTCATCCCCTTGTGC-3', Tm=55 °C, 39 cycles

AtEt2: 5'-CTATATCATCGGTTTTATCGAAATGGAATT-3'
5'-AAGTGATGCAGAGGTTAGGTGATTCTCATT-3', Tm=55 °C, 42 cycles

AtEt2 primers for detection of the 5'UTR and the beginning of the coding region:
5'-CTATATCATCGGTTTTATCGAAATGGAATT -3'
5'-CGAGGTAAGTTCTGGACTCTGTA-3', Tm=55 °C, 35 cycles

Stm (according to Cary et al., 2002):
5'-AGAGTGGTTCCAACAGCA-3'
5'-TTAGTTCCTTGGGGAGGA-3', Tm=55 °C, 38 cycles

Knat1: 5'-CACCGTCTGTCTCTGCCTCCTCTA-3'
5'-ATTCCGCCAACGCTACCTTCTCT-3', Tm=55 °C, 25 cycles

Knat2: 5'-CGAACTCGCTACCGCTTTGTCCT-3'
5'-TCGCGGTCATTGCTTCTTTGTTG-3', Tm=55 °C, 40 cycles

Knat3: 5'- CCGGCGGTGGAGAAAACAA-3'
5'- TCCCCCATCGAACATATTAGCATC-3', Tm=55 °C, 26 cycles

Knat6: 5'-CTCCGCCGGTGAAAATCGTGT-3'
5'-GGTTCCGTAGCTGCATCTCAATCT-3', Tm=55 °C, 26 cycles

Fil: 5’- ATGTCTATGTCGTCTATGTCC-3’
5’- TTAATAAGGAGTCACACCAACG-3’, Tm=55 °C, 37 cycles

AtGA20 oxidase (according to Biemelt et al., 2004):
5’-ATGGCCGTAAGTTTCGTAAC-3’
5’-TTAGATGGGTTTGGTGAGCC-3’, Tm=55 °C, 40 cycles

Gasa4: 5’-ATGGCTAAGTCATATGGAGC-3’
5’-TCAAGGGCATTTTGGTCCAC-3’, Tm=55 °C, 24 cycles

AtEf-1bα (according to Bauer et al., 2004):
5’-AGGAGAGGGAGGCTGCTAAG-3’
5’-AATCTTGTTGAAAGCGACAATG-3’, Tm=55 °C, 23 cycles

12. Extraction and analysis of phenolic constituents

Aliquots of powdered plant material were extracted repeatedly with methanol in Eppendorf tubes (final volume 1 ml). Extracts were centrifuged at 13,000 × g for 10 min at 4°C. Phenolic constituents were analyzed on an HPLC system consisting of a Waters 600 controller and pump unit and a Waters 996 photodiode array detector (Waters, Eschborn, Germany) combined with a fluorescence detector (FP-920, JASCO, Groß-Umstadt, Germany). Data acquisition and processing were performed with the Millenium software package (Waters, Eschborn, Germany).
Methanolic extracts were diluted with 0.25 volume of water and centrifuged again. Aliquots of the supernatants were injected and separated on a RP-18 phase column (LiChrospher, 5 µm, 125-4; Merck, Darmstadt, Germany). The mobile phase used consisted of 0.1% ammonium formiate in 2% formic acid (solvent A) and 90% methanol in solvent A (solvent B) as described previously (Heller et al., 1990), but with a slightly modified gradient profile. After 5 min elution with 100% solvent A, a gradient from 0 to

40% solvent B over 25 min was applied followed by a gradient from 40% solvent B to 100% B over further 20 min. Spectra from 250 to 400 nm were recorded (1/s) at maximal spectral resolution of the photodiode array detector (1.2 nm), and chromatograms of absorbance at 280 nm were extracted from these data sets. The wavelength settings for the fluorescence detector were 300 nm for excitation and 400 nm for emission.

13. Total lignin measurement

The lignin content was determined after thioacidolysis following the method described by Campbell and Ellis (1992). Recovery rates for each individual experiment were determined by analyzing parallel samples with appropriate amounts of authentic lignin.

14. Protoplast transformation for transient assay

Suspension cultures of *Nicotiana plumbaginifolia* and *Arabidosis thaliana* were used for protoplast isolation. Following cell wall digestion in a 1% cellulase R10 (Duchefa Biochemie) and 0.5% macerozym R10 (Duchefa Biochemie) solution, protoplasts were centrifuged and washed two times in W5 medium (0.9% NaCl, 1.8% $CaCl_2$, 0.04% KCl, 0.1% Glucose, pH 5.6). Next, they were concentrated in Mg Mannitol (0.45 M Mannitol, 15 mM $MgCl_2$, 0.1% MES pH 5.6) to a density of approximately $3x10^6$ cells/ml. To transform the resulting protoplasts, solution containing plasmid DNA (5 µg of each plasmid) and carrier DNA (160 µg) was added to 330 µl Mg Mannitol containing $1x10^6$ protoplasts. Equal amount of PEG solution (40% PEG 6000, 0.1 M $Ca(NO_3)_2$, 0.4 M Mannitol, 0.1% MES, pH 6.5) was added to the mixture after 10 minutes incubation. After 20 minutes, the transformed protoplasts were diluted into 4 ml K3 medium and transferred to small Petri dishes. Following a 16- 18h incubation time at 25°C in the dark, protoplasts were harvested and the GUS activity was determined by chemiluminescent assay using the GUS-Light™ Kit (Tropix, Bedford, USA) and a Lumat LB9501

luminometer (Berthold). A control construct consisting of the 35S CaMV promoter in front of the GUS reporter gene was efficiently expressed in this system and used to standardize the different experiments.

15. Transient expression of ET- GFP fusions in protoplasts

Protoplasts were transformed with pFF19g vector (free GFP control, Hofius et al., 2004), or pFF19 containing a C- terminal translational fusion of *AtEt1*, *AtEt2* or *AtEt3* with *Gfp*, following the above described protocol. The suspension was incubated for 16- 18 h in K3 medium, containing either 4.5 μM 6-benzylaminopurine, 10 μM 1-naphtalene acetic acid and 4.5 μM 2,4-dichlorophenoxyacetic acid (High Hormone Medium), or 0.9 μM 6-benzylaminopurine and 0,1 μM 1-naphtalene acetic acid (Low Hormone Medium). After the indicated period of incubation, the GFP expression was observed *in vivo* with a confocal laser- scanning microscope (CLSM Meta, Zeiss, Jena, Germany). The eGFP fluorophore was excited at 488 nm by an Argon laser. The detection was preformed between 505 nm and 520 nm.

16. Yeast Two- Hybrid Library Screening

AtEt2 open reading frame was cloned into the EcoRI restriction site of pGBKT7 (Clontech), containing GAL4 DNA binding domain. The construct was transformed into the yeast strain Y184 and transformants were selected on synthetically defined (SD) medium lacking the amino acid Tryptophane. One positive strain was mated to AH120 yeast cells transformed with pGADT7-REC, containing randomly amplified *Arabidopsis* cDNA library (kindly provided by Willem Reidt and Holger Puchta), fused to GAL4 activation domain. The transformation and screening procedures were done according to the Yeast Protocols Handbook (Clontech).

Interaction positives were selected on SD medium lacking Tryptophane (pGBKT7 presence selection), Leucine (pGADT7-REC presence selection) and Histidine (selection for interaction). Positive colonies were further verified by *LacZ* blue-white test.

17. Heterologous protein expression in *E. coli*

AtEt1 coding sequence was cloned in the expression vector pET29b (Novagen) ensuring a translational fusion with an S-tag. *AtEt2* was cloned into pET29a (Novagen). Competent *E. coli* cells of the expression strain HMS174 were transformed with the obtained constructs. Heterologous expression, driven by the T7 promoter, was induced by addition of 0.5– 1 mM isopropyl thiogalactoside (IPTG, Roche) for 5 hours at 37°C. Cells were harvested by centrifugation at 5000 x g and frozen until further use.

18. Protein purification

The collected pellets of *E. coli* cells, expressing the protein of interest, were lysed by 8 one- minute cycles of ultra- sonication by Sonopuls HD 200 (Bandelin) in 1x Bind/Wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100). The obtained extract was centrifuged and the pellet was resuspended in 1x Bind/Wash buffer containing 8M Urea (Sigma). After centrifugation, the two supernatants were united and the recombinant protein was affinity purified using the S-tag^{TM} Thrombin Purification Kit (Novagen). The purified proteins were eluted with 3x1 volume 3 M guanidine thiocyanate (Sigma) and dialyzed overnight against PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3). Protein concentrations were measured using Bradford Reagent (Sigma) at 595 nm with a BioPhotometer (Eppendorf).

19. PAGE and Western Blot

Proteins were separated on a 12.5% (w/v) SDS-polyacrylamide gel electrophoresis in Tris- Glycine buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS). Samples were visualized either by Coomassie blue staining or by Western blotting. After electrophoresis, the proteins were transferred in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol) to a Protran^{R} nitrocellulose membrane (Schleier & Schuell). The nitrocellulose filter was blocked by a 1 hour incubation in 3% Bovine Serum Albumin (BSA, Sigma), dissolved in TBST (20 mM Tris–HCl, pH 7.4, 180 mM NaCl, 0.1% Tween-20), to prevent non-specific binding of the antibody, followed by 1 hour incubation in an 1:10.000 dilution of the S-Tag conjugated antibody (Novagen). After three washes with TBST the protein/antibody complex was visualized by a color reaction (NBT/BCIP, Novagen).

20. ELISA based DNA binding experiment

DNA binding experiment was performed as previously described (Monke et al., 2004). In short, 5'-biotinilated double stranded oligonucleotides (Metabion) were immobilized on streptavidin coated microwell plates (Nunc) in TBST buffer (20 mM Tris–HCl (pH 7.4), 180 mM NaCl, 0.1% Tween-20) for 1 hour. Following washing and blocking with 3% Bovine serum albumin (Sigma) in TBST, incubation with the indicated amounts of either AtET1 or AtET2 was made in a reaction volume of 80µl for 1 hour. The signal from the bound protein was detected by S-protein– alkaline phosphatase conjugate (Novagen). The phosphatase activity was determined with p-nitrophenyl phosphate (p-NPP) dissolved in diethanolamine–HCl buffer (pH 9.8). After incubation at 37°C, the optical density at 405 nm was measured with a plate reader (Dynatech MR7000).

Oligonucleotides used in the experiments:

GARE (According to Raventos et al., 1998):

TCACCGTACCGGCCGATAACAAACTCCGGCCGACATATC

Destroyed GARE:

TCACCGTACCGGCCGAACCTGGGCTCCGGCCGACATATC

RY element (According to Monke et al., 2004):

AATTCTGACTCCATAGCCATGCATGCTGAAGAATGTCACACA

Arbitrary sequence (According to Monke et al., 2004):

GTACTCATACTGTCGCAATGAGTCATCTGTCACAGAGTTCAG

Central GARE, RY central elements and their mutated variants within the sequences are underlined.

21. Iron Binding

The experiment was performed following a procedure described by Krueger et al. (2004). The purified protein was spotted on a 40 µm nitrocellulose membrane (Schleier&Schuell) and incubated in 0.5525 µM $^{55}FeCl_3$ solution. If needed, the iron was reduced to Fe^{2+} with the addition of 55 µM Ascorbic acid. After 120 minutes, the membrane was washed three times with Metal Binding Buffer (0.025 M Tris pH 6.8, 0.15 M NaCl) and exposed to Phosphoimager screen (Fuji) for at least 5 hours before scanning.

22. Internet Searches and Alignments

Sequence searches were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) available on the NCBI web site

(http://www.ncbi.nlm.nih.gov/BLAST/). Alignments were done with the DNASTAR software (Lasergene) using the ClustalW algorithm or as one pair alignments by Martinez-NW method.

Results

1. Structure of the *Arabidopsis ET* family

The *Arabidopsis* genome contains three *ET*- like sequences (Figure 2a). Highest homology to the *BnET* shows the gene located on the fourth chromosome, named *AtET1* (At4g26170). The other two, located on the fifth chromosome, are obviously the result of a duplicaton event. One of them, *AtET2* (At5g56780), is a complete sequence, while the second one, *AtET3* (At5g56770), lacks the region, encoding the C- terminal ET repeats. *AtET3* is obviously an incomplete copy of *AtET2* due to their high homology in the N-terminal region.

The sequencing of the *Arabidopsis* genome, performed on the Columbia0 (Col0) ecotype, revealed *AtET1* as a pseudogene since the coding sequence was interrupted by stop codons and the whole C- terminus was in a different reading frame. Because our study was concentrated on an *AtET2* T-DNA insertion mutant in Wassilewskija2 (WS2) ecotype we cloned and sequenced the genomic and spliced versions of all three genes form this ecotype. While *AtET2* was identical in both ecotypes, there were significant differences in the other two genes.

AtET1 gene of WS2 is uninterrupted and completely in frame. As shown on Fig. 1b, an adenine deletion at position 114 relative to the ATG of the cDNA sequence leads to a frameshift and the destruction of the Col0 gene. The nucleotide deletion results in the formation of a GAATTC sequence, a recognition site for EcoRI restriction nuclease (Figure 4b). We used this advantage to create a **C**leaved **A**mplified **P**olymorphism **S**equence (CAPS) marker and screened several *Arabidopsis* accessions for the presence of the mutated allele. We amplified a genomic fragment of 1244 bp starting at the beginning of the coding sequence and containing an additional EcoRI site at position 162 after the ATG and the marker sequence. This additional EcoRI site was used as an internal digestion control. Following amplification and cleavage, an intact allele would give a major band at 1082 bp, while the mutant allele – at 897 bp. Of all investigated accessions, except Col0, only one more – Limeport – showed the presence of the mutant allele (Figure 4b).

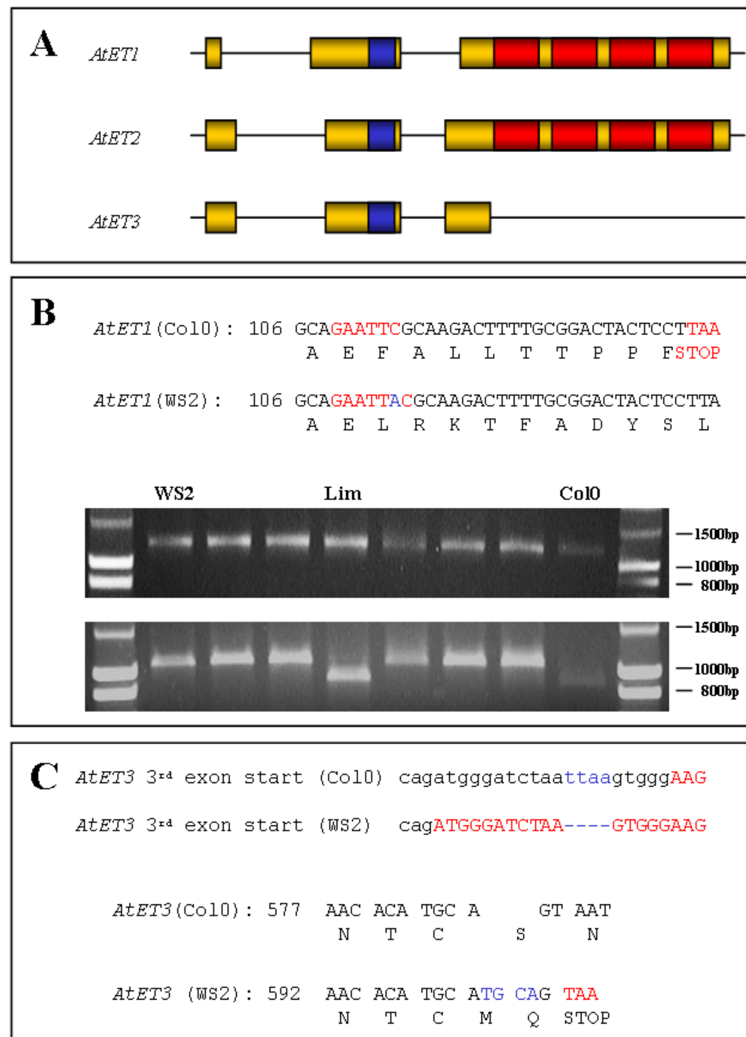

Figure 4. Structure of the *Atabidopsis ET* family

A. A schematic representation of the three *ET* genes in *Arabidopsis*: Yellow blocks represent the exones. Red - the regions encoding the ET repeats. Blue - region encoding for putative endonuclease domain, similar to the bacterial UVRC.
B. A polymorphism in the *AtET1* gene: In the Col0 allele, an adenine (marked in blue) is missing causing a gene inactivating frameshift. The deletion creates an EcoRI restriction site (in red), which was used as CAPS marker. Following PCR and EcoRI digestion, the mutated allele in Col0 and Limeport results in a smaller fragment, due to the presence of the additional EcoRI site.
C. Structure of the *AtET3* gene: Upper alignment – sequencing of the *AtEt3* cDNA revealed that the third exon (red letters) of the WS2 allele starts 15 bp earlier than in Col0 ecotype; in addition a 4 bp insertion (blue) is located in the elongated intron sequence of Col0. Lower alignment – a four- nucleotide duplication in the third exon of the WS2 allele of *AtET3* results in a premature stop codon at position 607 relative to the ATG of the cDNA.

The expression of the truncated *AtET3* gene was verified by RT-PCR, suggesting, that despite the lack of the C- terminal repeat it might be a functional gene. After successful cloning and sequencing, we discovered several polymorphisms between WS2 and Col0 ecotypes (Figure 4c). Two of the most significant points of difference between the two *AtET3* alleles were the beginning of the third exon (Figure 4c upper alignment), which is at an earlier position in WS2 allele, and a four- nucleotide duplication leading immediately to a frameshift and a stop codon. Thus, the WS2 allele has a 171 bp shorter coding sequence compared to the Col0 one (609 bp vs. 780 bp).

2. Molecular characterization of AtET

As ET was originally isolated in a South- Western hybridization and contained Cysteine-Histidine repeats, a function as metal coordinating and DNA binding factor has been proposed.

We expressed the *Arabidopsis* AtET1 and AtET2 in *E. coli* to prove the capability of the proteins to bind metal ions and specific DNA sequence and if possible to verify certain speculations on the molecular mechanism of their action.

Constructs containing full length *AtEt* cDNA translationally fused to an S-tag were expressed in *Escherichia coli*. Correct size (56.5 KDa for AtET1 and 58 KDa for AtET2) and purity of both proteins were verified by Western blot (Figure 5a).

As members of the ET family were already proven to bind Zn^{2+} (Ellerstrom et al., 2005, Raventos et al., 1998), we decided to go further and ask the question whether the ion binding was random or specific for bivalent ions. For a model, we used iron ions because they can be in both bivalent and trivalent form. Radioactive $^{55}Fe^{3+}$ was provided to AtET1 and AtET2 proteins immobilized on a nitrocellulose membrane as $FeCl_3$ (Figure 5b). For obtaining Fe^{2+}, ascorbic acid was added to the solution. AtET proteins were able to bind both forms, but displayed extremely high affinity to Fe^{2+}, much higher than the *Arabidopsis IRON TRANSPORT PROTEIN* (*ITP*) (Krueger et al., 2004) used as a positive control. Binding of Fe^{2+} was equal for both proteins while AtET1 showed higher strength in the interaction with Fe^{3+}. We suppose that the ET domains coordinate metal ions to acquire correct conformation in order to be able to bind DNA.

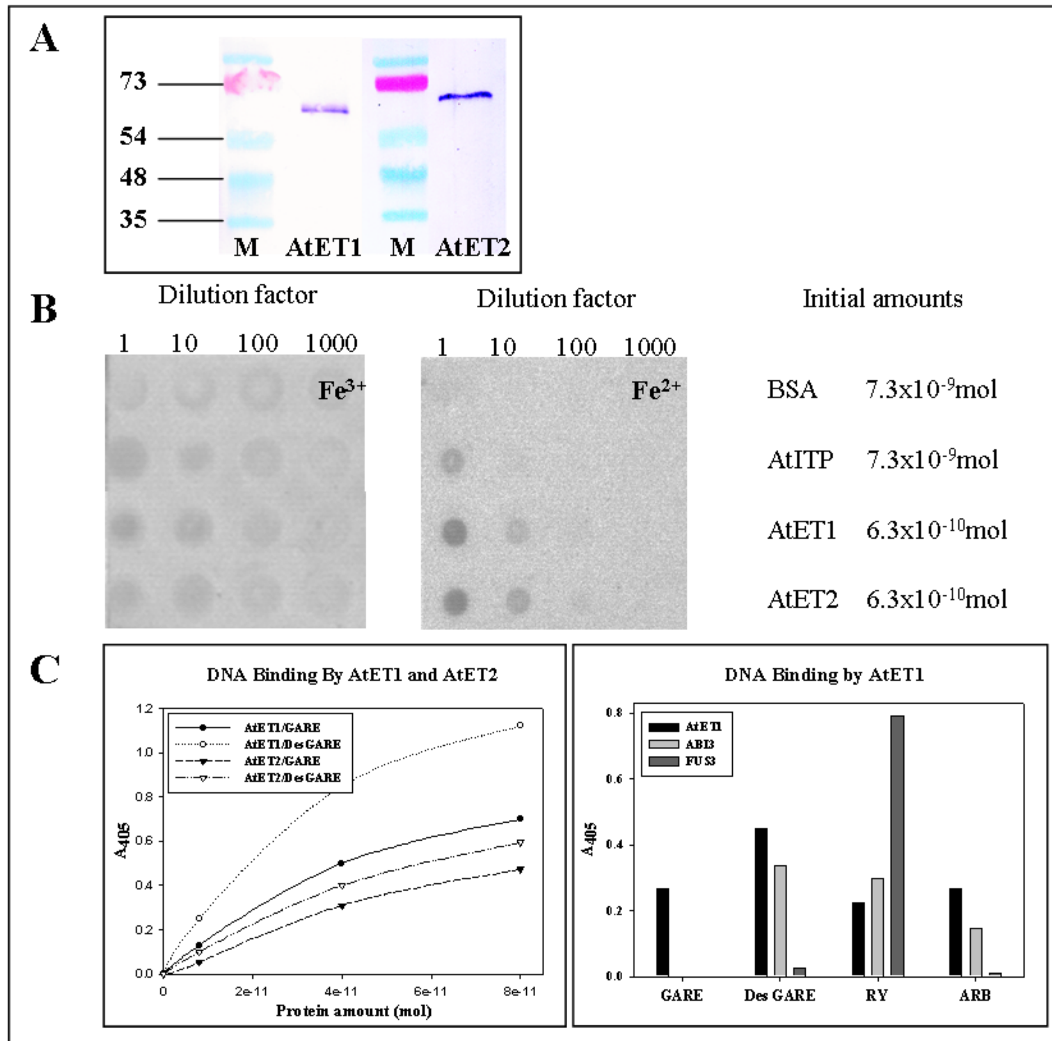

Figure 5. *In vitro* binding activities of the proteins AtET1 and AtET2

A. AtET1 and AtET2 proteins were expressed in *E. coli* and purified using S-tag. The correct size (predicted: 56.5 KDa and 60.2 KDa, respectively) and purity were verified by Western blot.

B. Iron binding capacity of the two expressed proteins. Four dilutions of the protein solution were immobilized on nitrocellulose membrane and incubated with $^{55}Fe^{3+}$ or $^{55}Fe^{2+}$ solution. AtITP was used as a positive control, bovine serum albumin (BSA) was used as a negative control.

C. DNA binding. Double stranded DNA oligonucleotides were immobilized on ELISA plates. Bound AtET1 and AtET2 were quantified using antibody against the S-tag. Both proteins could bind DNA without major differences in specificity (left). The background signal has been substracted. Specificity of AtET1 was further tested against several control oligonucleotides (right). GARE, GA response element; DesGARE, destroyed GA response element; RY, binding site of ABI3 and FUS3 (Reidt et al., 2000); ARB, arbitrary mouse genomic DNA fragment.

ETs were isolated as DNA binding proteins, but for most of them no binding to a specific sequence motif could be found. Only HRT from *Hordeum vulagare* has been demonstrated to target specifically the central sequence of the Gibberellin Response Element (GARE). We investigated the affinity of the *Arabidopsis* proteins to this sequence. Figure 5c shows the results of the ELISA based DNA binding experiment. As seen, both AtET1 and AtET2 were able to bind the provided DNA, but showed an even greater affinity to the oligonucleotide, containing a destroyed central element. AtET1 protein displayed a much higher binding activity and greater difference between binding GARE and Destroyed GARE compared to AtET2. Because it was the better binder, in an additional experiment we checked its ability to associate to any randomly chosen DNA fragments (Figure 5c, right). AtET1 showed high affinity to all DNA oligonucleotides it was provided with, including an RY- element (Reidt et al., 2000) and random mouse genomic sequence (Monke et al., 2004). The result was similar to the control experiment with the known random binder ABI3 (Monke et al., 2004).
Based on these results, we speculate that one of AtET's functions is to stabilize the protein complex, in which it acts, upon the DNA molecule before the specific binding occurs.

Additional indications on the molecular function were obtained by pattern searches, which revealed that all ET proteins possess a sequence with similarity to the 5'single strand endonuclease domain of the bacterial nucleotide excision repair protein UVRC (Figure 6). The homology includes a part of the domain present in several other known nucleases from Lambda and T7 phages and features the catalytic amino acid arginin. One significant difference between ET and the bacterial sequence is an elongation present in ET exactly at the position where the two β- strands of the bacterial domain are separated (Figure 6a). So, the domains in ET, with the exception of AtET1, contain a variable loop at this position. In case the functionality of this domain can be proven, this finding would allow us to speculate a more general function of ETs as transcriptional regulators not by recruiting other transcription factors, but changing the chromatin structure by introducing single strand DNA breaks.

Combined, the nuclease pattern and the random DNA binding may be an indication for a function of ET as a chromatin remodeling factor.

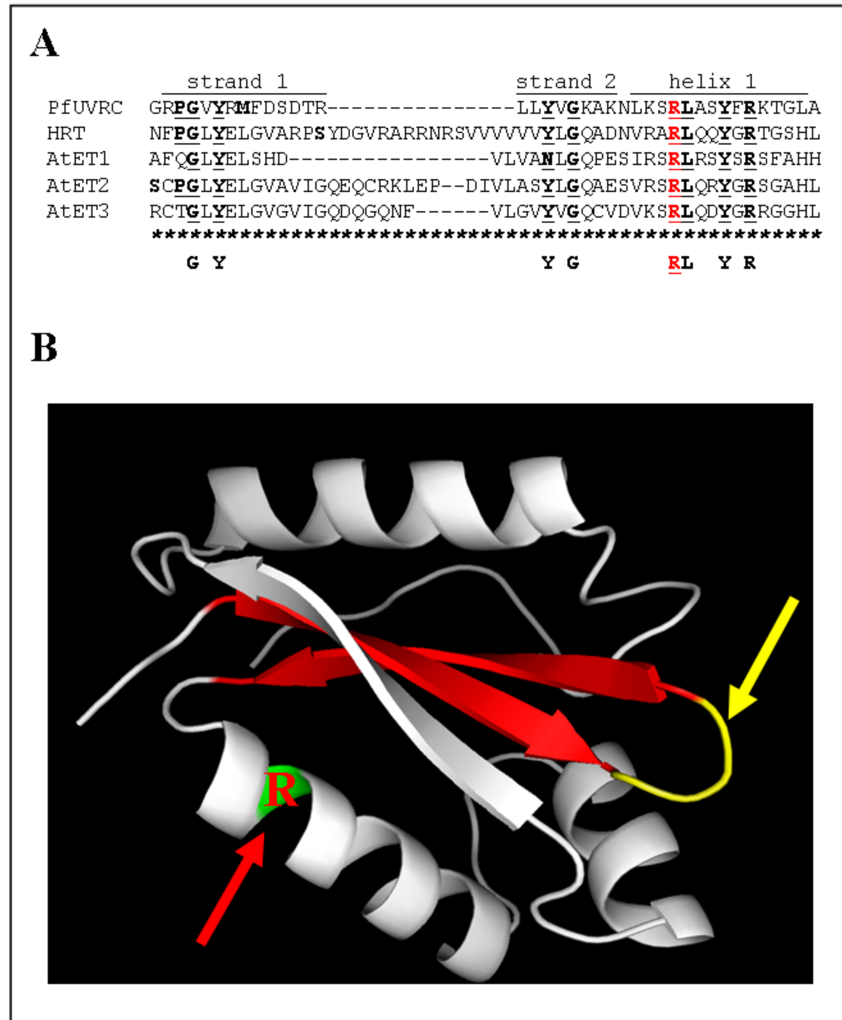

Figure 6. Structure of the proposed single strand nuclease domain of ET proteins

A. Alignment of the 5' single strand endonuclease domains of *Pseudomonas fluorescens* UVRC protein, *Hordeum vulgare* HRT and *Arabidopsis thaliana* AtET1, AtET2 and AtET3. The conserved amino acids are given in bold and underlined. The catalytic arginine is in red.

B. Three dimensional model of the endonuclease domain. Strands 1 and 2 are shown in red; the interstrand loop is given in yellow. This loop is extended in most ET proteins with the exception of AtET1. The position of the catalytic arginine is marked as R and a red arrow.

3. Expression profile of the *Arabidopsis ET* genes

To initiate the characterization of the *ET* gene family, we performed an expression analysis of the mRNA of the two genes *AtET1* and *AtET2*. Due to their extremely low expression levels, we chose an RT-PCR approach. Both genes are generally ubiquitously expressed in all tissues of the plant, with the one exception- *AtEt2* transcript is missing in the stipules (Figure 7a). Of special interest was the mRNA abundance during embryogenesis because of the initial identification of the family in seed libraries. From the expression patterns, we could conclude that *AtET2* is transcribed at higher levels during the stages of embryo formation (green siliques) and is seriously reduced in maturation, while *AtET1* is dominating in mature seeds. These data agree well with array experiments on *Arabidopsis* seed development performed by De Folter et al., 2004 (Figure 7c).

Furthermore, we have created and analyzed an *AtET2* promoter::GUS reporter gene line (Figure 7b). The promoter activity could be detected in all vascular tissues of stems, leaves and even flowers. This correlates well with the RT-PCR expression analysis. Additionally, several other zones were stained, notably the style of the gynoecium.

Combined, these data indicate a role of AtET factors during seed development and in specific zones of the mature plants as the vascular system and the flower. In order to elucidate their function, we analyzed a loss of function mutant for *AtET2*.

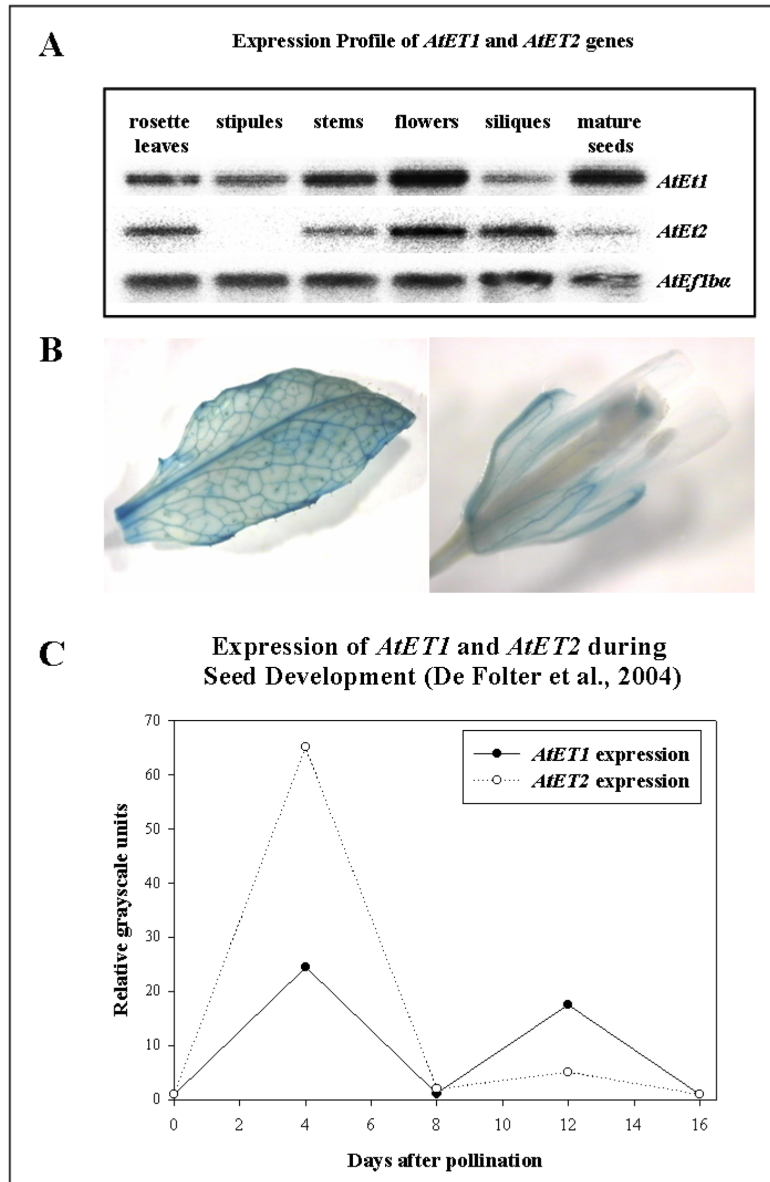

Figure 7. Expression of *AtET1* and *AtET2* genes

A. Expression of *AtET1* and *AtET2* as checked by RT-PCR. The profile showed a ubiquitous expression for both genes (*AtEt2* mRNA was not detected in stipules). Slightly enhanced expression was observed in flowers and at different stages of seed development. *Arabidopsis* elongation factor *EF1bα* was used as an amplification control.

B. Expression of *AtET2* in vegetative development. *AtET2* promoter::GUS lines were created and stained for reporter activity. *AtET2* expression was concentrated in vascular tissues (left) and at the flower style (right).

C. Array hybridization analysis (De Folter et al., 2004) showing expression profiles of *AtET1* and *AtET2* genes. *AtEt2* transcript is more abundant during early stages of development, whereas *AtEt1* is more strongly expressed during maturation. These data confirm our RT-PCR results shown in A.

4. Characterization of AtET2 in plant development

4.1 *AtET2* T-DNA insertion line

A line containing an insertion in the second exon of *AtET2* gene in Wassilewskija2 (WS2) ecotype was selected by pool screening from the collection of the *Arabidopsis* Knock- out Facility (AKF) at the University of Wisconsin Biotechnology center. The presence of a single T-DNA insertion was verified by Southern hybridization (Figure 8a). A homozygous line was created and verified by PCR analysis where two sets of primers- one for the amplification of the intact gene, and one for the presence of the insertion- were used (Figure 8b). Wild type plants were expected to be positive for the intact gene and negative for the insertion, while the homozygous insertion containing plants – negative for the intact gene and positive for the insertion. This was found to be true as shown on Figure 8b.

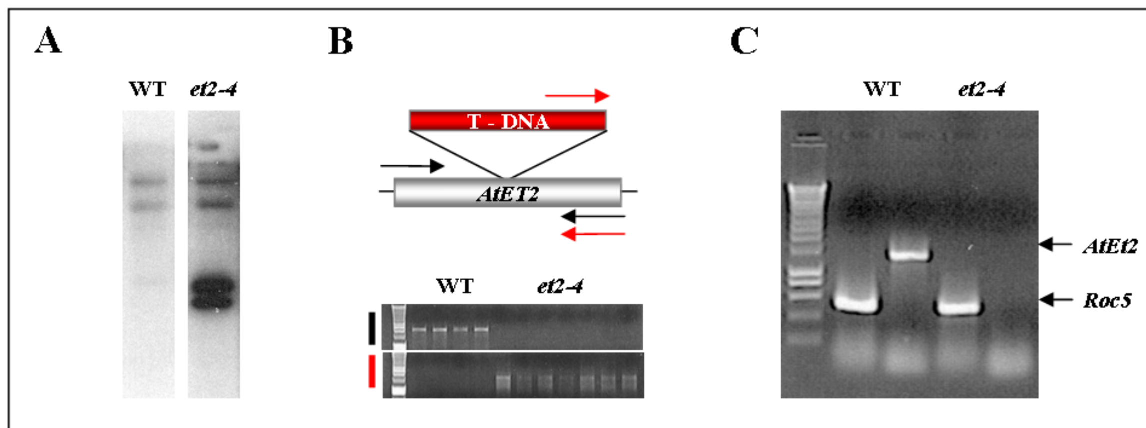

Figure 8. A T-DNA insertion mutant of *AtET2* in WS2 ecotype

A. A single T-DNA insertion in line *et2-4* was verified by Southern hybridization using a T-DNA probe. The presence of a HindIII site within the T-DNA explains the occurence of a double band.
B. PCR analysis proving that line *et2-4* is homozygous: a primer pair amplifying the wild type gene (black) gives a product only in wild type, whereas the primer pair amplifying the T-DNA insertion (red) gives a product only in single plants of the *et2-4* line.
C. Destruction of the *AtEt2* mRNA: Total RNA was isolated from wild type and *et2-4* plants and checked for the presence of *AtEt2* mRNA by RT-PCR. *AtEt2* message was not detected in the *et2-4* sample. *Roc5* mRNA was used as an amplification control.

The final step to verify the homozygous insertion requires demonstration of the absence of wild type *AtEt2* mRNA. We performed RT-PCR on total RNA taken from wild type and the homozygous T-DNA- containing plants. The *Roc5* mRNA was used to control the RNA quality and could be amplified from both samples. The *AtET2* product, though, could be amplified only from the wild type sample, demonstrating the destruction of the intact *AtEt2* mRNA (Figure 8c) in the homozygous T-DNA- containing plants.
This way, we have obtained a genetically well characterized *AtET2* T-DNA insertion line, *et2-4*, as a suitable tool for analysis of function and regulation of *ET* gene family in *Arabidopsis*.

Because our results show that *AtET1* gene is not a pseudogene in WS2 ecotype, and because of the reasonable possibility that certain phenotypes in the *et2-4* line might be hidden by overlapping functions of AtET1 and AtET2, we initiated a generation of a double mutant. For this purpose, we employed a strategy relying on the natural frameshift of the Col0 *atet1* allele. It involves crossing the *et2-4* line to Col0 and the second strategy is based on the isolation of a new AtET2 insertion line in the Col0 ecotype (Figure 9). A line was selected from the GABI- Kat collection (http://www.gabi-kat.de/db/showseq.php?seqid=27-K016060-022-333-C04-8409) with single insertion in the 3' untranslated region of the *AtET2* gene. Homozygous plants were selected by PCR from the segregating heterozygous population and are currently being analyzed.

4.2 Characterization of plant phenotypes and interactions of AtET2 during embryogenesis

The initial identification of the ET factors from *Brassica napus*, *Vicia faba* and *Hordeum vulgare* was done by South-Western screens on seed specific libraries, and the expression profile of the *Arabidopsis* genes suggested a function of ET during embryogenesis. Therefore, we investigated the ability of the *et2-4* seeds in two different stages of development to germinate.

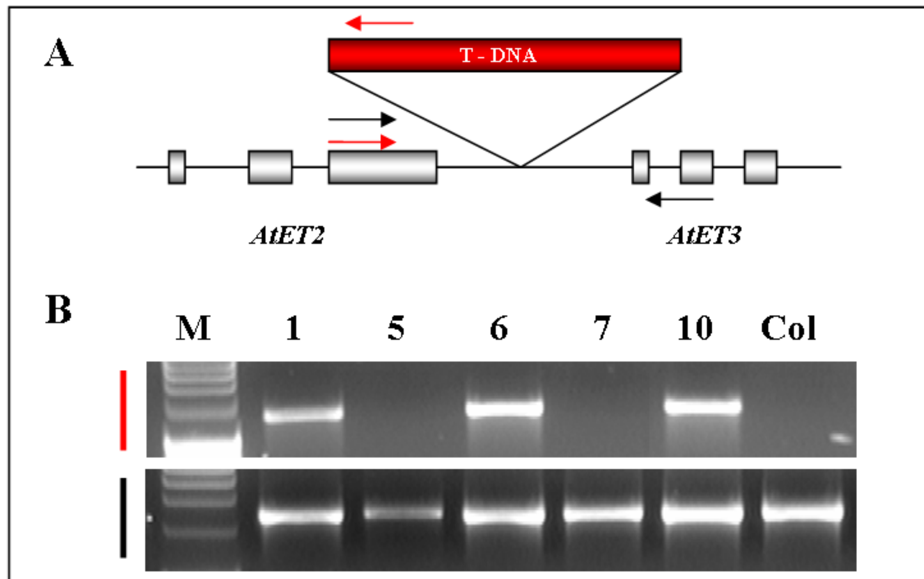

Figure 9. Generation of an *atet1 atet2* double mutant in Col0 ecotype

A. With the inactivated *AtET1* gene in Col0 an insertion in the *AtET2* gene of this ecotype was used to create a double mutant. Presence of the T-DNA insertion (red) and the wild type *AtET2* (black) was checked by PCR.
B. Three of the investigated plants (numbers 1, 6 and 10) were heterozygous for the insertion and their progeny is currently being investigated

At first, immature seeds were isolated from green siliques and grown on agar plates supplemented with basic Murashige and Scoog medium (see **Materials and Methods**). Wild type and *fus3* seeds were used as controls. The results, summarized in Figure 10a, show a highly increased germination potential of *et2-4* seeds compared to the wild type. The observed increase was similar to the one displayed by the seed maturation mutant *fus3*. This reveals an unnatural situation for *Arabidopsis*, in which in normal conditions premature germination is blocked by a so far poorly understood mechanism.

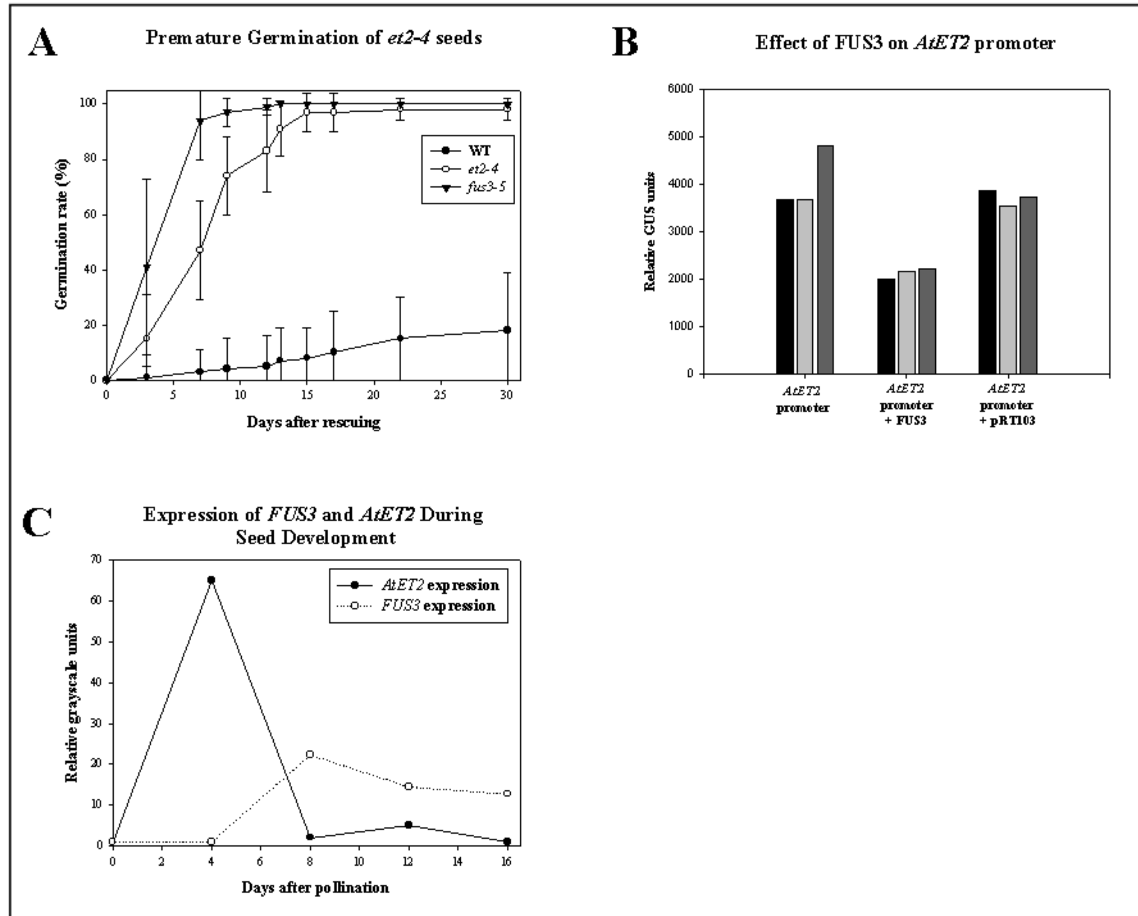

Figure 10. AtET2 in plant embryogenesis

A. Isolated immature seeds were placed on Petri dishes supplemented with MS medium. Germination rate was counted for one month. Seeds of the *et2-4* line showed strong precocious germination, comparable to the control seeds of the line *fus3-5*. Wild type seeds exhibit dormancy under these conditions and show a germination percentage below 20%.

B. In transient assay experiments, expression of FUS3 has negative effect on the promoter of the *AtET2* gene

C. Array hybridization data (De Folter et al., 2004) independently confirm the observation shown in B

As *FUS3* gene is encoding a transcription factor, we used a transient overexpression experiment in *Arabidopsis* protoplasts to test for a possible molecular interaction with ET (Figure 10b). Indeed, expression of FUS3 leads to downregulation of the *AtET2* gene promoter. These results were further confirmed by the recently published data on transcription factor expression during embryogenesis (De Folter et al., 2004), where the appearance of the *Fus3* transcript coincides with the dramatic reduction of *AtEt2* transcript levels (Figure 10c).

Once the seeds enter the maturation phase, lack of a functional AtET2 leads to an additional effect – seeds display a reduced ability to sense germination promoting signals following imbibition. Under normal conditions, without additional treatment, both wild type and *et2-4* seeds germinate to a rate of about 45%. This effect has already been demonstrated for the Wassilewskija2 ecotype (Debaujon and Koornneef, 2000) and can be overcome by cold treatment or by direct gibberellin application. *et2-4* seeds seem to be insensitive to these stimuli (Figure 11) as neither low temperature nor GA_3 application could significantly affect their germination potential.

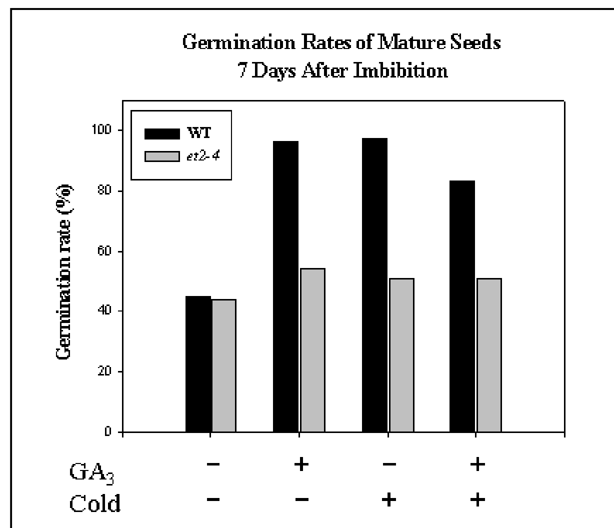

Figure 11. Germination of mature seeds

Seeds of the *et2-4* line had a low germination rate, which can be enhanced neither by GA stimulation, nor by cold treatment.

These results show a complex interaction of AtET2 with different regulatory pathways during the stages of seed development leading to the control of events like germination and seed maturation.

4.3 Phenotypes of *et2-4* in vegetative development

Our previous data show that overexpression of the BnET gene in tobacco leads to phenotypes associated with differentiation of different cell types, including the xylem tissues, visible by lignin accumulation (Ellerstrom et al., 2005). To confirm this ET activity in the available *Arabidopsis* knock- out mutant, we performed a metabolic profiling of methanol soluble phenylpropanes. The analysis revealed an increase in the amount of certain substances (Figure 12a), indicating a possible block in biosynthetic pathways that require them as substrates. Based on our data suggesting *AtET2* expression in vascular tissues, we hypothesized that the pathway affected in the mutant can be the lignin biosynthesis. This we could confirm by a total lignin content measurement. Compared to the wild type, the amounts of lignin in the mutant were reduced by 1/3 both in the stems and in the leaves (Figure 12b). This result indicated an altered cell differentiation in xylem tissues with cells not able to progress through the stages of differentiation and lignin accumulation.

For a further confirmation, we induced hypocotyl lignification by excising continuously the stem and shoots for five weeks. As a result, the wild type initiates a dramatic enlargement and lignification of the central cylinder while this effect in *et2-4* plants is much less pronounced (Figure 13). The final results for hypocotyl thickness are summarized on Figure 13b and show a similarity to the total lignin content data on Figure 12b.

Thus, we conclude that AtET2 is required for the proper differentiation of the xylem. Its absence makes xylem cells incapable of switching their differentiation program and therefore the normal flow of processes like cell wall thickening and lignin accumulation is disturbed. A further confirmation of this hypothesis requires an investigation of gene expression levels in the mutant plants.

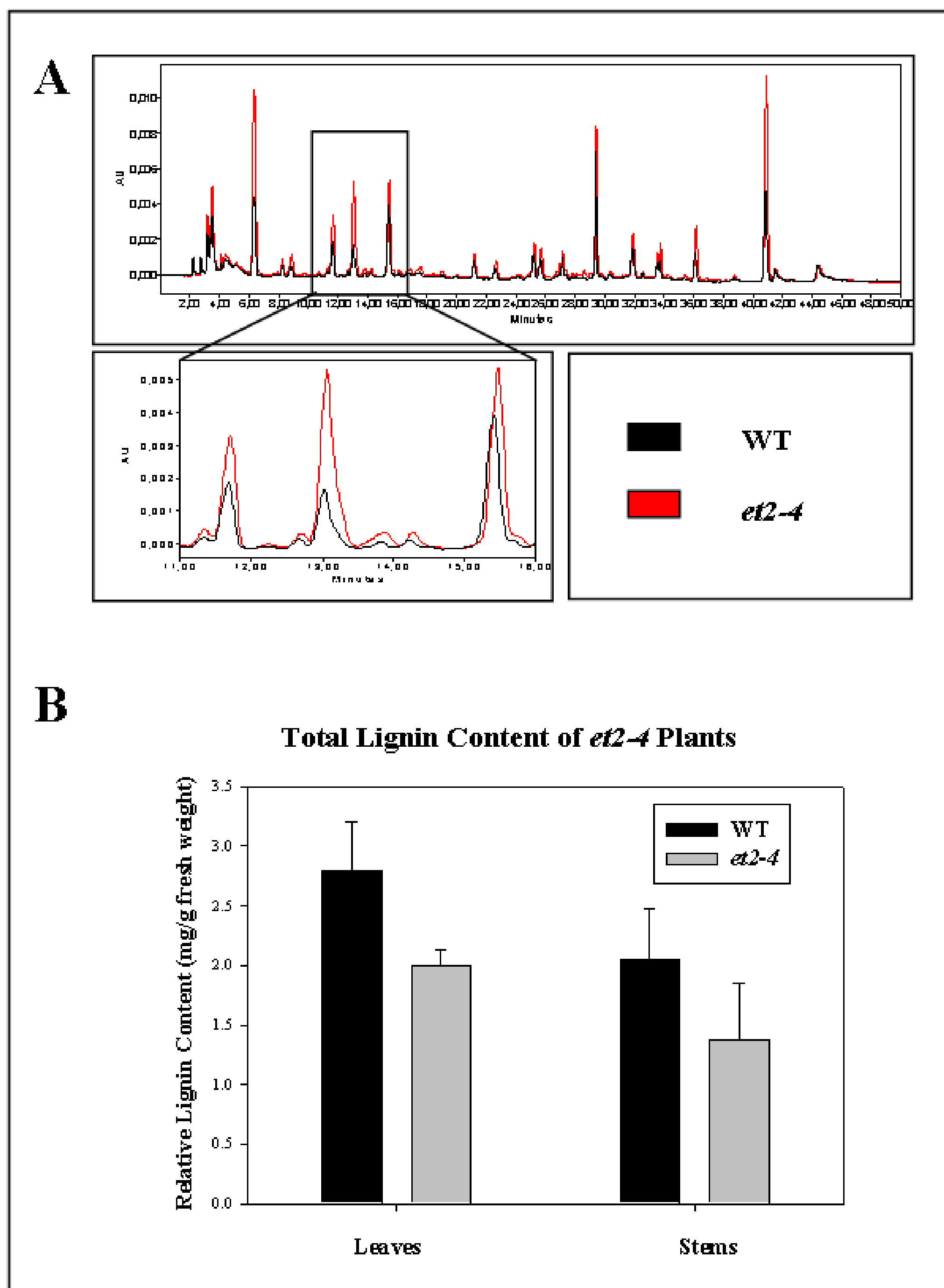

Figure 12. Influence of AtET2 on lignin content during vegetative development

A. Metabolic profiling of methanol soluble phenylpropanes in the *et2-4* line (red) in comparison to wild type (black)

B. Reduction of total lignin content in leaves and stems of *et2-4* plants (grey) compared to the wild type (black)

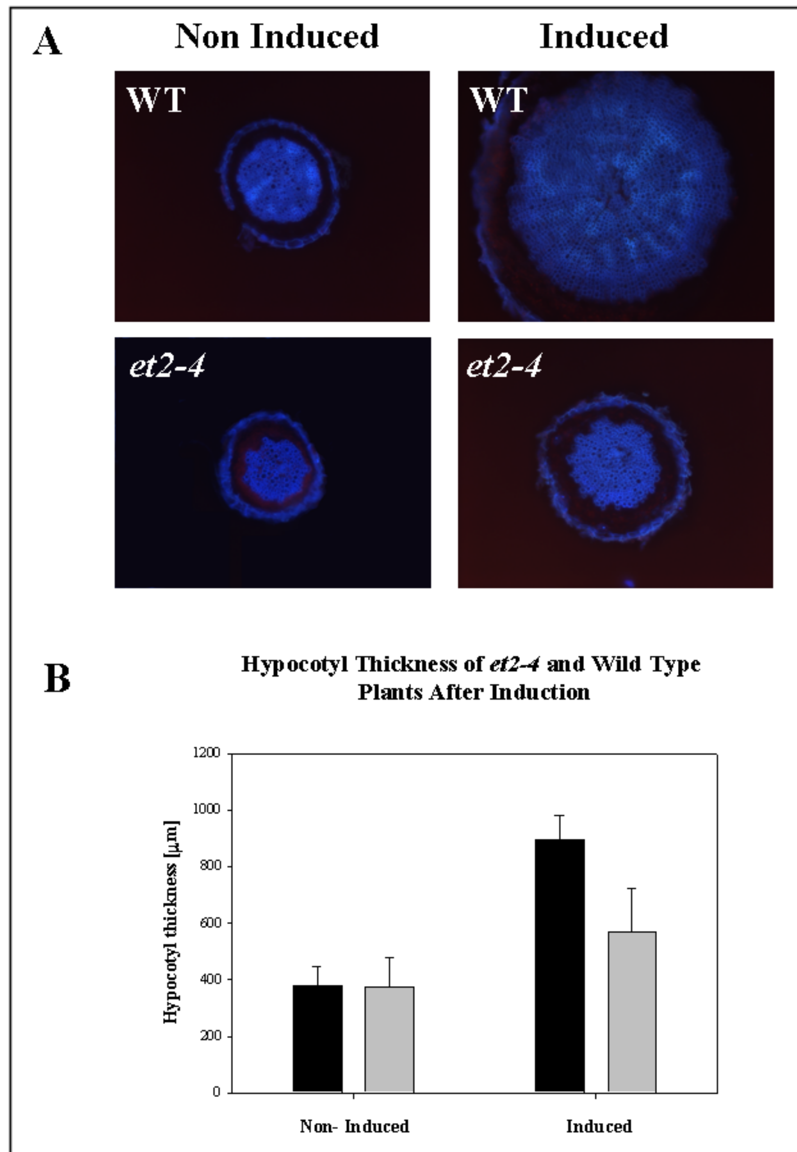

Figure 13. Influence of AtET2 on hypocotyl growth and lignification

A. Hypocotyl growth induction: without induction, hypocotyls of wild type and mutant plants are similar in size, whereas after induction by shoot clipping the wild type hypocotyls (upper pictures) increase in diameter more than twice. Hypocotyls of *et2-4* mutant plants (lower pictures) respond only weakly to this treatment

B. Quantification of the results given as hypocotyl diameter. Wild type is given in black, and the *et2-4* mutant is given in grey.

4.4 Expression profile of the *et2-4* line

To further investigate the molecular basis of AtET2 action in cell differentiation, an array filter hybridization approach was applied. At first, we used an 8000 *Arabidopsis* unigene array filter. The list of differentially regulated genes between *et2-4* and wild type plants included several differentiation associated genes, like a peroxidase, SAG12, chromatin remodeling proteins and transcription factors (Table 1).

Table 1a. Upregulated genes in *et2-4* mutant plants visualized by hybridization of an *Arabidopsis* 8000 unigene filter

AGI Number	Name	Predicted Function	Induction Factor
At1g17290	alanine aminotransferase, putative	Aromatic amino acid biosynthesis	23
At1g17360	CIP7 related, nucleus	Photomorphogenesis	11
At1g20570	Tubulin	Cell division	14
At1g22890	Unknown	Endomembrane system	23
At1g48520	glutamyl-tRNA(Gln) aminotransferase B family protein	Protein synthesis	11
At1g80500	Unknown	ER to Golgi transport	15
At2g01760	ARR14 (ARABIDOPSIS RESPONSE REGULATOR 14)	Cytokinin signaling	18
At2g13650	GDP-mannose transporter (GONST1)	Golgi	24
At2g14890	arabinogalactan-protein (AGP9)	Signaling	13
At2g16600	ROC3	Protein folding, heat stress response	28
At2g30390	ferrochelatase II	Heme biosynthesis	10
At3g11890	Unknown	Chromatin remodeling, DNA binding	46
At3g19540	unknkown		25
At3g20640	ethilen inducible	Ethilen response	11
At3g23390	60S ribosomal protein L36a/L44 (RPL36aA)	Protein synthesis	18
At3g59490	Unknown	Chloroplast	14
At4g14680	sulfate adenylyltransferase 3 / ATP-sulfurylase 3 (APS3)	Chloroplast sulfate assimilation	11
At4g21940	CPK15	Signaling	11
At4g35550	HB-2 homeodomain-leu zipper	Transcription factor	14
At5g02350	DC1 domain-containing protein	Transcription factor	11
At5g42560	abscisic acid-responsive HVA22 family protein	ABA response	11

Table 1b. Downregulated genes in *et2-4* mutant plants visualized by hybridization of an *Arabidopsis* 8000 unigene filter

AGI Number	Name	Predicted Function	Reduction Factor
At1g07210	30S ribosomal protein S18 family	Protein synthesis	13
At1g57790	F-box family protein	Protein degradation	53
At1g66430	pfkB-type carbohydrate kinase family protein		23
At1g75500	nodulin MtN21 family protein	Electron transport	10
At2g01620	Unknown		26
At3g08520	60S ribosomal protein L41 (RPL41D)	Protein synthesis	22
At3g16130	Unknown nuclear protein		72
At3g26510	Unknown		28
At3g45650	proton-dependent oligopeptide transport (POT) family	Transport	38
At3g53520	ATUXS1	Cell wall biosynthesis	14
At4g11220	octicosapeptide/Phox/Bem1p (PB1) domain-containing		22
At4g12080	AT-hook protein 2 - Arabidopsis thaliana	Transcription factor, mitochondrion	22
At4g31590	glycosyl transferase family 2 protein	Cell wall biosynthesis	15
At4g39680	SAP domain-containing protein	Chromatin remodelling, DNA repair	43
At5g40950	50S ribosomal protein L27	Protein synthesis	21
At5g42180	peroxidase 64 (PER64) (P64) (PRXR4),	Cell wall biosynthesis	22
At5g45890	SAG12	Leaf senescence	35
At5g59910	H2Blike	Chromatin structure	41

A second hybridization was performed using a 1200 transcription factors filter provided by the REGIA consortium (Figure 14). Two experiments were performed, and the results of them are summarized in Table 2a, b and c. As expected, several differentiation-associated genes appeared in the screen (Table 2), like members of the *TCP* gene family and the differentiation inducer REVOLUTA (Zhong and Ye, 2001).

Additionally, members of the *Arabidopsis KNOX* (*KNAT*) gene family, involved in induction and maintenance of meristem identity, were upreglated in the *et2-4* plants. Among the functions associated with them are the regulation of phytohormone response, embryo development and, importantly, control of xylem differentiation (Hake et al., 2004). The diferentially regulated genes on the array included also one of the regulators of the *KNAT* genes - FILAMENTOUS FLOWERS (FIL). Because of this, and the fact that some well characterized members of the family were not present on the filter, we investigated their expression pattern by the means of RT-PCR.

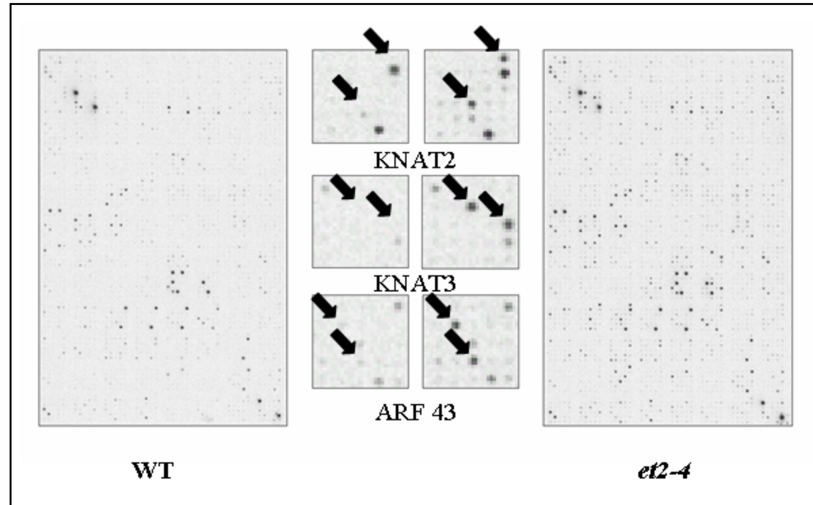

Figure 14. Expression profiling of wild type and *et2-4* mutant plants using a REGIA 1200 transcription factor filter array

Selected genes with differential expression between wild type and *et2-4* mutant plants are highlighted. The example shows the differential expression of the genes *KNAT2*, *KNAT3* and *ARF43*. All genes are double spotted.

Of special interest was the gene *KNAT1* (*BREVIPEDICELLUS1*, *BP1*). Previously, its role has been demonstrated as a maintainer of meristem identity and inhibitor of xylem lignification (Mele et al., 2003). Its expression was increased in the mutant, corresponding to the decrease in lignin content observed for *et2-4* plants (Figure 15). Other genes like SHOOTMERISTEMLESS (STM) and KNAT2 were regulated in the opposite way to the rest of the family members (Figure 15).

Table 2a. Short list of upregulated genes in *et2-4* mutant plants based on the REGIA 1200 transcription factors array filter. These genes were upregulated in the mutant in both experiments. Factors of induction below 3 were not considered.

AGI Number	Name	Function	First Array	Second Array
At1g23380	KNAT6	Meristem identity, Gibberellin response	33	711
At1g35540	ARF Protein	Auxin response	10	5
At2g40740	WRKY55		17	1446
At3g25710	AtbHLH 32		42	45
At2g45190	FIL (Filamentous Flowers)	Meristem identity, Regulation of KNAT	38	23
At3g15030	TCP4	Cell division, Leaf morphogenesis	42	33
At4g22070	WRKY31		19	239
At5g08330	TCP family bHLH protein	Auxin - induced protein	15	7
At5g12330	LRP1	Gibberellin response	14	59
At5g25220	KNAT3	Meristem identity, Gibberellin response	30	5
At5g53980	homeobox-leucine zipper protein		25	30

Table 2b. Long list of upregulated (A) and downregulated (B) genes in *et2-4* mutant line based on the REGIA 1200 TF array filter. These genes were differentially regulated only in the second experiment. Factors of up/downregulation below 3 were not considered.

A

AGI Number	Name	Predicted Function	Induction Factor
na	RG_TCP_8		95
At1g69780	Leu zipper homeobox		84
At2g40200	AtbHLH 51		16
At2g45270	AtbHLH 9 (PIF4)	Red light response	17
At3g03660	RG_NFYC1		36
At3g48590	HAP5a	CCAAT binding	6
At4g25490	C-repeat binding factor	Low temperature response	4
At5g45270	WRKY52		94

B

AGI Number	Name	Predicted Function	Reduction Factor
At1g01530	AGL 28		30
At1g08780	RG_CO2		5
At1g64380	RAP2 (AP2 domain)	Cell differentiation	4
At1g65330	PHE1	Embryo development. Target of MEA and FIE	18
At4g00980	RG_ZFCCHC4	zinc knuckle (CCHC-type) family protein	9
At4g28500	NAM family protein	Meristem identity	66
At5g15150	Leu zipper homeobox		23
At5g60690	REVOLUTA (REV)	Cell differentiation	4

The differential regulation of the *KNAT* gene family along with the induction of the *Fil* mRNA indicates that the regulation of *STM* and *KNAT2* expression through FIL is AtET2 independent, but the downregulation of *KNAT1* and *KNAT6* expression by FIL occurs only in the presence of AtET2.

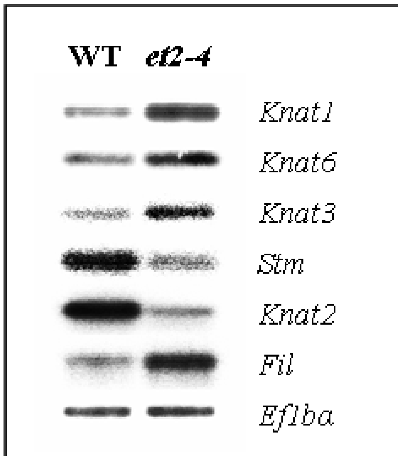

Figure 15. Regulation of *KNAT* genes and one of their regulators, FIL, in the *et2-4* line

Transcripts of *Knat1*, *Knat6* and *Knat3* were upregulated in the mutant, whereas *Stm* and *Knat2* were downregulated. Upregulation of *FIL* is consistent only with the effect on *STM* and *KNAT2*.

One of the most important developmental features of *KNAT* genes is their action as inhibitors of gibberellin biosynthesis and response. Because of this, and as GA has direct effect on xylem cell differentiation and seed germination, we investigated the interaction between AtET2 and GA.

4.5 Involvement of AtET2 in gibberellin response

The phenotype of the BnET overexpressing *Arabidopsis* and tobacco plants (Ellerstrom et al., 2005), combined with the observations for the *AtET2* knock-out mutant, indicated an action of ET proteins through modulation of GA response. To investigate this, we employed a protoplast based transient assay. The target promoter - that of the cell division associated gene *GASA4* – could be induced four fold by the application of GA_3, and presence of AtET2 could abolish this effect (Figure 16a). Similarly, the level of *Gasa4* mRNA is induced in the *et2-4* mutant plants (Figure 16b).

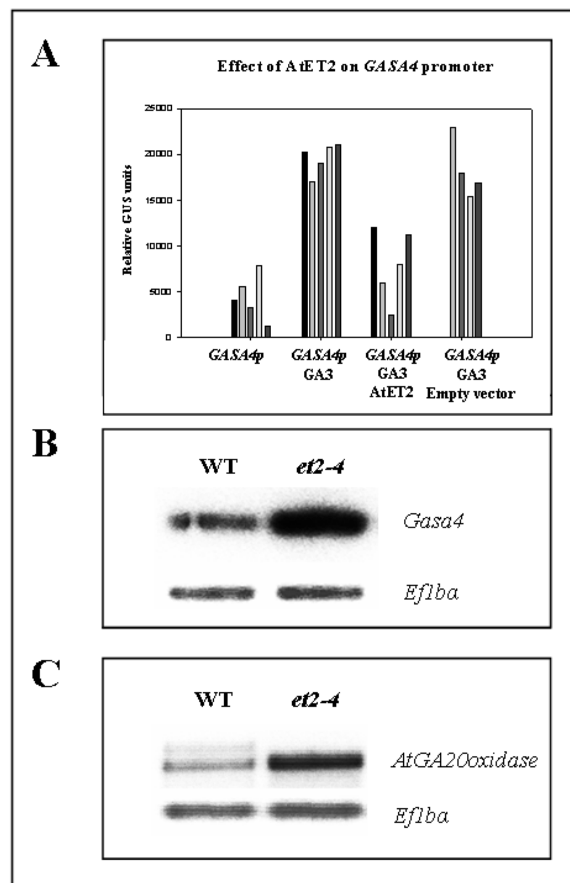

Figure 16. Involvement of AtET2 in GA biosynthesis and response

A. Transient assay demonstrating the downregulation of the GA responsive *GASA4* promoter by AtET2 counteracting the inductive effect of GA
B. Confirmation of the results given in A by RT-PCR. The lack of functional AtET2 in the *et2-4* mutant leads to an overaccumulation of *Gasa4* mRNA in seedlings.
C. Expression of the GA biosynthesis gene *GA20 oxidase* is enhanced in the leaves of the *et2-4* mutant plants.

Additionally, RT-PCR experiments in leaves showed an increase of the expression of the biosynthetic *GA20 oxidase* gene (Figure 16c), but that upregulation most probably does not lead to ectopic expression as there are stages during which the transcript is not detectable both in wild type and *et2-4* plants (data not shown).
Thus, regulation of cell differentiation by AtET2 (and possibly ET proteins as a whole) involves regulation of GA response and possibly GA biosynthesis. This is achieved by a complex mechanism, aiming for suppression of GA induced cell division.

5. Regulation of AtET

Being key developmental regulators, AtETs need to be strictly regulated to avoid negative effects of their presence in improper circumstances. We have evidence of several important checkpoints of AtET activity in the cells of *Arabidopsis*.

5.1 Autoregulation

We performed a transient assay to investigate the effect which AtET2 has on its own promoter. As a result, the overexpression led to downregulation of the promoter activity (Figure 17a). To verify this, we investigated the levels of the 5'end of the destroyed *AtEt2* mRNA in the *et2-4* mutant. The primer set is amplifying a portion of the 5'end coding region. In conditions with lack of functional AtET2, we could detect a significant induction compared to the wild type levels (Figure 17b). Combined, these results show that the expression of AtET2 is strictly dependent on its availability in the cell.

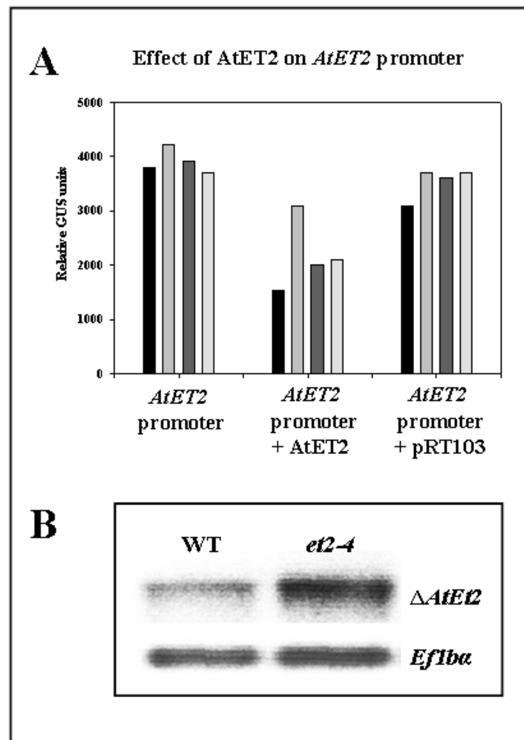

Figure 17. Autoregulation of AtET2

A. Transient overexpression of AtET2 in protoplasts leads to the inhibition of the *AtET2* promoter.
B. Confirmation of the data shown in A by RT-PCR analysis. In the *et2-4* mutant line, the remnants of the *AtEt2* mRNA are detected at a higher rate in the absence of AtET2 protein. Elongation factor *EF1bα* is used as an amplification control.

5.2 Regulation of ET expression by phytohormones

The effect of hormone treatment on the transcription of a certain gene can be masked by the internal levels of the same hormone and this way requires mutant analysis. Therefore, we developed a protoplast based system, in which cells can be directed to different developmental fate, by modulating their environment.

The protoplasts were cultured in two types of K3 medium – containing either high or low concentrations of the hormones auxin and cytokinin (see **Materials and Methods**).

When cultured in medium with high hormone levels, they preserve their meristematic identity and cell division abilities (Grafi, 2004). Phenotypically, they have the morphology of newly protoplasted callus cultures and can be seen as single spheric cells with large central vacuoles. On the contrary, under low hormone conditions, protoplasts begin differentiation, seen as formation of big and stable clumps, cell enlargement and appearance of distinct structures in the cytoplasm (Figure 18a). We used the expression of two meristematic identity genes of the *KNAT* gene family – *STM* and *KNAT1* - as markers for the molecular condition of the protoplasts. As expected, their expression was increased in "cell division" protoplasts while both were downregulated in the differentiated ones (Figure 18b).

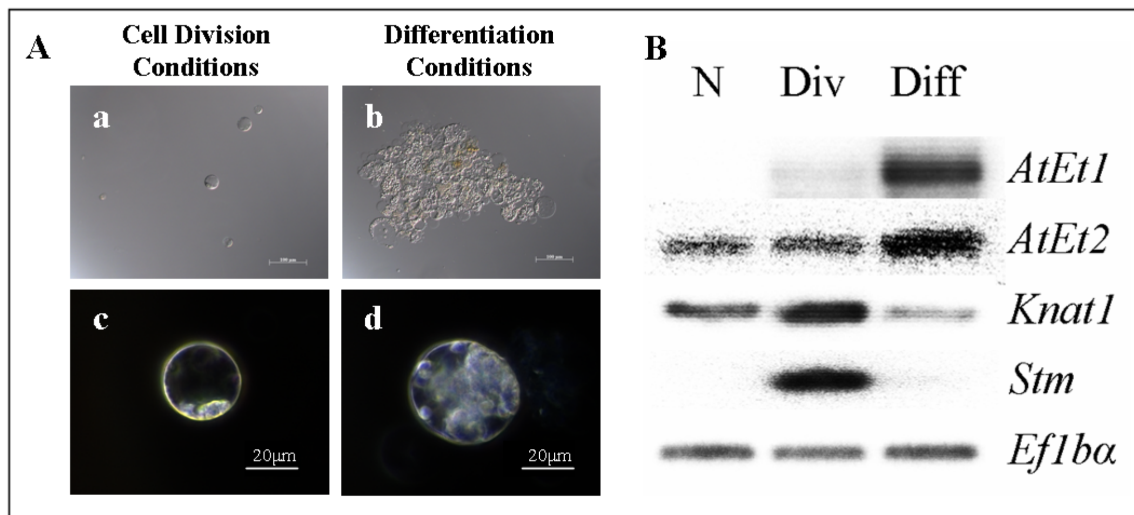

Figure 18. Transcriptional regulation of *AtET1* and *AtET2* genes

A. Protoplast cultures were triggered towards cell division or differentiation depending on the hormon supply. After 5 days of cultivation, there were structural differences between the two cell populations both at the level of cell clusters (a, b) and single cells (c, d).
B. The differences between the two populations were verified also by RT-PCR on the gene expression level. The expression of *KNAT1* and *STM* was used as a marker. Both of them become induced under cell division conditions confirming the observed phenotypes. *AtEt1* and *AtEt2* mRNAs are upregulated in differentiating cells. Elongation factor *EF1bα* is used as an amplification control.

As AtET1 and AtET2 had already a proposed function in regulation of differentiation, we followed their expression, expecting an appearance of their mRNA in the differentiating cells. Indeed, as seen on Figure 12k, there was a significant increase of the mRNA of both genes in the low hormone conditions, meaning that they are induced when the cell initiates differentiation and their regulation is involving the phytohormones auxin and cytokinin.
Despite that, expression of both *AtET1* and *AtET2* could be detected in meristematic protoplasts. Such a “leak” of expression could lead to big differentiation disturbances unless other means of post transcriptional regulation on the ET activity can be applied.

5.3 Subcellular localization of the AtET proteins

We studied AtET subcellular localization to verify their properties as transcriptional regulators and searched for additional levels of control. Using the protoplast system, we investigated the behavior of the *Arabidopsis* ET proteins fused to GFP in the cell. When cell division promoting medium was used for culturing the protoplasts after transformation, none of the three proteins was localized in the nucleus (Figure 19a, b, c). There were dispersed signals in the form of distinct spots around the cytoplasm, but excluding the nucleus and the central vacuole. None of the signals coincided with the position of the plastids or the mitochondria (data not shown). AtET1- and AtET2- GFP fusions could be located in the nucleus of the cells only in conditions of low hormone supply, which support cell differentiation (Figure 19d, e). Even then, there were detectable GFP signals in the cytoplasm, but they were fewer and weaker. It seems that AtET3 does not make the localization shift in the conditions used (Figure 19f). With the lack of the ET repeats in its structure, it is obviously subjected to a different type of regulation.

These results show that even if AtET1 and AtET2 become expressed in non differentiating cells, the protein is not allowed to enter the nucleus, keeping them this

way inactive. This represents an efficient way to inactivate a protein - by separating it from its target.

The localization shift opens a new dimension of questions in order to understand the way this process is achieved.

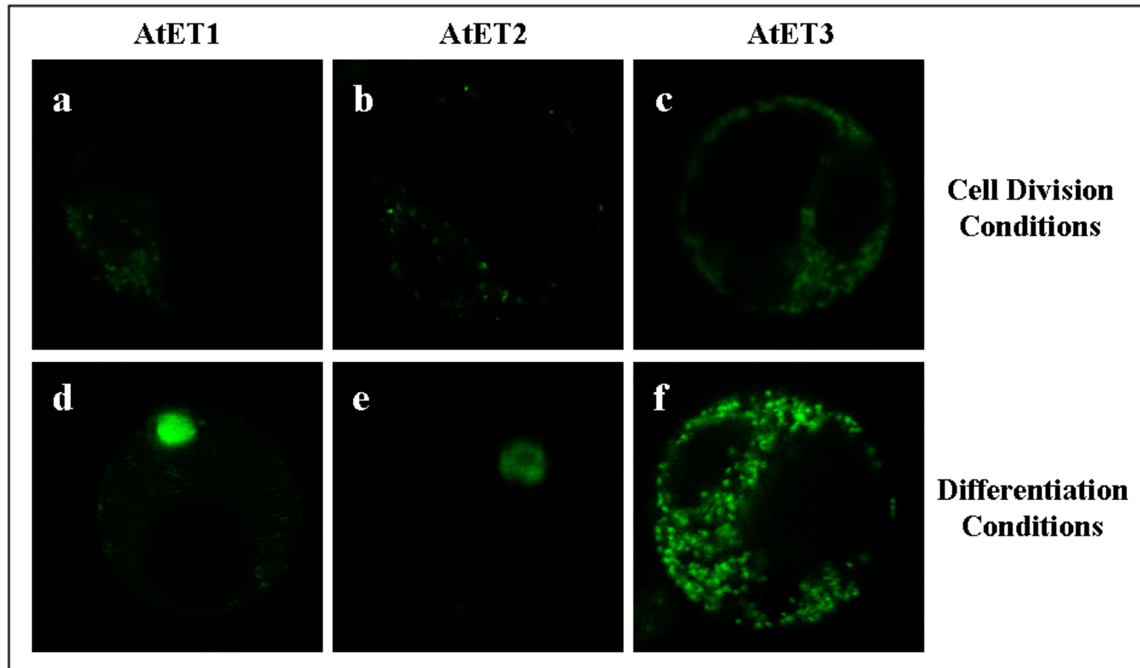

Figure 19. Subcellular localization of the *Arabidopsis* ET proteins

All three AtET proteins were translationally fused to GFP and expressed in the established protoplast system (see Figure 16). Under cell division conditions, none of the three fusion proteins could be detected in the nucleus (a, b, c). Only in differentiating cells, the AtET1- and AtET2-GFP fusions could be detected in the nucleus (d, e). This coincides with the upregulation of their genes under these conditions. This translocation into the nucleus could not be observed for the AtET3-GFP fusion (f), possibly due to the lack of the C- terminal ET repeats.

5.4 Interaction partners of AtET2

Absence of a distinguishable nuclear localization signal in the sequence of all three *Arabidopsis* ET proteins poses a question on the mechanism by which the cell is able to translocate them to the nucleus. Therefore, we performed a screening of a yeast two-hybrid library to identify AtET interaction partners, with which ET acts in the cell. The screen was based on the GAL4 system where the DNA binding domain and transcription activation domain of the yeast transcription factor GAL4 are physically separated.

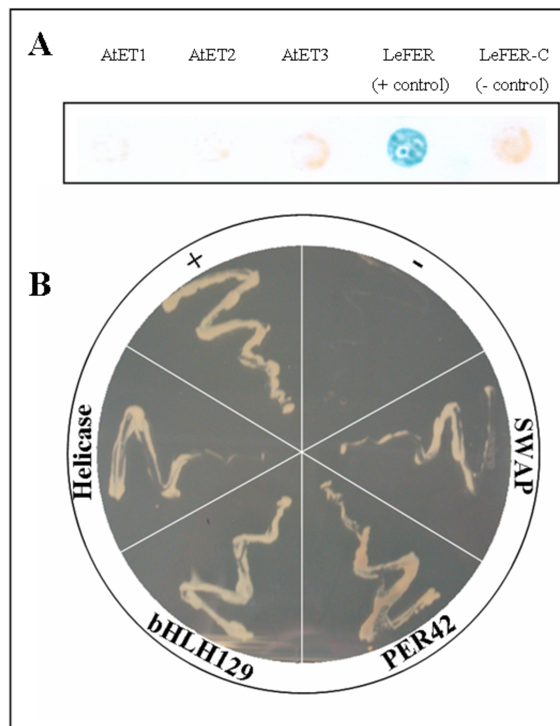

Figure 20. Yeast two- hybrid screening to isolate putative AtET2 interaction partners

A. The three *AtEt* cDNAs were fused to the DNA binding domain of the yeast transcription factor GAL4 and propagated in yeast cells. No self activation could be detected, which makes them suitable for the Yeast two- hybrid library screening. The tomato factor LeFER was used as a positive control (Brumbarova and Bauer, 2005).
B. Several putative AtET2 interaction partners were identified, including both nuclear (like a DNA helicase and an AtbHLH transcription factor) and cytoplasmic/ cell wall proteins (like a peroxidase and an arabinogalactan) as also given in table 3.

The bait proteins (AtET1, AtET2 and AtET3) were fused to the DNA binding domain and propagated in yeast cells, strain Y186. Absence of an internal activation domain was verified by *LacZ* screening, where presence of β-galactosidase activity in the cells would indicate its presence within the protein (Figure 20a). The whole plant library, which should provide the binding partners, had been fused to the activation domain and used to transform yeast strain AH109. The two strains were mated giving diploids in which interaction had occurred between the bait and the library protein, reconstituting a functional GAL4- like transcription factor. These were selected for the complementation of histidine auxotrophy, ensuring growth on histidine depleted medium.

The results presented on Figure 20b and Table 3, expectedly reveal both nuclear and cytoplasmic proteins. Notably, the nuclear ones include a DEAD box helicase and a bHLH transcription factor, which may be interpreted as transcriptional regulators. Interstingly, in the "cytoplasmic" group there is a peroxidase protein and a putative extensin connected with lignification and cell wall formation.

Table 3. Putative interaction partners of AtET2 detected by Yeast two- hybrid screening of an *Arabidopsis* whole plant library

	AGI code	Name	Subcellular localization/ Function
1	At1g18050	SWAP (Suppressor-of-White-APricot) surp domain-containing protein	Nucleus
2	At2g42280	AtbHLH129	Nucleus
3	At2g45470	fasciclin-like arabinogalactan-protein (FLA8)	Cell wall
4	At2g46630	Putative extensin	Cell wall
5	At4g21960	peroxidase 42 (PER42)	(EC 1.11.1.7) oxidative stress
6	At5g65900	DEAD/DEAH box helicase	Nucleus

These results have to be further verified before a more in depth analysis is done. It is very possible that AtET1 and AtET2 use one protein partner to enter the nucleus and than change it with a different one to be functionally active.

Discussion

Plant growth and development are controlled by a variety of factors in response to both endogenous programs and environmental stimuli. The signals are recognized by receptors and multiplied within the cell by cascades of signal transducers to inflict changes in structure, metabolic pathways and transcriptional activity. A number of transcriptional regulators with function in cell differentiation and plant development have been identified in the recent years.

One of these is the EFFECTOR OF TRANSCRIPTION (ET) gene family. Members from different plant organisms are known, but so far only two, the *Brassica napus* ET (BnET, Ellerstrom et al., 2005) and HORDEUM REPRESSOR OF TRANSCRIPTION (HRT, Raventos et al., 1998) have been partially characterized. Key feature of ET action is their involvement in regulation of cell differentiation as observed by phenotypes in BnET overexpressing *Arabidopsis* and tobacco plants (Ellerstrom et al., 2005).

1. Family structure and molecular mechanisms of AtET action

1.1 Structure of the ET gene family

Many ET- like sequences could be identified from the emerging plant databases of both mono and dicotyledonous species. Additional members of the family were discovered in more distantly related plant species, like the moss *Physcomitrella patens*. When searches were extended to outside the plant kingdom, though, no significant homology to the plant proteins could be observed. Pattern searches for the cystein containing motif revealed several proteins from bacteria, yeast and mammals but none of them shared any homology outside the pattern itself.

As plants have a life cycle different from the other organisms, they have evolved mechanisms of control specific only for them. Such plant specific regulatory cascades

would pose the recruitment of plant specific proteins. This way, the presence of unique gene families in plant genomes is not surprising.
The ET family has been previously demonstrated to interact with phytohormonal responses (GA, ABA, auxin and cytokinin) so it may have evolved as a requirement of the multicellular plant organisms to achieve a higher level of control through elaborated signaling networks.

To study the molecular basis of ET action and its interaction with the known regulatory pathways we concentrated our efforts on *Arabidopsis* where many advantages, among which the large collection of data, would allow us an in depth molecular investigation.
Arabidopsis contains a three member *ET* gene family. Two of them, designated *AtET1* and *AtET2*, represent full genes, while the third one, *AtET3*, although transcribed, is an incomplete copy of *AtET2* and lacks the region coding for the ET repeats.
Sequencing of the three genes from different accessions revealed valuable information on polymorphisms occurring in *Arabidopsis*. *AtET1* gene was identified as a pseudogene following the sequencing of the *Arabidopsis* genome. Our results showed that among the investigated accessions only Col0 and Limeprot contained the disrupted gene. Presence of a naturally occurring mutant of *AtET1* gives a hint for at least partial functional overlapping between its product and the AtET2.
The variations of *AtET3* between Col0 and WS2 are located towards the end of the gene and create a copy which is 150 bp shorter in WS2. The deleted region is not coding for any recognizable protein motif, so it might not be important for the overall functionality of the protein. We were not able to detect sequence differences in the rest of the *AtET3*.
AtET2 gene was completely identical in the two investigated ecotypes.

1.2 Molecular mechanisms of AtET action

Being isolated as DNA binding proteins, ETs were assigned a function as transcription factors. As not all DNA binders are acting as transcription factors and even transcription factors themselves show a variety of mechanisms to fulfill their function, a question was

whether we could identify the way by which ETs act on the molecular level. The DNA binding experiments performed for the AtET1 and AtET2 proteins did not reveal any specificity towards the provided oligonucleotides containing different target sequences. The interaction between the ET protein HRT and the central sequence of the GA response element has been demonstrated before (Raventos et al., 1998). We have included the same oligonucleotide in the experiment, but the detected signal did not reveal higher specificity of binding of the element, compared to other sequences, including random genomic DNA fragments (Figure 5c). As the binding activity of both AtET1 and AtET2 was quite high, it is possible that they do not target any specific sequence of the *Arabidopsis* genome. Similar random DNA binding was observed during the isolation of two other ET factors – from *Vicia faba* (VfET) and *Brassica napus* (BnET, Ellerstrom et al., 2005). This significant difference between HRT and the other factors may reflect the potentially different roles that ET proteins may have in monocotyledonous and dicotyledonous species.

A random DNA binding gives some indications on the way ET acts. A possible requirement is an additional protein/protein complex that can function as a target recognition factor. The role of ET during recognition might be to stabilize the complex upon the DNA. Studies of DNA repair in mammals have demonstrated that target recognition initially requires a non- specific binding in the target region, followed by a "one dimensional scanning", until the complex reaches its correct position (Feng et al., 1997). The same might be true for recognition of *cis* elements during the transcription initiation process and therefore the role of ET to support the protein- DNA complex during the movement process. A possible interaction partner of AtET2 acting as a target specifier could be the protein AtbHLH129 identified by the yeast two hybrid screening (Figure 18, Table 3, discussed below, see **4. Regulation of the AtET**)

An interesting feature of all ET proteins that provides serious indications on their molecular function is the presence of a single strand endonuclease domain, normally used by the bacterial nucleotide excision repair protein UVRC to create an incision in 5' direction of a damaged nucleotide. Presence of such domains has never been reported before in eukaryotic proteins making ET this way unique. Outside the UVRC proteins

only a few endonucleases, mostly phage T4 proteins, share this motif and interestingly one of them has been shown to target specific sequences in the genome (Verhoeven et al. 2000). Of special interest is the fact that all amino acids that are conserved between eukaryotes and prokaryotes are present in ET proteins, which includes also the catalytic arginine (Figure 6). Based on this we speculate a role of ET not as a transcription factor in the general sense, but a factor required for the modulation of the higher DNA structure to influence transcriptional activity. This notion is further supported by the *et2-4* mutant line where the gene expression profile analysis shows that AtET2 is involved in the transcriptional regulation of at least three other putative chromatin remodeling factors, among which histone H2B (Table 1a and b).

A known protein with similar function to the proposed one for ET is the *Arabidopsis* DNA glycosylase DEMETER (DME), which introduces a single strand cut to activate the transcription of the *MEDEA* (*MEA*) gene (Choi et al., 2004).

In order to initiate research in this direction, the nuclease properties of AtET1 and AtET2 must be first investigated. In future, we aim to mutate the catalytic arginine within the domain and perform relaxation of supercoiled plasmids, which requires single stranded cut. Comparison between the activity of the mutated and the wild type protein activity can bring a direct proof of this emerging new principle of gene regulation.

2. Involvement of AtET2 in seed development

As VfET, HRT and BnET were isolated from seed specific libraries their presence in *Arabidopsis* seeds was expected. Their expression pattern, shown on Figure 7a, suggests a role both in early and late stages of embryogenesis. Data from REGIA consortium, shown on Figure 7c, confirm this and give a better insight with both *AtEt1* and *AtEt2* following two waves of high abundance. Both sets of data are in good agreement and it is obvious that *AtEt2* is the more abundant of the two during the early stages and transition towards maturation. *AtEt1*, on the other hand, is the dominant factor during the maturation period.

2.1. Premature germination of *et2-4* seeds

Following the presented considerations, we expected a phenotype of the *et2-4* line related to the transition period between early and late embryogenesis. Additionally, germination experiments performed on tobacco and *Arabidopsis* seeds overexpressing BnET led to severe germination phenotypes with less than 20% of the seeds able to protrude and develop into plants (Ellerstrom et al., 2005). Having this in mind for the gain of function situation, a logical outcome from a loss of function would be the creation of a "super germinator". Premature germination experiments indeed confirmed this hypothesis. *et2-4* is one of the several known mutants whose seeds have the capability to germinate before entering maturation stage (Figure 10a).

The two major opposing factors controlling the fate of the developing seed are the phytohormones ABA and GA. While ABA is required for the transition to maturation, GA promotes germination (Koornneef et al., 1998, White and Rivin, 2000). This way, premature germination mutants fall into at least two groups – ABA- related (like the biosynthesis mutant *aba1* or the response regulator *abi3*), or GA- related (like *fus3*, *lec2*). Analysis of gene expression and transient overexpression experiments (Figure 16), show that AtET2, similarly to other proteins from the family (Raventos et al. 1998, Ellerstrom et al., 2005), is involved in control of both GA biosynthesis and response. On cellular level, this control may influence the reprogramming of the cells from cell division to differentiation (discussed below, see **4. Regulation of ET**). When this knowledge is applied to the premature germination phenotype of the *et2-4* seeds, it becomes evident that lack of functional AtET2 protein leads to release of the block on GA sensitivity/synthesis. This disturbs the ABA/GA balance in the seed increasing the influence of GA, which leads to a premature end of dormancy. As a result, seeds become sensitive to germination promoting signals and if put in suitable conditions can develop into viable plants.

Thus, the function of AtET2 during the transition phase towards seed maturation is to reduce the influence of GA, preventing germination before the seed is fully developed (Figure 21).

The process of inflicting dormancy is complex and requires the simultaneous action of many potentially redundant factors. That is why completely removing dormancy can rarely be achieved by destruction of a single gene. In *Arabidopsis*, it requires the destruction of at least two genes with the additional requirement that both ABA and GA influence should be disturbed (Figure 2, Raz et al., 2003). So vivipary, as the ultimate release of dormancy, is possible by crossing “ABA” and “GA” mutants. In this context, an important experiment for the future is to cross *et2-4* with mutants of both types, which will provide additional insight into the role of ET in support of seed dormancy. The first steps have already been made with the creation of an *et2/fus3* double mutant line (data not shown), whose characterization is in progress.

2.2 Function of AtET2 during seed maturation

An additional reason for crossing the available mutants of these two genes is an indication for possible interaction between them. Transient expression experiments show a decrease of the activity of *AtET2* promoter in the presence of FUS3 (Figure 10b, c). This observation is in agreement with REGIA gene expression data where increase of *Fus3* mRNA levels coincides with the rapid disappearance of *AtEt2*. It is very likely that the negative effect of FUS3 is not direct as neither the promoter of *AtET2* nor any of the other two *AtET* promoters has a recognizable RY element, the FUS3 binding site. It is more probable that there is either a direct protein- protein interaction between them or a signaling pathway monitoring the expression of both proteins within the cell. In any case, the relationship between FUS3 and AtET2 is evident and might be required for the proper accomplishment of the maturation. This observation does not exclude the possibility for interactions of ET and other factors during this period.

Absence of a functional AtET2 during the maturation phase does not lead to significant morphological changes in the seed. Despite that, the regulatory pathways seem to be

altered irreversibly as the seeds become insensitive to germination inducing stimuli. As shown on Figure 11, germination potential of both *et2-4* and its wild type, WS2, is reduced by more than 50%. This phenomenon, concerning WS2 ecotype, is known and has been reported previously (Debaujon and Koornneef, 2000). The effect can be reverted by gibberellin, leading to 100% germination. We tried to stimulate the seeds in two different ways – a direct application of GA_3 and cold treatment. Cold treatment stimulates seeds' sensitivity to the internal gibberellins, rather than the GA biosynthesis (Derkx and Karssen, 1993). This way both types of stimulation can be considered as two different ways, direct and indirect, of GA treatment. While, as expected, the wild type responded well to both GA_3 application and low temperature, *et2-4* seeds did not react to the treatment. In some way when seeds enter maturation phase, due to its interaction with maturation controlling transcription factors, the role of AtET2 evolves from a straightforward GA inhibitor to a more complex modulator of GA response.

Based on our results, the role of AtET2 in plant embryogenesis can be summarized as an inhibitor of premature germination and regulator of maturation (Figure 21). During the transition towards late embryogenesis, the embryo is in principle ready to germinate. In this stage, AtET2 is required to suppress premature germination by negatively regulating GA biosynthesis and response, and suppressing cell division (discussed below).

In the course of late embryogenesis, AtET2 interacts with maturation factors, among them FUS3, this way changing AtET2's role within the seed. Though it is still acting as repressor of GA and germination (visible from the BnET overexpression, Ellerstrom et al. 2005), AtET2 becomes a component of the cascade sensing external germination promoting stimuli, like low temperature.

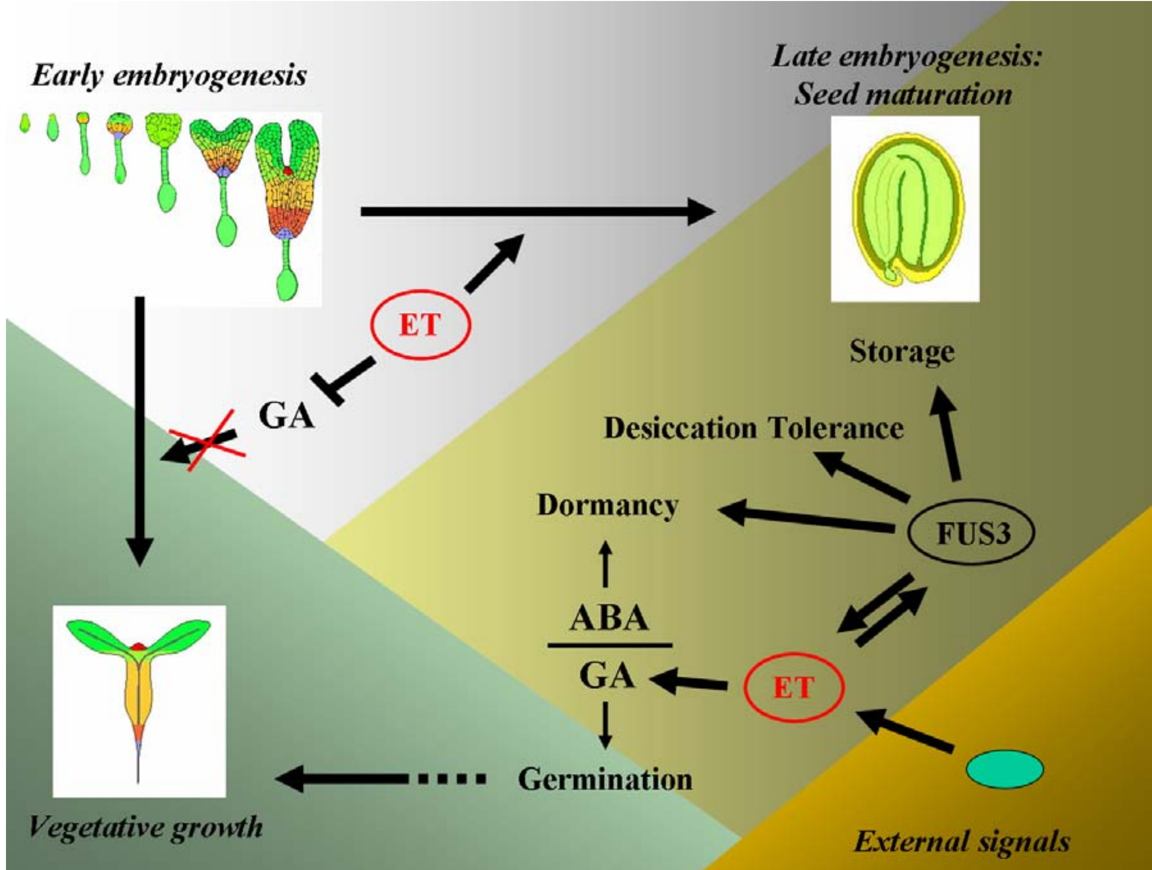

Figure 21. Schematic presentation summarizing the role of ET proteins during *Arabidopsis* embryogenesis.

ET proteins, together with other factors, act as repressors of GA-biosynthesis and responses in the transition phase from early to late embryogenesis. They prevent the immature seed from germination. During the maturation phase, ETs interfere with maturation promoting transcription factors, like FUS3. This interaction plays an important role to sensitze the seed to external germination promoting stimuli.

3. Role of ET in plant vegetative development

3.1 Regulation of cell differentiation by AtET2

Phenotypes observed in BnET overexpressing tobacco and *Arabidopsis* plants, suggested a role of ET proteins in the developing plants as inducers and maintainers of cell differentiation. By the use of the *et2-4* mutant line, we could confirm this and expand the knowledge into the molecular interactions by which this is achieved.
As *AtET2* gene is relatively highly expressed in the plant's vascular tissues (Figure 7b), we used the levels of xylem lignification as a reliable marker for cell differentiation. As suggested by the metabolic profiling experiments and expression of *AtET2* gene, the total lignin amount was decreased in the mutants, this way indicating lowered ability of the cells to accomplish their developmental program. Furthermore, *et2-4* plants were unable to respond to the induction of hypocotyl growth. How the absence of AtET2 makes the hypocotyl insensitive to the inductive signal is difficult to understand for the moment, but these results confirm that ET proteins act as differentiation promoting factors and, similarly to the situation in late embryogenesis, play a regulatory role in response to external stimulations.
This phenomenon is especially intriguing in terms of possible biotechnological applications. Removal of lignin in the paper industry is an expensive process and serious investigations are done to reduce the amount of lignin in the wood. We have initiated a project in this direction, based on overexpression of ET from *Arabidopsis* (*AtET2*) and *Brassica napus* (*BnET*) in poplar trees.

An additional observation was that ETs are not only promoting differentiation, but in parallel act as inhibitors of cell division. This was confirmed by the dwarf phenotype of BnET overexpressing plants, where longitudinal cell division activity was decreased (Ellerstrom et al., 2005). This may indicate a function of ET factors during the early stages of the cell reprogramming process.

The gene expression profiling experiments on the two different array filters and RT-PCR experiments were consistent with the proposed function of AtET2. Our expression experiments on the *et2-4* mutant, show an induction of the cell division induced genes, like *GASA4* (Figure 16a, b), two putative cell division regulators of the *TCP* family (Table 2), auxin and cytokinin regulated genes, and a tubulin gene (Table 1a).
GASA4 was demonstrated to be induced by gibberellin in actively dividing tissues of *Arabidopsis* (Aubert et al., 1998) and especially in xylem formation in wood species (Hertzberg et al., 2001, Israelsson et al., 2003). So there may be a direct connection between cell division and lignification of the xylem tissue. Its repression by the active AtET2 and increased expression in the *et2-4* mutant correlates well with the phenotypical observations for this line.
Though their role in xylem differentiation has not been demonstrated, the two upregulated *TCP* genes (Table 2) share a high homology to the rice *PCF1* and *PCF2*. The products of the latter two are positive regulators of the rice *PCNA* (Proliferating Cell Nuclear Antigene), a protein expressed during the S- phase in actively dividing cells (Kosugi and Ohashi, 1997) and absolutely required for the DNA replication process. Additionally, they are both strongly induced by the phytohormone auxin.
Regulation of/by auxin and cytokinin is important for induction of cell division and preventing differentiation. Therefore, the abundance of differentially expressed auxin and cytokinin regulated genes in the mutant line support the phenotype data for the *et2-4* mutant and correlate well with the phenotypes of the BnET overexpression lines. Such genes include also an Auxin Response Factor (ARF 43, Table 2) and the cytokinin signaling regulator *Arabidopsis* Response Regulator 14 (ARR14, Table 1a, Hwang et al., 2002).
A further cell cycle related gene induced in the mutant was tubulin, known to be upregulated in the late G2 phase and widely used as a marker for cell division potential (Vantard et al., 2000).

As these genes show an enhanced expression in plants lacking a functional AtET2, we postulate that to induce cell differentiation ET first functions as a division inhibitor, and this way might be one of the earliest acting factors in the process of reprogramming the

cell. Despite that, the appearance of more specialized genes in the screens, notably members of the *KNAT* family of meristem function maintainers, indicated a higher specificity of the ET action rather than as a common repressor of cell division.

For many years, members of the *KNAT* family in *Arabidopsis* and other species have been demonstrated to initiate and maintain plant meristems (Hake et al., 2004). The upregulation of two members of the family in the array hybridization and RT-PCR experiments, *KNAT3* and *KNAT6* (Figure 14, Table 2), provides a strong support for the proposed inhibitory role of ET on meristematic identity in favor of differentiation. Additional experiments revealed that the expression of another well characterized member of the family, *KNAT1*/*BP1*, is also repressed by AtET2 (Figure 15). This result is of great importance because of the demonstrated effects of KNAT1 on xylem differentiation. Lack of KNAT1 leads to accumulation of high amounts of lignin caused by enhanced xylem differentiation while its overexpression prevents xylem development and lignification (Mele et al., 2003). In the *et2-4* mutant plants, absence of AtET2 leads to induction of the *KNAT1* gene and accordingly an inhibition of the xylem differentiation, as visible by the mutant phenotypes (Figure 22).

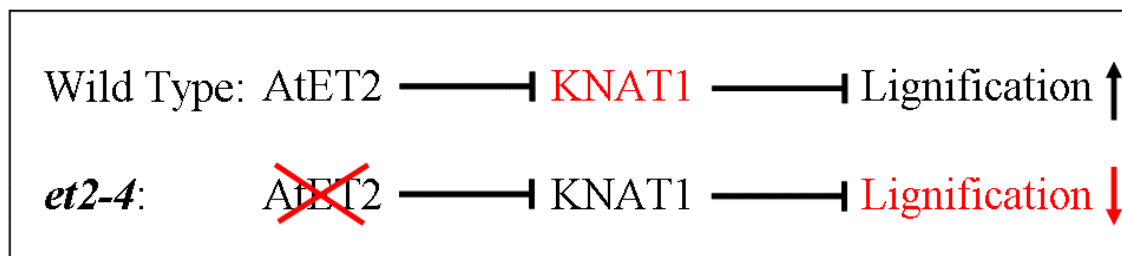

Figure 22. Influence of AtET2 on the lignification process

Suppression of *KNAT1* is a prerequisite for cell differentiation. In xylem cells, differentiation results in synthesis and accumulation of lignin, which is inhibited by KNAT1 (Mele et al., 2003). As a negative regulator of *KNAT1*, AtET2 promotes the differentiation and lignification of xylem cells. Therefore, in the *et2-4* mutant lack of AtET2 results in induction of *KNAT1* expression and reduced lignification.

Notably, two other *KNAT* genes, *KNAT2* and *SHOOTMERISTEMLESS* (*STM*) whose products have cellular function similar to KNAT1, were regulated in the opposite way in the *et2-4* plants. Despite this, as their expression levels were much lower than the one of *KNAT1* we conclude that the higher abundance of the latter is masking the potential opposite effects of STM and KNAT2.
The reasons for the differential regulation of the KNAT family members are discussed below (see **3.2 AtET2 mediated regulation of *KNAT* genes**).

The effects on the *et2-4* mutants are marked also by the appropriate expression differences of genes directly involved in the cell differentiation process. In wild type plants, the REVOLUTA (REV) protein acts to prevent auxin influence and trigger differentiation of the interfascicular xylem fibers. With the lack of AtET2 its expression is downregulated (Table 2c) and coincides with the loss of lignin in the mutant. Complementing this observation is the reduction of the expression of the SAG (SENESCENCE ASSOCIATED GENE) 12, a protease expressed in the terminal stages of cell differentiation and beginning of senescence. As xylem cells develop they need to lignify their cell wall and than perform programmed cell death (PCD). With the xylem in the *et2-4* mutant unable to complete its differentiation program it is expected that PCD is not triggered, therefore the gene is not expressed there. Interestingly, SAG12 is transcriptionally regulated by, at least, the protein WRKY53 (Miao et al. 2004). Our data show upregulation of three other members of the WRKY family - WRKY31, WRKY55 (Table 2a) and WRKY52 (Table 2b), though it is possible that they are not functioning as senescence regulators. For the moment, there is no demonstrated connection between them and SAG12.

In conclusion, phenotypic analysis and gene expression data are consistent with the function of AtET2 as important component required for the transition of the cell towards differentiation. This it achieved mainly by inhibition of cell division and meristem identity, and it is possible that AtET2 is required in the very early stages of the process. Its action may be the prerequisite for the activity of other differentiation promoting genes.

3.2 AtET2 mediated regulation of *KNAT* genes

Despite their similar function in maintaining meristem identity and function, the genes from the *KNAT* family reacted differently to the destruction of the *AtET2* gene. While *KNAT1*, *KNAT6* and the class II gene *KNAT3* showed an enhanced expression in the mutant, *STM* and *KNAT2* were downregulated (Figure 15). This indicates that AtET2 in some way is responsible for the regulation of only specific members of the family. The key observation, which can provide the answer to this, is the induction of the *KNAT* negative regulator *FILAMENTOUS FLOWERS* (*FIL*) by the absence of AtET2 (Table 2a, Figure 15). FIL has been demonstrated to repress three of the four class I *KNAT* genes - *STM*, *KNAT1* and *KNAT2* - and its effect on *KNAT6* has not been investigated (Kumaran et al., 2002). In the *et2-4* mutant the expression of FIL is strongly enhanced and this explains the downregulation of its targets *STM* and *KNAT2*. The fact that on the contrary, *KNAT1* and *KNAT6* (as a potential, but not proven target) are not affected shows that their regulation is not entirely dependent on FIL but requires also AtET2 (Figure 23). It has additionally been demonstrated that another YABBY family protein - YAB3 is also needed for complementing FIL's repression activity.

It is possible that there is a direct protein- protein interaction between FIL and AtET2 and the latter is excluded or included in the complex under different conditions, maybe different cell types, to ensure target specificity.

Such interactions need to be further verified by yeast two- hybrid experiments for the direct protein interaction, and genetic approaches through mutant crosses.

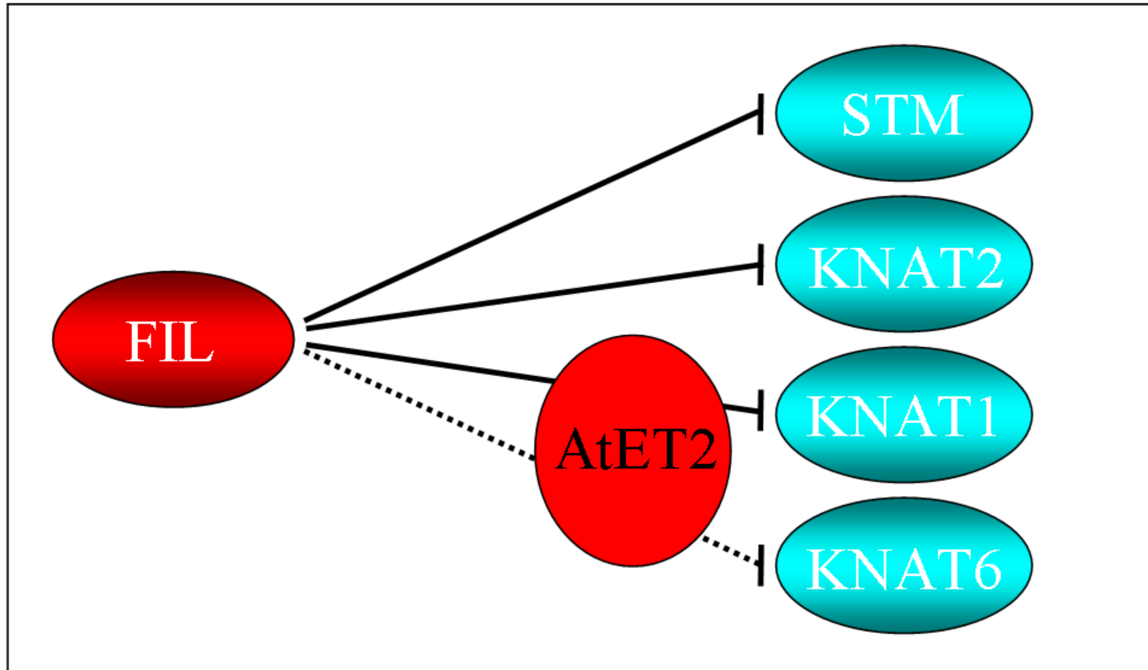

Figure 23. Working hypothesis for theAtET2 dependent regulation of KNAT genes

FILAMENTOUS FLOWERS (FIL) is a negative regulator of the *KNAT* family genes *STM*, *KNAT1* and *KNAT2*. Its influence on *KNAT6* has not been studied so far. In the absence of AtET2, upregulation of *FIL* results in the expected inhibition of *STM* and *KNAT2*, but not *KNAT1* and *KNAT6*. This suggests that the regulation of *KNAT1* and *KNAT6* is AtET2 dependent, whereas *STM* and *KNAT2* are repressed by FIL, independently of AtET2.

3.3 Involvement of AtET2 in GA regulation

The phytohormone gibberellin is an important regulator of plant development. Its presence is required for both induction of cell division and differentiation. As an inhibitor of cell division, we tested the relation of AtET2 to GA. We were able to demonstrate a negative regulation on the GA induced *GASA4* promoter (Figure 16a), where the four fold induction of the promoter by GA, correlating well with the literature data (Herzog et al., 1996), could be prevented by the addition of AtET2. This result was confirmed by the mutant analysis (Figure 16b), where we observed an increase of the abundance of the

Gasa4 mRNA. Despite the fact that the function of GASA4 is not known, it is strongly expressed in zones of active cell division, which includes also high abundance in germinating seedlings. Thus, regulation on genes like *GASA4* explains the premature germination activities of the *et2-4* plants where, with the absence of AtET2, the control on GA induced cell division is partially removed.
A further complexity of our understanding of ET's relationship with GA comes with the altered expression of the GA biosynthesis gene *GA20 oxidase* in leaves (Figure 16c). The gene itself is also GA regulated, like *GASA4*, and similarly upregulated in the leaves of the *et2-4* mutant. Despite that, the *Ga20 oxidase* transcript could not be detected neither in the seedlings of the mutant, nor in wild type. This shows that different mechanisms are involved in the regulation of GA biosynthesis in different tissues of the plant.
This result indicates that the induction of *GASA4* in the *et2-4* line is not a secondary effect of the enhanced GA biosynthesis, but the effect of AtET2 on the two genes is a result of two separate regulatory events. So, AtET2 seems to control both aspects of GA biosynthesis and response.

4. Regulation of AtET

As all developmental regulators, the expression of the *AtET1* and *AtET2* genes requires a precise temporal and spatial control. We have demonstrated different levels of regulation on AtET including transcriptional and post- transcriptional levels.

An interesting aspect of gene regulation is the impact that the presence of the product has on its own gene expression. We could demonstrate in two independent systems- transient assay and *et2-4* line- that presence of a functional AtET2 has inhibitory effect on the *AtET2* promoter. In transient assay, expression of AtET2 causes downregulation of the promoter activity (Figure 17a). Furthermore, the destruction of the protein in the *et2-4* mutant releases this block and results in an increased abundance of the interrupted *AtEt2* mRNA compared to the wild type (Figure 17b). With the lack of additional data, it is

difficult to speculate whether the effect of AtET2 is due to direct auto regulation or a feedback signaling loop, but nevertheless, this is a major factor that contributes to the extremely low expression activity of the *AtET2* promoter.

Transcription of *AtET1* and *AtET2* genes is restricted to differentiating cells which involves regulation by the hormones cytokinin and auxin, as seen in the protoplast system (Figure 18b). Combination of these phytohormones promotes cell division, so being inhibitors of this process *AtET* genes have to be downregulated by them (Figure 18). With the removal of the hormone influence, the block is released and the expression of both *AtET1* and *AtET2* is enhanced. From the RT-PCR analysis it is evident though, that background expression occurs in dividing cells, especially for the *AtEt2*, and shows that additional levels of regulation are required for complete inactivation of ET function. This expression noise can be also an artifact of the model system and needs to be verified by *in situ* hybridization. It cannot be excluded that it ensures a required activity of ET in the cytoplasm, which would explain the putative cytoplasmic proteins appearing on the AtET2 yeast two-hybrid screen (discussed below).
The results from the so developed protoplast system are applicable to explain our results on AtET2 in embryogenesis as well, where the cells of the embryo undergo a form of differentiation during the maturation phase.

There are indications that the low amount of the *AtEt2* mRNA is due also to additional regulatory processes preventing overexpression of the mRNA even if driven by the strong CaMV 35S promoter (data not shown). There might be a mechanism to destroy the surplus amounts in the cell, though currently we have no indications of how it may act.

Even if AtET1 and AtET2 are expressed in non differentiation competent cells, there is a mechanism to keep them inactive until differentiation begins. This is supported by the exciting finding of their subcellular localization shift between the cytoplasm and nucleus depending on the hormone supply and therefore the condition of the cell (Figure 19). In such a way, AtET1 and AtET2 are prevented from entering the nucleus and acting as

transcriptional regulators, in actively dividing cells. Translocation occurs and ETs become functional only if cells initiate differentiation process. At the current stage, it remains unclear whether the localization shift initiates the cell differentiation process or vice versa.
AtET3, unlike the two other members of the family, does not enter the nucleus in differentiation conditions. It is shorter than them and lacks completely the ET repeats (Figure 4a). Thus, we can speculate that the ET repeats have an important role in the translocation process. AtET3 may not be related in function to the AtET1 and AtET2, or can be their regulator on the level of protein- protein interaction. The latter, though, has not been verified yet.

As none of the three *Arabidopsis* ET proteins has a recognizable nuclear localization signal (NLS), the process of entering the nucleus would require an interaction with a second factor. This way the complex will use the NLS of the partner to translocate.

A search using the yeast two- hybrid system approach was done, and although these data need to be further confirmed by independent methods, several possible partners of AtET2 were identified. Generally, they were grouped into nuclear and non- nuclear proteins (Table 3). The nuclear ones included a bHLH transcription factor, a SUPPRESSOR OF WHITE APRICOT (SWAP) - like protein and a DNA helicase.
While none of these has been functionally characterized, the appearance of a transcription factor and a helicase was expected and, especially the latter one strongly supports the idea that ET proteins regulate gene expression by changing the chromatin structure. On the other hand, SWAP protein in *Drosophila* is associated with RNA splicing and might be in a contradiction with this concept. We cannot exclude, though, that ET can form only a temporary complex with it, or any of the other two proteins, just to use their nuclear localization signal, and dissociate once inside the nucleus.
In the screen, several sequences of non- nuclear proteins appeared as well (Table 3). They included a peroxidase, a GPI anchored arabinogalactan and a putative extensin. Interestingly, all three hits can be considered as involved in cell wall structure. Peroxidases are important elements of the cell wall biosynthesis and secondary growth as

they are required for the formation of phenolic cross- linkages between cell wall components. There are no indications, however, whether this one is involved in such processes. Despite that, the two other proteins, an arabinogalactan and an extensin, are important structural components of the cell wall (Keller, 1993, Johnson et al., 2003). It is difficult to explain the interaction of AtET2 with these proteins, but nevertheless it is indicative of its role in cell differentiation.

5. Conclusion

Based on the above presented data and comments, we have built a simple model for the way ET proteins are engaged by the plant organism to control cell differentiation processes (Figure 24).

The phytohormones auxin and cytokinin are involved in induction of cell division and meristematic cell identity. Their action strongly downregulates the expression of *ET* genes along with other differentiation inducing factors and in the same time induces meristem identity factors and participants in cell division. At that stage, ET proteins are either not expressed or expressed very weakly and either way are not permitted to enter the nucleus.

When the cell finds itself in the differentiation zone, it is no longer under the influence of auxin and cytokinin, and its gene expression is altered. ET genes are activated and the protein becomes abundant. Due to the action of an unknown mechanism, the ET proteins are translocated to the nucleus, where they negatively regulate meristem identity and cell division promoting genes, this way participating in the reprogramming of gene expression towards differentiation initiation (Figure 24b). How ET modulate gene expression is not clear for the moment, but there are serious indications that they may act in a more general way by influencing the chromatin structure.

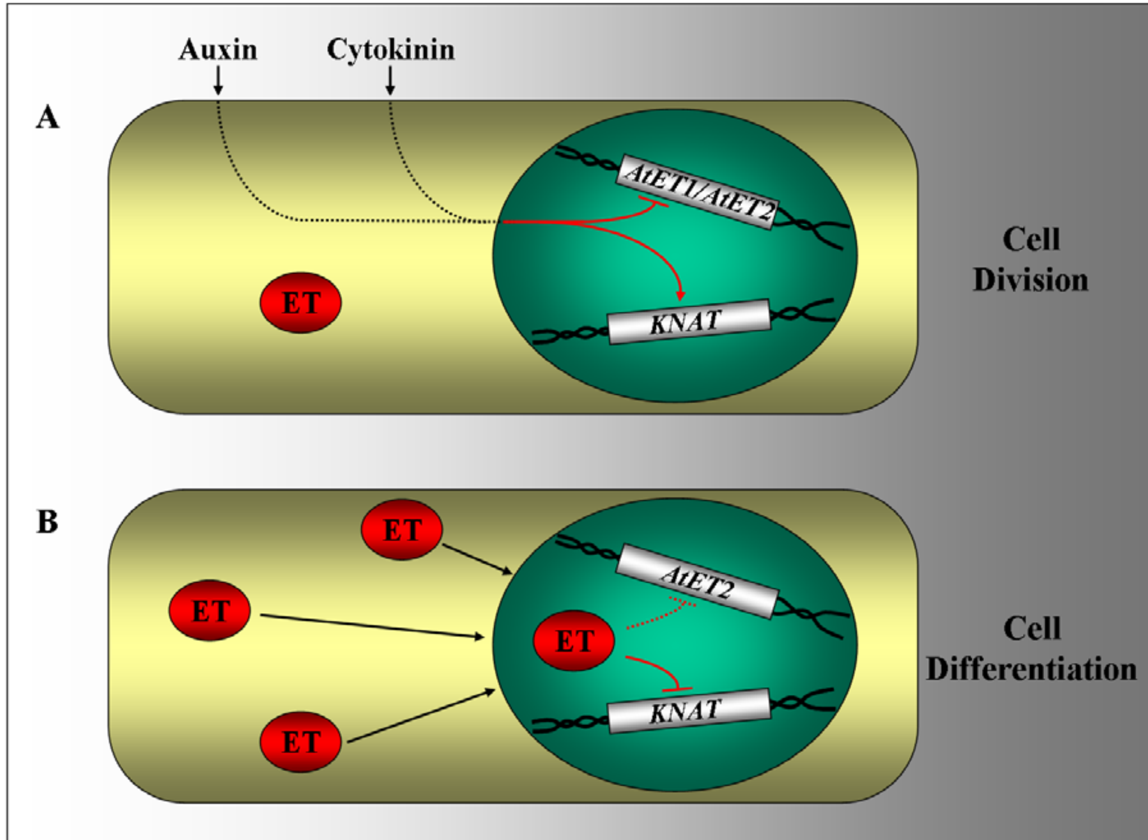

Figure 24. Putative role of ET proteins in plant cells

A. The phytohormones auxin and cytokinin promote cell division and meristem identity. Differentiation regulators AtET1 and AtET2 are kept inactive by at least two mechanisms a) repression of gene expression, b) prevention of their translocation to the nucleus.
B. Lowering the hormone level causes induction of AtET1 and AtET2 gene expression and translocation into the nucleus. There, AtET1 and AtET2 act as repressors of members of the *KNAT* gene family and thereby promote cell differentiation. The internal levels of ET proteins are further controlled by autoregulation.

Summary

Regulation of cell differentiation is the basic process that integrates growth and development of plants. It includes the synchronized transduction of phytohormone signals to control transcriptional and translational activity, metabolic pathways and the generation of cell and tissue structures.

Transcriptional regulation of cell differentiation is based on the function of various families of transcription factors. Members of a new family, *EFFECTOR OF TRANSCRIPTION* (*ET*), have been identified as differentiation inducers both during embryogenesis and vegetative development. Structurally, ET factors are characterized by 2-4 highly conserved C-terminal cysteine containing, zinc and DNA binding repeats, strictly confined to the plant kingdom. Members of the ET family are known from different plants, but so far only two, the genes *HORDEUM REPRESSOR OF TRANSCRIPTION* (*HRT*) and the *Brassica Napus ET* (*BnET*) have been partially characterized. Therefore, this study aims to the investigation of the molecular function of ET factors in the model plant *Arabidopsis thaliana.*

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The *Arabidopsis* genome contains three *ET* genes. Two of them, *AtET1* and *AtET2*, exhibit all structural features of the *ET* family, while the third, *AtET3*, is a truncated copy of *AtET2*, lacking the region which encodes the four C-terminal *ET* repeats. Polymorphisms have been identified in the *AtET1* and *AtET3* genes of different *Arabidopsis* accessions. Most notable is a gene inactivating frameshift mutation in the *AtET1* gene of the ecotypes Columia0 and Limeport.

Besides the ET repeats, all known ET factors are characterized by an N-terminal domain with faint structural similarity to the single strand cleavage domain of prokaryotic UVRC proteins. A catalytically essential arginine residue in the active site is highly conserved between bacterial and plant proteins, leading to the hypothesis that ET factors might regulate gene expression via a novel molecular mechanism, which involves single strand

cuts with affects on the higher order of DNA structure in the chromatin, as also suggested for the product of the *DEMETER* gene.

A T-DNA insertion mutant of the *AtET2* gene has been isolated and characterized in the Wassilewskija2 ecoytpe. Mutant seeds exhibit premature germination if removed from the silique, indicating that AtET2 is involved in the acquisition and/or maintenance of dormancy. Furthermore, AtET2 was shown to contribute to the regulation of the gibberellin biosynthesis gene *GA20-oxidase* and the GA responsive gene *GASA4*, further supporting the suggestion that AtET2 affects dormancy via the modulation of gibberellin functions.

During the vegetative development, absence of AtET2 leads to the reduction of the lignin content, indicating an altered differentiation of xylem cells. This phenotype correlates well with changes in the expression pattern of several meristem identity- and differentiation related genes, like *KNAT1*. Furthermore, expression data suggest the regulation of *AtET2* by the phytohormones auxin and cytokinin.

To investigate this further, a protoplast system has been established. High hormone concentrations maintain the meristematic idenity of the cells, whereas lowering the hormone concentration results in the initiation of cell differentiation. This is acompanied by the upregulation of *AtET1* and *AtET2* gene expression. Remarkably, both AtET1 and AtET2 proteins are detected in the nucleus only in differentiating cells, suggesting the existence of a shuttle mechanism as a component of ET-function. The trunctated AtET3 protein did not change its cellular localization under these conditions, possibly due the lack of the C-terminal ET-repeats.

Taken together, the data suggest that ET factors represent a novel class of gene regulators involved in the control of cell differentiation as a prerequiste for plant development.

Zusammenfassung

Die Regulation der Zelldifferenzierung ist von grundlegender Bedeutung für die pflanzliche Entwicklung und beinhaltet unter anderem die synchronisierte Übertragung von Phytohormonsignalen für die Kontrolle von Transkription, Translation und Metabolismus als Voraussetzung für das Entstehen von Zell- und Gewebestrukturen.

Die Transkriptionsregulation während der Zelldifferenzierung basiert auf der Funktion einer Reihe von Transkriptionsfaktor-Familien. Mitglieder einer neuen Familie, EFFECTOR OF TRANSCRIPTION (ET), wurden als Induktoren der Zelldifferenzierung während Embryogenese und vegetativer Entwicklung identifiziert. Strukturell sind ET-Faktoren durch 2-4 hoch konservierte, C-terminale *repeats* charakterisiert. Diese Zink- und DNA-bindenden Motive wurden bisher nur bei Pflanzen gefunden. Wenngleich ET-Faktoren von mehreren Pflanzenarten beschrieben wurden, liegen umfangreichere Daten nur für die Gene *HORDEUM REPRESSOR OF TRANSCRIPTION (HRT)* und *Brassica Napus ET (BnET)* vor. Die vorliegende Arbeit soll daher einen Beitrag zur Aufklärung der molekularen Funktion der ET-Genfamilie in der Modellpflanze *Arabidopsis thaliana* leisten.

Im *Arabidopsis* Genom liegen drei ET-Gene vor. Zwei davon, *AtET1* und *AtET2*, zeigen alle typischen Merkmale der ET-Faktoren, während das dritte Gen, *AtET3*, eine um die ET-*repeats* verkürzte Kopie von *AtET2* ist. Verschiedene Ökotypen weisen Polymorphismen in den Genen *AtET1* und *AtET3* auf, darunter eine das *AtET1*-Gen inaktivierende *frameshift*-Mutation in den Ökotypen Columbia0 und Limeport.

Neben den ET-*repeats* besitzen alle bekannten ET-Faktoren eine N-terminale Domäne mit schwacher struktureller Ähnlichkeit zu einer DNA-Einzelstrang schneidenden Domäne prokaryotischer UVRC-Proteine. Ein katalytisch essentieller Argininrest im aktiven Zentrum ist hoch konserviert zwischen bakteriellen und pflanzlichen Proteinen. Dies führt zu der Hypothese, dass Genregulation durch ET-Faktoren möglicherweise über

Einzelstrangschnitte und daraus folgende Einflüsse auf die höhere DNA-Struktur im Chromatin erfolgt, wie es auch für das Genprodukt *DEMETER* vorgeschlagen wird.

Eine T-DNA Insertionsmutante des *AtET2*-Gens im Ökotyp Wassilewskija*2* wurde isoliert und charakterisiert. Samen der Mutante zeigen vorzeitige Keimung, wenn sie aus den Schoten isoliert werden. Dies belegt einen Einfluss des *AtET2*-Gens auf Erwerb und/oder Ausprägung der Dormanz. Weiterhin wird gezeigt, dass AtET2 an der Regulation des Gibberellin-Biosynthese-Gens *GA20-Oxidase* und des GA-kontrollierten Gens *GASA4* beteiligt ist. Diese Daten weisen weiter darauf hin, dass AtET2 Dormanz offenbar über eine Modulation von Gibberellinfunktionen beeinflusst.

Während der vegetativen Entwicklung führt der Ausfall des *AtET2*-Gens zur Reduktion des Ligningehaltes. Dies wird als Einfluss von AtET2 auf die Differenzierung von Xylemzellen interpretiert. Dieser Phänotyp korreliert mit entsprechenden Änderungen des Expressionsmusters einer Reihe von Meristemidentitäts- und Differenzierungsgenen, wie z. B. *KNAT1*. Darüber hinaus belegen die Expressionsdaten die Regulation von *AtET2* durch die Phytohormone Auxin und Cytokinin.

Um dies näher zu untersuchen, wurde ein Protoplasten-System etabliert. Hohe Hormonkonzentrationen führen zur Aufrechterhaltung der meristematischen Identität der Zellen, während eine Erniedrigung der Hormonkonzentration zur Initiation der Zelldifferenzierung führt. Damit einher geht eine Hochregulation der Gene *AtET1* und *AtET2*. Bemerkenswert ist ferner, dass beide ET-Faktoren nur bei sich differenzierenden Zellen im Zellkern gefunden werden. Dies weist auf die Existenz eines *shuttle*-Mechanismus als eine Komponente der ET-Funktion hin. Das verkürzte AtET3 Protein zeigt keinen derartigen Wechsel in der Lokalisation, möglicherweise als Folge der fehlenden ET-*repeats*.

Zusammen genommen zeigen die Ergebnisse, dass ET-Faktoren eine neue Klasse von Genregulatoren repräsentieren, welche an der Kontrolle der Zelldifferenzierung als Voraussetzung für pflanzliche Entwicklung beteiligt sind.

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Appendix

Ectopic expression of *EFFECTOR OF TRANSCRIPTION* perturbs gibberellin-mediated plant developmental processes

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Abstract

The plant hormone gibberellin (GA) is known to modulate various aspects of plant cell differentiation and development. The current model of GA mediated regulation is based on a de-repressible system and includes specific protein modification and degradation. HRT, a zinc finger protein from barley has been shown to have GA dependent transcriptional repressing activity on the seed specific α-amylase promoter (Raventos et al., 1998). Here we report the characterization of a dicot zinc homologue from *Brassica napus* (BnET) and provide evidence for its role in GA response modulation suggesting that this could be a conserved feature of this gene family. When BnET is ectopically expressed in either *Arabidopsis* or tobacco the phenotypes include dwarfism due to shorter internodes and late flowering, reduced germination rate, increased anthocyanin content and reduced xylem lignification as a marker for terminal cell differentiation. Transient expression in protoplasts supports the notion that this most likely is due to a transcriptional repression of GA controlled genes. Finally, histological analysis showed that in contrast to other GA deficient mutants the shorter internodes were due to fewer but not smaller cells, suggesting a function of BnET in GA-mediated cell division control.

Introduction

Plant embryogenesis and the developmental phase transition from dormant embryo to the germinating and growing seedling are controlled by endogenous programs and environmental stimuli. At the molecular level this includes a complex interplay between various transcription factors like FUS3, LEC1, LEC2, ABI3 and precisely controlled ratios of phytohormones like gibberellin (GA) and abscisic acid (ABA) (Gazzarrini and McCourt, 2003). Thus, ABA promotes processes of late embryogenesis like storage compound synthesis, acquisition of desiccation tolerance and dormancy, whereas GA acts as a factor initiating cell division, -extension and -differentiation required for germination. The ratio of GA and ABA is known to control the decision between germination *versus* maturation (Koornneef et al., 2002) and the transcription factor FUS3 was recently shown to repress transcript levels of the GA biosynthetic gene AtGA3ox2 but upregulate ABA levels during early embryogenesis (Curaba et al., 2004). Moreover, the chromatin-remodeling factor PKL, a putative positive regulator of GA response, was found to inhibit the expression of the embryogenesis factors LEC1 and 2 as well as FUS3 to repress ectopic embryo formation during postembryonic development (Ogas et al., 1999). Although most of these regulators are obviously required to control embryogenesis, seed development and germination, their function is not necessarily restricted to these developmental stages and might pleiotropically function also in other developmental processes.

Thus gibberellins are key regulators of many aspects of vegetative and reproductive development, including stem elongation, leaf differentiation, photomorphogenesis, pollen development, flowering etc.. These diverse functions obviously require a tight control of synthesis and response (for review see Olszewski et al., 2002). Among the genes responsible for GA biosynthesis and degradation, GA20- and GA3-oxidase encoding genes are particular important for controlled synthesis of bioactive GA levels, whereas GA2-oxidases modulates GA levels *via* the catabolism. Feed back mechanisms lead to downregulation of GA20ox- and GA3ox- and upregulation of GA20ox-genes by elevated GA signaling or GA treatment. Moreover, several genes involved in GA signaling and response have been identified (Richards et al., 2001, Gazzarrini and McCourt, 2003,

Thomas and Sun, 2004, Fleet and Sun, 2005) with the DELLA proteins like GAI, RGA, RGL, SLN, SLR acting as central players in GA mediated gene regulation. Repression and its release seems to be a wide spread principle in GA response and includes at least two groups of negative regulators (Zhang et al., 2004). One group acts without binding to DNA and includes for instance the TPR domain containing SPY proteins (Jacobson et al., 1996), PKABA1 (Gomez-Cadenas et al., 1999), the huge DELLA family (for reviews see e. g. Richards et al., 2001; Olszewski et al., 2002; Fleet and Sun, 2005) as well as the GAMYB (Gubler et al., 1995) binding protein KGM (Woodger et al., 2003). Another group of repressors acts as promoter binding factors and includes for instance the RING-finger protein SHI (Fridborg et al., 2001), the MYB- and NAC-like HSI transcription factors involved in SPY regulation (Robertson, 2004), the WRKY factor OsWRKY71 (Zhang et al., 2004) as well as HORDEUM REPRESSOR OF TRANSCRIPTION (HRT), a zinc finger protein which represses the GA mediated induction of α-amylase *via* its binding to the GARE-element (Raventos et al., 1998).

Here we report a dicot homologue of HRT from *Brassica napus* (BnET). The data suggest a role of BnET in GA response modulation as a conserved feature of this gene family.

Materials and methods

South- Western screening and molecular biology techniques

South- Western screening was performed using amplified λZAPII cDNA expression libraries (Clontech, Pablo Alto, CA). For primary screening, approximately $6x10^5$ pfu (plaque forming units) were probed using both a library from immature *Brassica napus* and *Vicia faba* seeds. The oligonucleotides used as probes were synthesized according to sequences of the *napA* promoter from *B. napus* (EMBL/Genbank accession number J02798), the *USP* (EMBL/Genbank accession number X56240) or the legumin B4 (*LeB4*) promoters (EMBL/Genbank accession number X03677) from *V. faba*. The oligonucleotides were constructed to after annealing give the double-stranded phosphorylated probes either recessed 3’ ends or *BamH*I or *EcoR*I sticky ends to facilitate

oligomerization by ligation. The concatermerized oligonucleotides (average of 200 bp) were labeled by nick-translation using α-[^{32}P]-dNTPs (Amersham International, Amersham, UK). The South- Western screening was carried out using an incubation buffer composed of 25 mM HEPES (pH 8.0), 0.1 mM EDTA, 4 mM KCl, 7 % Glycerol, 1 mM $MgCl_2$, 0.5 mM DTT and 1.0 mM $ZnSO_4$. Sequencing of the insert of the excised plasmids was done on both strands using the Deoxy Terminator Cycle Sequencing kit and an Applied Biosystem 373A DNA sequencer (Applied Biosystem, Foster City, CA). Standard molecular techniques were used throughout the study (Ausubel et al., 1996; Sambrook et al., 1989).

Analysis of the zinc binding

A peptide corresponding to amino acid residues 363 to 395 of BnET was synthesized by F-Moc chemistry using an Applied Biosystems 430 peptide synthesizer operated with a FastMoc protocol (Perkin-Elmer/ABD, Foster City, CA). The peptide was purified by reverse phase chromatography and the correct fractions were collected after matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry analysis. The peptide was kept under acidic conditions until use to avoid formation of disulphides. MALDI-TOF mass-spectrometry for the determination of zinc binding was carried out according to Woods et al. (1995) using a Kompact MALDI IV mass-spectrometer (Kratos, Manchester, UK). Analyses were done with and without addition of equimolar amounts of Zn^{2+} to the peptide solution. Fluorescence measurements were performed at 25°C in a SLM 4800S spectrofluorometer (SLM-Instruments, Rochester, NY) in 0.1 M Hepes, pH 7.0, 50 mM NaCl. The excitation wavelength was 280 nM. Titration of the peptide with Zn^{2+} was monitored by measurement at the wavelength at which the maximal fluorescence emission change accompanying the interaction was observed (350 nm).

Plant transformation

An *Agrobacterium*-mediated leaf disc procedure was used for tobacco transformation as described previously (Bäumlein et al., 1991). Transformation of *Arabidopsis* was done by vacuum infiltration method as described by Bechthold et al. (1993).

Protoplast transformation and transient expression

Suspension cultures of *Nicotiana plumbaginifolia* and *Arabidopsis* were used for protoplast isolation. Following cell wall digestion in a 1% cellulase R10 and 0.5% macerozym R10 (Duchefa Biochemie, Haarlem, Netherlands) solution, protoplasts were centrifuged and washed twice in W5 medium (0.9% NaCl, 1.8% $CaCl_2$, 0.04% KCl, 0.1% glucose, pH 5.6). Next, they were concentrated in Mg mannitol (0.45 M mannitol, 15 mM $MgCl_2$, 0.1% MES, pH 5.6) to a density of approximately $3x10^6$ cells/ml. To transform the resulting protoplasts, solution containing plasmid DNA (5 µg of each plasmid) and carrier DNA (160 µg) was added to 330 µl Mg mannitol containing $1x10^6$ protoplasts. Equal amount of PEG solution (40% PEG 6000, 0.1 M $Ca(NO_3)_2$, 0.4 M mannitol, 0.1% MES, pH 6.5) was added to the mixture after 10 min incubation. After 20 min the transformed protoplasts were diluted into 4 ml K3 medium and transferred to small petri dishes. Following a 16-18 h incubation time at 25 °C in the dark, protoplasts were harvested and the GUS activity was determined by chemiluminiscence assay using the GUS-Light TM Kit (Tropix, Bedford, USA) and a Lumat LB9501 luminometer (Berthold, Germany). A control construct consisting of the 35S CaMV promoter in front of the GUS reporter gene and terminated was efficiently expressed in this system and used to standardize the different experiments. The test construct consists of the GASA4 gene promoter fused to the glucuronidase reporter gene and terminated with the nopaline synthase terminator (GASA4P-GUS-NOS). The BnET gene was cloned in the vector pRT103 (Topfer et al., 1988) and expressed under the control of the CaMV35S promoter and terminated with the nopaline synthase terminator (CaMV35SP-BnET-NOS). GA_3 was applied at a concentration of 10µM.

Transient expression of ET- GFP fusions in protoplasts

The GFP-vector pFF19 (Höfius et al., 2004) was used for transient expression in tissue culture derived *Arabidopsis* protoplasts (see above). Both full length BnET or BnET with the N-terminal 24 amino acids deleted (BnET ΔNLS) was translationally fused to GFP. The deleted 24 amino acids include a putative nuclear localization signal consisting of 6 lysine residues (positions 19-24). The cell suspension was incubated for

18 h in K3 medium, containing 4.5 µM 6-benzylaminopurine, 10 µM 1-naphtalene acetic acid and 4.5 µM 2,4-dichlorophenoxyacetic acid. The GFP signal was localized *in vivo* using a confocal laser-scanning microscope (Zeiss). The eGFP fluorophore was excited at 488 nm by an argon laser and detected between 505 and 520nm. The empty pFF19g vector was used as a negative control and shows an equal distribution.

RT-PCR

Total RNA from wild type and BnET overexpressing plants was isolated using the Total RNA Isolation Reagent (Biomol) following the manufacturers instructions. RNA was isolated from seedlings grown for 10 days in liquid culture and was treated with DnaseI (Fermentas) for 15 minutes at 37 °C. The enzyme was inactivated by the addition of 2mM EDTA and heat treatment at 70 °C. Single stranded cDNA was synthesized using a First strand cDNA Synthesis Kit (Fermentas) and subsequently used as PCR template. The annealing temperature and number of cycles for each primer pair was optimized in order to ensure quantitative measurement. PCR products reactions were separated on 1.5% Agarose gels, capillary transferred on HybondN+ membranes (Amersham) and hybridized. The labeling of the cDNA probe was carried out with [^{32}P]dCTP using the Rediprime^{TM} II Random Prime Labeling Kit (Amersham) The radioactive membranes were washed and exposed to Phosphoimager (Fujifilm). The primer pairs and their respective PCR conditions are as follows: BnET: 5'-ATGTTCTGTCTCAATACC-3' and 5'-AGATGTGATTCTCATCCC-3' (Tm 55 °C, 25 cycles); NtGA20oxidase: 5'-GGATCCATGGCAATTGATTGTATGATCACG-3' and 5'-GGATCCAGCCAATTT GAGAAAGTTTG-3' (Tm 55 °C, 37 cycles); NtActinAC1: 5'-ATGGCAGACGGTGAGGATATTCA-3' and 5'-GCCTTTGCAATCCACATCTGTT G-3' (Tm= 55 °C, 24 cycles).

Fixation, Substitution, Embedding, Histology, Light and Electron Microscopy

For the primary fixation 2 mm thick cross sections of the mid-region of the 7th internodes of tobacco plants were kept 10 h at room temperature in 50 mM cacodylate buffer (pH 7.2), containing 2.5% (v/v) glutaraldehyde and 2.0% (v/v) formaldehyde. For the secondary fixation samples were transferred after three washes for 15 min with the

used buffer to a solution of 1% (w/v) OsO_4 in 50 mM cacodylate buffer. The fixation of 2 h is followed by one wash with buffer, and three washes with distilled water. Dehydration of samples was done stepwise by increasing the concentration of ethanol. The steps were performed as follows: 30% (v/v), 50% (v/v), 60% (v/v), 75% (v/v), 90% (v/v) and two times 100% (v/v) ethanol for 2 h each. After 1 h dehydration with propylene oxide the samples were infiltrated subsequently with Spurr (Plano GmbH, Marburg, Germany) as follows: 33% (v/v), 50% (v/v) and 66% (v/v) Spurr resin in propylene oxide for 10 h each and then 100% Spurr overnight. Samples were transferred into BEEM capsules, kept there for 6 h in fresh resin and polymerized at 70 °C for 24 h. For light microscopical analysis semi thin sections with a thickness of approximately 3 µm were mounted on slides and stained for 2 minutes with 1 % methylene blue in 1% aqueous borax at 60 °C prior to examination in a Zeiss Axiovert 135 microscope. Fresh material of the mid-region of the 7th internodes was cut with a Leica Vibratome VT1000S into cross section with a thickness about 200 µm. Lignin autofluorescence was investigated using the Zeiss filterset 05 of an Axiovert 135 (Zeiss, Jena, Germany). Bright field and fluorescence images have been taken by using a Zeiss Axiocam provided with an Axiovision software package. For ultrastructural analysis ultra thin sections with a thickness of approximately 70 nm were cut with a diamond knife and contrasted with a saturated methanolic solution of uranyl acetate and lead citrate prior to examination in a Zeiss CEM 920A transmission electron microscope at 80 kV.

Anthocyanin and lignin quantification

Phenolic constituents including anthocyans were analyzed on an HPLC System (Alliance 2690, Waters, Eschborn, Germany) combined with a photodiode array detector (996, Waters) and a fluorescence detector (FP-920, JASCO, Groß-Umstadt, Germany). HPLC-methods and extraction of methanol-soluble phenols were as described (Mock et al., 1999). Profiles of anthocyans were obtained by extracting chromatograms at 535 nm from the photodiode-array data. The content of lignin was determined after thioacidolysis following the method described by Campbell and Ellis (1992). Recovery rates for each individual experiment were determined by analyzing parallel samples with appropriate amounts of authentic lignin.

GA measurement

Samples of 1g were ground in liquid nitrogen to a homogenous powder, and GAs were analyzed essentially as earlier described by Peng et al.(1999) by GC/MS-selected reaction monitoring (SRM, Moritz and Olsen 1995) besides that samples were methylated diazomethane prior HPLC. For all GA analyses, calibration curves were recorded from 0.5 to 40 pg GA with 10 pg [2H2]-GAs (Prof. Lew Mander, Canberra, Australia) used as internal standards. All data were processed by a JEOL MS-MP7010 data system.

Results

Isolation of the cDNA corresponding to BnET and VfET

Using South- Western screens with the aim of isolating transcription factors important for embryonal gene-regulation, several DNA-binding proteins from *Brassica napus* and *Vicia faba* were isolated from cDNA expression libraries made from developing seeds of both species. Factors from various classes of DNA-binding proteins were isolated including leucin zippers, zinc fingers, RING-fingers and high mobility group (HMG) proteins (M. Ellerström, T. Wohlfarth, P. Wycliffe, L. Rask and H. Bäumlein, unpublished). Moreover, two similar proteins were isolated in parallel from both expression libraries. Sequencing information revealed a conserved cysteine-histidine rich repeat in both factors from each species (Fig. 1a). The repeat, designated as ET *(EFFECTOR OF TRANSCRIPTION)* domain, was present twice in the *V. faba* protein (EMBL/Genbank accession number X97909) and four times in the *B. napus* protein (accession number AY533506) and exhibits sequence similarity to *HORDEUM REPRESSOR OF TRANSCRIPTION (HRT)* described by Raventos et al. (1998). The *B. napus* cDNA most likely is a full length clone due to stop codons both 5’ and 3’ of the open reading frame, whereas the *V. faba* sequence might be truncated due to the presence of stop codons only in the 3’ end. Blast searches showed that members of this strictly plant specific family occur in other dicots, monocots as well as in mosses. The conserved structure of the ET-domain contains the common pattern (C-$X_{8/9}$-C-X_9-R-C-X_2-H-K). So far, members with two, three or four domains have been identified. A factor found in

Physcomitrella (accession number: PPP_3786_C1, OSMOSS database) seems to contain only a single domain structure. Even if no functional data are available for the *Physcomitrella* protein, the presence of a similar domain also in such a distantly related phyle as mosses suggests an evolutionary conserved and therefore most likely important function in photosynthetic organisms.

The one member of the family characterized earlier is HRT (Raventos et al., 1998). Interestingly, this clone was also isolated by means of South- Western hybridization and shown to functionally repress transcription through the GA responsive *cis*-element of the *Hordeum vulgare* amylase promoter. This analysis included biochemical investigation of bacterial expressed protein including determination of the DNA-binding capacity of the ET domain and transient analysis for establishing function. In contrast to HRT, we have been unable to show a sequence-specific DNA-binding for BnET. Current experiments with a related *Arabidopsis* ET-factor show binding to DNA, however without specificity for the GARE motif. These results might be explained by differences between dicot and monocot factors, for instance concerning binding constants, requirement for hetero-dimerization or posttranscriptional modification, and suggest functional differences within this group of proteins. In this respect it is also worth pointing out that HRT has three ET domains whereas the other proteins either have two (VfET) or four (BnET and AtET) domains (Fig. 1a), as well as that HRT is the most distantly related of the four proteins (not shown).

Recently, another GA-induced gene, Os-GRF1, has been shown to be expressed in the intercalary meristem of deepwater rice (van der Knaap et al., 2000). The gene product shows sequence similarities to an *Arabidopsis* gene family of unknown function and contains a region with similarity to a protein interaction domain of SWI2/SNF2, which is a subunit of a chromatin-remodeling complex in yeast. A second domain designated WRC overlaps the core pattern of the ET-domain. Outside this domain WRC and the ET factor family do not display any sequence similarity. Heterologous expression of Os-GRF1 in *Arabidopsis* leads to the inhibition of stem elongation, and normal growth could not be recovered by the application of GA (van der Knaap et al., 2000).

Structure of the ET gene family in Brassica napus and Arabidopsis

Genomic DNA gel blot experiments using the portion encoding the ET domains of the *B. napus* cDNA as probe revealed that in both *B. napus* and *Arabidopsis* there exists a small family of related genes. This has been confirmed by blast searches of the *Arabidopsis* genome showing the existence of a small three-member gene family (accession numbers At4g26170, At5g56780 and At5g56770). Two have the nucleic acid interacting ET domain whereas the third lacks this domain. Similarity tree analysis (not shown) reveals that the dicot members of this family group together on a separate branch and that the closest homology to BnET is the *Arabidopsis* gene located on chromosome 4 (At4g26170).

Zinc binding of the BnET protein

The conserved spacing of the cysteine and histidine residues in the ET domain is reminiscent of the structure of zinc fingers, although the domain does not fit into previously described zinc finger subfamilies (Matthews and Sunde, 2002). For HRT one of the three cysteine-histidine domains has the capacity to interact with radioactively labeled Zn^{2+} (Raventos et al., 1998). To investigate if zinc binding is a common feature of this domain and if so to determine a dissociation equilibrium constant for the interaction, a peptide corresponding to amino acids 358 to 396 of BnET was synthesized. The peptide corresponds to the third BnET domain, which includes a tryptophan between the third cysteine and histidine residues. The complex formation between the peptide and Zn^{2+} was investigated by MALDI-TOF mass spectrometry (Woods et al., 1995). The analysis of the peptide alone generated a signal corresponding to the molecular mass of 4.291 Da, close to the expected mass of the peptide (Mw = 4.288). The addition of equimolar amounts of Zn^{2+} generated an additional signal with the mass of 4.353 Da (Fig. 1b). The differences between the signals (4353 Da – 4288 Da = 65 Da) correspond to the mass of one zinc ion (65 Da). The fact that additional signals corresponding to larger molecular weights were not observed indicates that each peptide binds one Zn^{2+} ion. To determine a dissociation equilibrium constant, fluorescence spectra of the peptide with and without the addition of zinc were measured. The maximum emission of the peptide alone was at 350 nm. A fluorescence increase of about 80% with no change of the emission maximum was observed in the presence of Zn^{2+} under the conditions of the

measurement. Titration of the peptide with Zn^{2+} at a peptide concentration of 10 μM gave a Zn^{2+}/peptide binding stoichiometry of 0.85:1, close to a 1:1 ratio. At a peptide concentration of 0.5 μM, which was found to be the lowest peptide concentration compatible with acceptable experimental precision, a dissociation equilibrium constant of $(5 \pm 1) \times 10^{-8}$ M was determined. Even if this figure is somewhat higher than those determined for individual zinc fingers from for example TFIIIA, K_d $Zn^{2+} = 3 \times 10^{-9}$ (Berg and Merkel, 1989), these data support the notion that in monocots, dicots and most probably also in mosses function of this domain is dependent on binding of Zn^{2+}.

Nuclear localization of BnET

Since an effector of transcription would be expected to be localized in the nucleus, we analyzed the subcellular localization of BnET. Tissue culture derived protoplasts of *Arabidopsis* were used for transient expression of BnET-GFP translational fusion constructs. The full length fusion protein could be clearly localized in the nucleus with low intensity signals remaining in the cytoplasm (Fig. 2). There is a characteristic stretch of 6 lysine residues in the N-terminal amino acid sequence (position 19-24) of BnET. To test whether this sequence feature might act as a nuclear localization signal, a deletion derivative with the first 24 amino acids missing has been constructed (BnET-GFPΔNLS), fused to GFP and tested by transient expression in protoplasts. As shown in Fig. 2, the deleted derivative is strictly localized in the cytoplasm, indicating that the poly lysine stretch might indeed act as a signal for nuclear transport.

BnET modulates the response of the GASA4 promoter to GA

Even though no sequence specific DNA binding has been detected, the isolation procedure due to affinity to nucleic acid of BnET and VfET and the nuclear localization shown for BnET-GFP suggest an effect on transcription. To further study the effect of BnET on GA signaling, we used a functional assay that measures the effect of transiently expressed protein on the GA-mediated transcriptional up-regulation of the *Arabidopsis* GASA4 gene promoter (Aubert et al., 1998) using *Arabidopsis* protoplasts. The same construct used to generate stable transgenic *Arabidopsis* and tobacco lines has been used in transient assays by means of the high copy number vector pRT103 (Topfer et al.,

1988). As shown in Fig. 2, the GASA4 gene promoter exhibits a reasonably low basic activity in this system. The application of GA3 results in an approximately four-fold induction of the promoter activity as measured by GUS activity. This induction is prevented by co-expression of the BnET protein. The empty vector was used as a control and did not affect the GA-induced promoter activity demonstrating the specificity of the repression. Supporting results have been obtained with a similar transient expression system based on *Nicotiana plumbaginifolia* protoplasts (not shown). Hereby, the aquaporin promoter PIP1b from *Nicotiana tabaccum* (Kaldenhoff et al., 1996) could be stimulated about two-fold by the application of GA. Co-expression of the BnET construct did prevent this stimulation. Thus, the data using transient expression assay support the notion that the ET factor family also in dicots most certainly plays a role in GA response and that this most likely is due to transcriptional repression.

Ectopic expression of BnET alters several aspects of plant development

To gain insight into the function of this gene family in dicots we expressed the *BnET* gene both in tobacco and *Arabidopsis* under the control of the viral 35S gene promoter. This leads to the obvious macroscopical phenotypes of dwarfism due to shorter internodes (Fig. 3 a, b). The phenotype was observed in seven of eleven independent transgenic tobacco lines and six of the nine transgenic *Arabidopsis* lines. Northern analysis showed that the transgene was expressed in the transgenic tobacco and *Arabidopsis* plants that had altered phenotypes indicating that the morphological changes of 35S:BnET were results of expression of the transgene. In both species the transgene transcript level roughly correlates with the strength of the dwarf phenotype (not shown). Moreover, their offspring inherited the phenotypes and neither of twenty transgenic *Arabidopsis* or tobacco lines transformed with either empty vector or the BnET cDNA in opposite orientation showed any phenotypes. As a first quantification of this phenotype the number of leafs at the onset of flowering was determined for all seven primary transgenic tobacco plants displaying the phenotype (12 $\pm$ 2) and for a set of five non-transformed tobacco plants (14 $\pm$ 1). These numbers are comparable and therefore indicative of that the dwarf phenotype is due to a reduction of the internode lengths. Three lines with similar medium severity phenotypes where chosen for further

characterization. As shown in Table 1 the number of internodes at the time of flowering is similar for wild type (WT) and the transgenic lines whereas the transgenic lines flower at a much later time point (30 *versus* 50 days). The reason for the dwarf phenotype is reduction in internode length as is shown clearly by the 7th internode being 68 mm in WT *versus* 52 mm in the transgenic plants (Table 1).

As the dwarf phenotype partially resembles that of GA-deficient mutants, we investigated if the phenotype was reversible by exogenous GA. By applying exogenous GAs we could not detect a restoration of the dwarf growth in both species. This indicates that dwarf phenotypes are not due to a strong impact on GA synthesis but rather result from a modulated GA response.

Since dwarfism could be the result of several different distortions, the feedback regulation of the GA-biosynthesis gene GA20 oxidase was used as the currently most reliable molecular marker to confirm the effect of BnET on GA response. As shown in Fig. 4 four lines ectopically expressing BnET exhibit an up-regulated expression of the GA20 oxidase gene in comparison to wild type. The level of up-regulation roughly correlates with the line specific expression level of the BnET transgene. In agreement with the increased GA20 oxidase transcript levels slightly increased levels of GA19 and GA20 could be measured in the transgenic tobacco plants. No clear changes could be detected for the levels of GA1 and GA8 (Fig.4).

Germination is a classical developmental process known to be regulated by GA levels. Assuming a role of the BnET factor in the GA response pathway we have analyzed the germination behavior of seeds derived from *Arabidopsis* plants ectopically expressing BnET. As shown in Fig. 5, the transgenic plants exhibit a strongly reduced and delayed germination in comparison to wild type. Four independent transgenic lines were tested in this experiment. This observation is consistent with the suggested function of BnET as a repressor of GA-dependent processes. Corresponding results have been obtained for tobacco indicating the species independent action of this factor.

Although anthocyanin accumulation is usually considered to be a response to unfavorable stress conditions, it has also been found to be an effect of shifting the balance between GA and ABA (White and Rivin, 2000). The ectopic expression of BnET in *Arabidopsis* plants leads to the accumulation of excessive anthocyanin as determined by

HPLC chromatography. In total nine independent transgenic lines transformed with the gene construct in sense orientation were compared to seven independent lines transformed with the otherwise identical antisense construct as well as non-transformed plants. As shown in Fig. 5, the sense plants accumulated about 10-fold higher amounts of anthocyanin compared to the antisense controls.

BnET prevents auxin and cytokinin induced in vitro organ development

To examine the effects of BnET on the response to other phytohormones, shoots, roots and callus were induced from tobacco leaf squares in medium containing different concentrations of auxin and cytokinin. The experiments were based on the classical experiment by Skoog and Miller (1957), showing that the auxin to cytokinin ratio controls the formation of shoots, roots and callus tissue *in vitro*. Whereas the growth of shoots, roots and callus of a control and antisense constructs of BnET was normal, ectopic expression of BnET resulted in a complete inhibition of all of these three processes, demonstrating that over-expression of BnET suppresses the capability of plant explants for *in vitro* organogenesis (Fig. 6). The cellular mechanism of GA action includes both the control of cell division (Sauter et al., 1995) as well as cell elongation (Fukazawa et al., 2000). These observations therefore suggest that an effect of ectopically expressing BnET is a disturbance in GA mediated cell cycle regulation and that this effect overrides the influences of auxin and cytokinin in the *in vitro* assay used. Despite this, it cannot be ruled out that the effect observed also is dependent on direct repression of the auxin and cytokinin responsiveness of the cells.

The shorter internodes of BnET plants are due to fewer cells

Studies of mutants with decreased GA signal transduction have demonstrated that the shorter internodes are due to smaller cells (Fridborg et al., 1999, Fukazawa et al., 2000), showing that one of the GA actions disturbed in these mutants is cell elongation. In contrast, our data point in the direction of disturbed GA mediated cell division. To further study this, longitudinal sections of three selected individual transgenic lines were used to determine the length of the epidermal and the cortical cells of the 7th internode of the BnET transgenic tobacco. Interestingly, no significant difference in length either for

epidermal or cortical parenchyma cells was observed (Fig. 7 a, b, Table 1). Thus it has been shown that these plants maintain their cell size while the internodes grow shorter, this problem only can be resolved if the total number of cells per internode is decreased, pointing to BnET effects on regulation of cell division.

Ectopic BnET expression perturbs lignification and secondary cell wall thickening

Analyses of transverse stem sections between the 7th and 8th nodes of tobacco plants ectopically expressing BnET revealed a severe loss of phenolic autofluorescence in comparison to wild type plants and reduced cell wall thickening (Fig. 7). The autofluorescense is due to lignin incrustation of the secondary cell wall of xylem vessels and fibers (Fig. 7 c). Quantitative analysis showed that the reduction of lignin content is in the order of one magnitude (Table 1). In plants ectopically expressing BnET, only the proto xylem vessels differentiating directly from cells of the procambial strands are lignified (Fig. 7 d, f) and show the normal patterns of secondary cell wall thickening. In contrast, the cells of the metaxylem do not show differentiation towards xylem vessels or xylem fibers independent of their origin either from fascicular or interfascicular cambia (Fig. 7 d, f). Besides loss of lignification electron-microscopical analysis revealed a living, widely undifferentiated state of these cells in BnET overexpressing plants (Fig. 7 f, h). While in wild type plants even directly beneath the cambial cells the xylem vessels and fibers show normal development (Fig. 7 e, g), the vessel adjacent to the protoxylem in BnET overexpressing lines still shows thickened secondary walls, but the integrity of plasma and organelles is still preserved. The next metaxylem vessel (one cambial division event younger) does not exhibit any differentiation towards vessel or fiber development.

Discussion

Transcriptional regulation is an essential basis for differentiation and development of multi cellular organisms. Transcription factor genes comprise a substantial fraction of all eukaryotic genomes and the majority of those genes can be grouped into different large families according to their type of DNA-binding domain. With the completion of the *Arabidopsis* genome sequencing project, the complete number of transcription factor genes has been estimated to be approximately 1700 of which only 7% have been characterized so far (Paz-Ares et al., 2002). Here, we describe the genetic and biochemical characterization of a sub-family of zinc- and nucleic acid- interacting nuclear proteins denoted the ET factor family, originally founded by HORDEUM REPRESSOR OF TRANSCRIPTION of barley (Raventos et al., 1998).

Ectopic BnET expression negatively modulates GA mediated developmental processes

The ectopic expression of *BnET* results in dwarf growth. This phenotype has been independently observed in two species, tobacco and *Arabidopsis* (Fig. 3) suggesting a conserved mode of function. Although dwarf phenotypes might be caused by failures in a variety of processes, dwarf plants often result due to changes in GA biosynthesis or response. Decreased GA responsive mutants comprise dwarf and semi-dwarf mutants that are phenocopies of plants with reduced GA biosynthesis. Further support for BnET disturbing GA perception comes from the quantification of the phenotype (Fig. 3) and Table 1), the reduced germination rate (Fig. 5) and the nuclear localization coupled with the block of transcription (Fig. 2). Finally, the feedback regulation of GA-biosynthesis genes is considered a reliable molecular marker for GA signaling. The clearly increased transcript level of the GA20-oxidase gene in independent BnET overexpressing plant lines and the good correlation between this upregulation and the level of BnET (Fig. 4) expression supports the conclusion that BnET is involved in the modulation of GA responses.

The germination of a seed is a dramatic developmental event that is dependent on the plant growth regulators GA and abscisic acid (ABA) besides multiple other factors (Koornneef et al., 2002). During germination GA is required to overcome the mechanical restraints conferred by the embryo-surrounding tissues, most likely by the induction of

genes encoding cell wall loosening enzymes (Leubner-Metzger et al., 1996). This has been concluded from the observation that non-germinating GA-deficient *Arabidopsis* mutants develop into dwarfs after the mechanical removal of the testa (Silverstone et al., 1997b). Furthermore, GA is essential to increase the growth potential of the embryo as demonstrated by the dwarf phenotype of GA-deficient mutants (Groot and Karssen, 1987). In general it is widely accepted that the GA/ABA balance governs germination *versus* seed maturation pathways (Koornneef et al., 2002). The original isolation of BnET as well as VfET from immature seeds suggests that ET factors contribute to the control of the ABA/GA balance as a basis for the developmental switch between dormancy and germination. Thus, the observed phenotype with a reduced germination rate as well as the dwarf growth is consistent with the proposed function of ET as a repressive modulator of GA mediated processes.

The results in Fig. 2 show that GA treatment increased the activity of the GASA4 promoter four-fold in the transient expression system used, and that this induction was blocked by BnET. The moderate induction rate is well comparable to the sixfold induction found under *in vivo* conditions (Herzog et al., 1995). Even though, the GA induction of the GASA4 promoter is moderate, the repression effect of BnET resembles that seen with other transcriptional repressors of GA action (Fridborg et al., 1999; Robertson et al., 1998) and is also similar to what has been determined for HRT (Raventos et al., 1998). Thus, the data support the conclusion that BnET just like HRT are involved in modulation of GA responses and by that showing conservation of this gene family between mono- and dicot plant species.

BnET overexpression induces anthocyanin synthesis

The ectopic expression of BnET in *Arabidopsis* leads to the accumulation of anthocyanin (Fig. 5). Although anthocyanin accumulation is usually considered to be a response to unfavorable stress conditions, alternative interpretations are conceivable. Extensive data are available concerning the regulation of anthocyanin biosynthesis in maize seeds by ABA and VP1. Both factors act synergistically to regulate the MYB-factor C1 which itself functions as a regulator of anthocyanin synthesizing enzymes (Hattori et al., 1992). Moreover, the GA-inhibitor paclobutrazol causes a strong increase

of the anthocyanin pigmentation of maize embryos, an effect that also can be induced by the application of ABA (White and Rivin, 2000). Therefore, the induced anthocyanin accumulation could be interpreted as that the ectopic expression of a GA-response repressor leads to a shift of the ABA/GA balance in favor of ABA.

The shorter internodes of BnET plants are due to fewer cells.

Growth and development of multicellular organisms is accomplished by orderly cell division and regulated cell expansion. It is generally assumed that GA promotes both cell division and cell elongation (Ogawa et al., 2003), but so far no genes involved in GA promoted cell division have been characterized. After germination, bioactive gibberellins are synthesized in and below the rapidly dividing meristems (Silverstone et al., 1997a) and promote stem elongation, leaf expansion and root growth (Yaxley et al., 2001). The cellular mechanisms include the control of cell division through transcriptional activation of cyclin-dependent protein kinases and mitotic cyclin genes (Sauter et al., 1995). For cell elongation, microfibrils must be oriented perpendicular to the growth direction and cellulose microfibrils transversely oriented in meristematic and young cells (Kende and Zeevart, 1997). Studies of mutants with decreased GA signal transduction have demonstrated that the shorter internodes are due to smaller cells, showing that one of the GA actions disturbed in these mutants is cell elongation (Fridborg et al., 1999, Fukazawa et al., 2000). In contrast to these findings analysis of the length of the epidermal and the cortical cells of the 7th internode showed that these cells in wild type and BnET transgenic plants are of similar length, demonstrating that cell number and not cell elongation is altered in these plants (Tab. 1; Fig. 7 a, b). Therefore, the repression of internode elongation of ectopically expressing BnET most certainly, and in contrast to other mutants disrupted in GA response, is due to disturbances of cell division. This suggests a putative function of BnET in GA mediated cell cycle control.

The suppression of *in vitro*-organogenesis (Fig. 6) might be interpreted in a similar direction. In controls the differentiated leaf disc cells start to dedifferentiate forming callus, which subsequently redifferentiates into shoots or roots depending on the hormone conditions. The ectopic expression of BnET prevents callus formation and subsequent

organogenesis, indicating the inability of the otherwise viable leaf cells to dedifferentiate and divide.

Ectopic BnET expression distorts xylem differentiation

Microscopic analysis of stem sections in the 7th internode of BnET tobacco plants revealed a strongly reduced cell wall thickening and lignification (Fig. 7, Table 1). Following the previous suggestion that BnET functions as a repressor of GA promoted processes, a literature search revealed only a few hints for a direct influence of GA on lignin biosynthesis. Therefore, it is difficult to decide whether the observed influence of BnET on xylem lignification is a direct or indirect consequence of dwarfism or *vice versa.* Previous reports describe an effect of GA and auxin on the stimulation of cambial activity in trees (Wareing, 1958; Digby and Wareing, 1966). Moreover, changes in lignin amount and composition due to altered GA-concentrations have been described (Aloni et al., 1990). Furthermore, an increased endogenous GA concentration stimulated secondary growth leading to more and longer xylem fibers (Eriksson et al., 2000). These data confirmed previous experiments described by Ridoutt et al. (1996). In that study it was shown, that the treatment of *Eucalyptus* shoots with a GA-biosynthesis inhibitor results in reduced fiber length and a decreased number of cambial cells and differentiating fibers. Finally, recent data demonstrate that tobacco dwarf plants, which result from the over-expression of the GA-degrading GA2-oxidase gene, show a very similar lignification phenotype as the one described for *BnET* expressing plants and have reduced transcript levels for lignin biosynthesis enzymes (Biemelt et al., 2004). Taken together, the data described are consistent with reports indicating a functional relation between GA and xylem lignification, but this clearly needs to be extended in further studies.

Tracheary elements are products of cambial activity. While protoxylem elements develop directly from procambial cells, metaxylem elements develop either from fascicular or interfascicular cambia. When the developing vessels reach their final size a specialized form of secondary cell wall formation begins. At the lateral cell wall, spiral or net-like thickening structures develop, accompanied by massive incrustations of lignin to gain air and nearly watertight insulation. Upon maturity the cells will undergo autolysis, a form of programmed cell death (PCD), accompanied by lysis of the transverse cell wall

(Fukuda, 2000). Apart from vessels, xylem fibers are also products of cambial activity and will undergo secondary cell wall development, lignin incrustation and finally programmed cell death during the process of differentiation. Several reports suggest that the developmental process of secondary cell wall formation and PCD not only are concurrent but molecularly interdependent (Fukada, 2000, Groover and Jones, 1999). Interestingly, the histological analysis of plants ectopically expressing BnET revealed that the differentiation and lignification of protoxylem vessels is unaffected, while all xylem vessels and xylem fibers of the metaxylem fail to differentiate. This is judged from the drastically reduced cell wall thickening (Fig. 7 c-f) and is confirmed by the overall decreased lignin content (Table 1), suggesting that metaxylem cells did not yet reach the mature stage and therefore do not start the process of secondary wall deposition. The effect becomes even more obvious in ultra structural analysis (Fig. 7 g, h). While the metaxylem cells of wild type stems are completely differentiated directly adjacent to the meristem even those close to the protoxylem in BnET transgenic plants are undiffentiated. Interestingly the oldest meristem derived metaxylem vessels still undergo cell wall thickening while the rest of the differentiation program, subsequently leading to PCD does not occur. This observation suggests that not only is BnET involved directly or indirectly in the regulation of lignin but also that at least for metaxylem there is no, and in contrast to what has been reported (Fukada, 2000, Groover and Jones, 1999), molecularly interdependency between the developmental programs of secondary cell wall formation and PCD.

Recently, another transcription factor, the *knotted1*-like homeobox gene BREVIPEDICELLUS (BP), has been described to be involved in the regulation of lignin biosynthesis. Lignification is here considered as an easily traceable differentiation marker. The data are consistent with that the modification of cell walls, and in particular the regulation of lignin deposition and quality, is one of the coordinated processes to regulate and to achieve proper cell differentiation (Mele et al., 2003).

In conclusion, although we cannot completely rule out the possibility that the morphological and molecular changes described for the plants ectopically expressing BnET could reflect the inhibition of more than one hormone pathways, all our data are

consistent with the suggested interpretation that the ectopic expression of BnET has repressive effects on GA promoted responses.

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Figure legends

Fig. 1 *Upper panel:* Sequence of the ET domain

Vf, *Vicia faba*, Bn, *Brassica napus*, At, *Arabidopsis thaliana*, HRT, Hordeum Repressor of Transcription from *Hordeum vulgare* (Raventos et al., 1998). The order of the domain, amino to carboxy terminal, in each individual protein are indicated by a to d. The consensus pattern $C\text{-}X_9\text{-}C\text{-}X_9\text{-}RC\text{-}X_2\text{-}HK$ is marked. OsGRF1 is a GA-induced protein from rice and AtWRC (AC006413) is one of several proteins from *Arabidopsis* containing the WRC domain.

Lower panel: Maldi-TOF mass-spectrometric analysis of a synthetic peptide corresponding to the third ET domain of BnET. Left hand panel displays peptide alone and right hand panel in the presence of equimolar amounts of zinc. RI, relative intensity

Fig. 2 *Upper panel:* Nuclear localization of a BnET-GFP fusion protein. The full length BnET-GFP fusion product was transiently expressed in *Arabidopsis* protoplasts and localized in the nucleus with some lower intensity signals remaining in the cytoplasm (A, B). A fusion product (BnET-GFP ΔNLS) lacking the N-terminal 24 amino acids, including a putative nuclear localization signal consisting of 6 lysine residues, is strictly localized in the cytoplasm (C, D.). Nucleus is indicated with an arrow and an N; the central vacuole is indicated with a V. The scale is 10µm.

Lower panel: Repression of GA induced promoter activity in a transient *Arabidopsis* expression system. The test construct consists of the GASA4 gene promoter fused to the glucuronidase reporter gene and terminated with the nopaline synthase terminator (GASA4P-GUS-NOS). The BnET gene was cloned in the vector pRT103 (Topfer et al., 1988) and expressed under the control of the CaMV35S promoter and terminated with the nopaline synthase terminator (CaMV35SP-BnET-NOS). GA_3 was applied at a concentration of 10µM. Three replicates are shown. 1, GASA4P-GUS-NOS; 2, GASA4P-GUS-NOS + GA_3; 3, GASA4P-GUS-NOS + GA_3 + CaMV35SP-BnET-NOS; 4, GASA4P-GUS-NOS + GA_3 + pRT103 vector.

Fig. 3 Dwarf phenotype of wild type control plants ectopically expressing BnET.

Upper panel: Arabidopsis, Lower panel: tobacco plants. Three individual transgenic plants with various grades of phenotype are shown.

Fig. 4 *Upper panel*: Feed back upregulation of the NtGA20-oxidase gene in the BnET transgenic plants. Note the correlation between BnET expression level and upregulation of the NtGA20-oxidase transcript.

Lower panel: Concentrations of various GA forms in wild type and BnET-571-10 line in pg GA per gram fresh weight.

Fig. 5 *Upper panel:* Reduced and delayed germination of BnET *Arabidopsis* seeds in comparison to wild type control plants. Four independent transgenic lines were used in the study.

Lower panel: Synthesis and accumulation of anthocyanin in *Arabidopsis* plants ectopically expressing BnET. WT, AS and S stands for nontransformed wild type, antisense construct and sense construct, respectively. Anthocyanin concentrations are given as relative units.

Fig. 6 Prevention of hormone induced *in vitro* embryogenesis due to ectopic expression of BnET. Leaf squares were incubated on media with different concentrations auxin and synthetic cytokinin, kinetin. The concentrations of the hormones are given above.

Fig. 7 Anatomy of stems of plants ectopically expressing BnET and wild type tobacco plants. Bright field (a, b, e, f), fluorescence microscopical (c, d) and transmission electron microscopical images (g, h) of stem sections between the 7th and 8th internode. Micrographs of transverse and longitudinal semi thin sections of fixed material demonstrate reduced secondary cell wall formation in BnET transgenic plants (b, f) compared to the wild type (a, e). Autofluorescence of lignified secondary cell walls of freshly cut transverse sections after violet light excitation

in the xylem of wild type (c) and BnET (d). Ultrastructure of meta xylem cells in wild type (g) and BnET (h) plants. C, cambium; Co, Cortex, ER, endoplasmatic reticulum; M, mitochondrion; mX, meta xylem; Ph, phloem; pCW, primary cell wall; pX, proto xylem; sCW, secondary cell wall. Space bars 50 µm in (a-f); 1 µm in (g, h).

Table 1 Phenotypic characterization of ectopic expression of BnET.
tr. = transgenic, WT = wild type,

	WT	BnET tr.	No. of plants
No. of leaves at flowering	14 +/-1	12 +/-2	5 WT / 7 plants of 7 tr.-line
Time of flowering (dag)	30 +/-3	50 +/-5	7 WT / 10 plants of 3 tr.-lines
No. of max. elongated Internodes at flowering	16 +/-1	16 +/-1	5WT/ 10 plants of 3 tr.-lines
Length of 7th internode at flowering (mm)	68 +/-5	52 +/-6	3 WT/ 10 plants of 3 tr.-lines
Length of epidermal cells of the 7th internode at flowering (µm)	103 +/- 5.8	106 +/-14.5	10 WT/ 10 plants of 3 tr.-lines
Length of cortical parenchyma cells in the 7th Internode at flowering (µm)	152 +/- 45.4	157 +/- 44	10 WT/ 10 plants of 3 tr.-lines
Lignin content (mg/g fresh weight)	26+/-5	3+/-2	3 WT/ 3 plants of 3 tr.-lines

Figure 1

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BnETa      VCGVLQEDGTTCLTAPVTGRKRCTEHKGQR
BnETb      ICGVILPEMVRCRSKPVSGRKRCEDHKGMR
BnETc      ICEATTKNGLPCTRSAPNGSKRCWQHKDET
BnETd      VCGVKLHNGSVCEKTPVKGRKRCQEHKGMR

AtET1a     ACGVLLEDGTTCTTTPVKGRKRCTEHKGKR
AtET1b     ICGVILPDMIRCRSKPVSRRKRCEDHKGMR
AtET1c     LCEATTKNGLPCTRSAPEGSKRCWQHKDKT
AtET1d     ICGFKLYNGSVCEKSPVKGRKRCEEHKGMR

VfETa      ICGVILDDGSICSKMPVGKRVRCNEHKGMR
VfEtb      ICGIVLEDGSTCRKEPVKGRKRCHEHKGKR

HRTa       VCGVMLEDGSSCLEDPMEGRKRCELHKGRR
HRTb       LCGVVTDNG-YCKLEPVIGRERCEEHRGIE
HRTc       VCGARASDGSPCKNQPIARRKRCALHKGQR

PhysET     ICGLKLLDGTVCPDPPRPDRKRCEAHKGLR
           ******************************
OsGRF1     RCRRTDGKKWRCSKEAYPDSKYCEKHMHRG
AtWRC      RCRRTDGKKWRCSKEAYPDSKYCEKHMHRG
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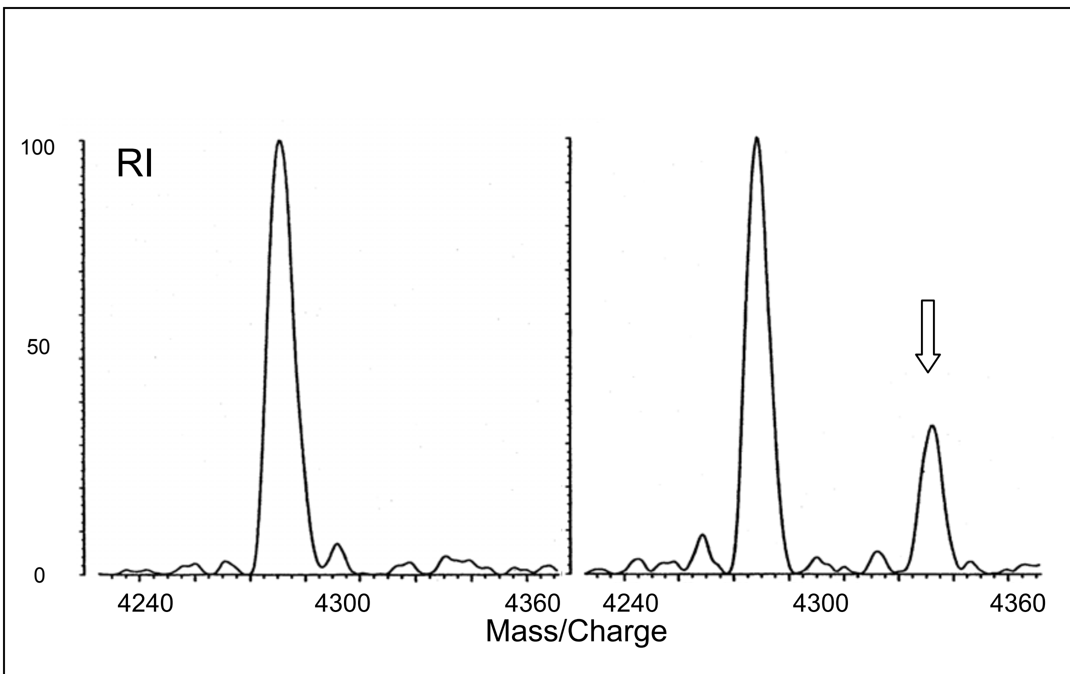
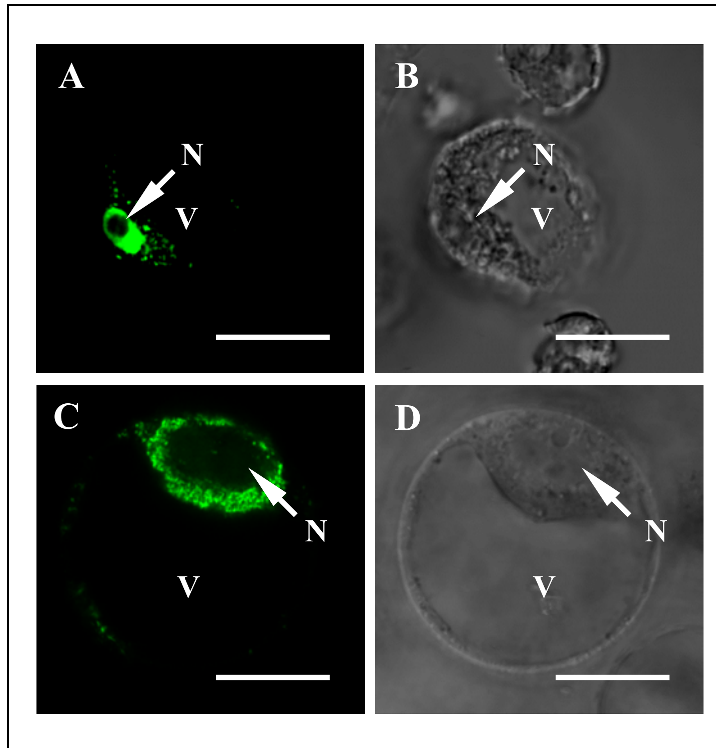
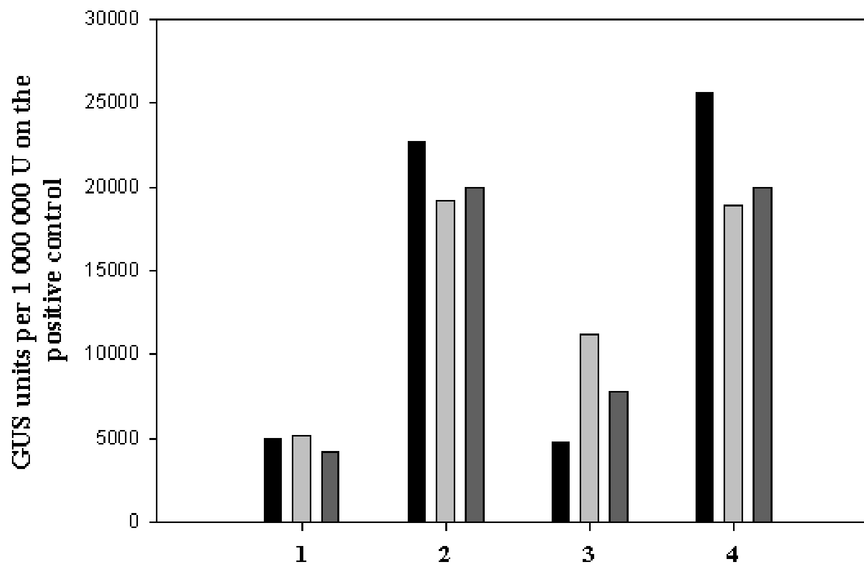


Figure 2





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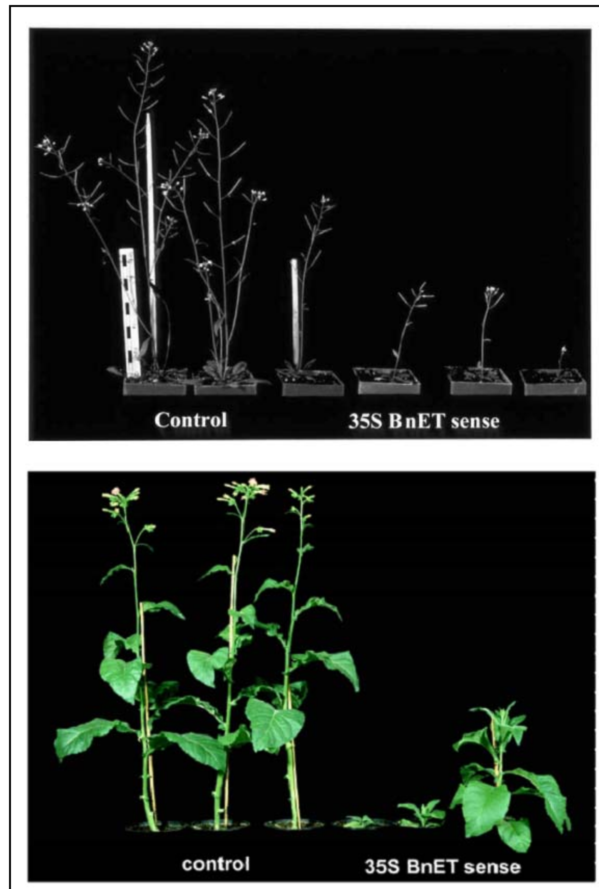

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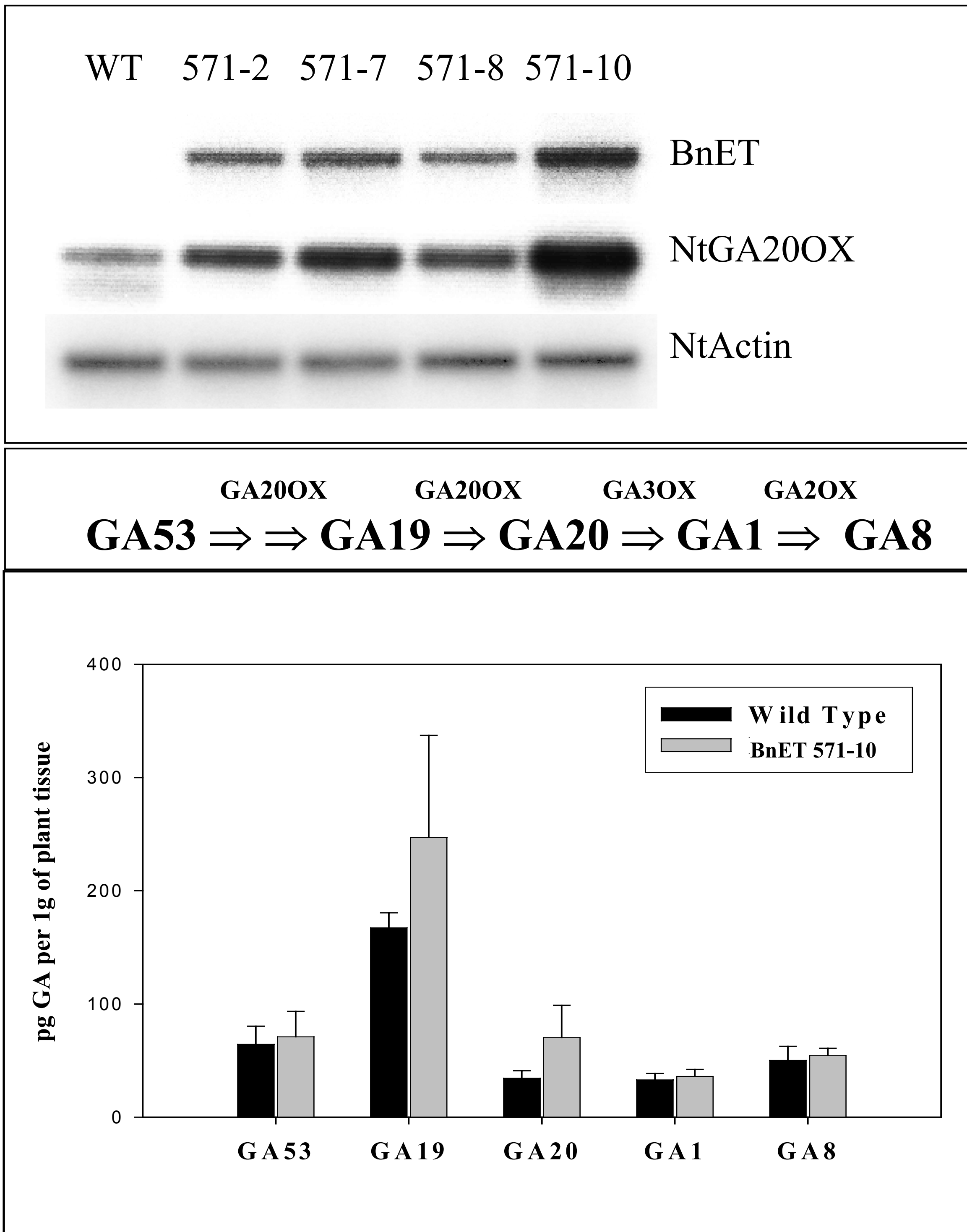


Figure 5

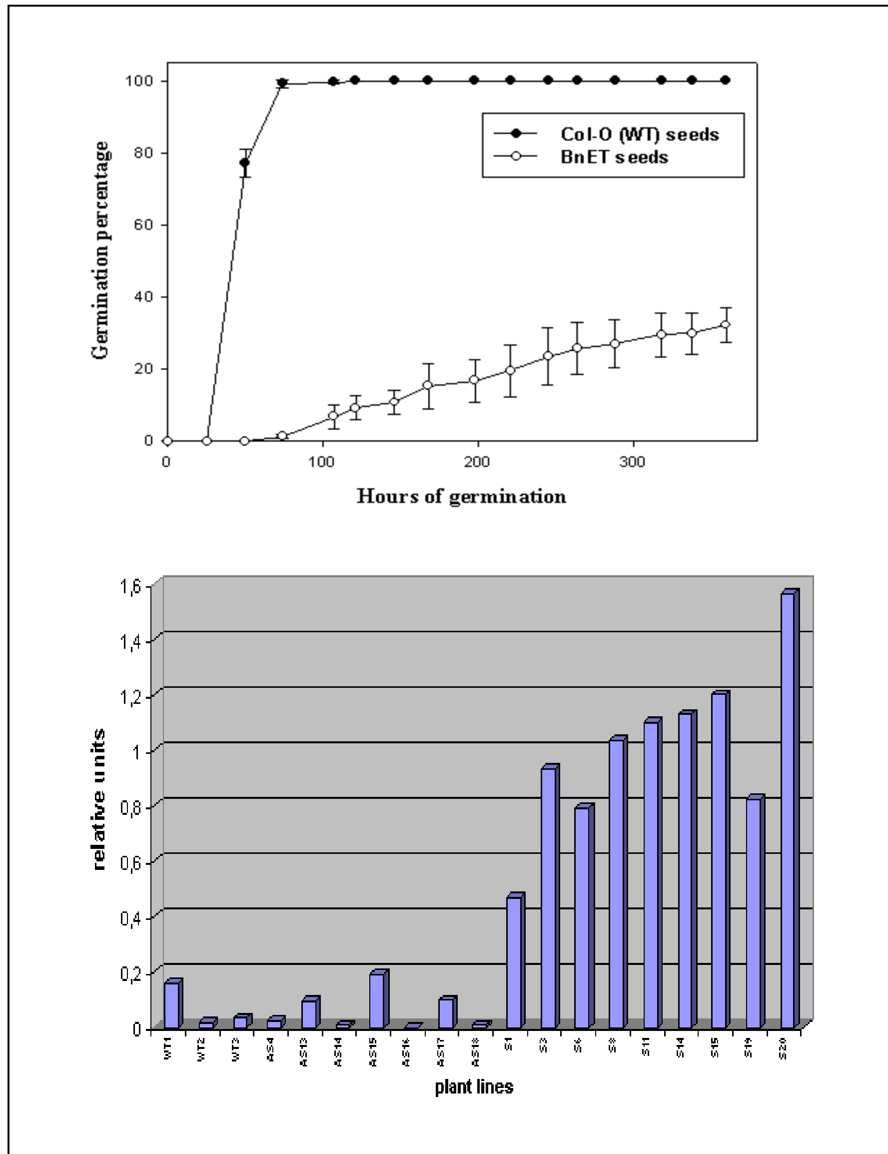


Figure 6

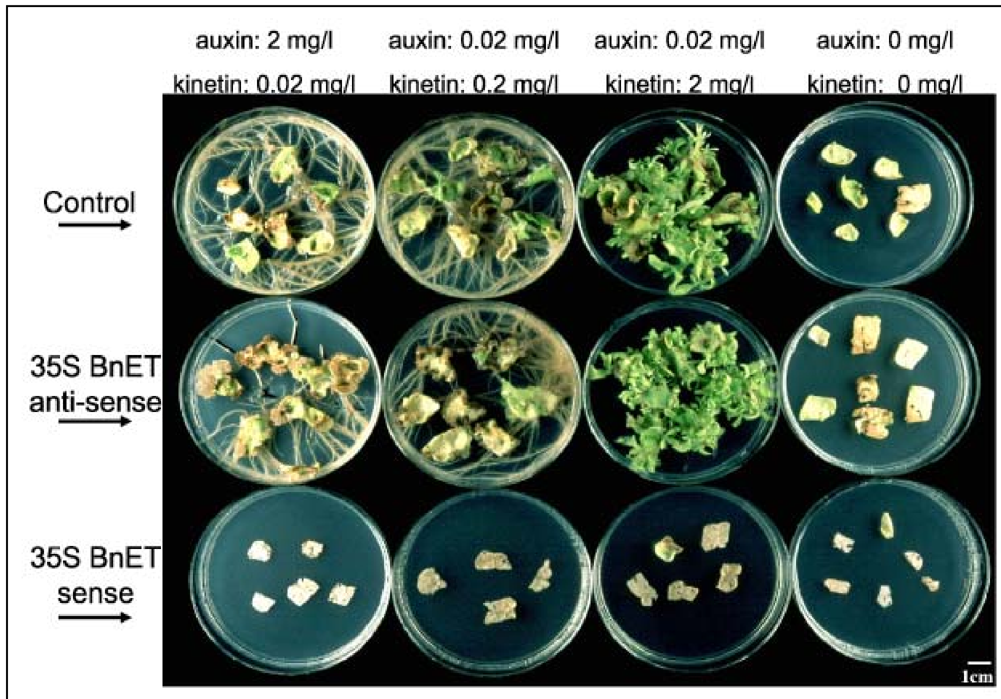
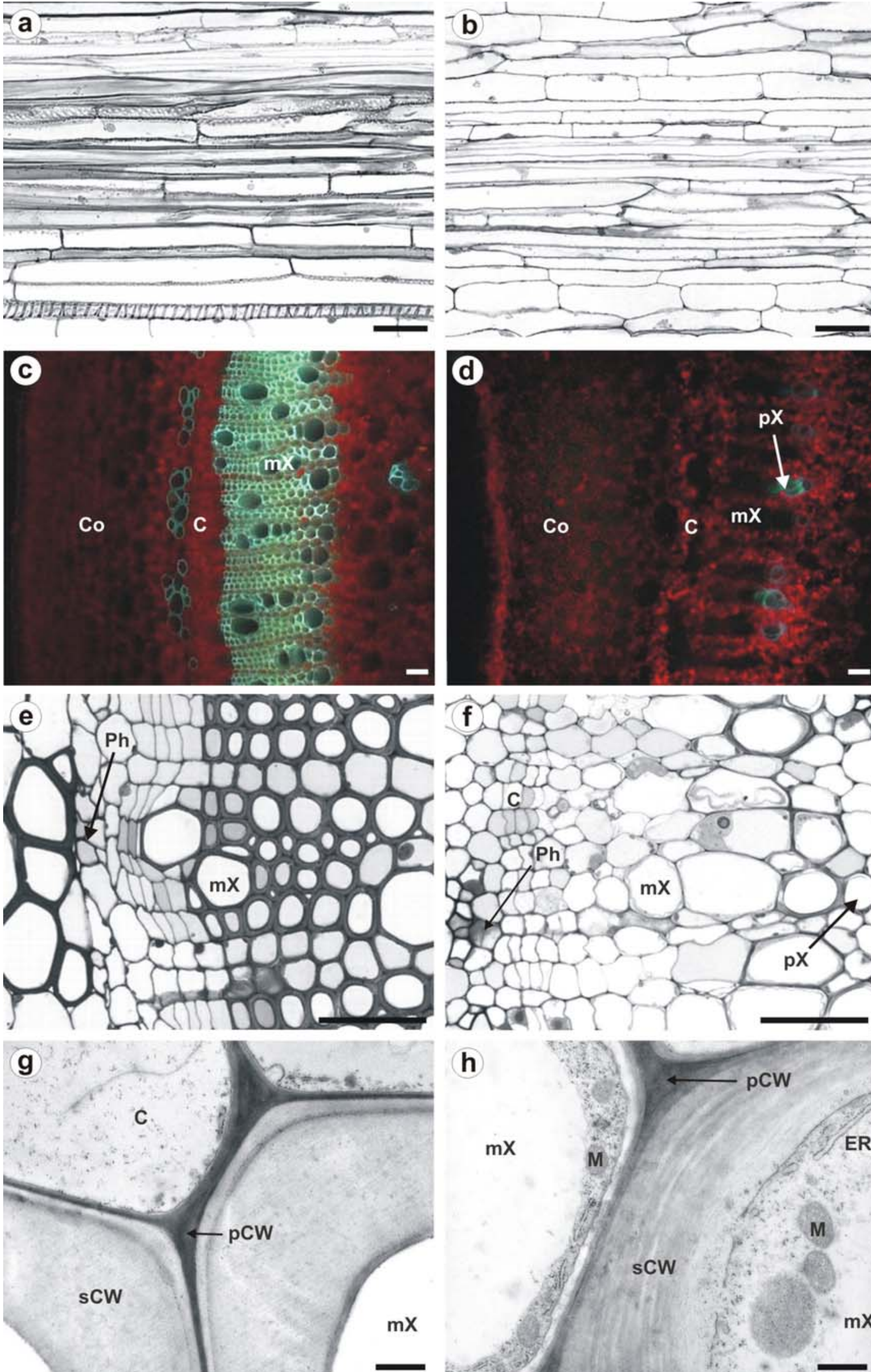


Figure 7 (next page)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