Analysis of transcription factors during late-embryogenesis:

the role of FUS3, LEC1, ABI3 and AtET

Dissertation

zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.)

vorgelegt der

Mathematisch-Naturwissenschaftlich-Technischen Fakultät

Martin-Luther-Universität Halle-Wittenberg

Fachbereich Biologie

von

Wim Reidt

geboren am 16. Dezember 1970 in Den Haag (NL)

Gutachter :

1. Prof. Dr. U. Wobus
2. Prof. Dr. M. Koornneef
3. Prof. Dr. K. Humbeck

Halle (Saale), den 16. Mai 2002

urn:nbn:de:gbv:3-000003844
[http://nbn-resolving.de/urn/resolver.pl?urn=nbn%3Ade%3Agbv%3A3-000003844]

Table of contents

List of abbreviations

ABA	abscisic acid
bp	base pair(s)
bHLH	basic helix loop helix
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
DAF	days after flowering
GA	gibberellic acid
GUS	β-glucuronidase
IAA	indol-acetic acid (auxin)
mRNA	messenger RNA
OD	optical density
WT	wild type
35S::XX	cauliflower mosaic virus promoter controlled overexpression

Erklärung

Ich erkläre hiermit, daß ich mit der vorliegende wissenschaftliche Arbeit erstmals die Erlangung des Doktorgrades bewerbe, die Arbeit selbständig und ohne fremde Hilfe verfaßt, nur die von mir angegebenen Quellen und Hilfsmittel benutzt und die benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Gatersleben, Dezember 2001

Wim Reidt

1. Introduction

Arabidopsis thaliana, a flowering cruciferae related to rapeseed, cauliflower and broccoli, is the model plant for studying plant development. The small size, short life cycle, prodigious seed production, availability of the whole genomic sequence and a large array of described mutants make it a valuable plant to study.

Embryogenesis is a vital process in the life cycle of a plant since it ensures the next generation and, with that, perpetuation of the species. During this time the body plan of the new plant is established, storage products are accumulated in readiness for germination, and the embryo acquires desiccation tolerance and dormancy that enables a prolonged survival in a dry state.

1.1 Embryogenesis in *A. thaliana*

In most plants, including *A. thaliana*, following a succesful pollination, the pollen grain germinates on the surface of the stigma and produces a tube that grows down through the style to reach an ovule inside the ovary. Through the pollen tube the sperm nuclei are discharged into the ovule. One sperm nucleus fuses with the egg to produce a diploid zygote that will develop into the embryo, marking the beginning of embryogenesis. Another sperm nucleus fuses with the two polar nuclei of the embryo sac, forming a triploid endosperm nucleus, which functions as a nutrient source for the developing embryo.

Four developmental processes during embryogenesis can be distinguished: (i) pattern formation, (ii) cell diversification and specification, (iii) growth and morphogenesis and (iv) maturation. The first three processes occur concurrently in the developing embryo and are also know as *early embryogenesis*, while maturation is a distinct process that begins later and is regarded as *late embryogenesis* (Jensen, 1968; Drews *et al*., 1989).

1.2 Early embryogenesis

Early embryogenesis includes the characteristic phases of pattern formation known as globular, heart, torpedo and cotyledon stages (Figure 1.1). After fertilisation, in the so-called preglobular stage, cells follow a consistent pattern of divisions and the embryo differentiates into a nearly spherical structure called the embryo proper (globular stage) and the suspensor. The suspensor is involved in absorption of nutrients from the endosperm, and has no function in the mature seed, while the embryo proper goes on to develop into a new plant (Yueng and Meinke 1993).

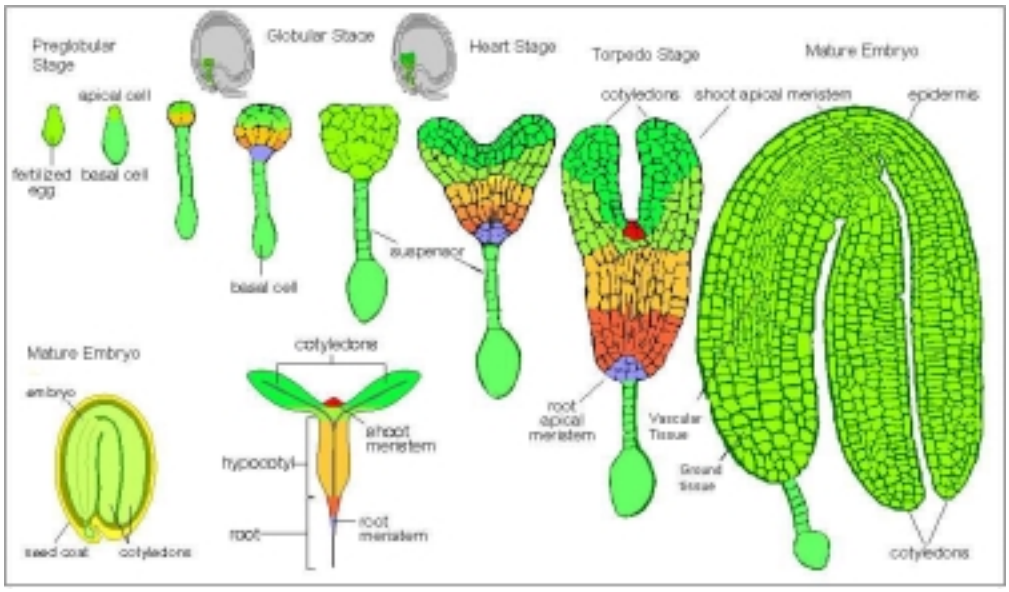


Figure 1.1: *A schematic drawing of the different stages of embryogenesis showing a progression from the preglobular stage through maturation (Wolpert, 1998).*

Through a series of regular cell divisions an outer protoderm layer is produced and two layers of inner cells with distinct developmental fates are established. The apical layer will produce cotyledons and shoot meristem, while the basal layer will produce the hypocotyl and root meristem. As the embryo enters the heart stage, the symmetry becomes bilateral. Several features of the developing embryo become apparent, including the enlarging cotyledons, the root meristem, the provascular tissue and, at the end of the heart stage, the shoot apical meristem. The latter are a highly organised group of quasi-embryonic cells that will give rise to the above ground structures of the plant after germination. During the torpedo stage, the embryo completes its growth and morphogenesis, elongates and enlarges to fill the seed. This stage is characterised by greening, rapid cell division and cotyledon expansion (reviewed by Meinke 1994; Goldberg *et al*., 1988). Early embryogenesis stops at this point. Although the embryo is in principle able to germinate it still has to undergo late embryogenesis.

1.3 Late embryogenesis

Late embryogenesis or seed maturation starts with the termination of cell division in the embryo, roughly at one-third to one-half through seed development. During this time, development is interrupted and the seed acquires dormancy, which is needed for a prolonged survival in a quiescent state. Two processes, embryo maturation and desiccation, characterise this period. Throughout seed maturation, the embryo is prevented from entering the germination pathway and is only able to germinate when maturation is completed. The developmental arrest of the embryo is then reversed upon germination, when proper environmental conditions are provided and the dry seeds imbibe water. Powered by materials stored in their cotyledons and by photosynthesis carried out by these organs, the seedling commences vegetative development from its meristems (reviewed by Harada, 1998).

1.3.1 Embryo maturation

The first process marking the beginning of seed maturation is the accumulation of storage products. The principal macromolecular storage reserve accumulating in maturating *A. thaliana* seeds are lipids, followed by proteins and carbohydrates (Bewley, 1995).

Since the highly reduced carbon in lipids generates much more energy upon oxidation as compared to proteins or carbohydrates, lipids are far more commonly found as a storage product in seeds rather than in vegetative tissues (Browse, 1998).

The major constituent of lipids in seeds are the triacylglycerols (TAG). TAGs accumulate in intracellular, spherical organelles called lipid bodies (oil bodies, oleosomes or spherosomes) which range in size from 0.2 - 2 μm in diameter (Herman, 1995). Lipid bodies consist of a core of TAGs surrounded by a monolayer of phospholipids in which are embedded specialised proteins called oleosins (Huang, 1994). The oleosin proteins, comprise the main component of oil bodies associated proteins. Oleosins are thought to prevent oil bodies from collapsing during desiccation rather than to be involved in oil synthesis itself. The main reason for this is because the expression of oleosin genes is delayed with respect to oil seed accumulation (Kater *et al*., 1991). When the time comes for the oil bodies to serve as a energy source for the seedling, oleosins are believed to interact with lipases initiating the breakdown of the triacylglycerols (Huang, 1996). Several studies have demonstrated that the seed-specific and developmental dependent expression of these genes is mainly regulated at the transcriptional level, and includes the action of abscisic acid (ABA, Hatropoulos *et al*., 1990; Keddi *et al*., 1994).

The most abundant seed proteins are designated as seed storage proteins. Generally they are packaged into protein bodies, which are modified vacuoles or extensions of the endoplamic reticulum (Tykarska, 1987). They accumulate either in the endosperm or in the cotyledons, although some deposition also occurs in the embryonic axis (Mansfield *et al*., 1992; Olsen *et al*., 1992), and mainly serve as carbon or nitrogen source during germination (Higgins, 1984; Shotwell *et al*., 1989). Seed storage protein genes have been extensively studied (e.g. Chlan and Dure; 1983; Shewry *et al*., 1995; Müntz, 1998). Their expression has been shown to be both temporally and spatially regulated mostly at the transcriptional level. Their transcripts accumulate and decay in specific places and at precise intervals during seed development. Furthermore, their expression seems to be embryo-specific because only negligible levels are found in non-embryogenic seed tissues or in vegetative plant organs (Goldberg *et al*., 1983; Okamuro *et al*., 1986).

1.3.2 Desiccation and late embryogenesis abundant proteins

In late stages of seed maturation, the water content in seeds drops dramatically during desiccation and the seeds become dormant.

This decrease in water content presumably results from the severing of the vascular connection between the seed and the fruit and evaporative drying (Harada, 1998). Late embryogenesis abundant (LEA) proteins accumulate before this desiccation period. Their mRNA appears in mature seeds as desiccation commences, becomes the most abundant mRNA species in dry seeds, and disappears shortly after imbibition (Baker *et al*., 1988; Galau *et al*., 1991). LEA proteins are widely distributed among monocot and dicot species and many different forms have been isolated, cloned and sequenced (Espelund *et al*., 1992; Dure, 1997). Predictions on the sequence and structure of these proteins suggest that they are rich in hydrophilic amino acid residues, possess domains with amphiphilic-helix structures and are boiling stable. Furthermore, they commonly have repeated amino acid sequence motifs (Baker *et al*., 1988; Dure, 1993). It has been demonstrated that many promoters of the LEA proteins are transcriptionally regulated by ABA, cellular water loss and osmotic potential (Baker *et al*., 1988; Mundy *et al*., 1988; Williamson *et al*., 1988; Bostock *et al*., 1992), and that the LEA proteins accumulate as well in vegetative tissues in response to environmental stimuli that have a dehydrative component or that are temporally associated with dehydration, such as drought, low temperature and salinity (Skriver and Mundy, 1990). The homology among different LEA proteins, the presence of highly conserved domains, specific developmental expression patterns and their inducibility in vegetative tissues after dehydration strongly imply a fundamental role of these proteins in desiccation tolerance.
Further evidences imply that these proteins play a structural role as desiccation protectants, i.e. their high concentration in the cell and biased amino acid compositions suggests that they do not function as enzymes. Moreover because these proteins are highly hydrophilic, it is unlikely that they occur in specific cellular structures (Baker *et al*., 1988). The randomly coiled moieties of some LEA proteins are more consistent with a role in binding water. Total desiccation is probably lethal and therefore such proteins could help maintain the minimum cellular water content (Ingram *et al*., 1988; Lane, 1991).

1.4 The RY element in seed-specific promoters

High tissue specificity and temporally regulated expression of the maturation specific proteins has attracted interest into their seed-specific regulation. Since it has been demonstrated that the expression of many of seed-specific genes is transcriptionally regulated, the attention was also focused on the promoter regions of seed-specific genes.

Several studies identified a conserved nucleotide sequence, characterised by the alternating purine and pyrimidine sequence CATGCATG, which is present in many seed-specific promoters (Dickinson *et al*., 1988; Bäumlein *et al*., 1992; Chamberland *et al*., 1992; Lelievre *et al*., 1992; Fujiwara and Beachy, 1994; Bobb *et al*., 1997). This motif was found in the seed-specific gene promoters of both dicots and monocots including the *Vicia faba* Legumin and USP (Unknown Seed Protein) gene promoter as well as the regulatory region of the *Brassica napus* napin genes (Bäumlein *et al*., 1986; 1991; Fiedler *et al*., 1993; Stalberg *et al*., 1993; Ellerström *et al*., 1996). Indications that the RY-element has an important function during seed maturation come from experiments in which the activity of an intact legumin promoter was compared to the activity of a legumin promoter containing a mutated RY element. These experiments revealed that deletion of the RY element abolishes most of the seed-specific promoter activity and results in a low level expression in leaves (Bäumlein *et al*., 1986; 1992). Similarly, experiments using the napin promoter demonstrated that deletion of its two RY-motifs drastically reduces promoter activity (Stalberg *et al*., 1993; Ellerström *et al*., 1996). These data and the analysis of several other seed-specific promoters (Morton *et al*., 1995) demonstrate the importance of the RY-motif for high level expression of several seed-specific genes as well as its potential role to function as a negative element repressing expression in non-seed tissues.

1.5 Regulators of maturation

In addition to the RY-motif other factors controlling seed maturation have been identified in *Arabidopsis*. They include the genes **Aba-Insensitive 3** (*ABI3)*, **FUSCA3** (*FUS3)* and the **Leafy cotyledon** genes (*LEC1* and *LEC2)*, as well as the plant hormone **abscisic acid** (ABA). Mutations in any of these genes or a reduced amount of ABA disrupts normal induction of late embryogenesis abundant genes and maturation, resulting in defects on storage protein synthesis, chlorophyll breakdown, anthocyanins biosynthesis and causing precocious germination and desiccation intolerance. Analysis of double mutants demonstrated that the genes *ABI3*, *FUS3* and *LEC1* function synergistically and are key members of a network controlling mid- and late-embryogenesis (Parcy *et al*., 1997).

1.5.1 Abscisic acid, ABA

ABA, a plant hormone, is a key factor involved in the regulation of seed development. It promotes seed maturation and dormancy, and inhibits seed germination (Crouch, 1987; Black 1991; Giraudat *et al*., 1994).

ABA levels are low in early seed development, become maximal during the first phase of seed maturation and decline again in mature seeds (Figure 1.2; Karssen *et al*., 1983; Hughes *et al*., 1989; Black, 1991).

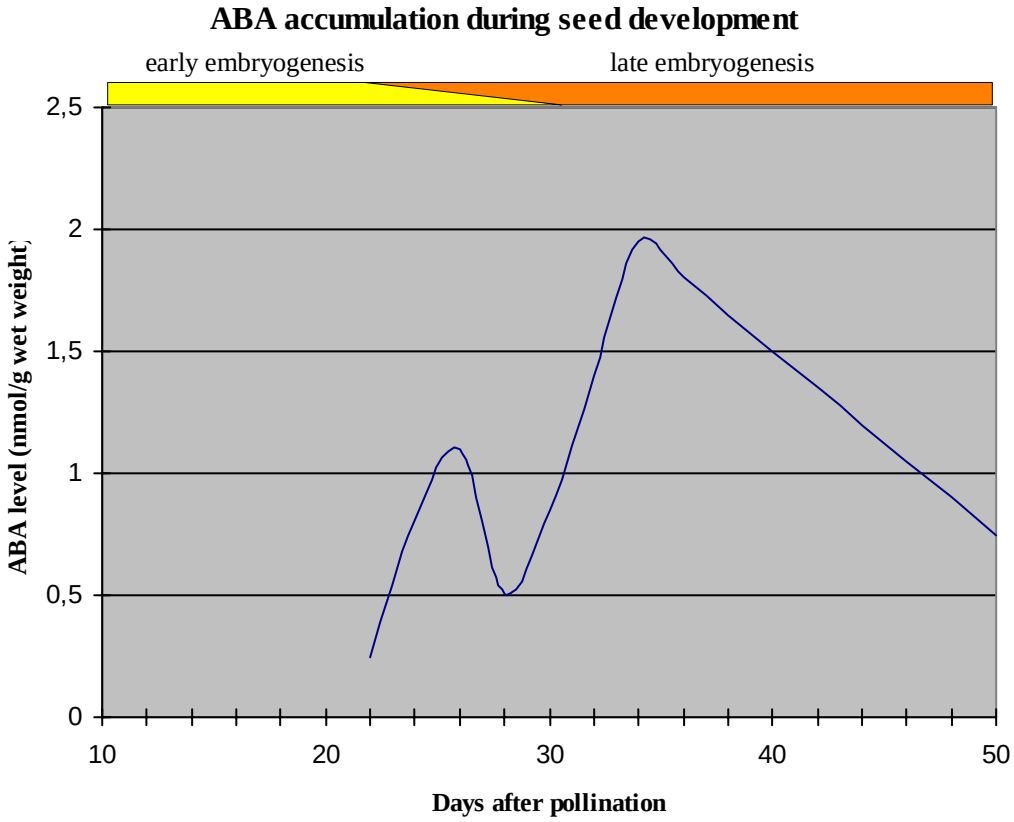


Figure 1.2: *ABA accumulation pattern during seed development in cotton. (Adapted from Galau et al., 1987).*

Several pieces of evidence indicate that ABA has a role in regulating seed protein gene expression and in preventing premature germination.

- developing wheat embryos removed from the seed and cultured on basal medium switch immediately to the germination programm. However when ABA is included in the medium the embryos still undergo a varied set of responses and appear to maintain many aspects of their seed maturation program (Quatrano, 1986).

- studies on mutants defective in ABA synthesis from maize, *Arabidopsis* and tomato showed that their embryos accumulated only some seed proteins, germinated precociously on the plant and were intolerant to desiccation. These observations suggested that ABA is needed for some, but not all, aspect of seed maturation (Koornneef *et al*., 1982; Wang *et al*., 1984; Neill *et al*., 1986; Kriz *et al*., 1990; Giraudat *et al*., 1994).
- seed-specific overexpression of anti-ABA single chain antibodies in tobacco lead to seeds containing less storage proteins and oil bodies. Seeds also germinated precociously if removed from the capsules during development and were unable to germinate after drying. This seed-specific immunomodulation approach resulted in a switch from the seed maturation programme to the germination programme (Phillips *et al*., 1997).

Although the function of ABA during seed maturation is intensively studied the exact mechanism in which it regulates seed maturation is unclear. It is clearly, however, not the only factor involved in either initiating or maintaining seed maturation.

1.5.2 *ABA Insensitive 3 gene, ABI3*

Maize and *Arabidopsis*, mutants that display a reduced sensitivity to ABA have been isolated. In *Arabidopsis*, five loci (*ABI1*, *ABI2*, *ABI3*, *ABI4* and *ABI5*) have been found, while in maize only one, the *Viviparous1* (*VP1)* locus has so far been identified (Robertson, 1952; Koornneef *et al*., 1984; Finkelstein, 1994). These mutants were selected based on the ability of seeds to germinate in the presence of inhibitory concentrations of ABA (Koornneef *et al*., 1984; Neill *et al*., 1986). *ABI3*, *ABI4* and *ABI5* seem to function primarily during seed development, since their mutants are altered in various aspects of seed development and germination, reflecting the decreased responsiveness to ABA. The *ABI1* and *ABI2* genes, on the other hand, have a minor effect on seed development and affect mainly vegetative processes (Koornneef *et al*., 1984; Finkelstein and Sommerville, 1990).

The maize *viviparous1* mutant displayed, as the *Arabidopsis* mutants, a reduced sensitivity to ABA. Kernels containing severe *vp1* mutant alleles were desiccation intolerant and germinated precociously. Furthermore anthocyanin biosynthesis and aleurone development were affected resulting in colourless seed (Robertson, 1955; Dooner, 1985). The *Arabidopis abi*- as well as the maize *vp1*-mutants were not affected in ABA synthesis, transport or metabolism (Robichaud *et al*., 1987).

Interestingly, after the isolation of the genes *VP1* and *ABI3* by McCarty *et al*. (1991) and Giraudat *et al*. (1992), respectively, both gene structure and amino acid sequences suggested that *VP1* and *ABI3* are orthologous genes. Their predicted protein sequences display three regions of high similarity, which were designated B1, B2 and B3 (Figure 1.3).

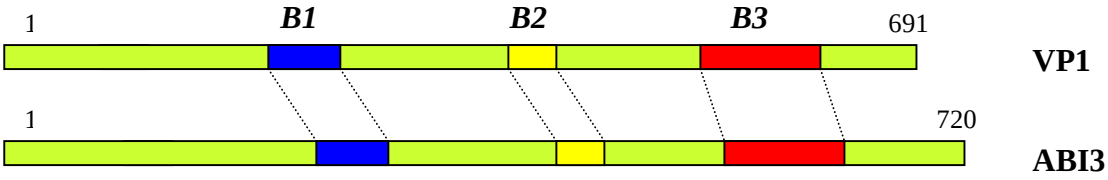
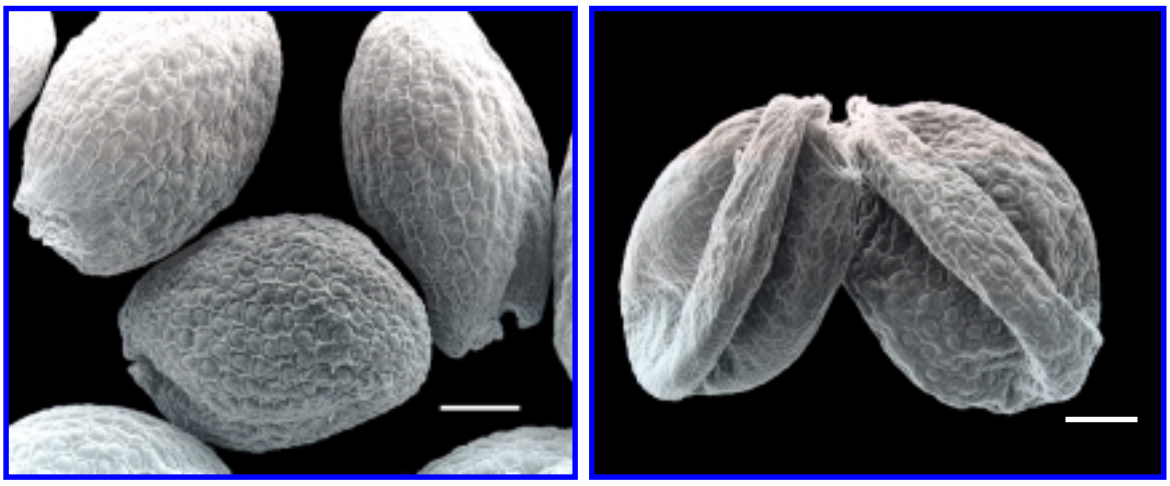


Figure 1.3: *Schematic representation of the VP1 and ABI3 predicted proteins from maize and Arabidopsis, respectively.*
The VP1 and ABI3 proteins are orthologous proteins involved in ABA perception. Both proteins contain discrete regions with high similarity (blue, yellow and red boxes) proteins designated B1, B2 and B3. The total number of amino acid residues is indicated.

A few years later, similar genes were isolated from rice, *Osvp1*, and French bean, *PvAlf*. Their predicted protein sequence displayed the same homologous regions, B1, B2 and B3 as found in the VP1 and ABI3 proteins (Hattori *et al*., 1994; Bobb *et al*., 1995). This indicated that these conserved regions are important for the function of the Vp1/ABI3 class of proteins.
Following the cloning of these genes, the functions of their proteins was studied. *Vp1* and *Osvp1* overexpression in protoplasts caused an increase of more than 100 fold in the activity of a LEA gene promoter and also in its responsiveness to ABA (McCarty *et al*., 1991). Furthermore, the N-terminal acidic regions of VP1 and PvAlf have been shown to function as transcriptional activation domains when fused to a DNA binding domain, while a truncated version of the VP1 protein containing the B3 domain had the capability to bind DNA (Bobb *et al*., 1995; Suzuki *et al*., 1997). Finally, ABA treatment of leaves overexpressing the *ABI3* gene caused the accumulation of several seed storage proteins. Together these observations indicate that the proteins belonging to the VP1/ABI3 family are transcriptional regulators with key roles during seed maturation.

1.5.3 *FUSCA3 gene, FUS3*

The *A. thaliana fus3* mutant displays a very similar, but still distinct, phenotype compared to the *abi3* mutant. The *fus3* phenotype includes desiccation intolerance and the production of excessive amounts of anthocyanin (Müller, 1963). Several endogenous, as well as, transgenic genes encoding seed-storage proteins, show strongly reduced expression in the *fus3* mutant (Bäumlein *et al*., 1994). Finally the development of trichomes on its cotyledons and the activation of transcription factors involved in meristem formation, which are considered to be vegetative traits, indicate the skipping of the seed maturation program and the switch to immediate germination (Bäumlein *et al*., 1994; Kirik *et al*., 1998).



wild type seed *fus3* mutant seed

Figure 1.4: *Scanning electron microscope picture of mature wild type (left) and fus3 mutant (right)* Arabidopsis *seeds.*
The A.thaliana *mutant* fus3 *seeds display desiccation intolerance, lack the accumulation of several seed storage proteins and accumulate anthocyanin. Bars represents 100µm.*

The *FUS3* gene encodes a predicted protein (312 amino acids residues) with sequence similarity to the ABI3 and VP1 gene products (Luerssen *et al*., 1998). The homology of FUS3 however is restricted to a stretch of 100 amino acids residues, which corresponds to the B3-domain of the VP1/ABI3-protein family (Figure 1.5). Since a truncated VP1-protein containing only the B3 domain binds DNA (Suzuki *et al*., 1997), it is likely that FUS3 has a similar capability.

Furthermore the FUS3 protein lacks the N-terminal part present in ABI3 and VP1, which has been suggested to provide a key interface for ABA signalling pathways (Ezcurra *et al*. 2000). Therefore FUS3 might act in an ABA independent matter. As the sequencing of the *Arabidopsis* genome progressed, several other proteins, among them the auxin response factor ARF (Ulmasov *et al*., 1997) and also the *LEC2* gene (Stone *et al*., 2001) turned out to contain the B3 domain. These proteins might represent a subgroup of the VP1/ABI3 protein family.
Kölle (1997), Kirik *et al*. (1998) and Nambara *et al*. (2000) demonstrated that the FUS3 protein not only up-regulates genes during seed maturation but can also down-regulate genes, probably to suppress vegetative growth and precocious germination. As with the ABI3 protein, all these results point to a central role of FUS3 protein as a transcription factor during seed maturation.

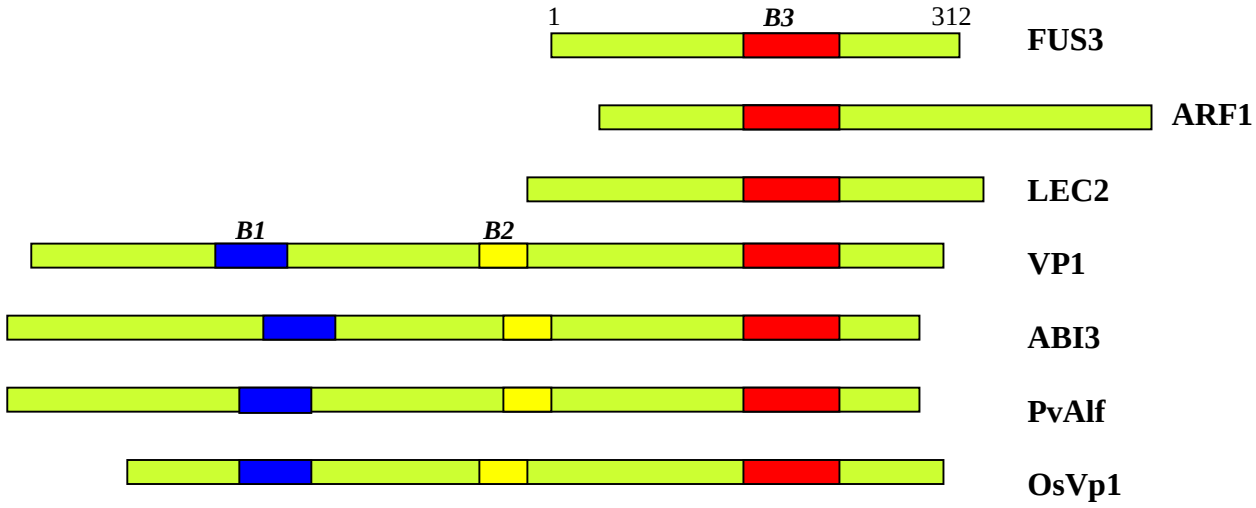


Figure 1.5: *Architecture of some B3 domain containing proteins.*
The FUS3 protein is characterised by a conserved stretch of approximately 100 amino-acids. This stretch was originally found in the ABI3/VP1-protein family and denominated as B3 domain. Several other plant proteins contain the B3 domain, among them the auxine respone factor ARF and the LEC2 protein.

1.5.4 *Leafy Cotyledon* genes, *LEC1* and *LEC2*

Another class of loci known to be important for seed maturation are the *Leafy Cotyledon* genes. Similar to the *abi3* and *fus3* mutants, mutant *lec* alleles cause defects in expressing some but not all maturation specific genes. Moreover the embryo becomes desiccation intolerant resulting in embryo lethality (Meinke, 1992; Meinke *et al*., 1994b). The LEC1 protein is, like FUS3 and ABI3, also involved in inhibiting premature germination (Meinke, 1992; Parcy *et al*., 1997).

Only little information is known about the *LEC2* gene but, as mentioned before, preliminary results indicate that the protein belongs to the subgroup of B3-domain family of transcription factors (Stone *et al*., 2001). The *LEC1* gene, on the other hand, has been characterised (Lotan *et al*., 1998). Based upon sequence homology with other proteins, the LEC1 predicted protein was divided into three regions A, B and C.

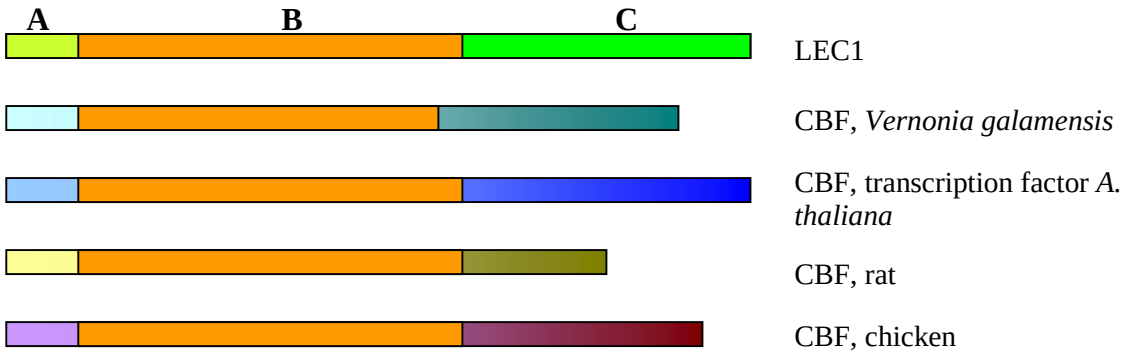


Figure 1.6: *Schematic representation of the LEC1 protein and other proteins containing the conserved CCAAT binding domain.*
The LEC1 protein can be divided into three regions (A, B and C). The B region is similar to the CCAAT box-binding factor (CBF) from several other organisms. Domain A and C contain no significant similarity to other characterised proteins

The B-region shares a high similarity with the HAP3 subunit of the CCAAT-box binding factor from several other organisms and partially with the transcription initiation factor IID (Lotan *et al*., 1998) whereas the A and C domain had no significant similarity (Figure 1.6).

The gene has shown to be expressed solely during embryo development with a peak on mRNA level during early embryogenesis. No expression could be detected in vegetative tissues (Lotan *et al*., 1998).

Overexpression of the *LEC1* gene in vegetative tissues leads to the co-expression of endogenous embryo-specific genes and to the formation of embryo-like structures in these tissues (Lotan *et al*., 1998). These results suggest that a specific set of genes involved in embryo development is activated by LEC1. Together with the phenotype of the *lec1* mutants, these obervations point to a central regulatory role of the *LEC1* gene during embryogenesis.

1.6 Aim of the project

Genetic studies with the help of *lec1*, *fus3* and *abi3* mutants demonstrated the importance of these genes during seed maturation. The LEC1, FUS3 and ABI3 gene products seem to control the acquisition of desiccation tolerance, dormancy, seed storage protein accumulation and inhibition of the anthocyanin biosynthesis (Bäumlein *et al*., 1994; Parcy *et al*, 1997; Kölle, 1998; Nambara *et al*., 2000; Raz *et al*., 2001). The exact way in which these proteins interact and their role in response to ABA is so far unknown. The present study was focused on the molecular characterisation of FUS3, LEC1, ABI3 in an attempt to clarify the interaction of these transcription factors and their role in late-embryogenesis. In addition, it is important to note that other genes, such as *ROM2*, *DET1*, *RDO1* and *RDO2*, are also known to have important regulatory functions during late-embryogenesis (Chern *et al*. 1996; Leon-Kloosterziel *et al*., 1996; Rohde *et al*., 2000; reviewed by Rock, 2000).

The *FUS3* gene, cloned by Luerssen *et al*., (1998) was the main focus of this study. Additionally, the seed-specific RY promoter element, which was also shown to be involved in seed maturation, was tested for its interaction with all three genes. Further screening of seed-specific promoter elements important for transcriptional regulation might enable the identification of additional factors involved in seed maturation and would contribute to a better understanding of the complex process of seed maturation and the function of the *FUS3* gene in this process.

2. Material and methods

2.1 Plant material

Two different plants *Arabidopsis thaliana* and *Nicotiana tabacum* were used as tools to study the function or regulation of certain kind of genes or promoters. The light regime included for all plants 16 hours light and 8 hours dark during the whole life cycle unless otherwise stated.

2.1.1 *Arabidopsis thaliana*

The *A. thaliana* ecotype 'Columbia' was used as wild type. Seeds of *A. thaliana* were sterilised by rinsing them in 70% ethanol followed by a rinse for 10 minutes in 50% sodium hypochloride and 0.05% Tween20. Before plating the seeds on **g**ermination **m**edium (GM) they were washed four times with sterilised water. Germinating seedlings were grown for two weeks on GM medium before transferring them to soil.

2.1.2 *Nicotiana tabacum*

Tobacco plants (*Nicotiana tabacum* cv 'Petit Havana SR1') were grown in green houses with additional light.

Seeds were surface sterilized for 5 min in 70% ethanol followed by an 30-45 minutes incubation in sterile water before transferring them to LS medium for germination. Germinating seedlings were grown for four weeks on LS medium before transferring them to soil. Leaf discs were sterilised in an identical way as described for the seeds.

2.2 Plant transformation

2.2.1 Construction of vectors used for plant transformation

Standard cloning, construction and sequencing techniques were performed according to Ausubel *et al.* (1996). To construct a *CaMV* promoter/FUS3 sense transcriptional fusion, the full-length FUS3 cDNA was amplified by PCR with primers containing additional *Asp*718 sites. The fragment was cloned into the *pCR-Script* vector (Stratagene) and sequenced. Following this step the error-free FUS3 cDNA was directly recloned into the binary vector *pBinAR* (Höfgens and Willmitzer, 1990).
BnET which was isolated from a seed specific cDNA library of *Brassica napus*, as described by Ellerström (1998), was amplified by PCR with primers containing additional *Nco*I sites. Following cloning steps were identical as described for the FUS3 *35S CaMV* construct.

2.2.2 Transformation of *Agrobacterium tumefaciens*

A. tumefaciens transformation was performed using a modified version of the freeze-thaw method (Hötgen and Willmitzer, 1988). Competent *Agrobacteria* 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 antibiotic of interest, they were diluted in 1 ml of YEB medium and shaken for 2 hours at 28°C.

2.2.3 Vacuum infiltration of *Arabidopsis thaliana*

Transformation of *Arabidopsis* was done according to the protocol of Bechthold *et al*. (1993). Plants of *A. thaliana* 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 a vector with a gene and a promoter of interest, 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 (100 μg/L). Infloresences 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 24h they were uncovered and set upright. Seeds were harvested from the dry siliques, sterilised and plated onto selection plates containing the suitable antibiotic. After two weeks viable plants were transferred to soil, grown up and their seeds were collected. Transgenic plants were analysed by PCR, Southern or Northern hybridisation.

2.2.4 Stable transformation of *Nicotiana tabacum*

For the generation of stably transformed tobacco plants, the recombinant binary vector *pGA472* was conjugated to the *Agrobacterium tumefaciens* strain pGV2260 and used for leaf disc transformation of *N. tabacum* cv. ‘Havana’ as described previously (Bäumlein *et al*., 1991). The presence of the transgene was confirmed by either Southern hybridization or PCR.

2.3 The transient assay

2.3.1 Vectors used for transient assay

The plasmid *pcabi3-4F* (Giraudat *et al*., 1992) containing the ABI3 cDNA was digested with *Hpa*I and *Eco*RV. The fragment containing the ABI3 cDNA was cloned between the *35S CaMV* promoter and the *NOS* termination signal from *pBI221* (Jefferson, 1987) in *pUC19* (Ezcurra *et al*., 2000). The FUS3 cDNA cloned in the plasmid *pHL1* (Luerssen *et al*., 1998) was subcloned as an *Asp*718 fragment behind the *35S CaMV* promoter of the plasmid *pRT103*. The LEC1 cDNA (obtained by a kind gift of J. Harada, Davis, USA) cloned in the plasmid *pBlueScript* was cut out of this vector with *Eco*R1/*Xho*1, blunted by *Klenow* polymerase and inserted as a blunt fragment behind the *35S CaMV* promoter of the plasmid *pRT103*.

2.3.2 Cloning and mutation of promoters containing the CATGCATG element

Both a 2.7 kb promoter fragment of the *legumin B4* gene (Bäumlein *et al*., 1991b) and a 0.76 kb promoter fragment of the *USP* gene (Bäumlein *et al*., 1991a) were blunt ended and cloned into the blunt *Sal*1 site in front of the *β-gluronidase* (GUS) reporter gene of the plasmid *pGUS1* resulting in the plasmids *Leg pGUS* and *USP pGUS*. Construction of the *napA pGUS* plasmid consisting of a PCR derived fragment (-309 to +45, position relative to CAP site) of the *napA* promoter (Josefsson *et al*., 1987) cloned in plasmid *A3* containing the *GUS* reporter gene and the *NOS* termination signal subcloned in *pUC19*, has been described (Stalberg *et al*.,1993). A 2.4 kb fragment of the *ent-kaurene synthetase A-like* promoter (-2562 to +1, position relative to CAP site; ATCHRIV5_183) and a 0.5 kb fragment of the *bHLH* promoter (-309 to +45, position relative to CAP site; AC005617_18) were amplified by PCR from genomic *Arabidopsis* DNA. The resulting fragments were blunt-ended and cloned into the vector *pT7Blue2* (Novagen). Next the cloned fragment was cut out by *Pst*I/*Hind*III and cloned into the *Pst*I/*Hind*III site of the vector *pGUS1*.

The chimaeric promoter/reporter constructs were either used directly for transient expression assays or subcloned into the binary vector *pGA472* for stable tobacco transformation.
Mutant promoter constructs of the *LegB4*, *napA* and *USP* promoters were generated by Thomas Wohlfahrt and Mats Ellerström as described in Reidt *et al*. (2000).

2.3.3 Isolation of protoplast from suspension cultures and transient expression

A suspension culture of *Nicotiana plumbaginifolia* was used for protoplast isolation. During an overnight incubation in a 1% cellulase and 0.5% macerozym solution, cell wall removal took place. Following cell wall digestion protoplasts were centrifuged and washed two times in W5 medium. Next the protoplasts were concentrated in Mg Mannitol to a density of $3.3x10^5$ – $3.3x10^6$ protoplasts/ml. To transform the resulting protoplasts, badges of 330 µl were heatshocked (45°C) before plasmid DNA (5 µg of each plasmid) and carrier DNA (160 µg) were added. To finally bring the DNA into the protoplasts PEG 6000 was added before transferring them to small petri-dishes. After a 18h incubation time in the dark protoplasts were harvested and the GUS activity was determined by fluorimetric assay (Jefferson, 1987) using the GUS-Light™ Kit (Tropix, Bedford, USA). 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.

2.4 *Escheria coli* strains

For general cloning techniques the *E. coli* stem DH5α was used (Sambrook *et al*., 1989) while PCR products were cloned using the *E. coli* host stem BL21(DE3). Finally the HMS(DE3)103 strain was used for the expression of proteins in bacteria.

2.5 Bacterial expression of fusion proteins

To produce FUS3 protein, the FUS3 cDNA was ligated into the *Asp*718 site of the *pET17b* vector (see appendix, Novagen). The resulting reading frame includes an T7 tag as an 11 amino acid leader sequence. The fusion protein was expressed in *E. coli* HMS(DE3)103 cells after induction with 0.5M isopropyl-1-thio-β-D-galactoside (IPTG) for 10 minutes at 37°C. Proteins were extracted after sonication in 1/10 volume washing buffer (4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.27 mM KCl, 0.137 M NaCl, 0.1% Tween-20, 0.002% sodium azide, pH pH 7.3). Following centrifugation, fusion proteins were isolated by T7-tag antibody agarose chromatography (Novagen). After buffer exchange (10mM Tris, 0.05mM EDTA) and concentration with polyethylene glycol, the protein concentration was determined by SDS-PAGE and Western blotting (Reidt *et al*., 2000).

2.6 DNA-protein interaction

The DNA binding reaction mix in 1x binding buffer (25mM Hepes-KOH, pH 7.4, 4 mM KCl, 5mM $MgCl_2$, 1mM EDTA, 7% glycerol, 0.05% Triton X-100) included 10 fmol of radioactively labelled oligonucleotide (end labelled with ^{32}P γ-ATP and polynucleotide kinase), 1 µg poly (dI-dC), 0-, 50-, 100- or 200-fold competitor DNA and 10 ng of the purified protein. The mixture was incubated at room temperature for 15 minutes and separated by PAGE on a 5% non-denaturating gel in 0.5xTBE at 4°C. After electrophoresis, the gel was dried and autoradiographed (Reidt *et al*., 2000).

2.7 RNA isolation and Northern blotting

Total RNA isolation was performed using the total RNA isolation agent from Biomol or Promega. RNA was separated in 1.2% agarose gels containing 15% formaldehyde, and blotted overnight onto Hybond-N^+nylon membranes (Amersham).

Various fragments obtained by enzymatic digestions, which excluded the polyA tail from the cDNAs, were used as probes after random prime labeling (Ready Prime Labeling Kit, Pharmacia) with [α-^{32}P]dCTP. Hybridizations were performed at 65°C, and washed at high stringency according to Church and Gilbert (1984). Signals on filters were quantified using a Fuji-BAS phospho-imager (Fuji Photo Film C., Tokyo, Japan).

2.8 Southern hybridisation

For Southern hybridizations, 10 µg DNA were digested and separated in a 1% agarose gel, which was blotted overnight onto a Hybond-N$^+$ nylon membrane. Complete cDNAs were used as probes after labelling with [α-^{32}P]dCTP. Hybridizations were performed at 65°C, and washed twice with 2xSSC/0.1% SDS, twice with 1xSSC/0.1% SDS, and once with 0.5xSSC/0.1% SDS at 65°C for 15 minutes each.

2.9 PAGE and Western blotting

Proteins were separated on a 12.5% (w/v) SDS-polyacrylamide gel electrophoresis. Samples were visualised either by Coomassie blue staining or by Western blotting. After electrophoresis proteins were transferred in blotting buffer (0.25M Tris, 0,192M glycine) to a nitrocellulose filter (Schleier&Schuell). The nitrocellulose filter was blocked by an 1 hour incubation in 3% BSA (dissolved in TBST) to prevent non-specific binding of the antibody, followed by 1 hour incubation in an 1:10.000 dilution of the conjugated antibody. After three washes with TBST the protein/antibody complex was visualised by a chemiluminescent reaction (NBT/BCIP).

2.10 Two hybrid system

2.10.1 Reporter gene constructs used in the two hybrid system

The yeast two hybrid assay is based on the fact that many eukaryotic transacting transcriptional regulators are composed of physically separable, functionally independent domains. These regulators often contain a DNA binding domain (BD) that binds to a specific promoter sequence and/or an activation domain (AD) that directs the RNA polymerase II complex to transcribe the gene downstream of the DNA binding site. Both domains are required to activate a gene. If physically separated by recombinant DNA technology and expressed in the same host cell, the DNA-BD and AD do not directly interact with each other and thus can not activate the responsive genes. However if the DNA-BD and the AD can be brought into close proximity in the promoter region, the transcriptional function will be activated. The DNA binding domain is provided by the prokaryotic LexA protein. The AD is an 88-residue acidic *E. coli* peptide that activates transcription in yeast. Two different cloning vectors are used to generate fusions of these domains to genes encoding proteins that potentially interact with each other, and the recombinant hybrid proteins are coexpressed in yeast (*Saccharomyces cerevisiae* EGY 48). An interaction between a target protein (fused to the DNA-BD) and a bait protein (fused to the AD) creates a novel transcriptional activator with binding affinity for the LexA operators. This factor than activates both the *LacZ* reporter gene having upstream 8 LexA operators and the *LEU2* nutritional reporter gene. When *LacZ* transcription is activated yeast cells produce β-galactosidase, whose activity can be monitered using a number of different assays.

The cDNA of all three transcription factors ABI3, FUS3 and LEC1 was amplified by PCR. In all cases sequencing was performed to select an error-free cDNA fragment. Primers pairs of FUS3 and LEC1 contained an *Eco*RI site at the 5’ end and a *Xho*I site at the 3’ end, the primer pairs of ABI3 contained an *Mfe*I and a *Sal*I site at the 5’ and 3’ end respectively. Primer pairs of ABI3 and LEC1 included the ATG start codon and the corresponding stop codon. The FUS3 forward primer contained the ATG start codon but the reverse primer was designed either just after the B3 domain (nucleotide position 598-610) to remove the activation domain, or at the end of the cDNA including its stop codon. Next the amplified DNA was cloned into the *Eco*R1/*Xho*1 site of the yeast two hybrid vectors *pB42AD*/*pLexA*.

2.10.2 Yeast transformation

Yeast cells were made competent following the Li^+ protocol developed by Ito *et al*.(1983), and modified by Schiestl and Gietz (1989), Hill *et al*. (1991) and Gietz *et al*. (1992).

2.10.3 Detecting two-hybrid interactions

Liquid cultures were used to assay β-galactosidase activity for the identification of two-hybrid interactions. Two different substrates were used to detect β-galactosidase activity, ONPG and CPRG, following the protocol of Clontech as described in the Yeast Protocol Handbook.

2.11 DNA analysis and software

DNA and protein sequence data were processed using the programmes PC/GENE version 6.85 (IntelliGenetics, Belgium) and the software blastn, blastp and blastx (basic local alignment search tool) available on the NCBI and Arabidopsis internet homepages (*www.ncbi.nlm.nih.gov*; *www.arabidopsis.org*). With the help of the database for plant cis-acting regulatory DNA elements (*www.dna.affrc.go.jp/htdocs/PLACE*) promoters were analysed.

2.12 Primers

The following primer pairs were used either for the amplification of promoters or genes from genomic DNA or plasmids:

bHLH-promoter primers:
forward - 5'- aaa ccg tgc att gaa cag gc - 3'
reverse - 5'- aca gag cgt tga ctc ttc ctt cc - 3'

AtET5 3-exon primers :
forward - 5´- tgg gat cta aga gag aag ctga ggc - 3’
reverse - 5’ - aag taa caa agt gga ggc atg gc - 3’

FUS3 primers for amplification until the B3 domain:
forward - 5’- cgg aat tcg tac cca tga tgg ttg atg - 3’
reverse - 5’ - tgc tcg agt gct ttt ctt gct tgt ata acg - 3’

ent-kaurene synthese A-like promoter primers:
forward - 5’- aga tga tga aac tat ttg gtg ag - 3’
reverse - 5’ - ccc tta tcc cca tta cct gg - 3’

2.13 Microscopy

2.13.1 Light microscopy

For histological examination plant samples were fixed in 1% (v/v) glutaraldehyde and 4% (v/v) formaldehyde dissolved in 50 mM sodium phospate buffer, pH 7.2 for 4 to 12 h at 4°C. After fixation, samples were treated with a series of ethanol and monomer solution A treatments for dehydration and infiltration and then embedded in solution A and B and sectioned as described by Chen *et al*. (1995). After rinsing with 95% (v/v) ethanol, the samples were transferred to 70% (v/v) ethanol, then moved to a glass slide and mounted in a clearing solution of 8 parts of chloral hydrate, 1 part glycerol and 2 parts of water (v/v) as described in Berleth *et al*. (1993) with some minor modifications. After 1 h of treatment at room temperature, the samples were observed and photographed with a microscope.

2.13.2 Electron microscopy

Samples for the EM were fixed overnight in 2.5% glutaraldehyde in sodiumcacodylate pH 7.2 at room temperature, post fixed in 0.1 mg/ml osmium tetroxide in the same buffer for 2 h at RT, dehydrated in a series of ethanol as described above and embedded in Spurr resin.

The sections were cut with a diamond knife, stained in uranyl acetate and lead citrate and viewed on an electron microscope.

3. Results

3.1 LEC1, FUS3 and ABI3 dependent activation of *napin* and *USP* promoters

The accumulation of large amounts of seed-specific storage proteins is one of the processes characterising the stage of late embryogenesis. Genetic studies demonstrated that the cooperative interaction of the transcription factors LEC1, FUS3 and ABI3 plays an important role in controlling seed-specific storage protein expression. Mutations, both single and double, in any of these three loci result in a strongly reduced expression of both seed-specific endogenous *Arabidopsis* genes as well as in the seed-specific heterologous transgenes (Bäumlein *et al*., 1994; Kölle, 1998; Parcy *et al*, 1998; Nambara *et al*., 2000).
Interestingly, along another line of investigation, a strong reduction in the activity of seed-specific gene promoters was also found as a consequence of the destruction of a defined promoter element.

The RY motif, which consists of the alternating succession of purine and pyrimidine nucleotides CATGCA(TG), represents a well-characterised, functionally important *cis*-motif found in many seed-specific gene promoters (Bäumlein *et al*., 1992; Dickinson *et al*., 1992; Lelievre *et al*., 1992; Bobb *et al*., 1997). The observation that both the destruction of the seed-specific RY motif as well as mutations in the genes of *LEC1*, *FUS3* and/or *ABI3* affects the level of mRNA accumulation of seed-storage protein genes suggested that the RY motif might be a direct target of these three transcription factors.

To assess both the potential interaction between these transcription factors and the RY motif, as well as the interaction between the transcription factors themselves, a transient *in vitro* expression system based on *Nicotiana plumbaginifolia* protoplasts was established. Using this system two different seed-specific gene promoters, i.e. the *napin* promoter from *Brassica napus* (Ellerström *et al*., 1996) and the *USP* promoter from *Vicia faba* (Bäumlein *et al*., 1991) were tested.

3.1.1 Transient expression of the *napin* gene promoter

To characterise the interaction of the transcription factors LEC1, FUS3 and ABI3 among themselves and with the *napin* promoter, these transcription factors were constructed behind a *35S* promoter, and the *napin* promoter was cloned in front of the *GUS*-reporter gene. Next, the *napin* promoter/*GUS* construct was co-transformed into *N. plumbaginifolia* protoplasts with either one of the transcription factors or with different combinations of them. GUS activities driven by the napin promoter were measured for each different combinations of constructs.This GUS activity was found to be negligible when the *napin* promoter construct was transformed alone (data not shown). Transient co-expression of the *35S* promoter-controlled *LEC1* gene led to a minor induction of the *napin* promoter (Figure 3.1). The co-expression of either FUS3 or ABI3 resulted in a much higher level of promoter activity, with an induction of up to 30 times (Figure 3.1). Interestingly, the co-expression of LEC1 or FUS3 with ABI3 resulted in a synergistic effect between these factors. In contrast, no synergism was observed between LEC1 and FUS3. Overexpression of all three transcription factors together resulted in the highest promoter activity of 90- up to 115-fold induction, displaying a strong synergism between all three transcription factors (Figure 3.1).

To define the function of the RY-element in these experiments, the same combinations of *LEC1*, *FUS3* and *ABI3* constructs were co-expressed with the *napin* promoter containing mutated RY-elements. Mutation of two of the three RY-elements at positions –78 and –50 from CATGCA to GTCGAC and GCTAGC, respectively, led to a drastic reduction of the promoter activity in all cases (Figure 3.1). This indicates that activation of the napin promoter by FUS3 and ABI3 in concert with LEC1 depends on two intact RY motifs.

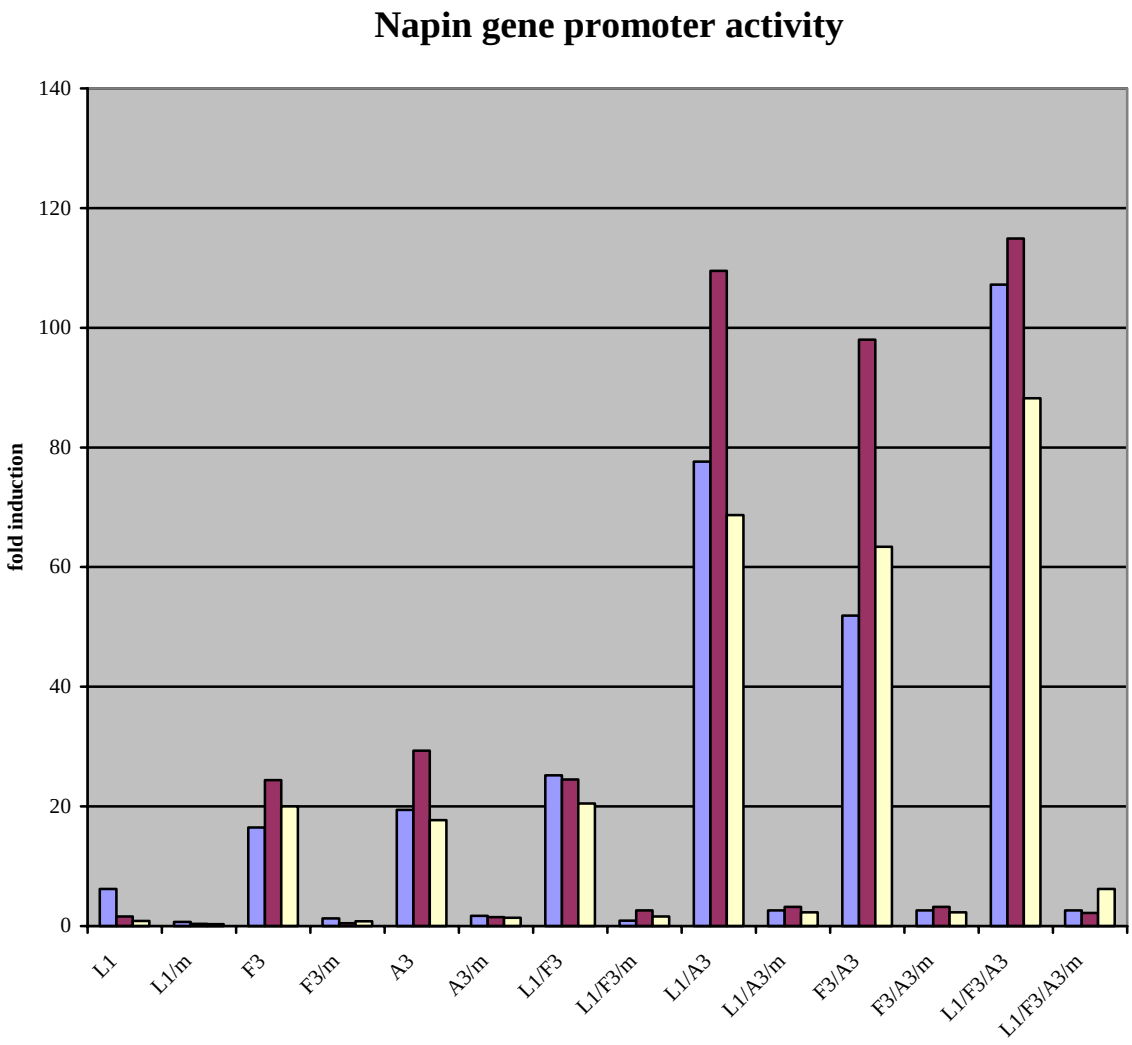


Figure 3.1: *Transient expression assay of the* napin *gene promoter.*
The napin *gene promoter-GUS construct was co-transformed into* N. plumbaginifolia *protoplasts with either one or with a combination of the transcription factors LEC1 (L1), FUS3 (F3) and ABI3 (A3) that were overexpressed under control of the* 35S *promoter. The effect of these transcription factors on the activation of the promoter is given as n-fold induction in comparison to the GUS activity driven by the promoter construct alone, i.e without transcription factors. The different coloured columns represent three independent experiments. The effect of mutations in a conserved nucleotide sequence, the RY element, of the promoter (m) on the transcriptional activation was measured.*

3.1.2 Transient expression of the *USP* gene promoter

Using the same protoplast system and experimental conditions, corresponding results were obtained with another seed-specific promoter, the *USP* promoter of *V. faba*.

This legume promoter was only slightly induced by the LEC1 gene product and stimulated 5- to 10-fold due to the co-expression of the FUS3 gene product (Figure 3.2). The *35S* controlled co-expression of the *ABI3* gene led to a higher induction of the *USP* promoter of up to 100-fold (Figure 3.2). Again both the LEC1 and ABI3 gene products and the FUS3 and ABI3 gene products displayed a strong synergistic effect on the *USP* promoter with an induction of 550- and 280-fold respectively. Overexpression of LEC1, FUS3 and ABI3 induced the *USP* promoter up to 700 fold, displaying the strong synergy between all three proteins (Figure 3.2). Also for the *USP* promoter the increase in the activity of the promoter by the three regulators could be almost abolished by the mutation of one of the two RY-motifs, ***CATGCATG***, into the sequence ***GGTTCCAA***, although the reduction was not as severe as with the *napin* promoter (Figure 3.2).

3.1.3 Transient expression of the legumin promoter

For unknown reasons, the protoplast expression system was not suitable for the analysis of the *legumin B4* gene promoter, although different culture conditions were tested. The promoter had a low basic activity and could not be stimulated by either the FUS3 or the ABI3 gene product or both. The *LEC1* cDNA was not included in the experiments with the *legumin B4* promoter.

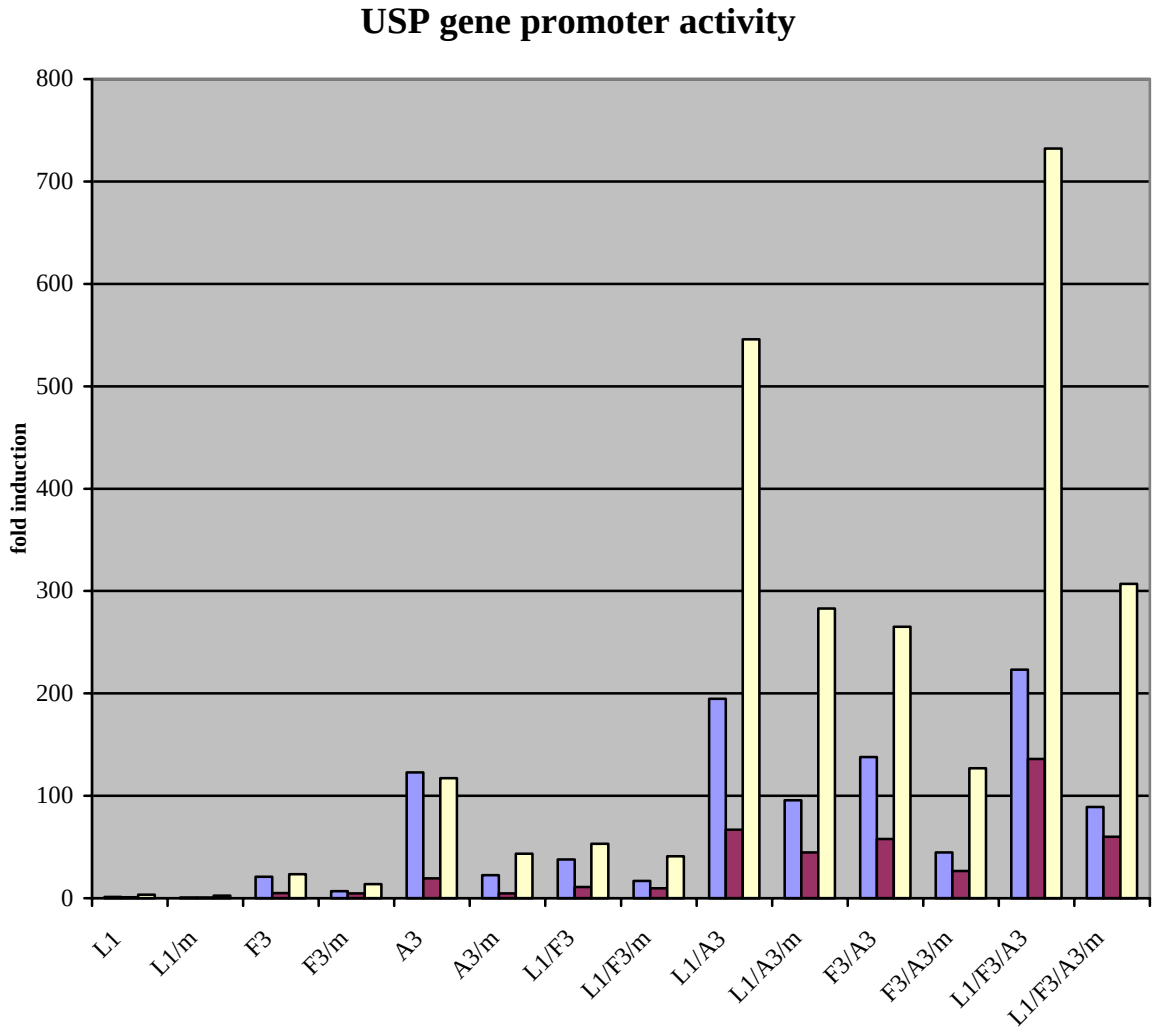


Figure 3.2*: Transient expression assay of the* USP *gene promoter.*
A USP *gene promoter-GUS construct was transformed into* N. plumbaginifolia *protoplasts alone or with different combinations of the transcription factors LEC1 (L1), FUS3 (F3) and ABI3 (A3) that were overexpressed under control of a* 35S *promoter. The effect of these transcription factors on the activation of the promoter is given as n-fold induction in comparison to the GUS activity of promoter construct alone. The different coloured columns represent three independent experiments. The effect of mutations in a conserved nucleotide sequence, the RY element, of the promoter (m) on the transcriptional activation was measured.*

3.1.4 The effect of ABA on the induction of the *napin* promoter by FUS3 or ABI3

Studies with Vp1, ABI3 and the *Phaseolus* orthologue PvALF in transient expression assays demonstrated that these transcription factors act in synergy with ABA (McCarty *et al*., 1991;

Bobb *et al*., 1995; Carson *et al*., 1997; Rojas *et al*., 1999). Since FUS3 contains a partial homology to all these transcription factors similar experiments were set up to know if a synergy could be observed between ABA and FUS3. In line with the previous experiments FUS3 and ABI3 both stimulated the *napin* promoter (Figure 3.3). However when ABA was added to the transformed protoplast the induction of the napin promoter was stimulated in the case of ABI3 as expected, but instead of a similar induction in the case of FUS3, a reduction occurred (Figure 3.3). It seems, therefore, that ABA has not the same cooperative effect on FUS3 as it has on ABI3.

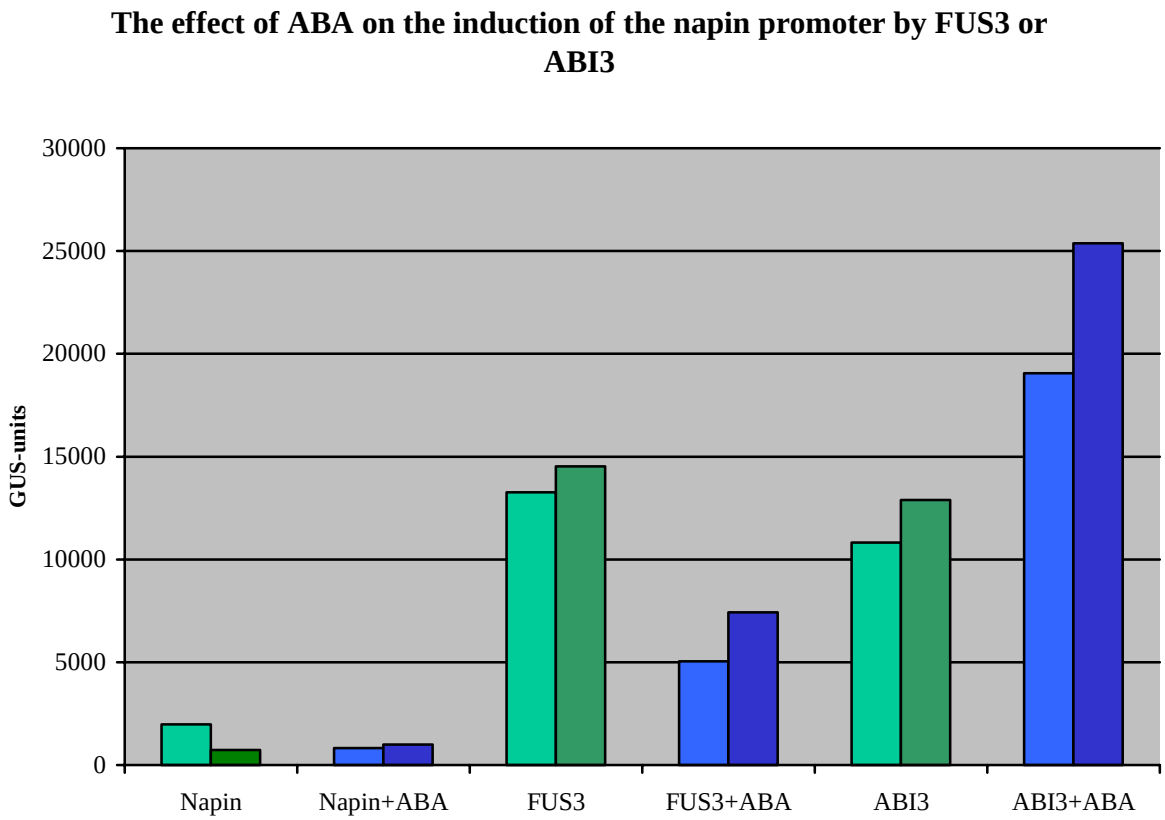


Figure 3.3*: Transient expression assay of the* napin *gene promoter in response to the transcription factors FUS3 and ABI3 and the effect of ABA.*

A napin *gene promoter GUS construct was transformed into* N. plumbaginifolia *protoplasts with either the transcription factor FUS3 or ABI3 under control of a* 35S *promoter in the presence or absence of ABA (10 µm). The FUS3 dependent activation is reduced in the presence of ABA while the ABI3 dependent activation is enhanced. The different colums represent different experiments.*

3.2 The two hybrid system

Results from both genetic studies and the transient assays suggested a protein-protein interaction between the combinations of the gene products of LEC1 and ABI3, FUS3 and ABI3 and LEC1, FUS3 and ABI3 all together. A well established method to investigate such interactions is the yeast two-hybrid system.

For the two-hybrid system in this study the FUS3 protein was used as a bait protein to identify the potential interacting partners, the ABI3, the LEC1 protein and FUS3 for homodimerisation. The presence of an activation domain at the C-terminal end of the FUS3 protein resulted in the autonomous activation of the *lacZ* reporter gene. Therefore only a truncated version of the FUS3 protein could be used. Using a truncated FUS3 protein (excluding all (122) amino acids after the B3 domain) an interaction with the ABI3 protein could not be detected (Figure 3.4). However an interaction between LEC1 and FUS3 was found, although both gene products did not display any synergism in the transient assay (Figure 3.4). Dimerisation of the FUS3 protein could also be confirmed using the two hybrid system as shown in Figure 3.4.

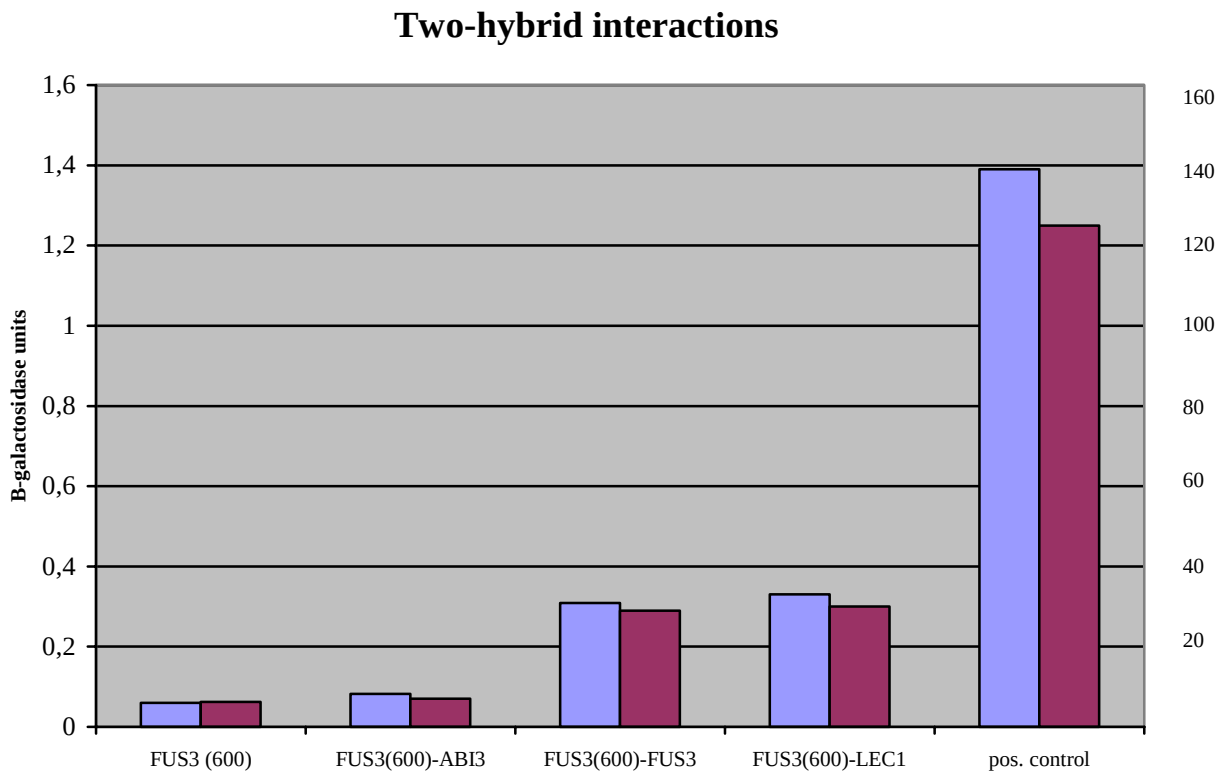


Figure 3.4*: Protein-protein interactions between FUS3 and LEC1, ABI3 and FUS3.*
A truncated version of FUS3 (FUS3(600)), in which the activation domain was removed, was used as bait in a two hybrid system with LEC1 (L1), ABI3(A3) and FUS3(F3) itself as prey. An interaction between two proteins activates the transcription of a lacZ reporter gene which results in the production of β-galactosidase. Different coloured bars represent two experiments. Completely right is shown a positive control of two interacting proteins from murine p53 and SV40 large T-antigen, with the β-galactosidase units on the right side.

3.3 Molecular interaction between the RY cis-motif and the FUS3 transcription factor

For a more definite characterisation of the interaction of the FUS3 transcription factor with the RY-cis motif, the FUS3 gene product was synthesised in *E. coli* as a fusion protein with the T7 tag. This fusion protein was used for band shift experiments with synthetic radiolabelled oligonucleotides, corresponding to the legumin box containing an intact RY-motif. As shown in Figure 3.5 the FUS3 fusion protein interacted with a legumin box oligonucleotide containing the RY-element as core (lane 1).

This interaction could be efficiently competed with the specific fragment (lanes 2-4) but not with the non-specific fragment (lanes 5-6). The protein-DNA complex could be supershifted with an antibody directed against the T7-tag of the recombinant FUS3 protein. The binding of an *E. coli* synthesised, cotyledon specific leucine zipper factor (Wohlfarth, 1996) to an oligonucleotide containing a G-box is shown as positive control.

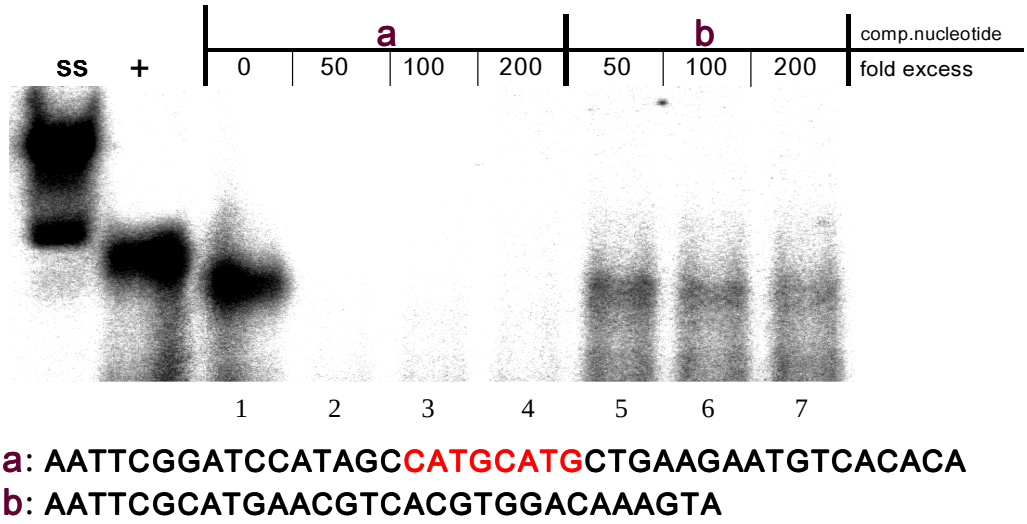


Figure 3.5*: Molecular interaction between the FUS3 protein and the RY-core motif of the legumin box.*

The FUS3 gene product synthesised as a fusion protein in E. coli *is binding to the wild-type legumin box oligonucleotide (lane 1). The sequence of the oligonucleotide is given as sequence 'a' in the lower panel. The binding specificity is demonstrated through competition by the addition of 50-, 100- and 200-fold excesses of the same non-labeled oligonucleotide (lanes 2, 3, 4) or the same amounts of a non-specific competitor (sequence 'b' in the lower panel). The FUS3/DNA complex can be super-shifted by an antibody directed against the T7 tag of the recombinant FUS3 protein (lane SS). An* E. coli *expressed leucine zipper protein binding to an oligonucleotide containing a G-box was included as a positive control (lane +).*

To determine the interacting nucleotide motif more precisely a scanning mutation analysis was performed. Purified FUS3 protein was incubated with the labelled legumin-box oligonucleotide containing the intact RY motif 'a' (Figure 3.6). The binding specificity was demonstrated by the addition of 50-, 100-, and 200-fold molar excesses of non-labelled competitor oligonucleotides 'a'-'g' (Figure 3.6). All nucleotides with the intact RY motif, CATGCATG, were efficient competitors at least when applied in 100- and 200-molar excess.

The destruction of the RY-motif (change from CATGCATG to ATCCTGTG) in oligonucleotides ‘f’ resulted in the loss of the competitor activity even at 100- and 200-fold excesses. The reduced competition by oligonucleotide ‘g’ may be explained by the overlap of this mutation with the two last nucleotides of the CATGCATG core motif. Interestingly one single base pair substitution in the RY-motif, oligonucleotide ‘b’, (change from CATGCATG to CATGGATG) also resulted into the loss of competitor activity while the substitution of two base pairs in oligonucleotide ‘c’ (change from CATGCATG to CATACCTG) did not affect the competitor activity. The partial loss of competitor activity of oligonucleotide ‘d’ indicates that sequences upstream of the core motif contribute to the interaction (see also Reidt *et al.*, 2000).

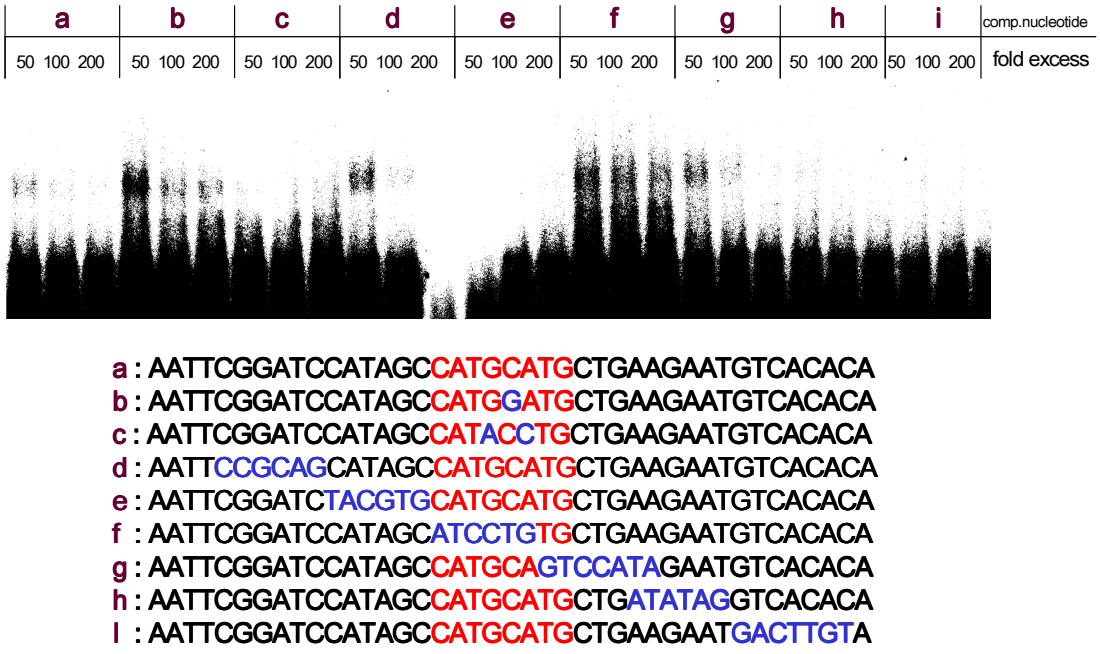


Figure 3.6*: Binding of the FUS3 protein to an oligonucleotide containing the RY-motif.*
To determine if the RY-motif, CATGCATG, was the direct target of the FUS3 gene product a scanning mutation analysis was performed. The FUS3 protein was incubated with a labelled oligonucleotide corresponding to a fragment of the legumin box (sequence ‘a’ in the lower panel). Next this binding complex was competed with 50-, 100- and 200-fold excesses of the same nucleotide but containing regions with an altered sequence (sequence ‘b-i’ in the lower panel, modified nucleotides are printed red). Competition (disappearance of the band-shift) in binding demonstrates that binding of the FUS3 protein to these ‘mutants’ occurs.

3.4 FUS3-regulated genes with RY containing promoters

The rather pleiotrophic phenotype of the *fus3* mutant with defects in storage, desiccation and dormancy has been interpreted as an indication for a central role of FUS3 in seed maturation. Using a subtractive hybridisation approach cDNA fragments of at least 50 FUS3 dependently expressed genes were isolated (Kölle, 1998). Via the annotated genomic sequence of *Arabidopsis* corresponding genes were identified and many of the potential promoters were shown to contain RY-motifs upstream of TATA-elements. This finding prompted the search for RY-motifs in the potential promoters on the chromosomes 2 and 4 of *Arabidopsis*. After the positional information for all 7781 annotated coding regions had been extracted from Genbank, potential promoters were searched for RY- and TATA-motifs. The RY-core motif CATGCA is found at least once in 521 potential promoters, whereas the TATA-motif TATAAA occurs 4106 times in 3054 different promoters. Under the constraint that functional RY-motifs usually occur 50-100 bp upstream of TATA-elements, 55 promoters remain. Table 3.1 lists 36 candidate genes, from chromosomes II and IV, with RY-motifs between 100 and 300 bp upstream of the start codon (Reidt *et al*., 2001). Out of this group, fourteen genes are known to have a function during late-embryogensis or germination, while the other genes are not yet properly characterised or it is not known whether they function during seed development. After the sequencing of the *Arabidopsis* genome was completed this search was extended to its whole genome and the results can be found in the appendix.

protein id	chr	position relative CATGCA	to ATG TATAAA	distance	functional assignment	functions during late embryogenesis or germination
AC002336_60	2	-295	-212	83	putative protein	unknown
AC005309_22	2	-293	-210	83	putative pectinesterase	yes
AC005499_62	2	-285	-233	52	putative cell division control protein kinase	yes
AC006248_83	2	-271	-174	97	putative retroelement pol polyprotein	unknown
ATCHRIV69_26	4	264	-206	58	NAM/CUC2-like protein	yes
ATCHRIV57_352	4	-255	-200	55	putative protein	unknown
AC005617_18	2	-255	-191	64	putative bHLH transcription factor <-----	unknown
AC002561_10	2	-237	-180	57	putative DNA polymerase delta small sub unit	unknown
ATCHRIV86_390	4	-213	-160	53	patatin like protein	yes
ATCHRIV27_150	4	-212	-115	97	gibberellin-regulated protein GASA2 precursor	unknown
ATCHRIV34_122	4	-208	-138	70	pEARL1-like protein	unknown
ATCHRIV29_99	4	-200	-106	94	NAM/NAP like protein	yes
ATCHRIV49_113	4	-199	-148	51	putative protein	unknown
ATCHRIV5_183	4	-197	-113	84	predicted protein of unknown function	unknown
ATCHRIV7_201	4	-193	-107	86	ent-kaurene synthetase A-like <-----	yes
ATCHRIV30_239	4	-183	-103	80	hypothetical protein	unknown
ATCHRIV91_492	4	-180	-103	77	myo-inositol-1-phosphate synthase	yes
AC004665_106	2	-177	-91	86	aquaporin (plasma membrane intrinsinc protein)	unknown
ATCHRIV90_310	4	-177	-80	97	putative endo-1, 4-beta-glucanase	yes
ATCHRIV69_408	4	-175	-85	90	xyloglucan endotransglycosylase-like protein	yes
AC007047_82	2	-167	-83	84	putative protein	unknown
AC004521_43	2	-166	-90	76	putative protein	unknown
ATCHRIV59_663	4	-156	-93	63	putative major latex protein	unknown
ATCHRIV67_279	4	-154	-68	86	hypothetical protein	unknown
AC004401_17	2	-153	-57	86	putative serine carboxypeptidase	yes
AC005168_48	2	-152	-62	90	putative protein	unknown
ATCHRIV76_352	4	-151	-57	94	cytochrome p450-like protein	unknown
AC007659_25	2	-145	-91	54	putative mitochondrial translation elongation	unknown
ATCHRIV60_344	4	-144	-47	97	putative protein	unknown
AC005311_5	2	-136	-80	56	putative protein	unknown
ATCHRIV72_415	4	-135	-82	53	xyloglucan endo-1,4-beta-D-glucanase-like	yes
ATCHRIV71_256	4	-135	-45	90	nucleotide pyrophosphatase-like protein	unknown
ATCHRIV66_452	4	-127	-74	53	NWMU3-2s albumin 3 precursor	yes
ATCHRIV68_531	4	-126	-56	70	tyrosine transaminase-like protein	unknown
ATCHRIV81_82	4	-117	-60	57	fatty acid elongase like protein	yes
ATCHRIV49_108	4	-116	-59	57	putative protein	unknown

Table 3.1: *Virtual screen for annotated genes on Arabidopsis chromosomes 2 and 4 with putative RY promoters.*

The table lists 36 candidate genes containing the RY motif CATGCA, a target sequence of the transcriptional activators FUS3, LEC1 and ABI3, and the TATA-box TATAAA within 300 bp of 5' flanking sequence. The fifth column indicates the distance between both motifs in the respective promoters. A putative functional assignment for the corresponding genes is based on sequence similarities. Also genes active during the stage of late-embryogenesis or germination are indicated. The promoters with red arrows were tested in a transient assay for their inducibility by LEC1, FUS3 and ABI3.

3.4.1 The induction of two RY containing promoters by LEC1, FUS3 and ABI3

Two of the RY-containing promoters, identified by the virtual screen on the *Arabidopsis* genome, were amplified by PCR from genomic DNA and fused to the *β-glucuronidase* gene. The first promoter was the promoter of the *ent-kaurene synthetase A-like* gene. The protein product of this gene plays an important role in gibberellin biosynthesis. It catalyses the first committed step in this pathway namely the conversion of geranylgeranyl diphosphate (GGDP) to ent-kaurene (Sun *et al*., 1994). While the second promoter, containing two RY-elements, belonged to a basic-helix loop helix transcription factor, bHLH.

Following protoplast transformation the effect of *CaMV*-controlled co-expression of LEC1, FUS3 and ABI3 on these two promoters was measured (Figure 3.7). Only a slight, but significant ($P<0.05$), induction of these promoters as a result of the simultaneous co-expression of LEC1, FUS3 and ABI3 was observed (Figure 3.7).

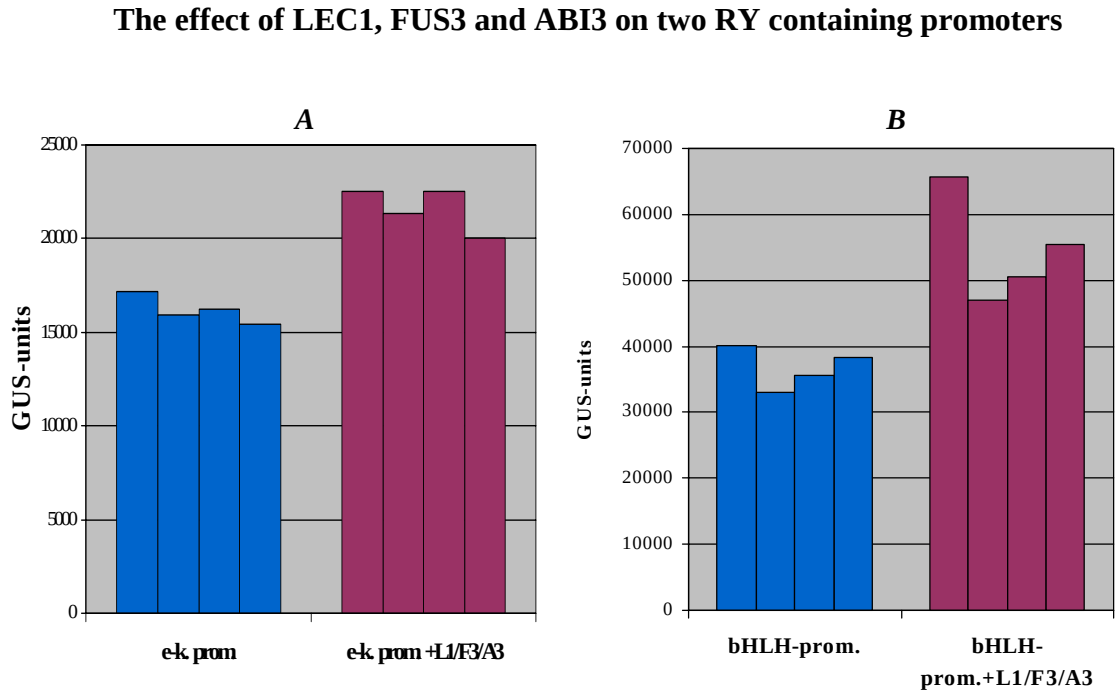


Figure 3.7*: The induction of two RY-containing promoters by LEC1(L1), FUS3(F3) and ABI3(A3). The promoters of the ent-kaurene synthetase A-like protein (**A**) and of a bHLH transcription factor (**B**) were individually fused to a* GUS *reporter gene. The effect of simultaneous overexpression of the LEC1, FUS3 and ABI3 transcription factors in a transient expression assay, on these promoters was tested. Each column represent a different experiment.*

3.5 Overexpression of FUS3 in *A. thaliana* and *N. tabacum*

To characterise the precise functions of the FUS3 protein, transgenic *Arabidopsis* and *N. tabacum* plants were generated overexpressing the *FUS3* cDNA under control of the *CaMV* promoter. Overexpressing lines were initially identified by PCR and by their ability to grow on kanamycin containing growth medium. As a second way of identifying overexpressing lines analysis of their RNA overexpression pattern by Northern blotting was used. Although the FUS3 cDNA was successfully overexpressed at the RNA level in both *Nicotiana* and *Arabidopsis* plants (Figure 3.8a+b) a phenotype could only be seen in the *N. tabacum* plants.

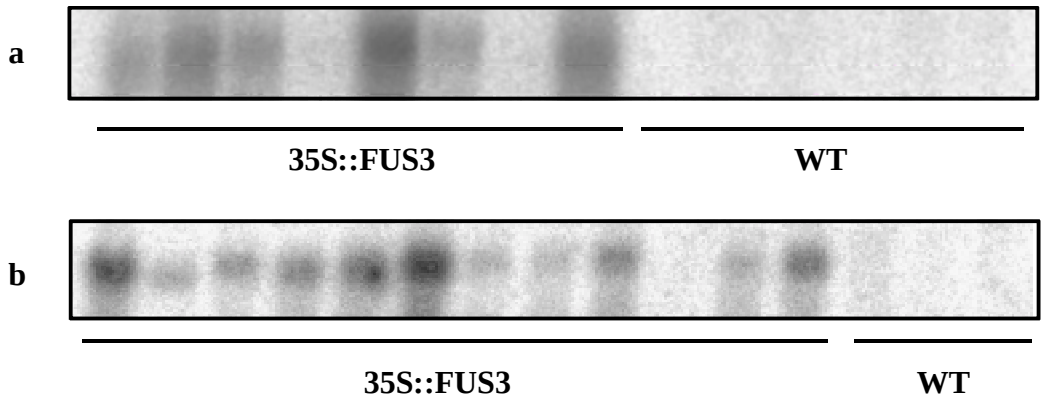


Figure 3.8a+b: *A Northern blot of 35S::FUS3 overexpressing* Arabidopsis*(a) and* Nicotiana*(b) plants. Total RNA was isolated from several different 35S overexpressing FUS3 (35S::FUS3) and wildtype plants (WT), separated on a 1.5% agarose gel and subsequently blotted onto a Nylon membrane. The resulting filter was hybridised with ^{32}P labelled FUS3 cDNA.*

These overexpressing plants displayed a dwarf phenotype (Figure 3.8) with slightly wilted light green leaves and were delayed in flowering. Further analysis by electron microscope showed an alteration in the morphology of the mitochondria as compared to the wild-type (Figure 3.9).

Figure 3.9: *Phenotype of 35S::FUS3 overexpressing* N. tabacum *plants.Overexpression of the FUS3 cDNA in* N. tabacum *leads to dwarf plants.*

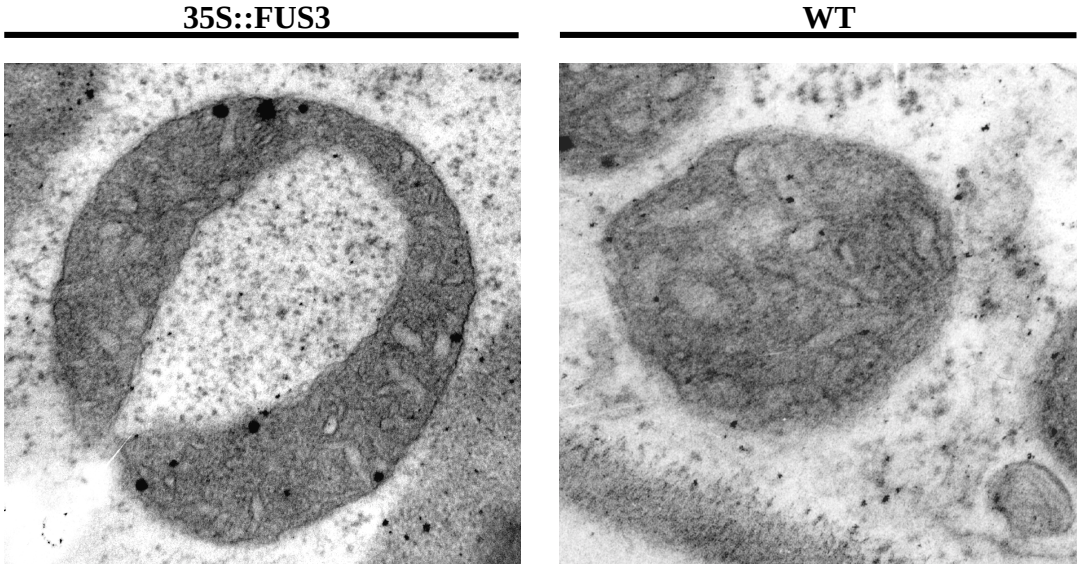


Figure 3.10: *An electron microscope picture of mitochondria in the mesophyll cells of wild-type and FUS3 overexpressing* N. tabacum *plants.* N. tabacum *was transformed with a* 35S *FUS3 construct. Transformants overexpressing the transgene were selected for analysis by electron microscope. Mitochondria in the FUS3 overexpressing plants display an altered structure.*

3.5.1 Downstream regulated genes in FUS3 overexpressing *Nicotiana tabacum* plants

The FUS3 overexpressing *Arabidopsis* plants not only lacked a discernible phenotype, but also showed no difference in the up- or down-regulation of 8000 spotted genes as compared to wild type plants. Contrary to this result was the identification of the seed-specific legumin protein in *N. tabacum* leaves by Western blotting (Figure 3.11). Furthermore it appeared that these legumin proteins were not only produced but also processed into α and β sububits (Figure 3.11). Further screening of downstream regulated genes with the help of Northern and Western blots could not detect the presence of oleosin or the HSP17 proteins in the leaves.

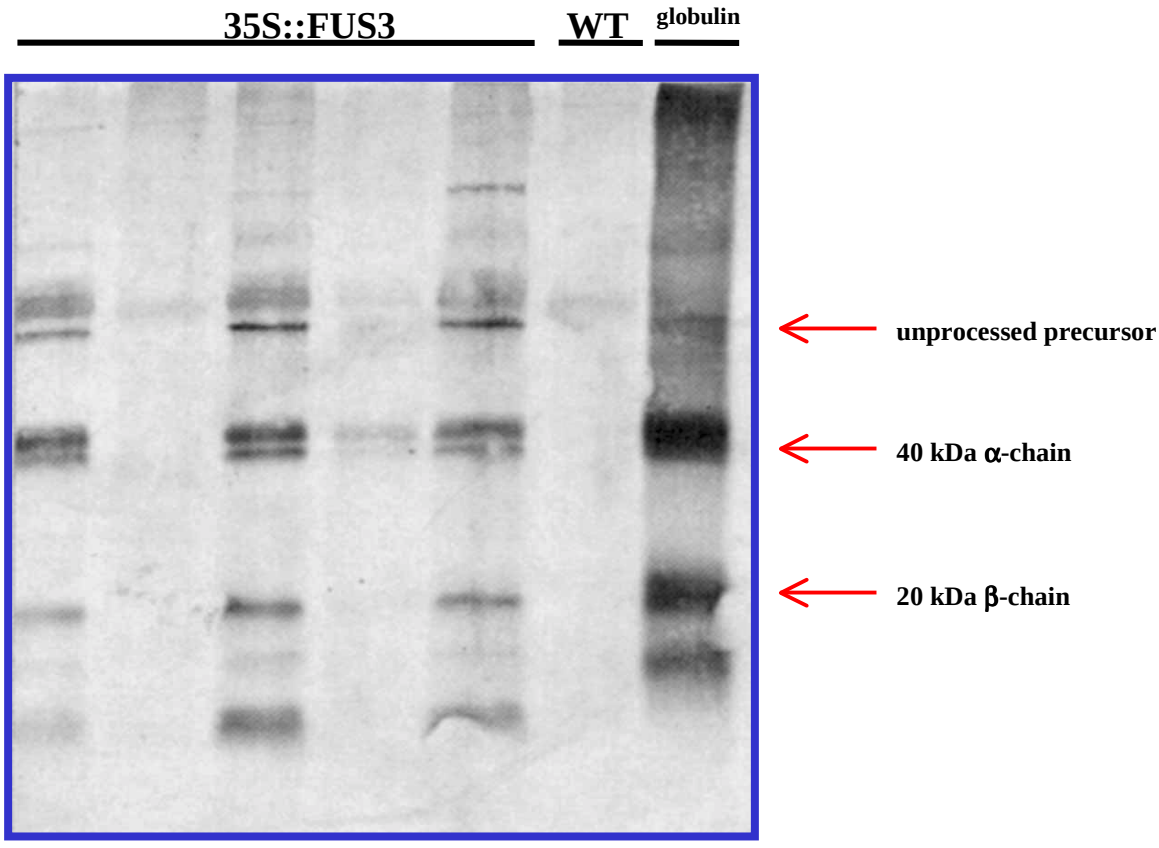


Figure 3.11: *A Western blot of FUS3 overexpressing* N. tabacum *plants.*
A Western blot of total leaf protein extracted from 5 transgenic N. tabacum *plants ectopically overexpressing FUS3 (***35S::FUS3***) was probed to a legumin antibody. The blot shows the detection of unprocessed and processed (***α-** *and* **β-chain***) legumin proteins in the leaves of several transgenic tobacco lines. WT - total leaf protein extract from wild type* N. tabacum*; globulin - positive control of globulin protein fraction extracted from tobacco seeds.*

3.6 Characterisation of the ET gene family

To isolate additional transcription factors involved in the regulation of seed-storage protein expression, expression cDNA libraries were constructed from both immature seeds of *B. napus* (Taipalensuu *et al*., 1997) as well as from *V. faba* seeds (Wohlfarth, 1996). Both libraries were screened, using a South-Western approach, with oligonucleotides corresponding to the E-box element of the napin promoter from *B. napus* and the USP and LegB4 promoter from *V. faba*. These fragments were chosen because they were known to be important for transcriptional activity (Ellerström *et al*., 1996; Stalberg *et al*., 1993, 1996).
The screening resulted in the isolation of two putative transcription factors, one from *B. napus* designated BnET and one from *V. faba* designated VfET (Ellerström, 1998; Wohlfarth, 1997). Comparison of the BnET and VfET proteins revealed a low but significant similarity especially in their carboxy terminal regions. Homology searches with the *Arabidopsis* genome sequence resulted in the identification of three additional genes with homology to the previous isolated ET-genes. Two of them are positioned on chromosome 5, designated *AtET51* and *AtET52* whereas the third one is positioned on chromosome 4 and was designated *AtET4*.

The genomic organisation of all three genes in *A. thaliana* is similar, they contain two introns on similar places on the genomic sequence (Figure 3.12). The total length of *AtET4* and *AtET51* is around 2.9 kB while *AtET52* is slightly smaller with 2.0 kB. A splicing mistake at the beginning of the second intron of *AtET4* shifts the reading frame of the second exon resulting in two in frame stop codons. Furthermore one in frame stop codon is present in the third exon. Therefore most likely *AtET4* represents a non-functional gene. The predicted amino-acid sequence of AtET51 shows, as compared to the BnET and VfET proteins, similarity throughout the whole protein sequence being more similar at their C-terminal end (Figure 3.13), whereas the homology of predicted amino acid sequence of AtET52 seems to be restricted to the N-terminal part.
Different studies demonstrated the presence of another homologous gene in *Hordeum vulgare* (Raventos *et al*., 1999). However this gene was not isolated due to its ability to bind a seed specific promoter element but to bind a gibberellic acid response element (GARE). They also demonstrated the ability of this gene to repress certain promoters and therefore named the gene, *HRT* (***H****ordeum* **r**epressor of **t**ranscription).

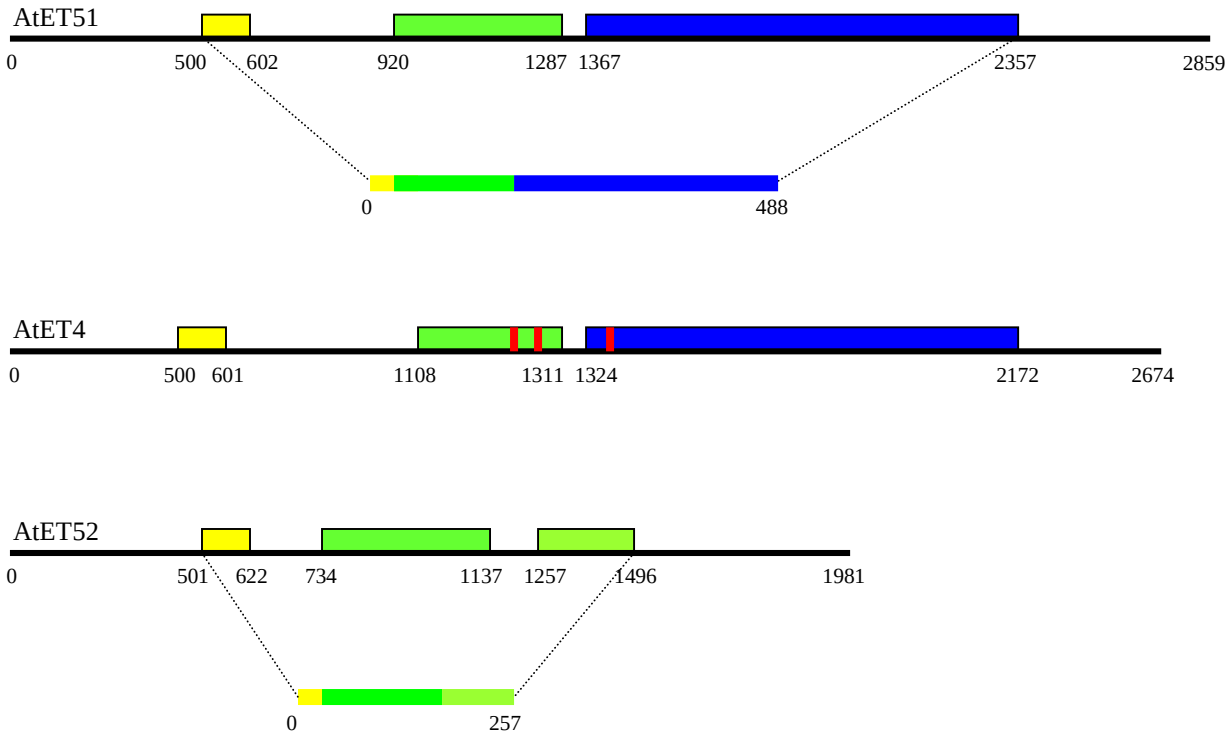


Figure 3.12: *Schematical representation of the genomic organisation of the three known* ET *genes from* Arabidopsis.
The genomic sequences, displayed as black bars, of the three ET *genes in* Arabidopsis *are shown. Exons are indicated by coloured bars and below each gene is shown the predicted protein. A splicing mistake at the beginning of the second exon of* AtET4 *results in two stop codons (red bars) in this exon, while one more is present in the third exon.*

A repeated amino acid sequence, $CX_9CX_9RCX_2HK$, is conserved in all ET proteins, except AtET52. This motif occurs four times in BnET, AtET51 and AtET4, three times in HRT and two times in VfET (Figure 3.13). In general an alternating structure of cysteine and histidine amino acids with the capability to bind Zn^{2+} is defined as a Zinc-finger. The capability of this repeated motif to bind metal ions was demonstrated by mass spectrophotometric analysis (Ellerström, 1998).

```
BNET        MF-----------------CLNTFKS-RWPRE-----------------FQNLKKKKKKTCV--
ATET51      MEFG--DG---VSFAVV----PTVFKREDYKRTKHD------------TVFSKWQFDAAQV----
VFET        ITFP--ATA------------QMLKREECKHTKHD------------SSFSHWKI---------
HRT         MP-A--VAA------------ARLKREDCPRTKHD------------SLFSPWKV---------
ATET52      MSFVLLSKMAKLKFNHIRHTIISTQKSTTIMPGISQLLNNRLIGKEFSSAVPTMFKREDYKLTIHD

BNET        -------K-----LSIPIRSVT----------GDAAS---LTVVVTM-YKRDDYVRNKPGGVFSR
ATET51      --------LIGSNDWEDFKNGKDGVGRYRVQNLPRKSCPGLYELGVAV--IGQEQCRKLEPDIVLA
VFET        --------LIGPSDWEDYSKGKEGSTRYRIQNLPQNSGPGVYELGVAMSTSGLGREIYKLATRVVV
HRT         --------LVGPSDWEDHSAGKEGVQRYHTRNLPDNF-PGLYELGVARPSYDGVRARRNRSVVVVV
ATET52      IAFSKWRNLIRHNDWKDFNNRKERVRRYRHEDLPPQRCTGLYELGVGVIGQDQGQNFDPDNN-VLG

BNET        W-QGFARSML--LP-KPFSETAELRRTVADYS---------LISRGLAPKILREAKG---NREDLR
ATET51      SYLGQAESVRSRLQRYGRSGAHLRNVNNLND-CETIESPVKAVTGGLFEDIFSKGGSILYRWAPMG
VFET        VYLGKADNVRTRLQSYGRNGAHLGNG------CSTFESSEEK-GHSLFHDIFFQSFPIVYRWAPMQ
HRT         VYLGQADNVRARLQQYGRTGSHLDTGNPLAAVCKAEMNALTA-GPGLFREVFSRGYSMMFRCALMG
ATET52      VYVGQCVDVKSRLQDYGRRGGHLPS-------------------GLYEDIFSEGYSVFYRWAP--

BNET        VGKDFVGSRYR----------VQESIQGLGVAVNIHDADDISHGQTESIRTRLRSYGRPVPLLKKL
ATET51      SKREAEATEGMLLSTFDYAWN-KGSNGERRQLDL--LKKLGDREFMSKR-------------KSG
VFET        NKGDALQTESQLLSTFDYAWNTINNGTRRPADILQMLNKISS----------GTRTFSEVA----
HRT         SKKAAEKTEGQLLGVFDYAWNKLQNGACRREEILLKLEQGSNRLSLLSRVRHLKQRVFGEKAGIKI
ATET52      ---EAAATEGMLLSTFDYAWNTCSNGERRH----LELQKLGDPEFMSKR----------------

BNET        GDNAS--------QTI-------TQKKTGGRSKDKKHGFEEERDVSRVEAEENNTNSVHASVLRLS
ATET51      IS----------RMLFP----FLRNQVGIRIKGEKHVLKEERKLTC--DVDEEKSNNFLTSILKLT
VFET        -------------KSLVP----FTQKKVGILIKARKLPMTDN-------KSDNDGYNFLSRVFKFN
HRT         NSSGSVEISSSSMKNMLPRVRTFVGFRPRLVNSGDDLNEASDIHRKCTPQANTAGKQAHRRSEGY-
ATET52      -----------KSQVLVPSIR---------------------DQVVTIKVEKSNYTFLTSTLKVM

BNET        RSRPQPVLERHDDIVDGSVSASVCGVLQEDGTTCLTAPVTGRKRCTEHKGQRITCAPPVKNPP--C
ATET51      RSRPQPVSDRFDEV-DGSCSDIVCGVLLEDGGCCIRSPVKGRKRCIEHKGQRVCRVSPEKQTPPKS
VFET        RSRKVVIHDTSDFAVEKN-----------------------------------------------
HRT         KVKKIDVIKRRTAPIREA--EAVCGVMLEDGSSCLEDPMEGRKRCELHKGRRVRVAYSRKVSSSSS
ATET52      RP------------------------------------------------------------

BNET        E-----------E---------ETEEICGVILPEMVRCRSKPVSGRKRCEDHKGMRV-------NA
ATET51      E----IFTGQDHHNH------KDSDVVCGVILPDMEPCNKRPVPGRKRCEDHKGMRI-------NA
VFET        ----GKI-------------------CGVILDDGSICSKMPVGKRVRCNEHKGMRINMVTTKAMR
HRT         T---AIPTVESIPQQTANPSKRDQAWQTSADQSKNLSTNAKEPSWQRNSFKANEMKIGEAPTEDEA
ATET52      ------------------------------------------------------------

BNET        F--------FFLLNPTERD--KILKEDKSKPKTRTSSTN----QEEPGESLICEATTKNGLPCTRS
ATET51      F--------LFLLNQTDRE--KTVKDEKPDPESHTESI------EEEALTRFCEATTKNGLPCTRS
VFET        RSKSESECQVNVFTAKEI----------------------RRSKSESEKVS-ESLVDESIT-K--
HRT         YGTSHAESQFHEDEPCGRKWFERLKAQKSANAPSSRGQGCQPREANNDASALCGVVTDNGY-CKLE
ATET52      FG*

BNET        APNGSKRCWQHKDETVDQKSSENVQTSTT---VCGVKLHNGSVCEKTPVKGRKRCQEHKGMRITS*
ATET51      SPKGSKRCWQHKEKTSSDTSPVYFQPEAAKNVACGVKLGNGLICERSPVKGRKRCEEHKGMRIT*
VFET        ----------------------------TVICGIVLEDGSTCRKEPVKGRKRCHEHKGKRVRAS
HRT         PVIGRERCEEHRGIEVTGASSAPCSGRSVLPSVCGARASDGSPCKNQPIARRKRCALHKGQRACCA

VFET        VSINQK*
HRT         SAPSVK*
```

Figure 3.13: *Amino acid sequence alignment of ET-like proteins.*

Perfectly conserved amino-acids are coloured red. A highly conserved amino acid sequence element, $CX_9CX_9RCX_2HK$, is underlined in each of the respective proteins.

An almost identical motif also occurs in a gibberellin-regulated protein from rice (Figure 3.14), OsGRF1, involved in the stem elongation of rice (van der Knaap *et al*., 2000) as well as in several undescribed *Arabidopsis* proteins. Homology searches show the presence of this structure also in bacteria and viruses.

CGVLLEDG-G**C**CIRSPVKGRKR**C**IE**HK**	AtET51 first zinc-finger from *A. thaliana*
CRRTDGKKWR**C**SKEAYPDSKY**C**EK**H**MR	OsGRF1 from rice
CSRVNGRGWR**C**CQQTLVGYSL**C**EH**H**LG	unknown protein *A. thaliana*
CNVVLRTGKL**C**SRRAC-VGQPL**C**WQ**HK**	EsV-1-13 *Ectocarpus siliculosus* virus
CEYTYSNGKK**C**RRKPL-PGSKY**C**SL**H**-	*Pyrococcus abyssi*
CIDIATHRHG**C**CVLQ------**RC**LD**H**G	Putative regulatory protein YGL023 *Saccharomyces cerevisiae*
CIDIATHRHG**C**CVLQ------**RC**LD**H**G	probable regulatory protein YGL014w *Saccharomyces cerevisiae*
PLDVSTHRHG**C**CVVQ------**RC**FD**H**A	serine rich pumilio family rna binding domain protein *Schizosaccharomyces pombe*
CIEIATTRHG**C**CVLQ------**RC**VSYS	putative protein *A.thaliana*

Figure 3.14*: Amino acid sequence alignment of the putative Zn-binding domain $CX_9CX_9RCX_2HK$ of 9 proteins from various organisms.*
The amino acid sequence $CX_9CX_9RCX_2HK$ has been detected as a conserved domain in a number of plant proteins. Homology searches using BLASTP demonstrate the presence of similar domains also in other organisms.

3.7 Analysis of the mRNA expression of AtET51 and AtET52

To study in which tissues AtET51 is expressed, a Northern blot with mRNA isolated from different tissues of *A. thaliana* was hybridised with a cDNA sequence corresponding to the third exon of AtET51 (Figure 3.15). Based upon this blot a ubiquitous expression pattern was observed with slightly lower expression levels during middle and late embryogenesis. This result was confirmed by RT-PCR (Tiedemann, personal communication).

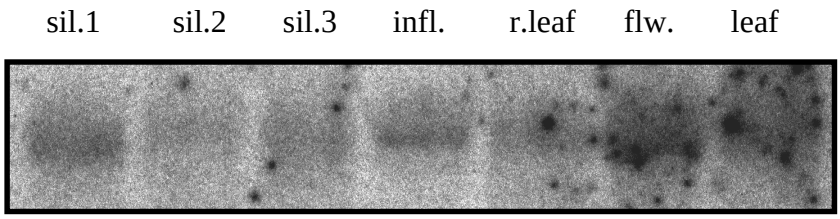
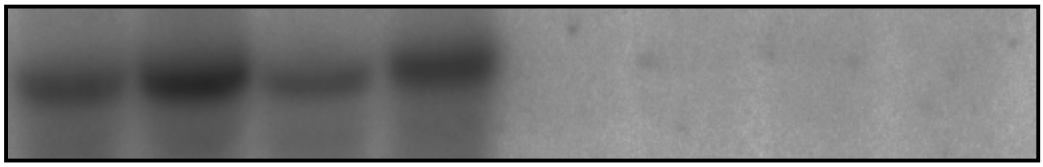


Fig 3.15*: Expression analysis of the gene* AtET51.

RNA (10 µg) isolated from different tissues was seperated in a gel and subsequently blotted to a filter. The resulting Northern blot was hybrised with a fragment (third exon) corresponding to the gene AtET51. *si1.1/3 – siliques from different developmental stages (1= young: 3= old) infl. - infloresence; r.leaf - rosette leaf; flw – flower; leaf – leaves.*

3.8 Functional characterisation of the ET-gene family

To study the function of the *BnET* gene transgenic tobacco plants were generated in which the BnET cDNA was expressed under the control of the *35S* promoter of the cauliflower mosaic virus promoter. In both *N. tabacum* and *Arabidopsis*, positive plants were initially identified by PCR (results not shown) and by their kanamycin growth resistance. RNA analysis by Northern blotting demonstrated the overexpression of the BnET cDNA (Figure 3.16). Seven of the eleven independent obtained transformants in tobacco exhibited a marked decrease in plant height (Figure 3.17). To see whether this decrease in height was a result of a reduction in internode length the number of leaves at the onset of flowering was counted. Wild-type *N. tabacum* plants had 13.9 $\pm$ 0.9 leaves at the onset of flowering. This was number was comparable to BnET overexpressing plants, 11.8 $\pm$ 1.5. Therefore the decrease in height seemed to be the result of a reduction in the internode length.



35S::BnET **WT**

Figure 3.16: *A Northern blot of wild type (***WT***) and* 35S *BnET (***35S::BnET***) overexpressing tobacco plants.*
Total RNA was isolated from 4 different 35S *overexpressing BnET plants and 4 wildtype plants. Next the isolated RNA (15 µg) was separated on a 1.5% agarose gel and subsequently blotted onto a Nylon membrane. The resulting filter was hybridised with ^{32}P labelled BnET cDNA.*

Since the previous results indicated that the dwarfism in the *N. tabacum* plants overexpressing BnET was most likely the result of a reduction in internode length, microscopical studies were focused on the stems. These studies showed that the middle of the stem, the pith, was missing (data not shown). Furthermore it became clear that the secondary cell wall thickening of the vascular tissue was severely reduced (Figure 3.18 a-h) probably as result of a reduced lignification (Figure 3.18 i-l).

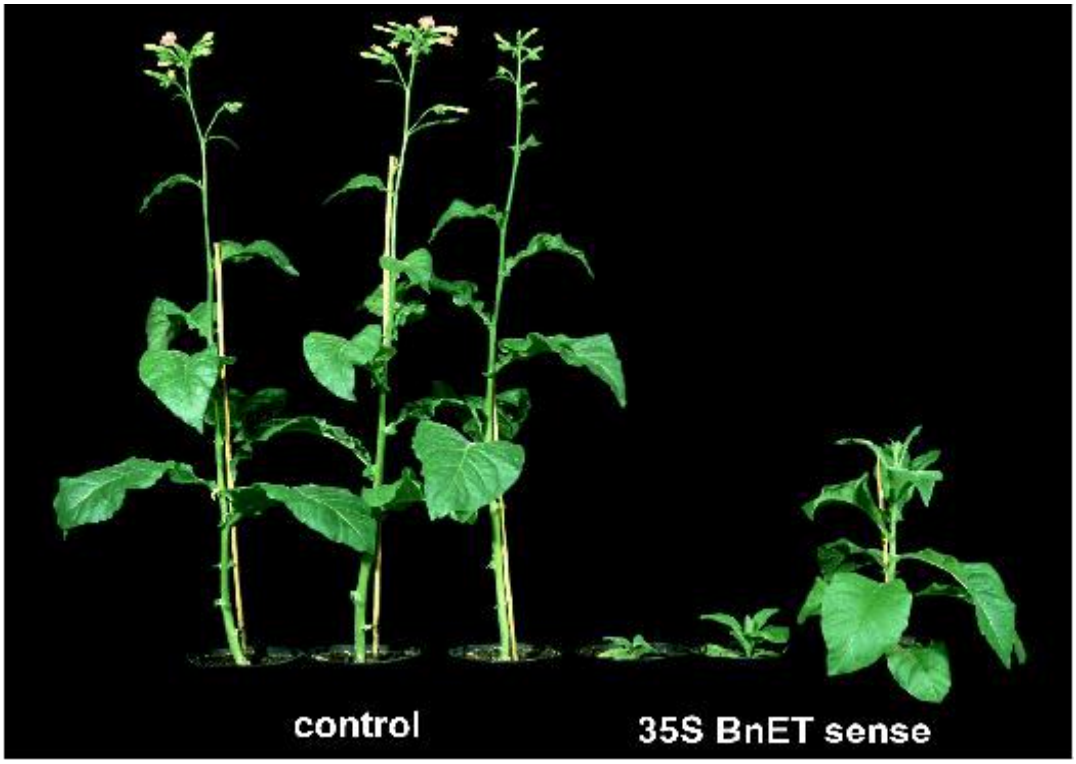


Figure 3.17: *Ectopical overexpression of BnET in* N. tabacum *results in dwarf plants.*

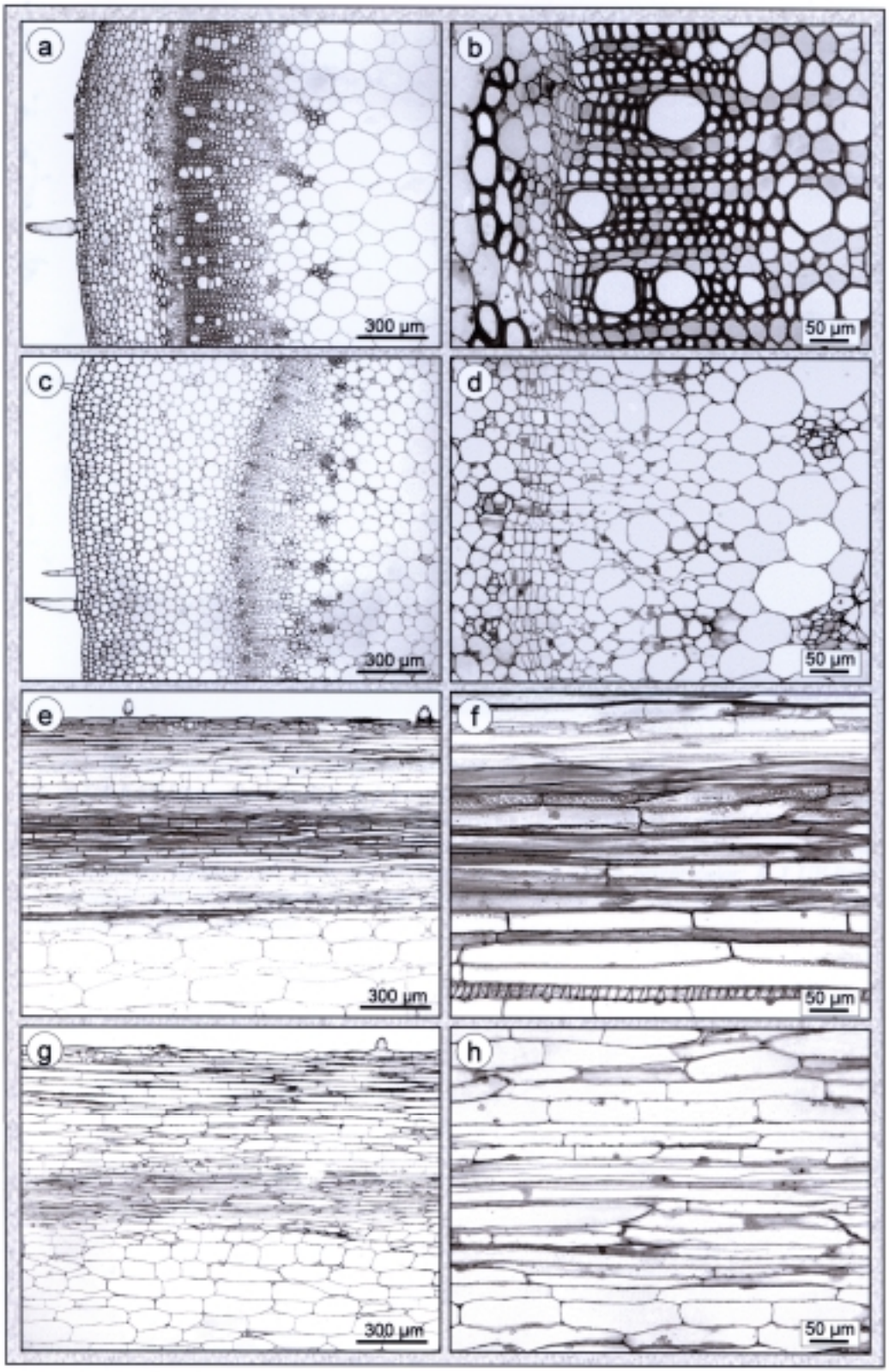


Figure 3.18 continued on next page

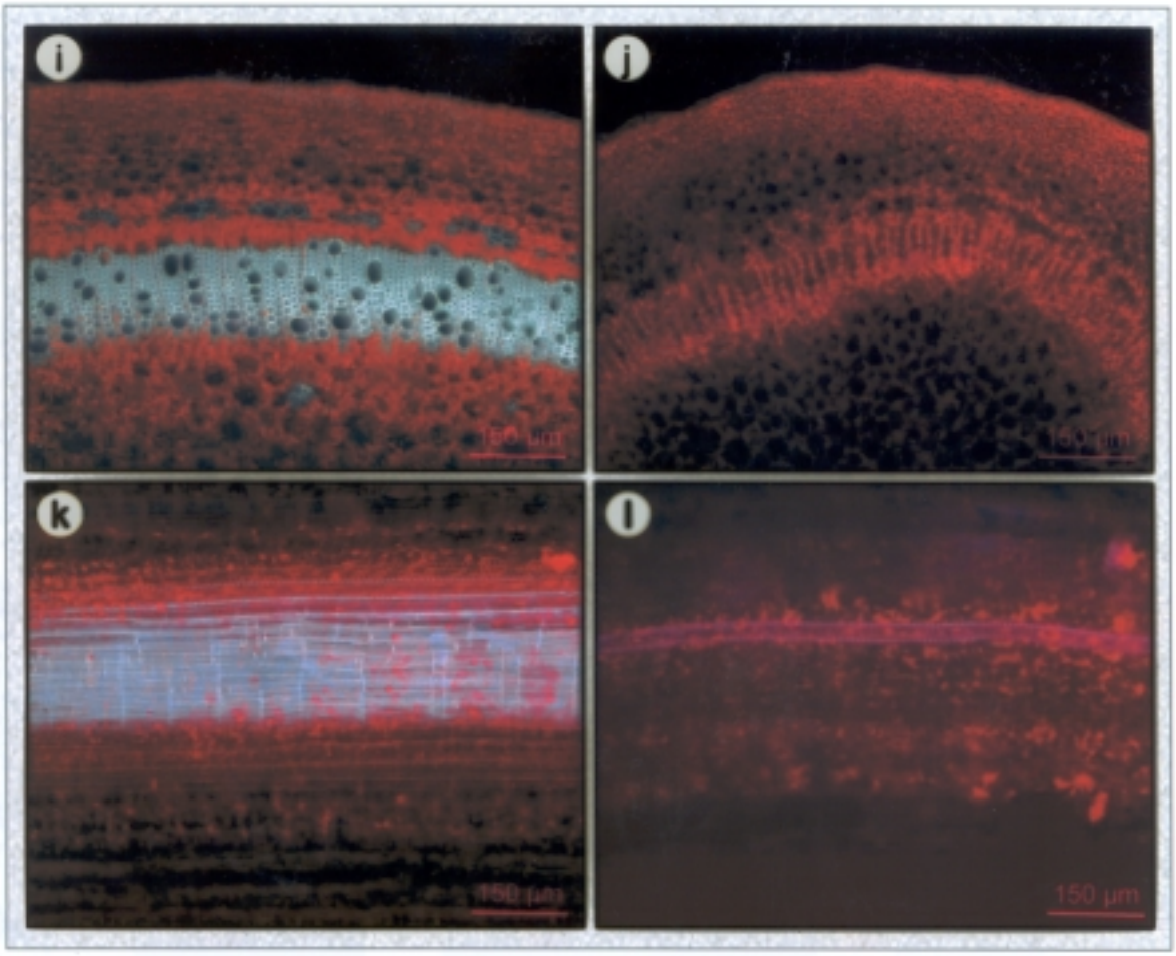


Figure 3.18: *Anatomy of stems of transgenic and wild-type tobacco plants.*
***a, b, e, f, i, k** wild-type plant; **c, d, g, h, j, l** ectopical overexpressing BnET plant; **a, c** transverse section of the stem; **b, d** enlargement of **a, c**, respectively; **e, g** longitudinal section of the stem; **f, h** enlargement of **e, g**, respectively; **i, j** UV-light microscopical view of a transverse section of the stem. Secondary lignified cell walls are autofluorescent and give a bright color; **k, l** UV-light microscopical view of a longitudinal section of the stem. All sections were taken from the stem between the 7th and 8th internode.*

The off-spring of the overexpressing dwarf *N. tabacum* plants displayed an additional phenotype. The germination frequency of the seeds was drastically reduced with a decrease as big as 70 percent as compared to wild-type plants (Figure 3.20).

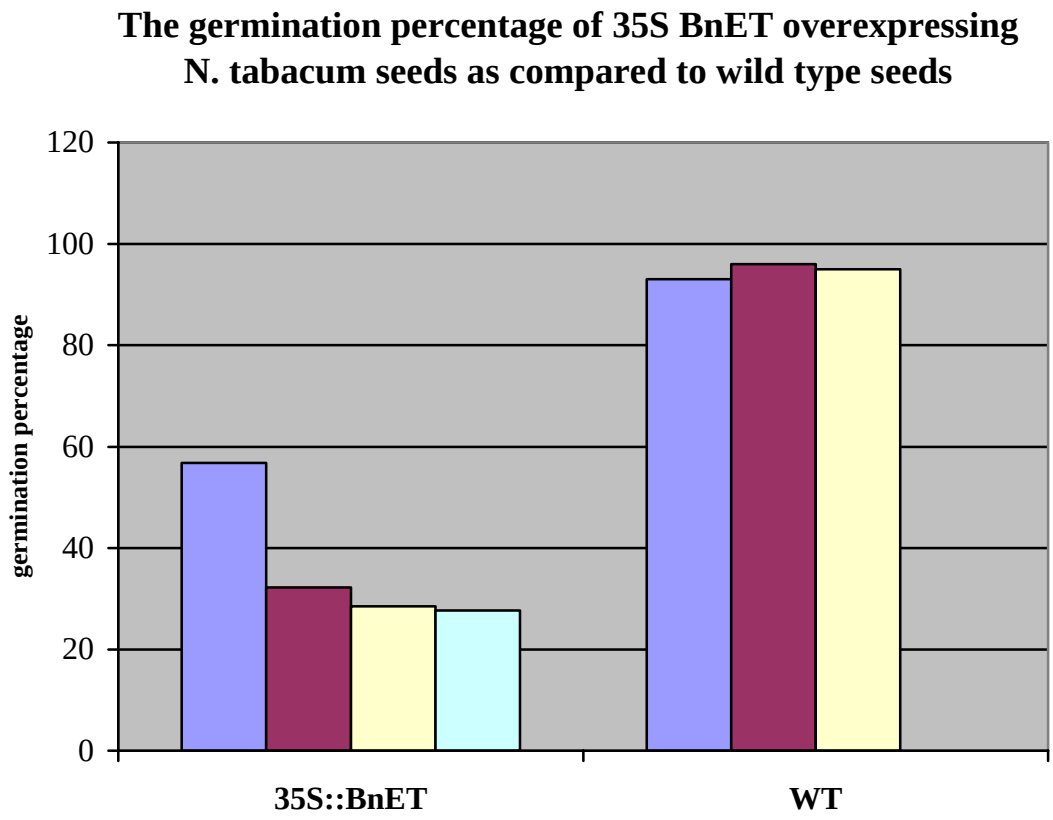


Figure 3.19: *Seed germination in BnET overexpressing lines from* N. tabacum*.*
*Ectopical overexpression of the BnET (***35S::BnET***) gene results in a reduced germination frequency as compared to wild type (***WT***) seeds. One hundred seeds each of three wild-type and four lines of* N. tabacum *overexpressing BnET were compared.*

To examine the effects of BnET on the growth of other parts of the plant, the root, callus and shoot were induced from leaf squares in a medium containing various concentrations of auxin and cytokinin. The auxin to cytokinin ration controls the formation of roots, shoots and callus tissue *in vitro* (Skoog *et al*., 1957). Whereas the growth of both root, shoot and callus of a control and an antisense construct of BnET was normal (Figure 3.20), overexpression of BnET resulted in a complete inhibition of all of these three processes. This demonstrated that the overall growth of tobacco was inhibited.

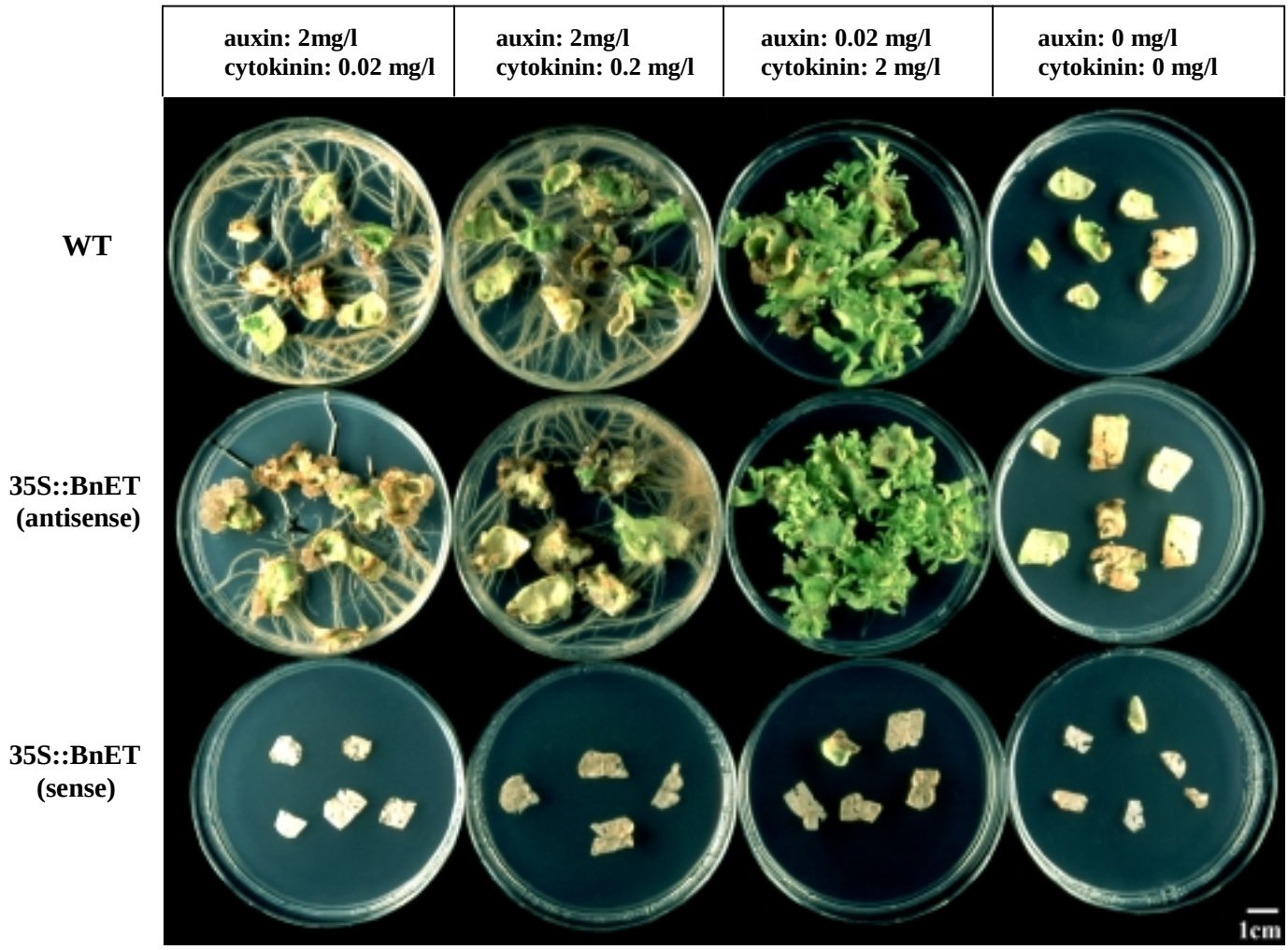


Figure 3.20: *Auxin and cytokinin dependent organ formation from leaves.*
Wild-type (**WT**) *leaves and transgenic tobacco leaves carrying a sense* (**sense**) *and antisense* (**antisense**) *construct of BnET under control of a* 35S *promoter* (**35S::BnET**) *were cut into squares and cultered for 8 weeks on MS medium containing various amounts of cytokinin and auxin. A high auxin concentration leads to root formation while a high cytokinin ratio leads to shoot formation. Both root and shoot formation are inhibited in* N. tabacum *ectopically overexpressing BnET.*

3.9 The effect of BnET on a gibberellin induced promoter

Both BnET and VfET were isolated due to their ability to bind to the E-box element of the *napin*- and *USP*-gene promoter. The function of these two promoters is well characterised due to the simultaneous overexpression of LEC1, FUS3 and ABI3 (see 3.1).

Therefore studying the effect of overexpression of BnET with or without the other combinations of transcription factors on these promoters might further define the function of the *ET*-gene family. Unfortunately no (clear) effect was observed. Also the addition of abscicic acid or gibberellic acid in combination with overexpression of BnET did not result into a difference in promoter activity. Raventos *et al.* (1998) demonstrated that a protein in barley, HRT, similar to the previously isolated ET proteins was able to repress the GA mediated activation of certain promoters. This prompted to investigate the effect of overexpression of BnET on a gibberrelin stimulated promoter. The aquaporin promoter *PIP1b* from *N. tabacum* has been shown to be stimulated by GA (Kaldenhoff *et al*., 1996) and was selected to study the effect of BnET on its gibberellin induced promoter activity. The *PIP1b* promoter was expressed at a low background level in *N. plumbaginifolia* protoplasts (Figure 3.24). The *35S* controlled overexpression of BnET had no significant effect ($P<0.05$) on the wild-type promoter activity (Figure 3.24). As expected the *PIP1b* promoter was induced in *N. plumbaginifolia* protoplasts by the addition of gibberellic acid. However the overexpression of BnET abolished this effect so that the activity of this promoter was as high as the uninduced promoter (Figure 3.24).

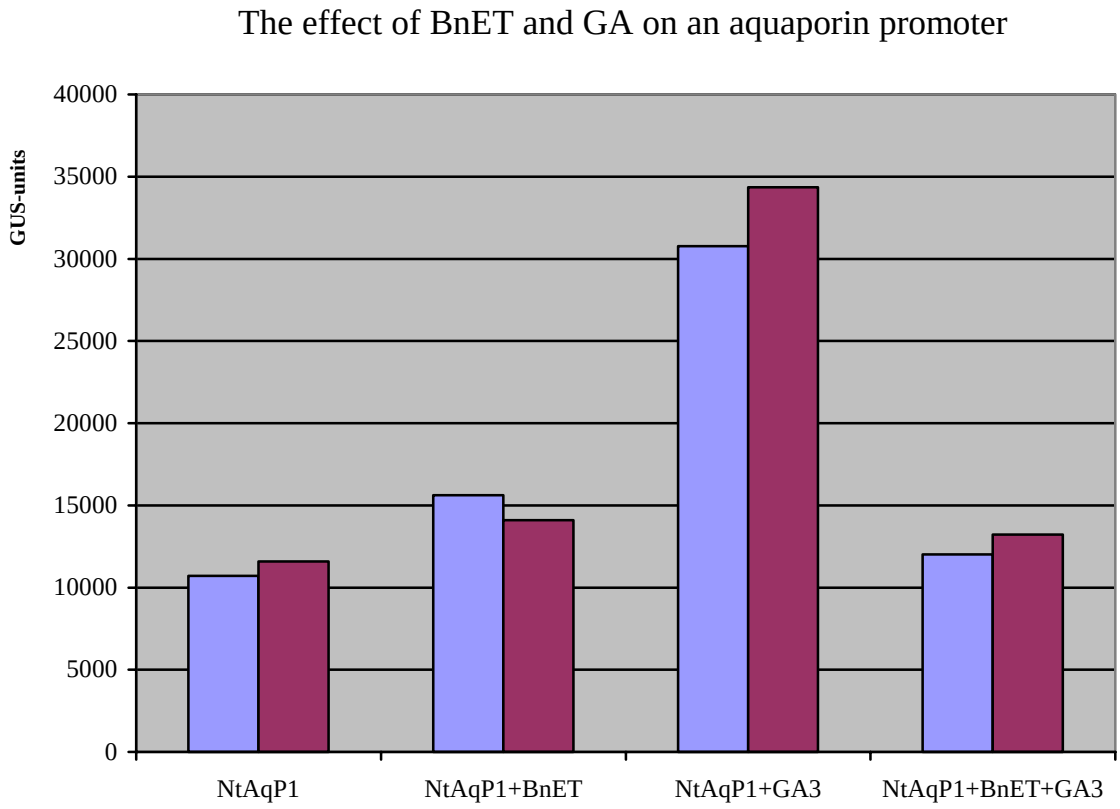


Figure 3.21: *The effect of gibberellic acid and the* 35S *controlled transcription factor BnET on a* aquaporin *promoter*-GUS *construct.*

The NtAqP1 *promoter* GUS *construct is stimulated by the addition of GA (10 μM) in* N. plumbaginifolia *protoplasts. However when the* 35S *controlled BnET cDNA is co-expressed in the protoplasts this stimulation is abolished. The different coloured bars represent two independent experiments.*

4. Discussion

4.1 Characterisation of FUS3

Late-embryogenesis involves the synthesis of large amounts of storage products, the acquisition of desiccation tolerance and the establishment of dormancy. Genetic studies demonstrated that important key-regulators of these processes are the protein products of the genes *LEC1*, *FUS3* and *ABI3*. The exact way in which these genes interact with each other and how they control late-embryogenesis becomes more and more clear as time progresses. In this study an insight into the regulation of late-embryogenesis on the molecular level is provided, focusing on the function of *FUS3*.

4.1.1 LEC1, FUS3 and ABI3 transcription factors interaction in the activation of seed-specific promoters

To investigate the potential interaction between the LEC1, FUS3 and ABI3 gene products and to investigate whether the RY motif is a possible target of these genes, a transient expression system using an embryonic cell suspension culture of *N. plumbaginifolia* was established. This system was chosen because it has been shown useful for the analysis of promoter transcription factor interactions (Feldbrugge *et al*., 1994) and since it was well established within the laboratory (Reidt *et al*., 2000).

Co-expression of either FUS3 or ABI3 strongly induced GUS activity driven by the seed-specific *napin* and *USP* promoters (Figures 3.1 and 3.2). On the other hand, co-expression of LEC1 is not sufficient for an induction of either of the two promoters (Figure 3.1 and 3.2). Increases in GUS activity due to the overexpression of either FUS3 or ABI3 demonstrates that these two transcription factors on their own are sufficient for the activation of both seed-specific gene promoters. In other words, the observed activation indicates that, at least for these promoters, FUS3 and ABI3 can act more or less independently of other seed-specific factors except for those that may naturally be present in this system. This is somewhat surprising since the pleiotropic seed phenotype of *fus3*, which includes disturbances in storage, desiccation and dormancy, has been interpreted as an indication of a rather central role in seed maturation. It is believed that FUS3 exerts its effect by promoting the expression of other transcription factors, which would in turn stimulate the expression of seed-specific genes. The LEC1 protein by itself in contrast is not sufficient for the activation of neither the *napin*- nor the *USP*-gene promoter and probably requires other seed-specific factors to work.

Even higher levels of promoter activity were observed due to the simultaneous co-expression of LEC1 and ABI3 or of FUS3 and ABI3 (Figures 3.1 and 3.2). LEC1 and FUS3 proteins seem to interact with ABI3, enhancing the activation of the promoters. In contrast, no synergism was observed due to the simultaneous overexpression of LEC1 and FUS3 (Figure 3.1 and 3.2), suggesting no interaction among themselves. Highest promoter induction was observed due to the simultaneous overexpression of all three factors together. It is not clear however whether this is the result of a synergistic effect between all three factors or of an additive effect (Figure 3.1 and 3.2).

The effect of ABI3 on the activation of the *USP* promoter is stronger than that of FUS3, while an equal effect is observed on the *napin* promoter, suggesting that the regulation mechanism of the *napin* and *USP* genes may be different.

The lack of a novel phenotype in the *lec1 fus3* double mutant led to the interpretation that LEC1 and FUS3 act in the same pathway (Bäumlein *et al*., 1994; Meinke *et al*., 1994; Vicient *et al*., 2000). Similarly, based on the lack of epistasis in the double-mutants *fus3 abi3* or *lec1 abi3*, it has been suggested that FUS3 and LEC1 regulate a different pathway than that regulated by ABI3 (West *et al*., 1994; Lotan *et al*., 1998; Raz *et al*., 2001). Assuming that the transient expression system reflects *in vivo* conditions, the present results provide further evidence that FUS3 and LEC1 act in overlapping pathways while ABI3 acts in a different non-overlapping pathway.

Although it has been shown that the expression of the *legumin B4* gene depends on the presence of the FUS3 gene product (Bäumlein *et al*., 1994) and that FUS3 is able to bind to the *legumin B4* promoter *in vitro* (Figures 3.5 and 3.6), the low level basic activity of this promoter could not be stimulated by either FUS3 or ABI3. Thus, the protoplast expression system was not suitable for the analysis of this promoter, and implies that additional factors that are absent in this system may be necessary for the activation of the *legumin B4* promoter.
In conclusion, the direct interaction of FUS3, ABI3 and LEC1 with seed-storage protein gene promoters shows that these transcription factors are necessary not only for triggering maturation processes, but also for the maintenance of seed maturation.

4.1.2 The RY motif is an essential target of the transcription factors FUS3 and ABI3

The FUS3- and ABI3-dependent increase in activity of either the *napin* or *USP* gene promoter could be strongly reduced when the RY motifs present in both promoters where mutated so that only one RY motif was left. This clearly demonstrates that the RY motifs are an essential target of the FUS3 and ABI3 proteins (Figure 3.1 and 3.2).
The ABI3 homologue in maize Vp1 is considered as the prototype of the B3-domain-containing group of transcriptional activators. For Vp1 it has been shown that the highly conserved C-terminal B3 domain is responsible for the activation of the *C1* gene that controls the anthocyanin pathway through binding to the RY motif of the *C1* promoter (Suzuki *et al*., 1997). The B3 domain does not affect seed maturation instead the maturation related functions of Vp1 are considered to be located in the N-terminal part of the molecule (McCarty *et al*., 1989). The FUS3 gene product which is much smaller in size could be seen as a natural truncation of the Vp1 protein lacking the N-terminal part of Vp1 (Luerßen *et al*., 1998). It is therefore noteworthy that the FUS3 protein can regulate maturation specific gene expression through the RY motif. Together, these data suggest that either seed maturation functions are mediated by different unrelated or uncharacterised regions of the two regulator molecules Vp1/ABI3 and FUS3, or that dicotyledonous and monocotyledonous plants are using the RY motifs for different functions during seed development.
McCarty *et al*., (1991) demonstrated that Vp1 expression in maize protoplasts activated the promoter of a *LEA* gene and increased the ABA responsiveness of this promoter.

Using a similar experimental strategy with the *ABI3* gene and the seed-specific *napin* gene promoter, an increase of the ABA responsiveness of the *napin* promoter was also found (Parcy *et al*., 1994). Furthermore, ectopic expression of ABI3 conferred the ability to accumulate several seed-specific mRNA markers only in response to spraying with ABA (Parcy *et al*., 1994). The N-terminal domain of Vp1/ABI3 has been suggested to provide a key interface for ABA signalling pathways (McCarty *et al*., 1991; Bobb *et al*., 1995; Carson *et al*., 1997; Rojas *et al*., 1999; Ezcurra *et al*., 2000).

As discussed before, the N-terminal region of Vp1/ABI3 is missing in FUS3, therefore it was interesting to examine what would be the effect of ABA on the FUS3 dependent activation of the *napin* promoter. Surprisingly, ABA has a repressive effect on the activation of the *napin* promoter by FUS3. It is tempting to speculate that an uncharacterised region of the FUS3 protein might therefore be also involved in ABA signalling, however, rather than having a similar enhancing effect as the N-terminal domain of ABI3, it might have a repressive effect. These data are in line with other experiments that demonstrate that FUS3 functions in the early steps of seed maturation when the ABA levels are relatively low, whereas ABI3 functions later in seed development when the ABA levels are higher (Figure 1.2; Karssen *et al*., 1983; Hughes *et al*., 1989; Raz *et al*., 2001). In addition, it has been suggested that FUS3 and ABI3 play a role in the developmental regulation of ABA synthesis (Nambara *et al*., 2000). Mutant *fus3* embryos had a lower ABA level at 10 DAF as compared to wild-type embryos, while mutant *abi3* plants had a much higher ABA level at this time point (Nambara *et al*., 2000). Taken together with the present study, these observations suggest that FUS3 up-regulates ABA synthesis but that high ABA levels, on its turn, repress the function of FUS3 and enhance the function of ABI3. However, ABI3 then down-regulates the ABA level.

4.2 Protein-protein interaction between FUS3 and ABI3 or LEC1

To investigate in more detail the interaction between the FUS3 and either the ABI3 or the LEC1 protein, the yeast two-hybrid system was used. Using this system an interaction between FUS3 and LEC1, as well as homodimerisation of the FUS3 protein was found. No interaction could be found between FUS3 and ABI3 (Figure 3.4).

These observations represent the first molecular evidence that the LEC1 and FUS3 proteins interact with each other. Homodimerisation of the FUS3 protein was also demonstrated using the two hybrid system. It is possible that FUS3 uses dimerisation as a means to assemble a multicomponent signalling complex. It could also use this self-interaction to regulate its own activity.

The interaction between FUS3 and LEC1 suggests that the activation of the *napin* and *USP* promoter in the transient assay due to the simultaneous overexpression of LEC1, FUS3 and ABI3 is the result of a synergism between all three factors rather than an additive effect. This is in line with genetic studies indicating that FUS3, LEC1 and ABI3 interact in concert in controlling several seed-specific processes (Bäumlein *et al*., 1994; Kölle 1998; Parcy *et al*., 1997; Nambara *et al*., 2000). The failure to detect an interaction between FUS3 and ABI3 could be the result of the truncation of the FUS3 protein indicating that a full-length protein is necessary to interact with ABI3. On the other hand it should not be excluded that both proteins do not interact. Further studies with for instance immuno-precipitation experiments or a different two-hybrid system could confirm this.

4.3 The direct molecular interaction of the FUS3 protein and the RY motif

The transient expression experiments suggested a direct interaction of the FUS3 transcription factor with RY-containing seed-specific promoters.

To precisely define this interaction band shift experiments, with oligonucleotides corresponding to the RY-containing promoter of the *legumin B4* gene from *V. faba* were performed. These experiments demonstrated not only the direct binding of the FUS3 protein to this promoter but scanning mutation analysis further defined the RY motif as the binding site of FUS3 (Figure 3.5 and 3.6). A similar strategy using BIAcore confirmed this result (G. Mönke, personal communication). This is the first demonstration of a direct molecular interaction between a natural B3-domain containing transcription factor and the RY motif. For the RY element in maize, it was shown that only a version of Vp1 with an artificial N-terminal truncation, exhibits co-operative interaction with the RY motif and only when high levels of recombinant protein were used (Suzuki *et al*., 1997).

This observation prompts the speculation that in contrast to FUS3, Vp1/ABI3 transcription factors interact with the RY motif *in vivo* only as part of a larger transcription factor complex and that FUS3, when bound to DNA, could serve as a docking partner for the other factors. This resembles what has been found for the mammalian co-activator OCA-B. This protein needs to interact with the transcription factor Oct1 to interact with an octamer motif as a complex (Chang *et al*., 1999). When OCA-B is truncated it gains the ability to interact with the same *cis*-motif without the need for Oct1 (Cepek *et al*., 1996).

In conclusion these data and the data from the transient assays define the structural requirements necessary for the function of the RY *cis*-motif found in many seed-specific gene promoters. This motif was shown to be an essential regulatory target of the FUS3- as well as the ABI3-gene products. Moreover, a direct interaction between the RY *cis*-motif and FUS3 was shown.

4.4 RY motifs are present in a number of seed-specific promoters

Both the transient assays and the DNA binding experiments defined the RY element as the FUS3 binding site. This finding prompted the search for RY motifs in the promoters of all annotated genes on the chromosomes 2 and 4 of *Arabidopsis*. Using this approach 55 RY-containing promoters were identified (Table 3.1). Keeping in mind the fact that the RY core element, CATGCA, occurs every 4^6 bp, also sequences in which the RY element occurs by chance are identified in this screen. Therefore probably only a subset of all genes found are targets for regulation by FUS3.

Genetic studies demonstrated that mutations in the *FUS3* gene leads both to the down- and up-regulation of certain genes. The down regulated genes are usually maturation-specific genes while many of the up regulated genes are important during germination (Bäumlein *et al*., 1994; Kölle, 1998; Nambara *et al*., 2000). Both maturation-specific and germination-specific genes are also identified by this virtual screen for RY containing promoters (Table 3.1) and might be regulated in a similar way as observed in mutant *fus3* plants.

Two of the promoters identified as having RY elements in their sequence were screened for activation by LEC1, FUS3 and ABI3.

The first promoter was chosen since the protein product of this gene, *ent-kaurene synthetase A*, plays an important role in the biosynthetic pathway of the hormone gibberellic acid (Sun *et al*., 1994). This gene catalyses the first committed step in the pathway namely the conversion of geranylgeranyl diphosphate (GGDP) to ent-kaurene. The second promoter, belonging to a bHLH transcription factor, was selected due to the presence of two RY elements in its sequence.

After cloning of both promoter sequences, promoter-*GUS* constructs were used in transient assays together with LEC1, FUS3 and ABI3 to measure the activity of the respective promoters. In both cases a minor increase in the activity was observed following co-transformation with FUS3, LEC1 and ABI3, suggesting that these transcription factors bind to the promoter (Figure 3.7). The effect however on the activity of the *ent-kaurene* and *bHLH* promoters was much smaller than that observed on the *USP* and *napin* promoters. This indicates that the RY motifs and overexpression of LEC1, FUS3 and ABI3 is not sufficient to drive an increase of the promoter activity and that possibly other factors must be involved in the activation. The exact regulation mechanism is however still highly obscure since both genetic and molecular data show that these transcription factors can also act as repressors (Bäumlein et al., 1994; Hoecker *et al*., 1995; Kölle, 1998; Nambara *et al*., 2000; Raz *et al*., 2001). It has been shown for instance that Vp1 can activate as well as repress promoters (Hoecker *et al*., 1995). Also the RY element seems to have this dual function. The presence in storage protein promoters is necessary for high level promoter activity in seed tissue while in non-seed tissue it prevents the expression of storage proteins (Bäumlein *et al*., 1992).

The ratio of ABA and GA plays an important role in determining the developmental state of maturing seeds (Koornneef and Karssen, 1994; Leon-Kloosterziel *et al*., 1996; Debeaujon and Koornneef, 2000; White *et al*., 2000). Generally speaking, ABA and GA play antagonistic roles in regulating seed dormancy and germination. ABA establishes dormancy during maturation whereas GA is needed to break ABA-induced dormancy. One may speculate that FUS3, ABI3 and LEC1 play a role in controlling the endogenous levels of GA and that the embryo is kept dormant by repression of the GA pathway. The presence of the RY motif in the *ent-kaurene* promoter whose protein is involved in the GA pathway supports this hypothesis.

4.5 Altered development caused by overexpression of FUS3 in *N. tabacum*

In order to provide further insight into the function of the *FUS3* gene, transgenic *Arabidopsis* and *N. tabacum* plants were generated in which the *FUS3* cDNA was ectopically overexpressed under control of the *35S* promoter. This promoter was chosen to aim for a strong ubiquitous expression in the plant. Overexpression of FUS3 in *N. tabacum* resulted in the accumulation of seed-specific legumin proteins in leaves of plants growing under normal conditions. Legumin proteins were not only deposited in the leaves of the transgenic plants, but they were also processed into α and β subunits. Preliminary results suggest that this is due to the presence and activity of seed-specific proteases. Oleosins and a seed maturation-specific heat shock protein were not found in these leaves, suggesting that the overexpression of FUS3 led to the transfer of only certain, but not all, aspects of seed maturation to non-seed tissue. It has been reported that over-expression of ABI3 in *Arabidopsis* also results in the accumulation of seed-specific proteins, however, this happened only after the exogenous application of ABA (Parcy *et al*., 1994). The differences in accumulation of seed-specific proteins in leaves as a result of ectopic overexpression of FUS3 or ABI3 supports the hypothesis that both transcription factors act in different pathways during seed maturation.

The detection of legumin proteins in leaves of *N. tabacum* is a surprising finding. In transient experiments neither FUS3 nor ABI3 were able to activate the *legumin B4* promoter (see 3.1.3), which may indicate that factors that are absent in the embryogenic cells used for the transient assay, but present in leaves, are necessary for the expression of legumin proteins in the latter tissue. Additional experiments should verify whether the USP proteins and napin proteins are also present in non-embryogenic tissue of tobacco.

The induction of dormancy is one of the main functions of the FUS3 protein during seed maturation. Raz *et al*. (2001) showed that this seed dormancy is secured via at least two sequential processes, embryo growth arrest and embryo dormancy. The embryo growth arrest is regulated by cell division activities which are gradually switched off at the mature embryo stage. Genetic studies showed that this is the result of the function of the FUS3/LEC type genes. Since the presence of legumin proteins demonstrates that the ectopic overexpression of *FUS3* transfers certain aspects of seed maturation to vegetative tissues it is not a surprise to see a dwarf phenotype in many of the overexpressing tobacco plants.

The overexpression of *FUS3* probably down-regulates or inhibits the cell division, in a similar way as during the embryo growth arrest, and results in the dwarf phenotype. Microscopic studies could confirm this hypothesis.

So far, ultra-structural studies to determine the localisation of the legumin proteins inside the cell have not been successful. However, these studies revealed that mitochondria in the leaves of *FUS3* overexpressing plants have a remarkable change in morphology. While mitochondria in wild type *N. tabacum* plants are round shaped, mitochondria in the transgenic plants were twisted around their own axis, forming what seems to be an invagination. Altered mitochondria morphology in transgenic tobacco plants have been previously reported (Farbos *et al*., 2001).

In addition to altered protein accumulation, the *fus3* mutant exhibits also defects in dormancy and desiccation. It is possible, therefore, that elements related to these processes have also been affected in the *FUS3* over-expressing plants, but this remains to be shown. Moreover, in *Daucus carota*, it has been found that ectopic expression of *ABI3* homologue in suspension cultures results in an increased desiccation tolerance (Shiota and Kamada, 2000).

Arabidopsis lines that ectopically contained higher levels of *FUS3* mRNA showed no altered phenotype. Additionally, an extensive screening for differences in the up- or down-regulation of 8000 genes spotted on a filter revealed no differences between transgenic and wild-type plants. It is unclear why the overexpression of *FUS3* in the homologous *Arabidopsis* background does not lead to similar alterations as those observed in the transgenic *N. tabacum* plants. So far, it remains unknown whether the over-expression of the *FUS3* cDNA in these *Arabidopsis* plants corresponds to an increase in the FUS3 protein.

4.6 The *ET* gene family

4.6.1 The structure of the *Arabidopsis ET* gene family

A South-western screening of seed-specific cDNA libraries from *B. napus* and *V. faba* using seed-specific promoter elements, resulted in the isolation of two similar proteins denoted as BnET and VfET, respectively (Wohlfarth, 1996; Ellerström, 1998). Searches in the available databases demonstrated that three similar genes are present in the *Arabidopsis* genome.

One of them positioned on chromosome 4 and denoted as *AtET4*, while the other two are positioned on chromosome 5 and denoted as *AtET51* and *AtET52*. The intron/exon structure of these genes is quite similar suggesting that they arose as result of a duplication event in the *Arabidopsis* genome (Figure 3.12). The three open-reading frames of *AtET4* all contain in-frame stop-codons and miss a start codon, therefore this gene most likely represents a non-functional pseudogene (Figure 3.12). The predicted protein sequences of AtET51 and AtET52 are highly similar to the previously isolated proteins BnET and VfET (Figure 3.13). However AtET52 is much shorter and misses a conserved C-terminal part present in the 3 other proteins and seems therefore to be a truncated version of these proteins.

4.6.2 A putative Zinc-finger domain

Interestingly the C-terminal part of all ET proteins, except AtET52, is characterised by having two or four repeats of the amino acid sequence, $CX_9CX_9RCX_2HK$. An alternating structure of cysteine and histidine residues with different length of amino acids (spacers) between them is commonly known as a Zn-finger binding protein. The common theme of these Zn-finger binding proteins is that a zinc-ion stabilises a scaffold, the Zn-finger, that holds a region of the protein, containing the cysteine and histidine residues, in the proper position for sequence-specific interaction with DNA, RNA or with another protein (Coleman, 1992). Because mass spectrophotometric experiments demonstrated that a synthetic peptide, corresponding to the alternating structure of three cysteine and one histidine residue, had the capability to bind Zn^{2+} with a high affinity (Ellerström, 1998), also this structure of the ET proteins was suggested to represent a Zn-finger.

Zn-finger proteins are classified according to the order and number of the cysteine and histidine residues in the finger (Schmiedeskamp *et al*., 1994; Takatsuji, 1998). The amino acid sequence structure as seen in the ET proteins probably represents a totally new class of Zn-finger binding proteins.

Proteins with a similar sequence are present in the *Arabidopsis* genome (Figure 3.14) but the sequence only occurs once in these protein sequences. Also in barley and rice proteins were identified containing the amino acid sequence $CX_9CX_9RCX_2HK$. Interestingly both the rice and barley proteins were able to repress GA-mediated promoter activation (Raventos *et al*., 1998; van der Knaap *et al*., 2000). Database searches show as well the presence of this structure in one bacteria, *Pyrococcus abyssi* and in one plant virus (Figure 3.14).

Another type of Zn-finger proteins with some similarity to the ET-family are the C_3H proteins including the embryonically active gene from *Drosophila*, *unkempt* (Mohler *et al*., 1992), and *Nup475* first described as an early gene, immediately induced in response to serum or mitotic agents (Worthington *et al*., 1996). This family is characterised by the consensus amino acid sequence $CX_8CX_5CX_3H$. This family shares with the ET family the order of cysteine and histidine residues as well as the preference for a conserved glycine residue between the first and second as well as between the second and third cysteine residue. Both *unkempt* and *Nup475* have, by genetic means, been shown to be physiologically important but their exact functions are not yet known (Mohler *et al*., 1992; Worthington *et al*., 1996).

4.6.3 Functional characterisation of BnET

Both the BnET and the VfET proteins were isolated by their ability to bind to a mixture of probes corresponding to seed-specific *napin*, *USP* or *legumin B4* promoters. The independent isolation from two different species using two different but seed-specific promoters resulting in similar factors indicates that both ET proteins are involved in seed-specific gene regulation. Therefore transient expression assays with BnET were performed to study a potential effect on seed-storage protein promoters. However no indications of an effect of BnET on the *napin* promoter was found also not when the well characterised transcription factors LEC1, FUS3 and ABI3 were included in these experiments. A better idea about the function of the ET proteins was obtained by the generation of transgenic *N. tabacum* plants overexpressing BnET under control of a *35S* promoter. Similarly as with the transgenic FUS3 plants the *35S* promoter was chosen to aim for a strong ubiquitous expression in the plant. Experiments to aim for a more seed-specific overexpression under control of the *USP* promoter are in progress.

The *N. tabacum* plants displayed, due to the overexpression of BnET, a dwarf growth habit (Figure 3.17). The dwarf *N. tabacum* plants had the same number of leaves as compared to wild-type plants which suggested that the decrease in height was the result of a reduction in internode length. Microscopical analysis of the morphology of the stem cells showed that this reduction in internode length was not the result of a reduction in cell elongation. Apparently cell size in the transgenic plants has not been drastically disturbed as compared to the wild-type plants (Figure 3.18e/g), suggesting rather a reduction in cell division. This reduction in cell division might also cause the ruptured pith of the stem.

The elongating non-dividing cells collapse as the internodes expand. This suggestion was further supported by an *in vitro* experiment investigating the effect of the transgene on the growth of other parts of the plant. The root, callus and shoot were induced in a medium containing various concentrations of auxin and cytokinin. Cytokinins promote (in synergism with auxins) cell division and auxins promote cell enlargement (Jacobs, 1997; Gray and Estelle, 1998). The ratio of cytokinin to auxin determines whether tissue grown in culture will be root tissue or shoot tissue: high cytokinin favours shoot formation, high auxin favours root formation (Skoog and Miller, 1957). The growth of roots, callus and shoots was comparable in wild-type tobacco and tobacco plants overexpressing an antisense construct of BnET (Figure 3.20). However no root, callus or shoot formation was observed in the tobacco plants ectopically overexpressing BnET. It seems that the ectopical overexpression of BnET either directly inhibits cell division or affects the responsiveness of the cells to either auxin or cytokinin or both plant hormones. This insensitivity results in the lack of cell division.

A further microscopical analysis of the stem of BnET overexpressing plants demonstrated that secondary cell wall thickenings of the tracheary elements were severely reduced (Figure 3.18). The differentiation of the tracheary elements begins as a parenchym cell where after the cell reaches maturity. Upon cell maturity secondary wall deposition begins, mainly in the form of lignin followed by wall lysis and cell autolysis also known as cell death (Torrey *et al*., 1971; Fukuda, 1996). The severe reduction in secondary wall deposition suggest that most cells of the tracheary elements are not yet in the mature stage and therefore do not start with the final process of secondary wall deposition. The inhibition of cell division observed from the leaf explants ectopically overexpressing BnET strongly accounts for this suggestion.

A similar protein of ET family in barley, HRT, was isolated due to its ability to bind a gibberellic acid response element (Raventos *et al*., 1998). This HRT protein was also able to repress the activation of several promoters, among them the GA-induced α-amylase promoter. This data suggested that the ET-family of proteins might have a similar function. Therefore in a transient assay the effect of overexpression of BnET on a GA-inducible aquaporin promoter from *N. tabacum* was tested. Remarkably overexpression of BnET resulted in a reduction of the GA-mediated activation of the promoter. It seems that the ET proteins can as well reduce the GA-mediated response of certain promoters.

Analysis of the expression pattern of the *ET* genes in *Arabidopsis* and *V. faba* (Wohlfarth, 1998) shows that both *AtET51* and *VfET* (Wohlfarth, 1996) have a very similar expression pattern.

Both have high expression levels during flower development and early embryogenesis and low expression levels during middle- and late-embryogenesis (Figure 3.15). During the later stages of flower development embryogenesis starts with the fertilisation of the egg cell. Following fertilisation the pattern formation of the embryo starts through a balance between regular cell divisions and cell elongation. The timing and rate of cell divisions are crucial for the pattern formation. Since it has been suggested that the ET protein is involved in regulating cell division this invites the speculation about a possible role of the ET proteins in the timing and rate of cell division. This speculation is supported by the fact that in late-embryogenesis no cell division takes place anymore and also a very low level of ET transcript can be detected. Interestingly the previously mentioned genes, *unkempt* (Mohler *et al*., 1992) and *Nup475* (Worthington *et al*., 1996), belonging to the C_3H (see 4.7.2) family are also hypothesised to play a role in cell division since they are induced in response to mitogenic agents (Mohler *et al*., 1992; Worthington *et al*., 1996).

Finally, the seed germination in the *35S* overexpressing BnET plants is severely reduced. After the induction of dormancy in the mature seed no cell division occurs anymore. Gibberellic acid plays an important role in breaking this induced seed dormany resulting in germination and continuation of cell division (Koornneef and Karssen, 1994; Leon-Kloosterziel *et al*., 1996; Debeaujon and Koornneef, 2000; White *et al*., 2000). The previous data show that overexpression of BnET inhibits both the cell division and the GA-mediated response of promoters. Therefore it is not unlikely that both of these processes might account for the observed reduced seed germination.

5. Conclusions and outlook

FUS3 is, in concert with ABI3 and LEC1, an important component of the regulatory pathway controlling late embryogenesis as proven by several genetic studies (Bäumlein *et al*., 1994; Kölle 1998; Parcy *et al*., 1997; Nambara *et al*., 2000; Raz *et al*., 2001). Taken all together, the data in this study demonstrate that the transcription factors FUS3, LEC1 and ABI3 act not only as switches between developmental pathways, but also continue to be necessary for the maintenance of the initiated pathway (Figure 4.1a). This is in contrast to the view propagated by Harada (Lotan *et al*., 1998), who suggested that these transcription factors act only as developmental switches and do not play a role in the further maintenance of seed maturation (Figure 4.1b). Furthermore, it was proven that FUS3 and ABI3 can act alone and through the RY-promoter element to control gene expression, as well as with each other and in concert with LEC1. The RY element was proven to be a direct target of the FUS3 protein. Therefore, the promoters of all annotated genes of the *Arabidopsis* genome were screened for the presence of a RY element in their sequence. In this way potential downstream regulated genes were identified. With the help of this list further investigations will focus on the hierarchic pattern of FUS3 dependent downstream genes.

The functions of FUS3 have been considered to be specific for the maturation pathway, since mutant seeds harvested prior to desiccation develop to normal vegetative plants (Müller, 1963). Due to the low abundance of the FUS3 message the transcript has been detected by Northern hybridisation mainly during seed development but also in other tissues (Luerssen *et al*., 1998). Using the more sensitive RT-PCR technique the FUS3 transcript can be clearly found in non-seed tissue (Reidt *et al*., 2001). These preliminary data invite the speculation that the *FUS3* gene is involved in additional functions in non-seed tissue. This idea is supported by recent evidence showing that ABI3 also functions in determining plastid identity in the shoot apex and in the regulation of flowering time (Rohde *et al*., 1999, 2000; Kurup *et al*., 2000). Present studies focusing on true-null mutations, in the form of a T-DNA insertion line, might shed some light on an additional function of FUS3 in vegetative processes.

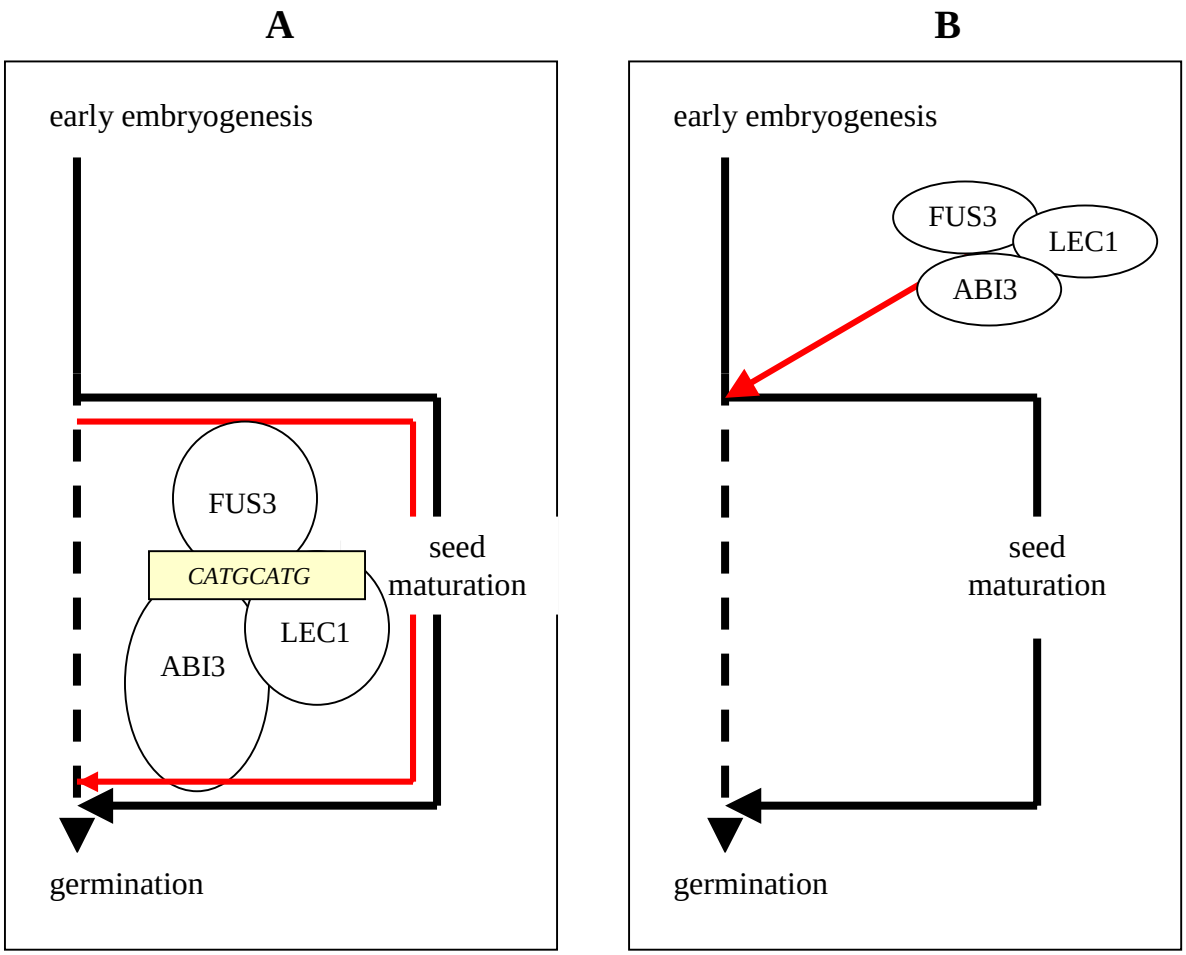


Figure 5.1: *Schematical representation of the regulation of seed maturation as demonstrated in this study (A) and a previous genetical view (B).*
FUS3, ABI3 and LEC1 prevent the embryo for directly germinating after completing the early embryogenesis by inducing seed maturation. The RY element is a direct target for these transcription factors. FUS3, ABI3 and LEC1 afterwards also maintain seed maturation (A) whereas in previous view (B) they were thought to be only necessary only for the start of seed maturation

The family of ET proteins, which were originally isolated by their capability to bind to certain seed-specific promoter elements, are characterised by having two or more repeats of the amino acid sequence $CX_9CX_9RCX_2HK$. This element can bind Zn^{2+} and is therefore hypothesised to represent a novel Zn-finger family. ET proteins may have a role in regulating cell division as demonstrated by overexpression of one of these proteins, BnET, in *N. tabacum*. It is not know how the ET proteins regulate cell division but it could be that the auxin/cytokinin sensing is affected. Leaf explants from overexpressing BnET plants were not able to regenerate *in vitro* on a medium with different concentrations of auxin and cytokinin.

It remains unclear what is the function of the ET proteins on storage protein promoters, their source of isolation. Seed-specific overexpression under the *USP* promoter with sense and antisense construct of the *ET*-gene might clarify this. Also ongoing experiments to identify a T-DNA insertion line would be helpful in solving this question.

6. Abstract

Late-embryogenesis is characterised by the accumulation of large amounts of storage proteins, the acquisation of desiccation tolerance and the induction of dormancy. Genetic studies demonstrated that these processes are controlled by the transcription factors LEC1, FUS3 and ABI3 and the plant hormone ABA. Mutations in any of these loci or a reduced amount of ABA result into disruption of late embryogenesis, resulting in defects of the storage protein synthesis, chlorophyll breakdown, anthocyanin biosynthesis, precocious germination and desiccation intolerance. The exact way in which these proteins interact and their role in response to ABA was unknown. Therefore the present study was focused on the molecular characterisation of FUS3, LEC1 and ABI3 in an attempt to clarify the interaction of these transcription factors and their role in late-embryogenesis.

To investigate the potential interaction between FUS3, ABI3 and LEC1 and their function on the activation of seed storage protein promoters a transient expression system in tobacco protoplasts was established. With the help of this system it was shown that the FUS3- and ABI3-gene products can induce independently of each other storage protein promoters. The LEC1 protein was not sufficient for an induction of storage protein promoters. Moreover, it was shown that the FUS3 and LEC1 proteins enhance the ABI3 mediated promoter activation. No synergism was detected between FUS3 and LEC1. The FUS3 and ABI3 mediated increase in activity of storage protein promoters could be strongly reduced when a conserved sequence element, the RY motif, was mutated. The suggestion that this motif could therefore be a direct target of the FUS3 protein was confirmed by band-shift assays.

Analysis of the response of FUS3 and ABI3 to ABA demonstrated that in contrast to the enhancing effect of ABA on the ABI3 mediated promoter activation, ABA had an repressive effect on the FUS3 mediated promoter activation. A further characterisation of the interaction between the FUS3, LEC1 and ABI3 proteins with the two hybrid system showed that the FUS3 and LEC1 proteins interact with each other. This in contrast to FUS3 and ABI3. The identification of the RY element as a direct target of the FUS3 protein prompted the screening of promoters from the annotated genes on chromosomes two and four of the *Arabidopsis* genome. Using this approach fifty-five RY containing promoters and their corresponding genes were identified. Two of these promoters were tested for their activation by FUS3, LEC1 and ABI3. However only a minor induction of both promoters was observed and possibly other factors must be involved in the activation. In order to provide further insight into the function of the FUS3 protein, transgenic *N. tabacum* plants were generated in which the FUS3 cDNA was ectopically overexpressed. This overexpression resulted into the accumulation of legumin proteins in the leaves. Furthermore mitochondria had a change in morphology. They were twisted around their own axis, forming what looked like an invagination. The transfer of other aspects of 'seed maturation syndrome' to the leaves is under investigation.

Screening for additional factors regulating seed storage protein expression resulted into the isolation of two proteins one from *B. napus*, BnET and one from *V. faba*, VfET. Both proteins had a low but significant homology to each other and contained a repeated conserved amino acid sequence. Three genes displaying sequence similarity to the cDNA sequence of BnET and VfET are also present in the *Arabidopsis* genome. The predicted protein sequence of one of this genes, *AtET51*, has the highest homology as compared to the predicted protein sequences of BnET and VfET. From the other two genes, one seems to represent a pseudogene while the predicted protein from the other gene, *AtET52*, looks like a truncated version of the BnET and VfET proteins.The C-terminal part of all ET proteins, except AtET52, is characterised by having two or four repeats of the amino acid sequence, $CX_9CX_9RCX_2HK$. This structure of the ET proteins is suggested to represent a novel uncharacterised Zn-finger. Analysis of the expression pattern of AtET51 and VfET showed that both genes are ubiquitous expressed but have lower expression levels during late embryogenesis. Ectopical overexpression of BnET in *N. tabacum* resulted into plants with a strong dwarf phenotype. Microscopical analysis demonstrated that the secondary cell wall thickenings of the tracheary elements from these dwarf plants was severely reduced.

Investigations of the effect of ectopical overexpression of BnET on the overall growth of the plant showed that the auxin and cytokinin depedent root and shoot formation was severely disturbed. Moreover this overexpression resulted into a severe reduction of seed germination. Although the function of this protein is still speculative it may play a role in the repression of cell division.

7. Zusammenfassung

Die späte Embryogenese und Samenreifung ist im Wesentlichen durch drei Prozesse gekennzeichnet: a) Akkumulation von Speicherstoffen, wobei artabhängig Proteine, Kohlenhydrate oder Fette dominieren, b) Erwerb der Austrocknungstoleranz aller Gewebe und c) Ausprägung der Samenruhe (Dormanz). Genetische Untersuchungen belegen, dass die Transkriptionsfaktoren (TF) FUS3, ABI3 und LEC1 sowie das Pflanzenhormon Abscisinsäure (ABA) wesentlich an der Kontrolle der Prozesse der späten Embryogenese beteiligt sind. Mutanten in den Genen aller drei TF sowie erniedrigte ABA-Konzentrationen führen z. B. zu verringerter Akkumulation von Speicherproteinen und aberranter Anthocyanin-Synthese, zur Verzögerung des Chlorophyllabbaus im reifenden Samen, zu vorzeitiger Keimung sowie zum Verlust der Austrocknungstoleranz.

Die regulatorischen Interaktionen der drei TF untereinander bzw. mit ihren *target*-Genen sowie die Wechselwirkung mit ABA waren und sind weitgehend unklar. Ziel der vorliegenden Arbeit ist es, einen Beitrag zu deren Aufklärung leisten. Frühere Arbeiten haben gezeigt, dass in den Samen der *fus3*-Mutante (ebenso wie in denen der *abi3*- und *lec1*-Mutanten) die Synthese und Akkumulation der Samenproteine weitgehend reduziert ist. Auch war bekannt, dass die Zerstörung eines in den Promotoren samenspezifischer Gene vorkommenden, konservierten *cis*-Elements (RY-Element) zum Verlust der Embryogenese-spezifischen Genexpression führt. Beide Befunde zusammen führten zu der Hypothese, dass RY-Promotorelemente das direkte Bindungsmotif des FUS3 TF sind. Für die Überprüfung diese Hypothese wurde ein transientes Expressionsystem auf der Basis von isolierten Tabak-Protoplasten etabliert und angewandt. Die Ergebnisse zeigen, dass in diesem

Expressionssystem die TF FUS3 und ABI3 unabhängig voneinander die Aktivität von Samenprotein-Genen induzieren. Als Einzelfaktor ist LEC1 dazu nicht in der Lage. Allerdings sind die TF FUS3 und LEC1 fähig, die ABI3 induzierte Promotoraktivierung synergistisch weiter zu erhöhen. Kein Synergismus war zwischen FUS3 und LEC1 nachweisbar. Die FUS3- und ABI3-abhängige Promotoraktivierung konnte durch die mutative Zerstörung des RY-Elements weitgehend reduziert werden. Dies führte zu der Schlussfolgerung, dass FUS3 und ABI3 ein intaktes RY-Element für die Regulation von *downstream*-Genen benötigen. Die physische Interaktion zwischen dem FUS3-TF und dem intakten RY-Promotorelement wurde durch *band shift*-Experimente gezeigt. Die Nutzung des transienten Expressionssystems bestätigt frühere Befunde, dass die ABI3 induzierte Promotoraktivierung durch ABA-Zugabe weiter erhöht wird. Bemerkenswert ist aber der Befund, dass die FUS3 induzierte Promotoraktivierung durch ABA reprimiert wird. Die genetischen Analysen der Mutanten (*fus3, abi3, lec1*) legen eine enge Interaktion der drei Faktoren untereinander nahe. Um dies näher zu untersuchen, wurde die Interaktion der Faktoren im *yeast-two hybrid*-System untersucht. Die bisherigen Ergebnisse belegen eine direkte Wechselwirkung zwischen den TF FUS3 und LEC1, nicht aber zwischen FUS3 und ABI3.

Die Charakterisierung von RY-Elementen als direkte Bindungsstellen des TF FUS3 sowie die Verfügbarkeit der vollständigen genomischen Sequenz von *Arabidopsis* initiierte die Nutzung der Bioinformatik für die Identifizierung RY-enthaltender Promotoren aller der zunächst auf Chromosom II und IV annotierter Gene. Bisher wurden 55 Gene identifiziert. Zwei dieser Promotoren wurden im transienten Expressionssystem auf Induktion durch die drei TF getestet. Die geringe Induktion lässt die Notwendigkeit weiterer Faktoren vermuten.

Für eine weiterführende funktionelle Analyse des FUS3 TF wurde die cDNA in transgenen Tabakpflanzen unter Kontrolle des CaMV 35S-Genpromotors exprimiert. Diese ektopische Expression führt bei Tabak zur Synthese und Akkumulation des 12S-Samenproteins Legumin in Blättern. Bemerkenswert ist, dass dabei der 60-kD *precursor* korrekt in die α- und β-Untereinheit prozessiert wird, was die Anwesenheit einer entsprechenden *processing*-Protease voraussetzt. Elektronenmikroskopische Untersuchungen zeigen darüber hinaus, dass es zu bisher nicht näher interpretierbaren Veränderungen in der Morphologie der Mitochondrien (Invagination) kommt. Gegenwärtig noch andauernde Arbeiten sollen klären, ob durch die ektopische Synthese von FUS3 noch weitere Aspekte eines postulierten „Samensyndrom“ auf Nichtsamen-Gewebe übertragbar sind.

Das gleiche durch den CaMV 35S-Genpromotor gesteuerte FUS3-cDNA-Konstrukt wurde in transgenen *Arabidopsis*-Pflanzen untersucht. Bei *reversed northern*-Experimente mit 8000 *Arabidopsis*-Gensequenzen konnte in drei unabhängigen transgenen Linien keine Veränderung der Genexpression durch die ektopische Expression des FUS3 TF nachgewiesen werden. Möglicherweise verhindert ein nur im homologen System wirksamer Repressor die Funktion von FUS3 in Nichtsamen-Geweben.
Unabhängige *southwestern* Experimente mit Promotorelementen samenspezifischer Gene und Kotyledonen-spezifischen cDNA-Expressionsbanken aus *Vicia faba* und *Brassica napus* führten zur Isolierung von Mitgliedern einer neuen, Zink-bindenden TF-Familie. Die beiden Proteine VfET und BnET haben charakteristische C-terminale Cystein-Histidin (CH) *repeats* mit einem als HRT (*hordeum repressor of transcription*) bezeichneten Protein aus Gerste gemeinsam. Der Gerstenfaktor ist an der Repression Gibberellin (GA)-induzierter Genexpression beteiligt. Das Protein aus *Vicia faba* besitzt zwei, das Protein aus Gerste drei und das Protein aus *Brassica napus* vier CH-repeats mit der hochkonservierten Struktur $CX_9CX_9RCX_2HK$.

Im *Arabidopsis*-Genom werden drei ET-ähnliche Gene gefunden. Eines dieser Gene auf Chromosom IV ist offensichtlich ein durch mehrere Stopcodonen unterbrochenes inaktives Pseudogen. Die beiden anderen Gene liegen in enger Nachbarschaft auf Chromosom V. Eines davon repräsentiert ein funktionsfähiges Gen. Das andere Gen kodiert für eine um die CH-repeats verkürzte Version des Proteins. Gegenwärtige Arbeiten konzentrieren sich auf die Analyse einer T-DNA-Insertionslinie.

Die ektopische Expression von BnET-cDNA unter Kontrolle des CaMV 35S-Genpromotors in Tabak- und *Arabidopsis*-Pflanzen führt in beiden Arten übereinstimmend zu ausgeprägtem Zwergwachstum. Die mikroskopische Analyse zeigt, dass in den transgenen Tabakpflanzen die sekundäre Zellwandverdickung der Gefäßelemente beträchtlich reduziert ist. Darüber hinaus wird in den transgenen Tabakpflanzen die Auxin- bzw. Cytokinin-induzierte Wurzel- bzw. Sprossbildung vollständig unterdrückt, und schließlich führt die Überexpression des BnET-Gens zur Unterdrückung der Keimung. Wenngleich die Funktion des ET-TF noch weitgehend unklar ist, wird eine Funktion bei der Repression GA-vermittelter Induktion der Zellteilung postuliert.

8. References

Aryan A. P., An G. and Okita T. O. (1991). Structural and functional analysis of promoter from gliadin, an endosperm-specific storage protein gene of *Triticum aestivum* L.. *Mol. Gen. Genet.* **225,** pp. 65-71.

Ausubel F. N., Brent R., Kingston R. E., Moore D. D., Seidman J. G., Smith J. A., Struhl K. (1996). *Current Protocols in Molecular Biology*, eds. John Wiley and Sons, New York.

Baker J. C. Steele C. and Dure, L. S. (1988). Sequence and characterisation of 6 LEA proteins and their genes from cotton. *Plant Molecular Biology* 11, pp. 277-291.

Bard J. (1994). Embryos: Color Atlas of Development. London: Wolfe.

Bäumlein H., Wobus U., Pustell J. and Kafatos (1986). The legumin gene family: structure of a B-type gene of *Vicia faba* and a possible legumin gene specific regulatory element. *Nucleic Acids Research* **14**, pp. 2707-2720.

Bäumlein H., Boerjan W., Nagy I., Bassüner R., van Montagu M., Inzé D.and Wobus U. (1991a). A novel seed protein gene from *Vicia faba* is developmentally regulated in transgenic tobacco and *Arabidopsis* plants. *Mol. Gen. Genet.* **225**, pp. 459-467**.**

Bäumlein H., Boerjan W., Nagy I., Panitz R., Inzé D. and Wobus U. (1991b). Upstream sequences regulating legumin gene expression in heterologous transgenic plants. *Mol. Gen. Genet.* **225**, pp. 121-128.

Bäumlein H., Nagy I., Villarroel R., Inze D. and Wobus U. (1992). *Cis*-analysis of a seed promoter: The seed protein gene specific RY repeat CATGCATG is essential for expression of a legumin gene. *Plant Journal* **2**, pp. 233-239.

Bäumlein H., Miserá S., Luerßen H., Kölle K., Horstmann C., Wobus U. and Müller A. J. (1994). The *FUS3* gene of *Arabidopsis thaliana* is a regulator of gene expression during late embryogenesis. *Plant Journal* **6**, pp. 379-387.

Bechthold N., Ellis J. and Pelletier G. (1993). *In planta Agrobacterium* mediated transfer by infiltration of adult *Arabidopsis thaliana* plants. *CR Academy of Sciences Paris* **316**, pp. 1194-1199.

Berleth T. and Jürgens G. (1993). The role of *MONOPTEROUS* gene in organizing the basal body region of the Arabidopsis embryo. *Development* **118**, pp. 575-587.

Bobb A. J., Eiben H. G. and Bustos M. M. (1995). PvAlf, an embryo specific acidic transcriptional activator enhances gene expression from phaseolin and phytohemagglutinin promoters. *Plant Journal* **8**, pp. 331-343.

Bobb A. J. M., Chern S. and Bustos M. M. (1997). Conserved RY repeats mediate transactivation of seed-specific promoters by the developmental regulator PvALF. *Nucl. Acids Res*. **25**, pp. 641-647.

Black M. (1991). Involvement of ABA in the physiology of developing and mature seeds. In: *Absiscic Acid: Physiology and Biochemistry*, Davies W. J.and Jones, H. G., eds. (Oxford: Bios Scientific Publishers Ltd.), pp. 99-124.

Bostock R. M. and Quatrano R. S. (1992)**.** Regulation of Em expression in rice: interaction between osmotic stress and abscisic acid. *Journal of Plant Physiology* **98**, pp. 1356-1363.

Berleth T. and Jürgens G. (1993). The role *monopteros* gene in organising the basal body region of the *Arabidopsis* embryo. *Development* **118**, pp. 575-587.

Bewley J. D. (1995). *Seeds. Physiology of development and Germination. Second edition* (New York : Plenum Press).

Bowman J. (1993). Arabidopsis: An Atlas of Morphology and Development. New York: Springer-Verlag.

Browse J. (1998). Synthesis and storage of fatty acids. In: *Cellular and Molecular Biology of Plant Seed Development*, Larkins B.A. and Vasil I. K. (eds).

Carson C. B., Hattori T., Rosenkrans L., Vasil V., Vasil I. K., Peterson P. A. and McCarty D. R. (1997). The quiescent/colourless alleles of viviparous1 show that the conserved B3 domain of VP1 is not essential for ABA-regulated gene expression in the seed. *Plant J*. **12**, pp. 1231-1240.

Chang J. F., Phillips K., Lunback T., Gstaiger M., Ladbury J. E. and Luisi B. (1999). October-1 POU and octamer DNA cooperate to recognise the Bob-1 transcription co-activator via induced folding. *Journal of Molecular Biology* **288**, pp. 941-952.

Cheng J. V., Lertpiriyapong K., Wang S. and Sung R. Z. (2000)**.** The role of the Arabidopsis *ELD1* gene in cell development and photomorphogenesis in darkness. *Plant Phyiology* **123**, pp. 509-520.

Church G. M. and Gilbert W. (1984). Genomic sequencing. *Proceedings of the National Academy of Sciences USA* **81**, pp. 1991-1995.

Cepek K. L., Chasman D. I., Sharp P. A. (1996). Sequence-specific DNA binding of the B-cell specific coactivator OCA-B. *Genes Dev*. **10**, pp. 2079-2088.

Chamberland S. N., Daigle and Bernier F. (1992). The legumin boxes and the 3` part of a soybean beta-conglycinin promoter are involved in seed gene expression in transgenic tobacco plants. *Plant Mol. Biol*. **19**, pp. 937-949.

Chang J. F., Phillips K, Lundback T., Gstaiger M., Ladbury J. E., Luisi B. (1999). Oct-1 POU and octamer DNA co-operate to recognise the Bob-1 transcription co-activator via induced folding. *J. Mol. Biol*. **288**, pp. 941-952.

Cheng J-C., Seeley K. A., Sung Z. R. (1995). *RML1* and *RML2 Arabidopsis* genes required for cell proliferation at the root tip. *Plant Physiology* **107**, pp. 365-376.

Chern M. S., Bobb A. J., Bustos M. M. (1996). The regulator of MAT2 (ROM2) protein binds to early maturation promoters and represses PvALF-activated transcription. *Plant Cell* **8(2)**: pp. 305-321.

Chlan C. A. and Dure L. (1983). Plant seed embryogenesis as a tool for molecular biology. *Mol. Cell. Biochem.* **55(1)**: pp. 5-15.

Crouch M. L. and Sussex, I. M. (1981). Development and storage protein synthesis in *Brassica napus* L. embryos in vivo and in vitro. *Planta* **153**, pp. 64-74.

Coleman J. E. (1992). Zinc proteins: Enzymes, storage proteins, transcription factors and replication proteins. *Annual review of Biochemistry* **61**, pp. 897-946.

Debeaujon I. and Koornneef M. (2000). Gibberellin requirement for *Arabidopsis* seed germination is determined both by testa characteristics and by embryonic abscisic acid. *Plant Physiology* **122**, pp. 415-424.

Dickinson C. D., Evans R. P. and Nielsen N. C. (1988). RY repeats are conserved in the 5´-flanking regions of legume seed-protein genes. *Nucleic Acids Research* **16**, pp. 371.

Dooner H. K. (1985). *Viviparous-1* mutations in maize conditions pleiotrophic enzyme deficiencies in the aleurone. Plant Physiology 77, pp. 486-488.

Dure, L. S. (1993). A repeating 11-mer amino acid motif and plant desiccation. *Plant Journal* **3(3)**, pp. 363-369.

Dure L. S. (1997). Lea proteins and desiccation tolerance in seeds. In: Cellular and Molecular Biology of Plant Seed Development, Larkins B.A. and Vasil I. K. (eds).

Drews D. G. and Goldberg R. B. (1989). Genetic control of flower development. *Trends in Genetics* **5**, pp. 256-261.

Ellerström M., Stålberg K., Ezcurra I. and Rask L. (1996). Functional dissection of a napin gene promoter: identification of promoter elements required for embryo and endosperm specific transcription. *Plant Mol. Biol*. **32**, pp. 1019-1027.

Ellerström M. (1998). Studies on mechanisms of plant seed-specific gene regulation. Doctoral thesis. Swedish University of Agricultural Sciences Uppsala.

Espelund M., Saeboe-Larssen S., Hughes D. W., Galau G. A., Larsen F., Jakobsen K. S. (1992). Late embryogenesis-abundant genes encoding proteins with different numbers of hydrophilic repeats are regulated differentially by abscisic acid and osmotic stress. *Plant Journal* **2(2),** pp**.** 241-252.

Ezcurra I., Wycliffe P., Nehlin L., Ellerström M. and Rask L. (2000). Transactivation of the *Brassica napus* napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different *cis*-elements: B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G box. *Plant Journal* **24**, pp. 57-66.

Farbos I., Mouras A., Bereterbide A., Glimelius K. (2001). Defective cell proliferation in the floral meristem of alloplasmic plants of Nicotiana tabacum leads to abnormal floral organ development and male sterility. *Plant Journal* **26(2)**, pp.131-142.

Feldbrugge M., Sprenger M., Dinkelbach M., Yazaki K., Harter K., Weisshaar B. (1994). Functional analysis of a light-responsive plant bZIP transcriptional regulator. *Plant Cell* **6(11),** pp. 1607-1621.

Fiedler U., Filistein R., Wobus U. and Bäumlein H. (1993). A complex ensemble of *cis*-regulatory elements controls the expression of a *Vicia faba* non-storage seed protein gene. *Plant Mol. Biol*. **22**, pp. 669-679.

Finkelstein R. R. (1994). Mutations at two new Arabidopsis ABA response loci are similar to the abi3 mutations. *Plant Journal* **5**, pp. 765-771.

Finkelstein R. R. and Sommerville, C. R. (1990). Three classes of absiscic acid (ABA) insensitive mutations in *Arabidopsis* define genes that control overlapping subsets of ABA responses. *Plant Physiology* **94**, pp. 1172-1179.

Fujiwara T. and Beachy B. N. (1994). Tissue-specific and temporal regulation of a beta-conglycinin gene: Roles of the RY repeat and other *cis*-acting elements. *Plant Mol. Biol.* **24**, pp. 261-272.

Galau G. A., Jakobsen, K. S. and Hughes A. D. (1991). The controls of late dicot embryogenesis and early germination. *Plant Physiology* **81**, pp. 280-288.

Gietz D., St Jean A., Woods R. A. and Schiestl R.H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Research* **25;20(6)**, pp. 1425.

Giraudat J., Hauge B. M., Valon C., Smalle J., Parcy F. and Goodman H. M. (1992). Isolation of the *Arabidopsis* ABI3 gene by positional cloning. *Plant Cell* **4**, pp. 1251-1261.

Giraudat, J., Parcy F., Bertauche N., Gosti F., Leung J., Morris P. C., Bouvier-Durand M. and Vartanian, N. (1994). Current advances in absiscic acid action and signaling. *Plant Molecular Biology* **26**, pp. 1557-1577.

Goldberg R. B., Hoschek G., Vodkin L.O. (1983). An insertion sequence blocks the expression of a soybean lectin gene. *Cell* **33(2)** , pp. 465-475.

Goldberg R. **B., de Paiva G., and Yadegari R.** (1988). Plant embryogenesis, body plan elaboration and preparation for life after the seed. *Science* **240**, pp. 1460-1466.

Hake S. and Meyerowitz E. M. (1998). Growth and development: growing up green. Current Opinions in Plant Biology **1(1)**, pp**.** 9-11.

Harada, **J. J.** (1998). Seed maturation and control of germination. In: *Cellular and Molecular Biology of Plant Seed Development*, Larkins B.A. and Vasil I. K. (eds).

Hatzopolous P., Franz G., Choy L. and Sung R. Z. (1990). Interactions of nuclear factors with upstream sequences of lipid body membrane protein from carrot. *Plant Cell* **2**, pp. 457-467.

Hattori T., Terada T. and Hamasuna S. T. (1994). Sequence and functional analyses of the rice homologous to the maize *Vp1*. *Plant Molecular Biology* **24**, pp. 805-810.

Helliwell C. A., Sheldon C. C., Olive, M. R., Walker A. R., Zeevaart J. A. D., Peacock W. J. and Dennis E. S. (1998). Cloning of the Arabidopsis ent-kaurene oxidase gene GA3. *Proceedings of the National Academy of Sciences USA* **95**, pp. 9019-9024.

Herman E. **M.** (1995). Cell and molecular biology of seed oil bodies. In: *Seed development and Germination*. Kigel J. and Galili G. (eds.). M. Dekker Inc. New York, pp. 195-214.

Higgins T. J. V. (1984). Synthesis and regulation of major proteins in seeds. *Annual review of Plant Physiology* **35**, pp. 191-221.

Hill J., Donald K. A., Griffiths D. E. and Donald G. (1991). DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Research* **25;19(20)**, pp. 5791

Hoecker U., **Vasil I. K., McCarty D. R.** (1995). Integrated control of seed maturation and germination programs by activator and repressor functions of Viviparous-1 of maize. *Genes and Development* **9**, pp. 2459-2469.

Hötgen R. and Willmitzer L. (1988). Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Research* **16(20)**, pp. 9877.

Höfgens R. and Willmitzer L. (1990). Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (*Solanum tuberosum* L.). *Plant Science* **66**, pp. 221-230.

Huang A. H. C. (1992). Oil bodies and oleosins in seeds. *Annual review of Plant Physiology and Plant Molecular Biology* **43**, pp. 177-200.

Huang A. H. C. (1994). Structure of plant seed oil bodies. *Current Opinion in Structural Biology* **4**, pp. 493-498.

Hughes D. W. and Galau G. A. (1989). Temporally modular gene expression during cotyledon development. *Genes Dev*. **3**, pp. 358-369.

Ingram J., and Bartels D. (1996). The molecular basis of dehydration tolerance in plants. *Annual review of Plant Physiology and Plant Molecular Biology* **47**, pp. 377-403.

Ito H., Fukada Y., Murata K., and Kimura A. (1983). Transformation of intact yeast cells treated with alkali cations. *Journal of Bacteriology* **153**, pp. 163-168.

Jefferson A. J. (1987). Assaying chimaeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep*. **5**, pp. 387-405.

Jensen W. A. (1985). Cotton embryogenesis. Polysome formation in the zygote. *Journal of Cellular Biology* **36(2)**, pp. 403-406.

Josefsson L. G., Lenman M., Ericson M. L. and Rask L. (1987). Structure of a gene encoding the 1.7S storage protein, napin from *Brassica napus*. *J. Biol*. *Chem*. **262**, pp. 12196-12201.

Jürgens G. (1995). Axis formation in plant embryogenesis: cues and clues. *Cell* **81**, pp. 467-470.

Kaldenhoff R., Kolling A. and Richter G. (1996). Regulation of the *Arabidopsis thaliana* aquaporin gene AthH2 (PIP1b). *Journal of Photochemistry Photobiology* **36(3)**, pp. 351-354.

Karssen C. M., Brinkhorst-van der Swan D. L. C., Breekland A. E. and Koornneef M. (1983). Induction of dormancy during seed development by endogenous abscisic acid: Studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L). Heynh. *Planta* **157**, pp. 158-165.

Kater M. M., Konigstein G. M., Nijkamp H. J. J. and Stuitje A. R. (1991). cDNA cloning and xpression of Brassica napus enoyl-acyl carrier protein reductasein Escheria coli. *Plant Molecular Biology* **17**, pp. 895.

Keith K., Kraml M., Dengler N. G. and McCourt P. (1994). *Fusca3*: a heterochronic mutation affecting late embryo development in *Arabidopsis*. *Plant Cell* **6**, pp. 589-600.

Keddi J. S., Tsiantis M., Piffanelli P., Cella R., Hatzopolous P., and Murphy D. J. (1994). A seed-specific *Brassica napus* oleosin promoter interacts with a G-box specific protein and may be bi-directional. *Plant Molecular Biology* **24**, pp. 327-340.

Kirik V., Kölle K., Wohlfarth T., Miséra S. and Bäumlein H. (1998). Ectopic expression of a novel MYB gene modifies the architecture of the *Arabidopsis* inflorescence. *Plant J.* **13**, pp. 729-742.

Knaap van der E., Kim J. H., Kende, H. (2000). A nnovel gibberellin-induced gene from rice and ist potential regulatory role in stem growth. *Plant Physiology* **122,** pp. 695-704.

Kölle K. (1996). Analyse von Genexpressionsprogrammen während der Embryogenese von *Arabidopsis thaliana* (L.) *PhD. Thesis*, Halle University.

Koornneef M., Jorna M. L., Brinkhorst-van der Swan D. L. C. and Karssen C. M. (1982). The isolation of absiscic acid (ABA deficient mutnats by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. *Theoretical Applied Genetics* **61**, pp. 385-393.

Koornneef M., Reuling G. and Karssen C. M. (1984). The isolation and characterisation of abscisic acid-insensitive mutants of *Arabidopsis thaliana*. *Plant Physiol.* **90**, pp. 463-469.

Koornneef M. and Karssen C. M. (1994). Seed dormancy and germination. In: *Arabidopsis*, Sommerville C.R. and Meyerowitz E.M. (eds). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 313-334.

Kriz A. R., Wallace M. S. and Paiva R. (1990). Globulin gene expression in embryos of maize vivparous mutants: Evidence for regulation of the Glb1 gene by ABA. *Plant Physiology* **92**, pp. 538-542.

Kurup S., **Jones H. D., Holdsworth M. J.** (2000). Interactions of the developmental regulator ABI3 with proteins identified from developing *Arabidopsis* seeds. *Plant Journal* **21**, pp. 143-156.

Lane B. G. (1991). Cellular desiccation and hydratation: developmentally regulated proteins and the maturation and germination of seed embryos. *FASEB Journal* **5,** pp. 2893-2901.

Lelievre J. M., Oliveira L. O. and Nielsen N. C. (1992). 5´-CATGCATG-3´ elements modulate the expression of glycinin genes. *Plant Physiol.* **98**, pp. 387-391.

Leon-Kloosterziel K. M., van de Bunt G. A., Zeevaart J. A., Koornneef M. (1996). Arabidopsis mutants with a reduced seed dormancy. *Plant Physiology* **110(1),** pp. 233-240.

Lotan T., Ohto M., Yeem K. M., West M. A., Lo R., Kwong R. W., Yamagishi K., Fischer R. L., Goldberg R. B. and Harada J. J. (1998). *Arabidopsis LEAFY COTYLEDON1* is sufficient to induce embryo development in vegetative cells. *Cell* **93**, pp. 1195-205.

Luerßen H., Kirik V. and Miséra S. (1998). The *FUSCA3* gene of *Arabidopsis thaliana* encodes a product with partial homology to Vp1/ABI3-like regulatory proteins. *Plant J.* **15**, pp. 755-764.

Mansfield S. G. and Briarty L.G. (1992). Cotyledon Cell development in *Arabidopsis thaliana* during reserve deposition. *Canadian Journal of Botany* **70**, pp. 151-164.

McCarty D. R., Carson C. B., Stinard P. S. and Robertson D. S. (1989). Molecular analysis of viviparous-1: an abscisic acid insensitive mutant of maize. *Plant Cell* **1**, pp. 523-532.

McCarty D. R., Hattori T., Carson C. B., Vasil V. and Vasil V. K. (1991). The viviparous1 developmental gene of maize encodes a novel transcriptional activator. *Cell* **66**, pp. 895-905.

McCarty D. R. (1995). Genetic control and integration of maturation and germination pathways in seed development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, pp. 71-93.

Meinke D. W. (1992). A homeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. *Science* **258**, pp. 1647-1650.

Meinke D. W. (1994a). Seed development in *Arabidopsis thaliana*: In *Arabidopsis*. E Meyerowitz and C. Sommerville (eds). Cold Spring Harbor, NY. Cold Spring Harbor Laboratory Pres, pp. 253-295.

Meinke, D. W., Franzmann, L. H., Nickle, T. C. and Yueng, E. C. (1994b). *Leafy cotyledon* mutants of *Arabidopsis*. *Plant Cell* **6**, pp. 1049-1064.

Meinke D. W. (1995) .Molecular genetics of plant embryogenesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **46**, pp. 369-394.

Morton R. L., Quiggin D. and Higgins T. J. V. (1995). Regulation of seed storage protein gene expression. *In*: Seed development and germination, J. Kigel and G. Galili (eds.), Marcel Dekker, Inc., New York, Basel, Hong Kong, pp. 103-138.

Müller A. J. (1963). Embryonentest zum Nachweis rezessiver Letalfaktoren bei Arabidopsis thaliana. *Biol. Zentralbl*. **82**, pp. 133-163.

Mundy J. and Chua N. H. (1988). Abscisic acid and water-stress induce the expression of a novel rice gene. EMBO Journal **7(8)**, pp. 2279-2286.

Müntz, K. (1998). Deposition of storage proteins. *Plant Molecular Biology* **38(1-2)**, pp. 77-99.

Nambara E., Hayama R., Tsuchiya Y., Nishimura M., Kawaide H., Kamiya Y. and Naito S. (2000). The role of ABI3 and FUS3 loci in Arabidopsis thaliana on phase transition from late embryo development to germination. *Developmental Biology* **15;220(2)**, pp. 412-423.

Neill S. J., Horgan R. and Parry A. D. (1986). The carotenoid and absiscic acid content of viviparous kernels and seedlings of *Zea Mays* L. *Planta* **169**, pp. 87-96.

Neill S., Horgan J. R. and Rees A. F. (1987). Seed development and vivipary in *Zea mays* L. *Planta* **169**, pp. 358-364.

Okamuro J. K., Jofuku K. D., Goldberg R. B. (1986). Soybean seed lectin gene and flanking nonseed protein genes are developmentally regulated in transformed tobacco plants. *Proceedings of the National Academy of Sciences USA* **83(21)** , pp. 8240-8244.

Olsen O-A., Potter R. H. and Kalla R. (1992). Histo differentiation and molecular biology of developing cereal endosperm. *Seed Science Research* **2,** pp. 117-131.

Parcy F., Valon C., Raynal M., Gaubier-Comella A., Delsney M. and Giraudat J. (1994). Regulation of gene expression programs during seed development: Roles of ABI3 locus and of endogenous abscisic acid. *Plant Cell* **6**, pp. 1567-1582.

Parcy F., Valon C., Kohara A., Miséra S. and Giraudat, J. (1997). The *ABSCISIC ACID-INSENSITIVE3*, *FUSCA3* and *LEAFY COTYLEDON1* genes act in concert to control multiple aspects of *Arabidopsis* seed development. *Plant Cell* **9**, pp. 1265-1277.

Pepper A., Delaney T., Washburn T., Poole D., Chory J. (1994). DET1, a negative regulator of light-mediated development and gene expression in *Arabidopsis*, encodes a novel nuclear-localized protein. *Cell* **15;78(1)**, pp. 109-116.

Phillips J., Artsaenko O., Fiedler U., Horstmann C., Mock H. P., Muntz K. and Conrad U. (1997). Seed-specific immunomodulation of abscisic acid activity induces a developmental switch. *EMBO Journal* **1;16(15)**, pp. 4489-4496.

Putterill J., Robson F., Lee K., Simon R. and Coupland G. (1995). The CONSTANS gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell* **24; 80(6)**, pp. 847-857.

Quatrano, R. S. (1986). Regulation of gene expression by abscisic acid during angiosperm embryo development. *Oxford Surv. Plant Mol. Cell. Biol*. **3**, pp. 467-477.

Raventos D., Skriver K., Schlein M., Karnahl K., Rogers S. W., Rogers J. C. and Mundy J. (1998). HRT, a novel zinc finger, transcriptional repressor from barley. *Journal of Biological Chemistry* **273(36)**, pp. 23313-23320.

Raz V., Bergervoet J. H., Koornneef M. (2001). Sequential steps for developmental arrest in *Arabidopsis* seeds. *Development* **128(2)**, pp. 243-252.

Reidt W., **Wohlfarth T., Ellerström M., Czihal A., Tewes A., Ezcurra I., Rask L., and Bäumlein H.** (2000). Gene regulation during late-embryogenesis: the RY motif of maturation specififc gene promoters is a direct raget of the FUS3 gene product. *Plant Journal* **21**, pp. 401-408.

Reidt W., **Ellerström M., Kölle K., Tewes A., Tiedemann J., Altschmied L. and Bäumlein** (2001). FUS3-dependent gene regulation during late-embryogenesis. *Journal of Plant Physiology* **158**, pp. 411-418.

Robertson, D. S. (1952). The genotype of the endosperm and the embryo as it influences vivipary in maize. *Proceedings in the National Academy of Sciences U.S.A* **38**, pp. 580-583.

Robertson, D. **S.** (1955). The genetics of vivipary in maize. *Genetics* **40**, pp. 745-760.

Robichaud, C. S. and Sussex, I. M. (1987). The uptake and metabolism of 2[carbon-14] ABA by excised wild type and *Viviparous-1* embryos of *Zea Mays* L. *Journal of Plant Physiology* **130**, pp. 181-188.

Rock C. (2000). Pathways to abscisic acid-regulated gene expression. *New Phytologist* **148**, pp. 357-396.

Rohde A., De Rycke R., Beeckman T., Engler G., Van Montagu M., Boerjan W. (2000)**.** The *Abscisic ACID-INSENSITIVE 3* (ABI3) gene is expressed during vegetative quiesence processes in *Arabidopsis*. *Plant, Cell and Environment* **22**, pp. 601-610.

Rojas A., Almoguera C. and Jordano J. (1999). Transcriptional activation of a heat shock gene promoter in sunflower embryos: synergism between ABI3 and heat shock factors. *Plant Journal* **20**, pp. 601-610.

Sambrook J., **Maniatis T., Fritsch E. F.** (1989). Molecular Cloning: A laboratory manual. Cold Spring Harbour, Cold Spring Harbour Laboratory Press, New York.

Schmiedeskamp M. **and Klevit R. E.** (1994). Zinc-finger diversity. *Current Opinion in Sturctural Biology* **4**, pp. 28-35.

Schiestl R.H. and Gietz R.D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16**, pp. 339-346.

Shewry P. R., Napier J. A., Tatham A.S. (1995). Seed storage proteins: structures and biosynthesis. Plant Cell **7(7)**, pp. 945-956.

Shotwell, M. A. and Larkins, B. A. (1989).The molecular biology and biochemistry of seed storgae proteins. In *The Biochemistry of Plants*, A. Marcus, ed. (San Diego: Academic Press), pp. 297-345.

Shiota H. and Kamada H. (2000). Acquisation of desiccation tolerance by cultured carrot cells upon ectopic expression of C-ABI3 of ABI3, a carrot homolog of ABI3. *Journal of Plant Physiology* **156(4)**, pp. 510-515.

Skriver, K. and Mundy J. (1990). Gene expression in response to absiscic acid and osmotic stress. *Plant Cell* **2**, pp. 503-513.

Skoog F., Miller C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Soc. Exp. Biol.* 11: pp. 118.

Stålberg K., Ellerström M., Josefsson L. G. and Rask L. (1993). Deletion analysis of a 2S seed storage protein promoter of *Brassica napus* in transgenic tobacco. *Plant Mol. Biol.* **23**, pp. 671-683.

Stålberg K., Ellerström M., Ezcurra I., Ablov S. and Rask L. (1996). Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage protein promoter in transgenic *Brassica napus* seeds. *Planta* **199**, pp. 515-519.

Stone S. L., Kwong L. W., Yee K. M., Pelletier J., Lepiniec L., Fischer R. L., Goldberg R. B., Harada J. J. (2001). LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development. Proceedings of the National Academy of Sciences USA **98(20)**, pp.11806-11811.

Sun T. P. and Kamiya Y. (1994). The Arabidopsis GA1 locus encodes the cyclase ent-kaurene synthetase A of gibberellin biosynthesis. *Plant Cell* **6**, pp. 1509-1518.

Suzuki M., Kao C. Y. and McCarty D. R. (1997). The conserved B3 domain of VIVIPAROUS1 has a co-operative DNA binding activity. *Plant Cell* **9**, pp. 799-807.

Taipalensuu J., Falk A., Ek B. and Rask L. (1997). Myrosinase-binding proteins are derived from a large wound-inducible and repetitive transcript. *European Journal of Biochemistry* **243**, pp. 605-611.

Takatsuji H. (1998). Zinc-finger transcription factors in plants. *Cellular Molecular Life Sciences* **54(6)**, pp. 582-596.

Torrey J. G., Fosket D. E., Hepler P. K. (1971). Xylem formation: a paradigm of cytodifferentiation in higher plants. *American Sci.* **59**, pp. 338-352.

Tykarska, T. (1987)**.** Rape embryogenesis. VI. Formation of protein bodies. *Acta SOC Bot. Pol*. **56,** pp. 585-598.

Ulmasov T., Hagen G. and Guilfoyle T. J. (1997). ARF1, a transcription factor that bind to auxin response elements. *Science* **276**, pp. 1865-1868.

Vicient C. M., Bies-Etheve N., Delseny M. (2000). Changes in gene expression in the leafy cotyledon1 (lec1) and fusca3 (fus3) mutants of Arabidopsis thaliana L. *Journal of Experimental Botany* **51(347)**, pp. 995-1003

Wang T., Donkin M. and Martin E. (1984). The physiology of a wilty pea: absiscic acid production under water stress**.** *Journal of Experimental Botany* **35,** pp. 1222-1232.

West, M. A. L., K. M. Yee, Danao J., Zimmerman J. L., Fischer R. L., Goldberg R. B. and Harada J. J. (1994). *LEAFY COTYLEDON1* is an essential regulator of late embryogenesis and cotyledon identity in *Arabidopsis*. *Plant Cell* **6**, pp. 1731-1745.

White C. N. and Rivin C. J. (2000). Gibberellins and seed development in maize. II Gibberellin synthesis inhibition enhances absiscic acid signaling in cultured embryos. *Plant Physiology* **122,** pp. 1089-1097.

Williamson J. D., Scandialos J. G. (1994). The maize (*Zea mays L.*) *Cat1* catalase promoter displays differential binding of nuclear proteins isolated from germinate and developing embryos and from embryos grown in the presence and absence of abscisic acid. *Plant Physiology* 106, pp. 1373-1380.

Wobus U. and H. Weber (1999). Seed maturation: genetic programmes and control signals. *Currunt Opinions in Plant Biology* **2**, pp. 33-38.

Wohlfarth T. (1996). Regulation samenspezifischer Gene der Ackerbohne *Vicia faba*: Analyse von *cis*-Elementen sowie Isolierung und Charakterisierung von Transkriptions-faktoren. PhD thesis, Martin-Luther-Universität, Halle Wittenberg, Germany.

Wolpert L (1998). Principles of Development. Oxford University Press, NY.

Yueng, E. C. and Meinke, D. W. (1993). Embryogenesis in angiosperms: development of the suspensor. Plant Cell 5, pp. 1371-1381.

Curriculum vitae

Personal data

Name:	Wim Reidt
Place of birth:	Den Haag (Netherlands)
Date of birth:	16 december 1970
Nationality:	Dutch

Education and employment

1989-1995	Master studies at the Agricultural University Wageningen Diploma thesis subjects: 1. Characterisation of SERK gene expression in *Daucus carota* 2. Sex determination of the nematode *Aphelenhus avanea* 3. Infection of lettuce with TSWV by mechanical inoculation or transmission by thrips
1995-1997	Scientific co-worker at the Plant Disease Institute Wageningen
1997-2001	Post graduate student at the Institute für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany Ph.D thesis: Analysis of transcription factors during late-embryogenesis: the role of FUS3, LEC1, ABI3 and AtET

Acknowledgements

This work was done at the Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben. I would like to thank all the co-workers of the Institute, especially from the groups of Gene Regulation and Gene expression.

I am particulary grateful to Elke Liemann for her endless help in transforming *Arabidopsis* and tobacco as well her assistance in a lot of the other things concerning plant work, Christel Ernst and Hannelore Weiss for all their help in preparing plasmid DNA and Andreas Czihal for his advice and help in hydrisation experiments. I also want to thank Annegret Tewes and Sabine Skiebe for their help in the protoplast preparation and Angela Stegmann for some practical assistance. Furthermore I would like to thank Heike Ernst, Brigitte Schäfer, Ursula Tiemann, Anett Kilian and Karin Lipfert for photography or help with the computer presentation of the results, Michael Melzer for his microscopy work and preparation of the pictures and Lothar Altschmied for screening of the *Arabidopsis* genome for RY containing promoters. For scientific discussion and suggestions concerning this manuscript I would like to thank Manoela Miranda, Felicia Hosein and professor Ulrich Wobus. Finally, last but not least Helmut Bäumlein for his supervision, discussion and for critical reading of the manuscript.

Publication of results

Reidt W., **Ellerström M., Kölle K., Tewes A., Tiedemann J., Altschmied L. and Bäumlein** (2001). FUS3-dependent gene regulation during late-embryogenesis. *Journal of Plant Physiology* **158**, pp. 411-418.

Reidt W., **Wohlfarth T., Ellerström M., Czihal A., Tewes A., Ezcurra I., Rask L., and Bäumlein H.** (2000). Gene regulation during late-embryogenesis: the RY motif of maturation specififc gene promoters is a direct target of the FUS3 gene product. *Plant Journal* **21**, pp. 401-408.

.

Posters

Reidt W., Tiedemann T., Melzer M., Tewes A., Ellerström M., Wohlfarth T., and Bäumlein H. (2001). A novel class of transcriptional activators: Gene structure, ectopic expression and insertion mutagenesis. *Institutstag IPK-Gatersleben*, October 10 2001.

Reidt W., **Ellerström M., Kölle K., Czihal A., Tewes A. and Bäumlein** (2000). Gene regulation during late embryogenesis: The RY motif is a direct target of the FUS3 gene product. *EMBO course* 1998 Paris.

Reidt W., **Wohlfarth, T., Czihal A., Tewes A., Zimmerman G., Adler K., Hähnel U., Altschmied L. and Bäumlein H.** (1999). Gene regulation during late embryogenesis: The role of B3 and MYB transcription factors. *Institutstag IPK-Gatersleben*, October 11 1999.

Mönke G., Reidt W., Bäumlein H. and Conrad U. (2000). Production of recombinant antibodies against FUS3. *8th International Symposium on Plant Seeds, Seed Development, Metabolism and Biotechnology*. Gatersleben/Meisdorf, 27-31 August 2000

Appendix

-*Arabidopsis* genes with RY containing promoters-

gene id	length promoter	# of patterns	pos RY	X	pos TATA	description of gene
At1g03170	503	1	-264	51	-207	hypothetical protein
At1g04250	503	1	-139	61	-72	putative auxin-induced protein, IAA17/AXR3-1
At1g05650	503	1	-75	62	-7	putative polygalacturonase
At1g06080	503	1	-204	70	-128	delta 9 desaturase
At1g06590	503	1	-221	72	-143	unknown protein
At1g06650	503	1	-337	74	-257	oxidoreductase, putative
At1g06800	503	1	-112	51	-55	lipase, putative
At1g08150	503	1	-308	88	-214	hypothetical protein
At1g08220	503	1	-420	70	-344	hypothetical protein
At1g11370	503	1	-203	53	-144	similar to flower-specific pectin methylesterase precursor
At1g11510	503	1	-194	95	-93	hypthetical protein
At1g13710	503	1	-200	82	-112	hypothetical protein
At1g14640	503	1	-468	84	-378	splicing factor, putative
At1g16500	503	1	-211	49	-156	hypothetical protein
At1g17060	503	1	-355	78	-271	putative cytochrome P450
At1g18860	503	1	-124	76	-42	hypothetical protein
At1g22620	503	1	-308	83	-219	unknown protein
At1g26920	503	1	-211	52	-153	unknown protein
At1g28650	503	1	-377	57	-314	lipase, putative
At1g29790	503	1	-282	76	-200	hypothetical protein
At1g30040	503	1	-280	75	-199	unknown protein
At1g30870	503	1	-171	82	-83	F17F8.26
At1g34390	503	1	-494	100	-388	auxin response factor, putative
At1g35030	503	1	-369	74	-289	hypothetical protein
At1g35310	503	1	-400	83	-311	hypothetical protein
At1g35540	503	1	-421	44	-371	auxin response factor, putative
At1g43160	503	1	-488	93	-389	hypothetical protein
At1g43950	503	1	-416	44	-366	hypothetical protein
At1g47990	503	1	-150	54	-90	dioxygenase, putative
At1g48650	503	1	-398	75	-317	hypothetical protein
At1g49360	503	1	-236	69	-161	hypothetical protein
At1g49570	503	1	-153	64	-83	peroxidase, putative
At1g49800	503	1	-154	95	-53	hypothetical protein
At1g50650	503	1	-154	70	-78	stig1-like protein
At1g53990	503	1	-139	71	-62	hypothetical protein
At1g54680	503	1	-91	61	-24	
At1g54940	503	1	-146	56	-84	hypothetical protein
At1g55650	503	1	-398	78	-314	unknown protein
At1g56230	503	1	-464	87	-371	hypothetical protein
At1g56430	503	1	-294	53	-235	hypothetical protein
At1g61800	503	1	-285	81	-198	hypothetical protein
At1g62440	503	1	-477	45	-426	putative extensin-like protein (gnl|PID|e1310400
At1g64710	503	1	-335	73	-256	hypothetical protein
At1g64850	503	1	-419	88	-325	hypothetical protein
At1g66020	503	1	-433	87	-340	hypothetical protein
At1g66270	503	1	-157	79	-72	beta-glucosidase
At1g69600	503	1	-124	93	-25	hypothetical protein
At1g69760	503	1	-202	85	-111	unknown protein
At1g70720	503	1	-159	89	-64	hypothetical protein
At1g71890	503	1	-180	70	-104	putative sucrose transport protein
At1g72690	503	1	-381	91	-284	unknown protein
At1g73560	503	1	-200	72	-122	lipid transfer protein, putative
At1g74550	503	1	-155	78	-71	putative cytochrome P450
At1g75930	503	1	-130	68	-56	anter-specific proline-rich -like protein (APG-like)
At1g76210	503	1	-448	66	-376	hypothetical protein
At1g76830	503	1	-185	86	-93	hypothetical protein
At1g80390	503	1	-322	46	-270	unknown protein
At2g05080	503	1	-309	91	-212	putative helicase
At2g10040	503	1	-105	86	-13	pseudogene; TNP2 protein of the Tam1 family of transposons [Anti
At2g13900	503	1	-204	69	-129	hypothetical protein
At2g15020	503	1	-377	93	-278	hypothetical protein
At2g15050	503	1	-492	67	-419	putative lipid transfer protein
At2g15640	503	1	-270	91	-173	hypothetical protein
At2g16400	503	1	-166	78	-82	putative homeodomain transcription factor
At2g18450	503	1	-355	70	-279	putative succinate dehydrogenase flavoprotein subunit
At2g21070	503	1	-379	59	-314	unknown protein
At2g21900	503	1	-391	44	-341	putative WRKY-type DNA binding protein
At2g22770	503	1	-254	58	-190	putative bHLH transcription factor
At2g22980	503	1	-152	90	-56	putative serine carboxypeptidase
At2g23440	503	1	-404	100	-298	unknown protein

Arabidopsis genes with RY containing promoters (cont.)

gene id	length promoter	# of patterns	CATGCAN(X)TATAAA pos RY	X	pos TATA	description of gene
At2g23440	503	1	-404	100	-298	unknown protein
At2g25980	503	1	-394	58	-330	similar to jasmonate-inducible proteins from Brassica napus
At2g26420	503	1	-396	68	-322	putative phosphatidylinositol-4-phosphate 5-kinase
At2g26870	503	1	-151	84	-61	putative phospholipase C
At2g28110	503	1	-423	83	-334	hypothetical protein
At2g31030	503	1	-135	50	-79	putative oxysterol-binding protein
At2g31120	503	1	-346	56	-284	unknown protein
At2g32830	503	1	-481	56	-419	putative phosphate transporter
At2g34070	503	1	-317	57	-254	hypothetical protein
At2g34240	503	1	-187	44	-137	hypothetical protein
At2g38620	503	1	-284	46	-232	putative cell division control protein kinase
At2g39850	503	1	-155	96	-53	putative serine protease
At2g40530	503	1	-294	77	-211	hypothetical protein
At2g42110	503	1	-236	51	-179	hypothetical protein
At2g42200	503	1	-482	91	-385	putative squamosa-promoter binding protein
At2g43520	503	1	-314	92	-216	putative trypsin inhibitor
At2g43830	503	1	-451	83	-362	putative protein kinase
At2g44300	503	1	-165	70	-89	unknown protein
At2g44990	503	1	-144	48	-90	hypothetical protein
At2g45960	503	1	-176	80	-90	aquaporin (plasma membrane intrinsic protein 1B)
At2g46730	503	1	-339	62	-271	unknown protein
At2g47030	503	1	-207	43	-158	putative pectinesterase
At2g47040	503	1	-215	43	-166	putative pectinesterase
At2g47660	503	1	-292	77	-209	hypothetical protein
At3g01130	503	1	-204	63	-135	unknown protein
At3g01670	503	1	-360	94	-260	unknown protein
At3g01870	503	1	-391	70	-315	hypothetical protein
At3g02220	503	1	-415	72	-337	unknown protein
At3g03540	503	1	-122	62	-54	unknown protein
At3g05590	503	1	-362	54	-302	putative 60S ribosomal protein L18
At3g10720	503	1	-470	60	-404	putative pectinesterase
At3g11480	503	1	-248	43	-199	hypothetical protein
At3g13130	503	1	-214	73	-135	hypothetical protein
At3g13840	503	1	-460	96	-358	hypothetical protein
At3g14300	503	1	-338	55	-277	putative pectin methylesterase
At3g15370	503	1	-224	77	-141	putative expansin S2 precursor
At3g15870	503	1	-413	77	-330	putative delta 9 desaturase
At3g19030	503	1	-393	79	-308	hypothetical protein
At3g20940	503	1	-359	59	-294	cytochrome P450, putative
At3g21360	503	1	-203	88	-109	unknown protein
At3g26390	503	1	-113	58	-49	hypothetical protein
At3g29390	503	1	-285	47	-232	unknown protein
At3g44920	503	1	-215	70	-139	putative protein
At3g46200	503	1	-213	69	-138	putative protein
At3g47170	503	1	-498	49	-443	hypersensitivity-related protein-like protein
At3g47750	503	1	-166	88	-72	ABC-type transport protein-like protein
At3g48670	503	1	-166	55	-105	putative protein
At3g49340	503	1	-123	74	-43	cysteine protease - like protein
At3g51590	503	1	-108	54	-48	lipid transfer protein-like protein
At3g52930	503	1	-158	54	-98	fructose bisphosphate aldolase - like protein
At3g53810	503	1	-327	67	-254	serine/threonine-specific kinase like protein
At3g54940	503	1	-183	97	-80	cysteine proteinase precursor-like protein
At3g55030	503	1	-349	41	-302	phosphatidylglycerophosphate synthase - like protein
At3g57490	503	1	-249	87	-156	40S ribosomal protein S2 homolog
At3g59760	503	1	-211	95	-110	cysteine synthase
At3g61620	503	1	-358	74	-278	exonuclease RRP41
At3g61910	503	1	-133	85	-42	NAM-like protein
At3g62310	503	1	-480	62	-412	ATP-dependent RNA helicase-like protein
At3g62930	503	1	-220	99	-115	glutaredoxin -like protein
At4g00120	503	1	-306	70	-230	hypothetical protein
At4g00670	503	1	-355	62	-287	hypothetical protein
At4g01340	503	1	-443	69	-368	hypothetical protein
At4g01950	503	1	-196	78	-112	predicted protein of unknown function
At4g02780	503	1	-192	80	-106	ent-kaurene synthetase A - like protein
At4g07370	503	1	-96	49	-41	
At4g08910	503	1	-247	99	-142	hypothetical protein
At4g09610	503	1	-211	91	-114	gibberellin-regulated protein GASA2 precursor
At4g10250	503	1	-421	77	-338	heat shock protein 22.0
At4g10350	503	1	-199	88	-105	NAM/NAP like protein

-*Arabidopsis* genes with RY containing promoters (cont.)

gene id	length promoter	# of patterns	CATGCAN(X)TATAAA pos RY	X	pos TATA	description of gene
At4g10910	503	1	-182	74	-102	hypothetical protein
At4g11340	503	1	-129	65	-58	putative protein
At4g12470	503	1	-207	64	-137	pEARLI 1-like protein
At4g16220	503	1	-417	88	-323	hypothetical protein
At4g16270	503	1	-161	60	-95	peroxidase like protein
At4g16970	503	1	-490	98	-386	kinase like protein
At4g18020	503	1	-341	88	-247	putative protein
At4g18370	503	1	-348	74	-268	putative protein
At4g18650	503	1	-115	51	-58	putative protein
At4g18660	503	1	-198	45	-147	putative protein
At4g19150	503	1	-451	87	-358	ankyrin-like protein
At4g22620	503	1	-254	49	-199	putative protein
At4g23680	503	1	-155	57	-92	putative major latex protein
At4g24030	503	1	-143	91	-46	putative protein
At4g26000	503	1	-458	58	-394	putative nucleic acid binding protein
At4g27160	503	2	-493	44	-443	NWMU3 - 2S albumin 3 precursor
At4g27160	503	2	-126	47	-73	NWMU3 - 2S albumin 3 precursor
At4g27330	503	1	-369	47	-316	NOZZLE/SPOROCYTELESS
At4g27590	503	1	-153	80	-67	hypothetical protein
At4g28420	503	1	-125	64	-55	tyrosine transaminase-like protein
At4g28520	503	1	-108	44	-58	12S cruciferin seed storage protein
At4g28530	503	1	-263	52	-205	NAM / CUC2 -like protein
At4g28850	503	1	-174	84	-84	xyloglucan endotransglycosylase - like protein
At4g29700	503	1	-134	84	-44	nucleotide pyrophosphatase -like protein
At4g30290	503	1	-134	47	-81	xyloglucan endo-1,4-beta-D-glucanase-like protein
At4g32170	503	1	-150	88	-56	cytochrome p450 - like protein
At4g34250	503	1	-116	51	-59	fatty acid elongase - like protein
At4g36700	503	1	-374	77	-291	globulin-like protein
At4g37050	503	1	-212	47	-159	patatin-like protein
At4g39000	503	1	-176	91	-79	putative endo-1,4-beta-glucanase
At4g39800	503	1	-179	71	-102	myo-inositol-1-phosphate synthase
At5g01110	503	1	-328	46	-276	putative protein
At5g02170	503	1	-484	94	-384	putative protein
At5g03930	503	1	-354	68	-280	putative protein
At5g05730	503	1	-219	92	-121	anthranilate synthase component I-1 precursor (sp|P32068)
At5g08100	503	1	-413	76	-331	asparaginase
At5g10120	503	1	-204	49	-149	transcription factor TEIL/ethylene-insensitive - like protein
At5g12940	503	1	-196	90	-100	putative protein
At5g13790	503	1	-257	94	-157	floral homeotic protein AGL15 (sp|Q38847)
At5g14360	503	1	-145	45	-94	putative protein
At5g15100	503	1	-463	59	-398	auxin transport protein - like
At5g17980	503	1	-337	84	-247	phosphoribosylanthranilate transferase-like protein
At5g19430	503	1	-412	77	-329	putative protein
At5g20630	503	1	-163	83	-74	germin-like protein
At5g22490	503	1	-150	93	-51	putative protein
At5g23940	503	1	-253	100	-147	acyltransferase
At5g24090	503	1	-157	78	-73	acidic endochitinase (dbj|BAA21861.1)
At5g24820	503	1	-125	55	-64	putative protein
At5g25100	503	1	-392	82	-304	putative multispanning membrane protein
At5g33230	503	1	-82	49	-27	putative protein
At5g39580	503	1	-435	83	-346	peroxidase ATP24a
At5g41090	503	1	-250	83	-161	putative protein
At5g41430	503	1	-186	81	-99	unknown protein
At5g42160	503	1	-259	62	-191	putative protein
At5g46360	503	1	-340	91	-243	KCO-like protein 3 (emb|CAB40380.1)
At5g46830	503	1	-111	62	-43	bHLH transcription factor
At5g49050	503	1	-190	45	-139	unknown protein
At5g49550	503	1	-263	54	-203	phytoene dehydrogenase-like
At5g49660	503	1	-434	80	-348	receptor protein kinase
At5g49690	503	1	-268	49	-213	anthocyanidin-3-glucoside rhamnosyltransferase-like
At5g52160	503	1	-197	85	-106	putative protein
At5g64240	503	1	-150	80	-64	putative protein
At5g66430	503	1	-346	81	-259	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase-
At5g66960	503	1	-258	81	-171	protease-like
