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The HDZip Class I Transcription Factors in *Arabidopsis thaliana*

Characterisation of HDZip Genes Involved in the Mediation of Environmental Signals

BY

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Abstract
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Homeodomain leucine zipper (HDZip) proteins constitute a large family of transcription factors characterised by the presence of a DNA-binding homeodomain and an adjacent leucine zipper motif, which mediates protein-dimer formation. The HDZip genes of Arabidopsis have been divided into four classes, HDZip I-IV. This thesis describes the characterisation and phylogeny of the class I HDZip genes and focuses on the expression and function of four HDZip I genes, *ATHB5*, *-6*, *-7* and *-16*.

The phylogenetic analyses of the 17 HDZip I sequences defined six subclasses, supported by the intron patterning and the traced duplication history of the genes. The members within each subclass showed diversification in expression, suggesting that the *cis* regulatory regions of the closely related genes have undergone evolutionary changes. However, similarities in the gene expression patterns between genes also exist and external factors like the availability of water and quality of light directs the expression of a subset of HDZip I genes. Expression analyses revealed that the plant hormone abscisic acid (ABA) is involved as a systemic signal for the salt or osmoticum induced *ATHB7* expression, whereas light signals mediated through the blue light photoreceptors was found to direct the expression of *ATHB6*.

Phenotypic analyses of plants with altered levels of *ATHB6* or *ATHB16* suggested that these paralogous genes encode proteins with very similar functions. ATHB16 was shown to act as a negative regulator of leaf cell expansion, as a suppressor of the flowering time sensitivity to photoperiod and as a positive regulator of blue light dependent inhibition of hypocotyl growth. A similar role for ATHB6 in the regulation of hypocotyl elongation was recorded. Further, analyses of multiple loss-of-function plants demonstrated that *ATHB5*, *-6* and *-16* function at least in part redundantly in mediating light effects on hypocotyl elongation.

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This thesis is based on the following papers, which will be referred to in the text by their corresponding Roman numerals

I. **EVA HENRIKSSON**, ANNA OLSSON, HENRIK JOHANNESSON, HENRIK JOHANSSON, JOHANNES HANSON, PETER ENGSTRÖM AND EVA SÖDERMAN. HDZip class I genes in Arabidopsis: expression profiles and phylogenetic relationships. In manuscript.

II. **EVA HENRIKSSON** AND KERSTIN NORDIN HENRIKSSON. Salt stress signalling and the role of calcium in the regulation of the Arabidopsis *ATHB7* gene. Plant, Cell and Environment. In press.

III. YAN WANG, **EVA HENRIKSSON**, EVA SÖDERMAN, KERSTIN NORDIN HENRIKSSON, EVA SUNDBERG AND PETER ENGSTRÖM (2003) The Arabidopsis homeobox gene, *ATHB16*, regulates leaf development and the sensitivity to photoperiod in Arabidopsis. Developmental Biology 264:228-239.

IV. **EVA HENRIKSSON**, KERSTIN NORDIN HENRIKSSON HENRIK JOHANNESSON, EVA SÖDERMAN AND PETER ENGSTRÖM. The HDZip class I genes *ATHB5*, *-6* and *-16* act redundantly to mediate light effects on hypocotyl elongation. In manuscript.

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INTRODUCTION

Arabidopsis thaliana as a model plant

Arabidopsis thaliana, a member of the *Brassicaceae* family, is a widely used organism within plant research. Arabidopsis (Fig. 1) is small in size, has a short generation time, produces a large amount of offspring and can easily be cross-pollinated even though it is naturally a self-pollinator. These features make Arabidopsis a suitable organism for laboratory studies. A relatively small genome size (125 Mb) as well as low content of repetitive DNA further increases the value of Arabidopsis as a model for molecular genetic studies. The ability of the plant pathogen *Agrobacterium tumefaciens* to infect Arabidopsis by transferring part of the tumor inducing plasmid, the T-DNA, into the plant genome enables the use of easily applied transformation techniques on Arabidopsis (Bechtold *et al.* 1993). The development of genetic transformation techniques has been crucial for the use of reverse genetic methods, like disruption or enhancement of gene function. Further, the publicly available mutant collections, mutagenized by inserted transposons or T-DNA of *Agrobacterium*, have contributed to the characterisation of a vast amount of lines with disrupted gene function.

With the completion of the Arabidopsis genome sequence during the year 2000 (Arabidopsis Genome Initiative 2000), the research on Arabidopsis has gained new opportunities. The Arabidopsis genome became the first representative of the plant kingdom to be entirely sequenced, enabling comparisons of genome structure and evolution of plants, yeast and animals. The Arabidopsis genome analyses unexpectedly revealed the presence of a substantial amount of structural redundancy (Arabidopsis Genome Initiative 2000). About 70 % of the genome has been estimated to be duplicated through several polyploidization and segmental duplication events (Arabidopsis Genome Initiative 2000; Blanc *et al.* 2000; Vision *et al.* 2000; Ziolkowski *et al.* 2003). The duplication events have been ordered in different age classes; the most recent and best defined event occurred

between 20 and 60 million years ago, whereas the two earlier events occurred more than 100 million years ago (Blanc and Wolfe 2004 and references therein). Gene pairs, arisen through these duplication events have significantly contributed to the evolution of Arabidopsis as the paralogs can encounter different faiths. If both gene copies are retained in the genome, which appears to be a more rare outcome (Lynch and Conery 2003), functional divergence can have occurred by neofunctionalization or by subfunctionalization. In the case of neofunctionalization a gene copy acquires a new function, whereas in the case of subfunctionalization the copies retain different subsets of the functionality of the ancestral gene (Blanc and Wolfe 2004). If a dosage effect provides selective advantages, functionally completely redundant genes might also be retained (Osborn *et al.* 2003). Large amounts of different types of genomic changes, e. g. chromosomal rearrangements and sequence losses, as well as epigenetic changes, resulting in heritable alterations in gene expression, have contributed to the function of the Arabidopsis genome present today.

Figure 1. *Arabidopsis thaliana*

Plant growth and development in response to environmental conditions

Arabidopsis has been successfully used for studying responses of plants to environmental conditions. As sessile organisms, unable to escape from unfavourable surroundings, plants have evolved a substantial developmental plasticity in order to cope with the changing environmental conditions. Together with internal information, environmental factors such as light, water availability, temperature, gravity, mechanical stimuli, nutrients, oxygen and CO_2, affect plant development. Embryogenesis establishes the radial and axial patterns as well as the primary meristems that are responsible for generating most of the plant structure after germination. Switches between developmental patterns or programmes, in response to environmental cues, cause the plastic development of the plant. Co-ordination of growth and development rely on signalling, often composed of complex networks, at the cellular as well as the whole plant level.

Light effects on plant growth and development

Light is one of the most important environmental cues regulating plant development. In addition to acting as an energy source for photosynthesis, light regulates developmental processes throughout the post-embryonic growth and affects all major developmental transitions such as germination and flowering. Plants monitor the quantity, quality, periodicity and the direction of light by use of photoreceptors that transduce the light signals into physiological and developmental responses.

Plant photoreceptors

Three types of photoreceptors have been characterised in plants; cryptochromes and phototropins absorbing UV-A/blue light (320-500 nm) and phytochromes absorbing red/far-red light (600-750 nm). The cryptochromes and the phytochromes have important and partially overlapping functions in the control of photomorphogenesis, transition to flowering and in photic entrainment of the circadian clock, whereas

phototropins mainly regulate movement responses like phototropism, chloroplast movement and stomatal opening (reviewed by Frankhauser and Staiger 2002).

Cryptochromes are photoreceptors found throughout the higher eukaryotes. In Arabidopsis there are two cryptochrome genes, *CRY1* and *CRY2*, which show high similarity to each other and to genes encoding the bacterial DNA repair enzyme photolyase (Ahmad and Cashmore 1993; Lin *et al.* 1998). The photolyase related domain of the cryptochromes is responsible for the chromophore binding, but it lacks the DNA repair activity. *CRY1* and *CRY2* are expressed throughout the whole plant and their expression levels have been found to oscillate with the circadian rhythm (Ahmad and Cashmore 1993; Lin *et al.* 1998; Harmer *et al.* 2000). Also the protein levels of CRY2 cycle; diurnally and dependent on the photoperiod (El-Din El Assal *et al.* 2001). Degradation of CRY2, under high blue light intensities, contributes to the oscillation of CRY2 (Ahmad *et al.* 1998a; Lin *et al.* 1998). Phototropins, also encoded by two genes, *PHOT1* and *PHOT2* (nomenclature by Briggs *et al.* 2001) in Arabidopsis, are distinct from the cryptochromes. Phot1 and Phot2 have two LOV domains (for light, oxygen and voltage-regulated proteins), that are responsible for chromophore binding, and a serine/threonine kinase domain that undergoes light-dependent autophosphorylation (Sakai *et al.* 2001).

The red/far-red light absorbing phytochromes are encoded by a small gene family, composed of *PHY A* to *PHY E* (recently reviewed by Nagy and Schäfer 2002). In addition to the apoprotein encoding genes, *PHY A* to *PHY E*, the chromophore encoding genes, *HY1* and *HY2*, are required for the production of functional phytochromes (Kohchi *et al.* 2001 and references therein). *In vivo,* phytochromes exist in two photoreversible forms: in the biologically inactive Pr (for red-light-absorbing) form and in the biologically active Pfr (for far-red-light-absorbing) form. Upon light perception the phytochromes translocate from the cytosol to the nucleus. Light also causes degradation of PHY A, whereas the other phytochromes, PHY B-PHY E, are stable in light (Quail, 1997). Natural light environments simultaneously

activate several photoreceptors, which co-operate to direct light regulated physiological processes.

Photoreceptor interactions in the regulation of photomorphogenesis

Following germination, light signals direct the photomorphogenic (de-etiolation) development of Arabidopsis seedlings, characterised by short hypocotyls, expanded and unfolded cotyledons, open apical hooks and the onset of photosynthesis. The regulation of de-etiolation involves a complex interplay of both phytochromes and cryptochromes (reviewed by Franklin and Whitelam 2004). The characterisation of lines mutant in one or several photoreceptor genes, showing deficiencies in de-etiolation, have provided functional information of the photoreceptors. Under low light intensities, PHY A and CRY2 have pronounced effects on de-etiolation whereas their levels decrease as light intensities increase (Lin *et al.* 1998; Sullivan and Deng 2003). Under high light intensities the CRY1 and the photostable phytochromes, predominantly PHY B, have more dominant roles (Lin *et al.* 1998; Quail 2002). Crossings between photoreceptor mutants have revealed functional redundancy within and between the cryptochromes and the phytochromes (Mockler *et al.* 1999; Mazzella *et al.* 2001; Franklin *et al.* 2003b) as well as interactions between these photoreceptors. The precise nature of the co-operations has been difficult to interpret. However, functional interactions have been shown to exist between CRY2 and PHY B (Más *et al.* 2000), CRY1 and PHY B (Casal and Mazzella 1998) and possibly between CRY2 and PHY C (Franklin *et al.* 2003a). Further, a physical *in vitro* interaction of CRY1 and PHY A proteins has been shown (Ahmad *et al.* 1998b). In addition to the interactions, the phenotypes of the photoreceptor mutants grown in different light conditions have been interpreted to mean that also phytochromes possess blue-light photoreceptor activity (Casal 2000).

Photoreceptor functions in the transition from vegetative to reproductive growth

Arabidopsis is a facultative long day plant, but flowers eventually even under short days. Several regulatory pathways have been identified which regulate flowering of Arabidopsis in response to environmental signals (reviewed by Mouradov *et al.* 2002). The autonomous, vernalization, gibberellic acid (GA) and photoperiod response pathways co-regulate the transition to flowering. The photoperiod response pathway involves the circadian clock function. The cryptochromes and the phytochromes are involved in the measurement of photoperiod and influence the circadian clock entrainment. CRY2 and PHY A are the photoreceptors likely to be involved in the measurement of the photoperiod (Mouradov *et al.* 2002; Mockler *et al.* 2003), and PHY A, PHY B, CRY1 and CRY2 all influence the clock entrainment under specific light conditions, even though a *phyA phyB cry1 cry2* quadruple mutant can still entrain to different photoperiods (Frankhauser and Staiger 2002). In addition, roles for PHY D and PHY E have also been shown in the regulation of flowering (Casal 2000).

Signalling downstream of the photoreceptors

The light perceived by the cryptochromes and phytochromes trigger diverse signalling components downstream of the photoreceptors (reviewed by Lin and Shalitin 2003; Quail 2002). At least two kinds of mechanism are involved; second messengers and direct interactions with transcription factors. Large-scale changes in gene expression, as a part of signalling or as a result of it, have been detected upon light perception. During de-etiolation the expression of approximately 30 % of the Arabidopsis genes is changed (Ma *et al.* 2001; Tepperman *et al.* 2001). The different photoreceptors control distinct as well as overlapping sets of downstream genes (Ma *et al.* 2001). Blue light induces rapid and slow responses in gene expression suggesting that transcriptional cascades are triggered by blue light (Jiao *et al.* 2003).

Water availability effects on plant growth

The availability of water has a significant impact on plant growth and development. Often the rate of transpiration exceeds the water uptake and plants suffer from water deficit, which causes osmotic stress. Under natural conditions, osmotic stress can be caused by water deficit as well as by high salinity. High salinity, in addition to osmotic stress, causes ionic stress, as the concentration of ions, most commonly Na^+ and Cl^-, exceeds optimal levels. Plants respond to drought and salt stress with a number of changes at the molecular, cellular and the whole plant level. These changes are involved in control and repair of the damage, establishment of ion and osmotic homeostasis and growth and development re-programming to suit the new environmental conditions (reviewed by Zhu 2001; Xiong & Zhu 2002). The responses as well as the signalling during salt and osmotic stress share many similarities (reviewed by Zhu 2002).

Signal transduction during salt and osmotic stress

During salt stress, an excess of Na^+ and Cl^- ions activate the ionic stress pathway whereas a change in turgor is likely to activate the osmotic stress pathway during salt and drought stress. These pathways regulate the maintenance of ion and osmotic homeostasis. The primary signals, Na^+ and Cl^- ions and the turgor changes, cause injuries in the plant cells and provoke production of secondary signals, e. g. reactive oxygen species (ROS), the hormones abscisic acid (ABA) and ethylene and second messengers like phospholipids. The injuries and the secondary signals function as cues for the control and repair of the damage as well as for the regulation of cell division and expansion. The secondary signals are likely to be involved in long-distance signalling to co-ordinate the whole plant responses to salt and osmotic stresses. ABA has been shown to function as a systemic signal during drought stress (Zhang and Davies 1991) and ROS as a signal during other types of stresses, like wounding, pathogen attack and excess light (Alvarez *et al.* 1998; Karpinski *et al.* 1999; Lopez-Huertas *et al.* 2000).

Transient changes in cytosolic free calcium ($[Ca^{2+}]_{cyt}$) has been recorded in response to salt and osmotic stress (Knight *et al.* 1997) as well as

in response to a wide range of other stimuli (reviewed by Reddy 2001). In unstimulated cells, the free $[Ca^{2+}]_{cyt}$ is maintained low and upon stimulus, the influx of calcium from the intra- and extracellular sources increases the $[Ca^{2+}]_{cyt}$. The influx and efflux of calcium determine the form of the calcium signal that is potentially specific to the decoders, calcium sensors. Four major type of calcium sensors have been characterized in plants; calmodulin, EF-hand containing Ca^{2+}-binding proteins, including the calmodulin like proteins, Ca^{2+}-regulated protein kinases and Ca^{2+}-binding proteins without EF-hand motifs (reviewed by Reddy 2001). Even though calcium signals have been detected in response to many different stimuli, the evidence that the calcium signal is an essential intermediate of downstream responses is sparse.

Calcium has been proposed to trigger the ionic stress signalling pathway, controlling ion homeostasis under salt stress. This pathway is defined by the *SOS* (*Salt Overly Sensitive*) genes, *SOS1* to *3* (reviewed by Zhu 2002). The most upstream component of the SOS pathway known is SOS3, a myristoylated EF-hand type calcium-binding protein, suggested to sense $[Ca^{2+}]_{cyt}$ changes (Ishitani *et al.* 2000; Liu and Zhu 1997; Liu and Zhu 1998). SOS3 activates the subsequent components of the pathway, a serine/threonine protein kinase, encoded by *SOS2* (Halfter *et al.* 2000; Liu *et al.* 2000) and a plasma membrane Na^+/H^+ antiporter, encoded by *SOS1* (Shi *et al.* 2000).

The role of calcium during osmotic stress signalling is less well characterised. The Ca^{2+} sensitive response elements might be calcium-dependent protein kinases (Pei *et al.* 1996; Sheen 1996; Hwang *et al.* 2000; Saijo *et al.* 2000) or kinases associated with calcineurin B-like proteins (Cheong *et al.* 2003; Kim *et al.* 2003). Dependent on or independent of the calcium signal, the osmotic stress signalling, however, activates phospholipid-based signalling, ROS, different types of protein kinases and the accumulation of ABA (reviewed by Xiong *et al.* 2002; Zhu 2002). ABA is an important and fairly well studied mediator of osmotic stress responses, even though an ABA independent osmotic stress signalling pathway appears also to exists (Shinozaki and Yamaguchi-Shinozaki 2000; Seki *et al.* 2003).

ABA is synthesised mainly in the roots of water stressed plants and transported in the xylem to the different parts of the shoots (reviewed by Hartung *et al.* 2002). The catabolism of ABA also has an effect on the ABA levels in different parts of a plant (reviewed by Finkelstein and Rock 2002). Genetic screens have led to the isolation of genes involved in ABA biosynthesis (reviewed by Finkelstein and Rock 2002), and genes responsible for ABA degradation have been identified (Kushiro *et al.* 2004). The ABA receptor(s) is unknown, even though indirect evidence argues for intracellular and extracellular ABA perception sites. Early steps of ABA signalling include the participation of GTP-binding proteins, phospholipases, protein kinases and phosphatases (reviewed by Finkelstein and Rock 2002). The class 2C serine/threonine protein phosphatases have been defined as negative regulators of ABA signalling (Gosti *et al.* 1999; Merlot *et al.* 2001; Tahtiharju and Palva 2001; Saez *et al.* 2004). The first described type PP2C protein phosphatase, ABA INSENSITIVE (ABI) 1, but not the very similar ABI2, contain a Ca^{2+}-binding EF-hand motif (Leung *et al.* 1994, 1997; Meyer *et al.* 1994; Rodriguez *et al.* 1998), though a role of calcium binding for the function of ABI1 has not been shown. The other cloned ABA insensitive loci, *ABI3*, *ABI4* and *ABI5*, encode transcription factors of the B3-, APETALA2- (AP2), and basic leucine zipper (bZIP) domain families, respectively (reviewed by Finkelstein *et al.* 2002), and *ABI8* encodes a protein with unknown function (Brocard-Gifford *et al.* 2004). To date, over 50 loci affecting ABA signalling have been described (reviewed by Finkelstein *et al.* 2002) demonstrating the presence of a complex ABA signalling network, possibly interacting with other signalling pathways.

Several *cis* and *trans*-acting factors have been defined that are involved in transcriptional regulation of different genes by ABA (reviewed by Rock 2002; Finkelstein and Rock 2002). The *cis*-acting sequences fall into four main groups: the G-box elements designated ABREs, the coupling element (CE)-like sequences functionally equivalent to ABREs, the RY/Sph elements and the recognition sequences for the MYB and MYC class of transcription factors. The ABRE elements have been shown to be bound by bZIP transcription factors and the RY elements by B3-domain proteins.

ABA regulates transcription of various types of genes, including genes encoding homeodomain leucine zipper (HDZip) transcription factors (Söderman *et al.* 1996; Lee and Chun 1998; Söderman *et al.* 1999; Johannesson *et al.* 2003).

HDZip transcription factors

HDZip transcription factors are characterised by the presence of a DNA-binding homeodomain and an adjacent leucine zipper motif, which mediates protein-dimer formation. In Arabidopsis the HDZip genes constitute a rather large gene family with 47 members (I; Schrick *et al.* 2004). The homeodomain and the leucine zipper domains are found in other Arabidopsis transcription factor families in combinations with domains with different functions. The combination of the homeodomain and the leucine zipper domain adjacent to each other appears to be specific for plants as proteins with a similar domain organisation have not been found in yeast or animals. However, the homeodomains of the HDZip transcription factors are related to the homeodomains of other eukaryotic transcription factors. In many eukaryotes the homeodomain proteins, encoded by the homeobox genes, act as key developmental regulators (reviewed by Gehring 1993). Thus, attempts to isolate homeobox genes from Arabidopsis were taken. The conservation of the coding sequence of the homeodomain was exploited in studies resulting in the isolation of the first HDZip genes in Arabidopsis (Ruberti *et al.* 1991; Mattsson *et al.* 1992; Schena and Davies 1992). In these studies a strategy previously used for the isolation of the homeodomain encoding genes in the nematode *Caenorhabditis elegans* (Bürglin *et al.* 1989) was applied. Degenerate oligonucleotide probes, designed to match all possible codon combinations corresponding to conserved amino acids within the helix 3 of the homeodomain, were designed and used to screen Arabidopsis cDNA libraries. The isolated HDZip genes were denoted as *Arabidopsis thaliana* homeobox genes; *ATHB1*, *-2* and *-3* (Ruberti *et al.* 1991; Mattsson *et al.* 1992) and homeobox from *Arabidopsis thaliana*; *HAT4*, *-5* and *-22* (Schena and Davies 1992).

Exploiting the sequence similarity of the first isolated HDZip genes, additional members of the HDZip family have been identified and characterized in Arabidopsis (Table 1). A classification of HDZip genes into four different classes; HDZip I-IV, based on sequence similarity and supported by the intron/exon patterns, was suggested by Sessa *et al.* (1994). The homeodomains of the HDZip class I and II genes are most similar and genes of these classes are likely to share a common origin (Chan *et al.* 1998). The HDZip III and IV genes, distantly related to HDZip I and II (Chan *et al.* 1998), in addition to the homeodomain and the leucine zipper domain, encode a START domain, hypothesised to bind a steroid-like ligand (Pontig and Aravind 1999; Schrick *et al.* 2004). The divergence of the HDZip genes appears to have an early origin in the plant lineage, as HDZip I, II, IV genes and HDZip I-III genes have been isolated from the fern *Ceratopteris richardii* and the moss *Physcomitrella patens*, respectively (Aso *et al.* 1999; Sakakibara *et al.* 2001).

Table 1. HDZip genes identified in Arabidopsis.

HDZip class	Gene name	Gene code	Reference
I	*ATHB1/HAT5*	At3g01470	Ruberti *et al.* 1991; Sessa *et al.* 1993; Aoyoma *et al.* 1995; Sessa *et al.* 1997
I	*ATHB3/HAT7*	At5g15150	Mattsson *et al.* 1992
I	*ATHB5*	At5g64310	Söderman *et al.* 1994; Johannesson *et al.* 2001; Johannesson *et al.* 2003
I	*ATHB6*	At2g22430	Söderman *et al.* 1994; Söderman et al. 1999; Himmelbach *et al.* 2002; IV
I	*ATHB7*	At2g46680	Söderman *et al.* 1994; Söderman *et al.* 1996; Hjellström *et al.* 2003; Olsson *et al.* in press
I	*ATHB12*	At3g61890	Lee and Chun 1998; Lee *et al.* 2001; Olsson *et al.* in press
I	*ATHB13*	At1g69780	Hanson *et al.* 2001; Hanson *et al.* 2002
I	*ATHB16*	At4g40060	III
I	*ATHB20*	At3g01220	Hanson 2000
I	*ATHB21*	At2g18550	I
I	*ATHB22*	At2g36610	I
I	*ATHB23*	At1g26960	Hanson 2000
I	*ATHB40*	At4g36740	I

I	*ATHB51*	At5g03790	I
I	*ATHB52*	At5g53980	I
I	*ATHB53*	At5g66700	I
I	*ATHB54*	At1g27050	I
II	*ATHB2/HAT4*	At4g16780	Ruberti *et al.* 1991; Carabelli *et al.* 1993; Schena *et al.* 1993; Sessa *et al.* 1993; Sessa *et al.* 1997; Steindler *et al.* 1999; Ohgishi *et al.* 2001
II	*ATHB4*	At2g44910	Carabelli *et al.* 1993
II	*ATHB17*	At2g01430	Ruberti *et al.*, unpublished
II	*HAT1*	At4g17460	Schena and Davies 1994
II	*HAT2*	At5g47370	Schena and Davies 1994
II	*HAT3*	At3g60390	Schena and Davies 1994
II	*HAT9*	At2g22800	Schena and Davies 1994
II	*HAT14*	At5g06710	Schena and Davies 1994
II	*HAT22*	At4g37790	Schena and Davies 1992
III	*ATHB8*	At4g32880	Baima *et al.* 1995; Sessa *et al.* 1998a; Baima *et al.* 2001; Kang and Dengler 2002
III	*ATHB9/PHB*	At1g30490	Sessa *et al.* 1998a; McConnell *et al.* 2001; Emery *et al.* 2003
III	*ATHB14/PHV*	At2g34710	Sessa *et al.* 1998a; McConnell *et al.* 2001; Emery *et al.* 2003
III	*ATHB15*	At1g52150	Ohashi-Ito and Fukuda 2003
III	*REV/ILF1*	At5g60690	Talbert *et al.* 1995; Zhong and Ye 1999; Ratcliffe *et al.* 2000; Otsuga *et al.* 2001; Emery *et al.* 2003
IV	*ATHB10/GL2*	At1g79840	Di Cristina *et al.* 1996; Masucci *et al.* 1996a; Masucci *et al.* 1996b; Ohashi *et al.* 2002; Ohashi *et al.* 2003; van Hengel *et al.* 2004
IV	*ANL2/AHDP*	At4g00730	Kubo *et al.* 1999
IV	*PDF2*	At4g04890	Abe *et al.* 2003
IV	*ATML1*	At4g21750	Abe *et al.* 2001; Abe *et al.* 2003
IV	*FWA*	At4g25530	Soppe *et al.* 2000
IV		At1g05230	Schrick *et al.* 2004
IV		At1g17920	Schrick *et al.* 2004
IV		At1g34650	Schrick *et al.* 2004
IV		At1g73360	Schrick *et al.* 2004
IV		At2g32370	Schrick *et al.* 2004
IV		At3g03260	Schrick *et al.* 2004
IV		At3g61150	Schrick *et al.* 2004
IV		At4g17710	Schrick *et al.* 2004
IV		At5g17320	Schrick *et al.* 2004
IV		At5g46880	Schrick *et al.* 2004
IV		At5g52170	Schrick *et al.* 2004

HDZip dimers as transcriptional regulators

DNA-binding studies of the members of HDZip I and II have shown that ATHB1 (HDZip I) and ATHB2 (HDZip II) interact as homodimers with the pseudopalindromic binding sites, CAAT(A/T)ATTG and CAAT(G/C)ATTG, respectively, consisting of two five bp half-sites that overlap at the central position (Sessa *et al.* 1993). A theoretical model of DNA binding of a HDZip dimer is illustrated in figure 2. In subsequent *in vitro* DNA binding studies, other HDZip I proteins were also found to bind to the CAATNATTG sequence; ATHB5, -6 and -16 with preference for the central position A/T or G/C and ATHB3 and -13 with preference for the central position A/T (Johannesson *et al.* 2001). HDZip I and II proteins from other species, from rice and the resurrection plant *Craterostigma plantagineum*, have also been found to have binding site preferences similar to the Arabidopsis HDZip I and II proteins (Meijer *et al.* 1997; Frank *et al.* 1998; Meijer *et al.* 2000). The HDZip III and IV proteins have been found to bind to slightly different sequences (GTAAT(G/C)ATTAC) and (TAAATG(C/T)A) respectively (Sessa *et al.* 1998a; Abe *et al.* 2001; Abe *et al.* 2003; Ohashi *et al.* 2003).

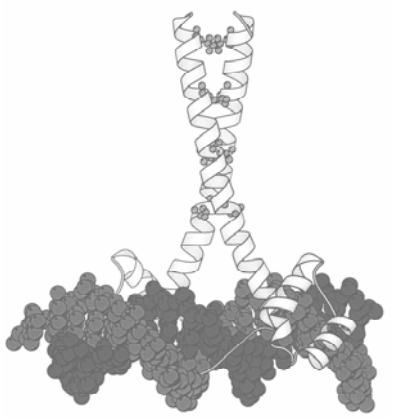

Figure 2. A theoretical model of a HDZip dimer binding to DNA. The model is based on the three-dimensional structures of the *Drosophila* engrailed homeodomain and the yeast GCN4 leucine zipper motif (K. Johansson, H. Johannesson and E. Söderman, unpublished).

In vitro protein-protein interaction studies have shown HDZip proteins to form homodimers and heterodimers (Sessa *et al.* 1993; Meijer *et al.* 1997; Frank *et al.* 1998; Meijer *et al.* 2000; Johannesson *et al.* 2001). Heterodimer formation has been detected between HDZip I and II proteins, although only within each class (Gonzalez *et al.* 1997; Meijer *et al.* 1997; Frank *et al.* 1998; Meijer *et al.* 2000; Johannesson *et al.* 2001). Heterodimer formation within a class is selective as e. g. the Arabidopsis HDZip I protein ATHB5 is able to heterodimerize with ATHB6 and -16, but not with ATHB1 (Johannesson *et al.* 2001).

Both transcriptional activators and repressors have been found among the HDZip proteins. In transient expression assays on plants three class I proteins have been shown to activate gene expression in a binding site dependent manner; the ATHB1 (Aoyama *et al.* 1995, Sessa *et al.* 1998b) and the rice HDZip proteins Oshox4 and -5 (Meijer *et al.* 2000), whereas the class II proteins, ATHB2, Oshox1 and -3, were found to be repressors of gene expression (Meijer *et al.* 1997; Steindler *et al.* 1999; Meijer *et al.* 2000). However, in yeast, Oshox1 was found to activate transcription, indicating that HDZip proteins might function as activators or repressors depending on other factors or conditions (Meijer *et al.* 2000).

Biological roles of HDZip genes

The proteins encoded by HDZip genes have been found to function in a wide range of processes. The HDZip III includes the five relatively well characterised genes, *REVOLUTA* (*REV/IFL1*), *PHABULOSA* (*PHB/ATHB9*) and *PHAVOLUTA* (*PHV/ATHB14*), which direct the development of adaxial domains of lateral organs, the apical meristem and the vascular bundles (Zhong and Ye 1999; McConnell *et al.* 2001; Otsuga *et al.* 2001; Emery *et al.* 2003), and *ATHB8* and *-15*, which have been suggested to direct vascular development (Baima *et al.* 2001; Ohashi-Ito and Fukuda 2003). Among the members of the HDZip IV, *GLABRA2* (*GL2/ATHB10*), *Arabidopsis thaliana MERISTEM LAYER1* (*ATML1*) and *PROTODERMAL FACTOR2* (*PDF2*), appear to be involved in establishing cell fates in epidermal cells and to regulate cell-layer-specific gene expression (Rerie *et al.* 1994; Di Cristina *et*

al. 1996; Lu *et al.* 1996; Masucci *et al.* 1996a; Ohashi *et al.* 2002; Abe *et al.* 2003) and *ANTHOCYANINLESS 2* (*ANL2*) to affect anthocyanin accumulation in the leaf subepidermal layer and cell identity in the root (Kubo *et al.* 1999).

The HDZip I and II genes are less well characterised, as regards function. The available functional information on HDZip I and II genes, however, suggests that at least some of the genes are involved in mediating the effects of external conditions on plant growth and development. The expression of *ATHB2* and *-4* (HDZip II) genes are induced by far-red-rich light and the phenotypic analyses of plants with increased levels of *ATHB2* suggest a function as a mediator of the red/far-red light effects on leaf cell expansion in the shading response (Carabelli *et al.* 1993, 1996; Steindler *et al.* 1999). Phenotypic analyses of plants with increased levels of *ATHB1* (HDZip I) or *ATHB13* (HDZip I) expression suggest that these genes are involved in the regulation of cotyledon and leaf development (Aoyama *et al.* 1995; Hanson *et al.* 2001), *ATHB13* potentially as a mediator of sugar signalling (Hanson *et al.* 2001). Several HDZip I genes have been proposed to be involved in ABA related responses. *ATHB5, -6, -7,* and *-12* are either up- or down-regulated by water deficit conditions or externally applied ABA (Söderman *et al.* 1996; Lee and Chun 1998; Söderman *et al.* 1999; Johannesson *et al.* 2003) and the phenotypic analyses of Arabidopsis plants with elevated levels of *ATHB5, -6, -7*, or *-12* suggest that these genes regulate growth in response to ABA and/or water deficit conditions (Himmelbach *et al.* 2002; Hjellström *et al.* 2003; Johannesson *et al.* 2003; Olsson *et al.* in press). Further evidence for the involvement of *ATHB6* in ABA related responses was provided by Himmelbach *et al.* (2002) as ATHB6 was identified as an interaction partner for ABI1.

RESULTS AND DISCUSSION

Identification of HDZip I genes in Arabidopsis (I and III)

A search of Arabidopsis databases allowed the identification of 26 different genes with homeobox sequences with a high degree of similarity to the HDZip class I homeoboxes, and that encode leucine zipper domains located in a position typical for the HDZip proteins (I). Of these, 19 corresponded to the previously characterised HDZip I and II genes (HDZip I: *ATHB1, -3*, *-5, -6, -7*, *-12*, *-13*, *-16*, *-20, -23* and HDZip II: *ATHB2, -4* and *-17, HAT1*, *2, 3, 9, 14, -22* (Table 1), whereas 7 represented novel putative HDZip genes. The novel genes were denoted *ATHB21*, *-22*, *-40*, *-51*, *-52*, *-53* and *-54*, respectively. The deduced amino acid sequences of these 26 genes were compared over the HDZip region. With the exception of ATHB22, which has an 8 amino acid insertion between helix 1 and 2, all HDZip sequences aligned perfectly over the homeodomain (Fig. 3). Despite the atypical architecture of the ATHB22 homeodomain, the protein might be able to adopt a similar three-dimensional structure of the homeodomain as the other HDZip proteins.

The homeodomains of the HDZip proteins contain the five invariant amino acids (L_{16}, W_{48}, F_{49}, N_{51}, R_{53}), except that of ATHB22 (K_{53}), and seven out of eight of the highly conserved residues (F_{20}, L_{26}, L_{40}/ M_{40}, I_{45}/V_{45}, I_{47}/V_{47}, R_{55}, K_{57}) of the homeodomain consensus sequence, defined on the basis of a compilation of 346 homeodomain sequences from a range of different eukaryotes (Bürglin, 1994). A number of amino acid positions distinguish HDZip I from HDZip II (Fig. 3). The amino acid at position 46 of helix 3 is invariant within the HDZip I and II but distinct between these classes. Several other amino acids, e. g. the ones at positions 6, 25, 29, 30, 58 and 61 are invariable within the HDZip II and differ from HDZip I amino acids, which show some degree of variation at these positions. ATHB21, -22, -40, -51, -52, -53 and 54 are more similar to the HDZip I proteins than to HDZip II proteins and were thus suggested to belong to the HDZip I.

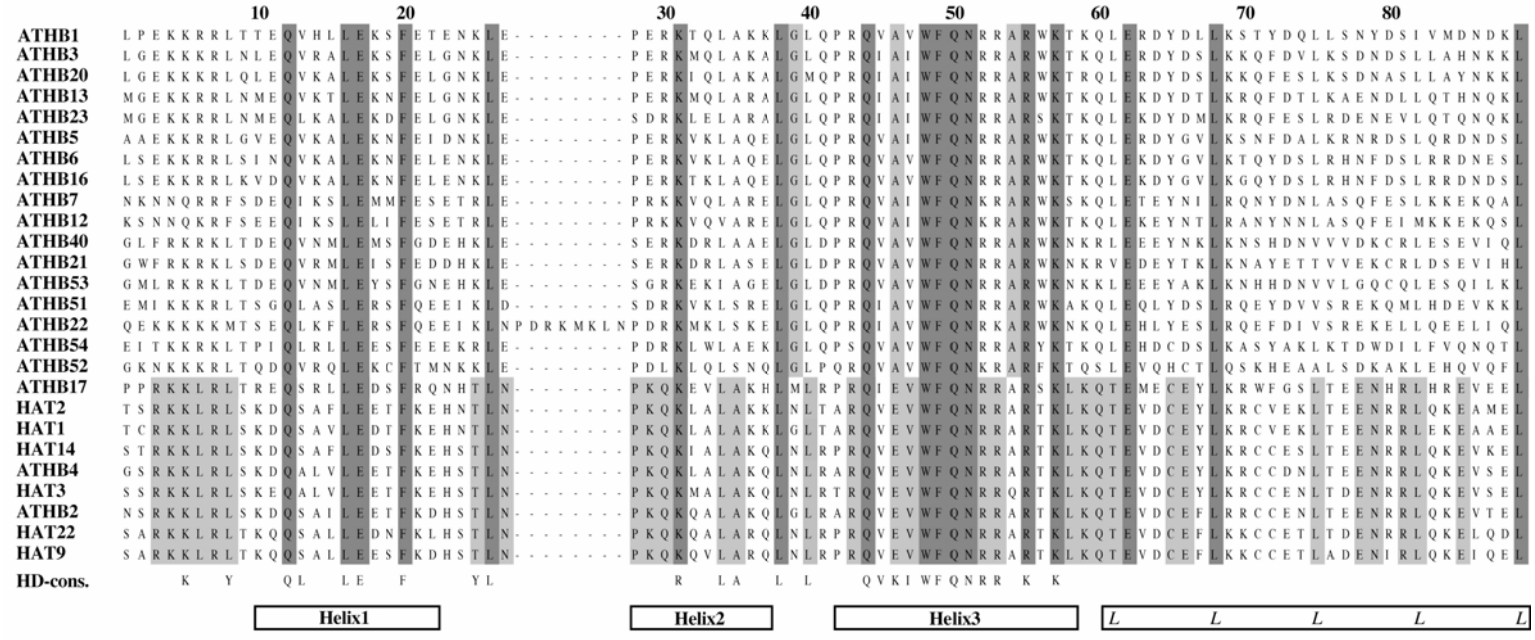

Figure 3. Alignment of the amino acid sequences of HDZip class I and II proteins (I). Gaps are indicated by dashes. Highly conserved positions in a homeodomain consensus sequence (Bürglin 1994) are indicated below the alignment. Residues conserved in both classes are depicted in dark grey, residues conserved only within HDZip I or II are depicted in light grey and residues showing variation between and within the classes are depicted in white. The three α-helices of the homeodomain and the leucine residues of the leucine zipper are shown below the alignment.

Phylogeny and duplication history of HDZip I genes (I)

Phylogenetic analyses of the Arabidopsis HDZip I and II proteins confirmed that HDZip I and II, as defined by Sessa *et al.* (1994), are monophyletic (Johannesson 2000). To assess the phylogeny of the HDZip I proteins, a maximum parsimony analysis based on the HDZip domain amino acid sequences, shown in figure 3, was performed. The resulting phylogenetic tree defined six HDZip I subclasses; α (*ATHB3*, *-13*, *-20*, *-23*), β (*ATHB1, -5*, *-6* and *-16*), γ (*ATHB7* and *-12*), δ (*ATHB21*, *-40* and *-53*), ε (*ATHB22* and *-51*) and ϕ (*ATHB52* and *-54*) (Fig. 4). The subclasses α, γ, δ and ε were well supported, whereas the subclasses β (*ATHB1, -5*, *-6* and *-16*) and ϕ (*ATHB52* and *-54*) received low support. However, the association of *ATHB5*, *-6* and *-16*, within the subclass β received high support values. A bayesian analysis, based on the nucleotide sequence of the HDZip domain, resulted in a tree with major topologies very similar to the tree in Figure 4. However, the phylogenetic association of *ATHB1* within subclass β and the subclass ϕ was not well supported in this analysis either.

The HDZip I genes have intron/exon patterns distinct from the genes of HDZip II. In each subclass (α-ϕ) the members share an intron/exon pattern within the HDZip domain, with the exception of *ATHB1* that has an additional intron, absent from the other members of the β subclass (Fig. 4). The position of one intron is, however, shared by all members of the subclass β. Thus, the intron distribution pattern within the HDZip domain strongly supports the presented phylogenetic relationships of the HDZip I genes.

Figure 4. Phylogeny of the Arabidopsis HDZip class I (I). A) The most parsimonious tree from a maximum parsimony analysis based on the amino acid sequences of HDZip domains. The HAT1 (HDZip II) sequence was used as an outgroup. The subclasses within the HDZip I are denoted α, β, γ, δ, ε and ϕ. Bootstrap support is indicated on the branches. The letters (A-E) indicate the intron/exon pattern within the HDZip domain illustrated in 4B. The branching points supported by data on the duplication history of the HDZip I genes, presented in paper I, are marked with (*). B) An illustration of the intron positions within the region corresponding to the HDZip domains of HDZip I and II (triangles indicate intron positions).

Further support for the presented phylogenetic relationships is provided by the traced duplication history of the HDZip I genes. By comparing the chromosomal locations of the HDZip I genes in relation to the segmental duplication events identified by the Arabidopsis Genome Initiative (2000) and by Blanc *et al.* (2003), *ATHB3* and *-20, ATHB13* and *-23, ATHB6* and *-16*, *ATHB5* and *-6, ATHB1* and *-5, ATHB7* and *-12, ATHB21* and *-40*, *ATHB40* and *-53*, as well as *ATHB21* and *-53* were found to constitute pairs of paralogous genes (I). Importantly, the duplication history of the HDZip I genes supported the association of *ATHB1* within the subclass β, which gained low support in both maximum parsimony and the bayesian analyses. The duplication history of the HDZip I genes as well as the intron/exon patterning of the HDZip domain thus provided independent support for the phylogenetic associations of the HDZip I genes. In addition, the traced duplication history of the HDZip I genes revealed that only a few of the HDZip I genes have lost the paralogous gene. The average half-life for a gene duplicate, in different species including Arabidopsis, has been estimated to be approximately 4 million years (Lynch and Conery 2000). The majority of the HDZip I gene pairs, however, seem to have evolved 20-60 or over 100 million years ago thus suggesting positive selection for HDZip I paralog retention. In consistens with this notion, Blanc and Wolfe (2004) found unequal gene loss between functional categories of duplicated genes in Arabidopsis. Gene pairs involved in signal transduction and transcription, e.g. transcription factors, have preferentially been retained whereas genes involved in e.g. DNA repair and defence have more often lost the paralogous gene (Blanc and Wolfe 2004).

HDZip I genes have wide expression profiles (I)

All identified HDZip I genes were found to be expressed, as determined by reverse transcriptase (RT) mediated PCR analysis (I). Thus no pseudogenes are present in the HDZip I assuming that the transcripts produce a functional protein. The expression analyses showed that the HDZip I genes are expressed in most organs in different developmental stages. Transcripts of subclass α (*ATHB20*), β (*ATHB1, -5*, *-6* and *-16*), γ (*ATHB7* and *-12*), δ

(*ATHB21*) and ε (*ATHB51*) genes were detected from all examined samples; 5 and 12 days old seedlings, roots, leaves, stems, siliques and flowers of adult plants, consistent with III and the previous reports (Söderman *et al.* 1994; Lee and Chun 1998; Söderman *et al.* 1999). The subclass α (*ATHB3*, -*13*, -*23*), δ (*ATHB40* and -*53*), ε (*ATHB22*) and ϕ (*ATHB52* and -*54*) genes showed more restricted expression profiles. However, none of these genes showed expression restricted to only one organ. In general the subclass members seemed to have only partially overlapping expression patterns. Consistent with this notion the promoter reporter gene (GUS) fusions have shown that expression of *ATHB5*, -*6* and -*16* differs at the tissue level (Söderman *et al.* 1999; Wang 2001; Johannesson *et al.* 2003). The subclass γ is an exception among the HDZip I subclasses since the members of this subclass, *ATHB7* and -*12*, have very similar expression patterns even at the tissue level, as detected by the use of promoter reporter gene (GUS) fusions (Hjellström *et al.* 2003; Olsson *et al.* in press). Thus apart from *ATHB7* and -*12,* it appears as the *cis*-regulatory regions of the closely related HDZip I genes have undergone functional diversification. This conclusion is in accordance with previous reports describing rapid divergence in the expression of duplicated genes in Arabidopsis, yeast and human (Blanc and Wolfe 2004 and references therein). The divergence of expression patterns has been suggested to be progressive as a fraction of transcriptionally divergent gene pairs was found to be larger among the older than the newer paralogs (Blanc and Wolfe 2004).

Changes in the *cis*-regulatory regions of transcription factor genes, rather than in protein coding regions, have been argued to be the principal mechanism of evolutionary change (e. g. Doebley and Lukens 1998 and references therein; Wang *et al.* 1999). This conclusion seems to be applicable to the class I HDZip transcription factors, though detailed sequence comparisons of the promoters to date are missing. The expression analyses showed that *cis*-regulatory regions of the HDZip I genes have undergone diversification, whereas the proteins encoded by the closely related genes appear to be functionally analogous as plants with elevated levels of *ATHB6* or -*16* and *ATHB3*, -*13*, -*20* or -*23,* respectively, result in

similar phenotypes (I; III; IV; Hanson 2000). Further, the DNA binding specificities of the different HDZip I proteins are very similar (Johannesson *et al.* 2001).

The paralogous genes *ATHB7* or *-12* differ from the rest of the HDZip I paralogs as they have almost identical expression patterns (Söderman *et al.* 1996; Lee and Chun 1998; Olsson *et al.* in press) suggesting that *cis*-regulatory regions of these genes have not undergone extensive functional divergence. As deduced from the phenotypes of plants with elevated levels of *ATHB7* or *-12* (Hjellström *et al.* 2003; Olsson *et al.* in press), also the proteins appear to be functionally very similar. The preservation of an apparently functionally conserved gene pair in the Arabidopsis genome might be explained by the dose effect of these genes.

External factors regulate the expression of HDZip I genes (II and IV)

Previously, water deficit conditions and externally applied ABA have been reported to up- or down-regulate the transcript levels of *ATHB6, -7,* and *-12* and *ATHB5* and *-16,* respectively (Söderman *et al.* 1996; Lee and Chun 1998; Söderman *et al.* 1999; Johannesson *et al.* 2003; unpublished observation). Further, the expression of these genes has been found to be dependent on the products of *ABI* loci, *ATHB6, -7,* and *-12* on ABI1 and ABI2 and *ATHB5* on ABI3 and ABI5 (Söderman *et al.* 1996; Söderman *et al.* 1999; Johannesson *et al.* 2003). The biological roles of *ATHB5, -6, -7* and *-12* have been suggested to be in water deficit responses, e. g. *ATHB7* has been suggested to regulate leaf and inflorescence stem growth in response to water deficit conditions (Hjellström *et al.* 2003).

Salt and osmoticum induced expression of ATHB7 (II)

The expression of *ATHB7,* as detected by northern blot analyses, was rapidly and strongly induced in the roots of a seedling by a direct contact with salt (II). In the shoots, a direct contact with salt solution did not induce *ATHB7* expression. Instead, when the seedlings were exposed to salt via the root system, the expression of *ATHB7* in the shoots was rapidly induced. These

results demonstrate that a root-derived signal causes the induction of *ATHB7* expression in the shoots.

Mannitol treatments, mimicking the osmotic stress caused by the salt, induced *ATHB7* in a similar way as the salt treatments (II). As these treatments caused very similar changes in *ATHB7* expression, the osmotic, rather that the ionic effect of the salt stress was deduced to be responsible for the *ATHB7* induction. In accordance with this conclusion, salt induction of *ATHB7* was not affected by a mutation in *SOS3*, the known most upstream component of the SOS pathway involved in ionic stress signaling.

The induction of *ATHB7* was impaired in the roots and shoots of the ABA-deficient mutant, *aba3-1*, when stress treated via the root system (II). This indicates that ABA is needed for full induction of *ATHB7* in the roots, directly in contact with salt or osmoticum, as well as in the shoots, sensing the salt or osmoticum only via the root system. ABA or an ABA dependent component was thus suggested to function as a systemic signal for the induction of *ATHB7* expression in the shoots. Differential xylem transport of ABA, in seedlings stress treated in different ways, might explain the differences in *ATHB7* induction in the shoots. Seedlings soaked in salt or mannitol solution have limited transpiration as well as low levels of *ATHB7* expression in the shoots, whereas the seedlings grown on solid growth medium likely have higher transpiration rates and also high levels of *ATHB7* expression in the shoots. Thus potentially a higher rate of xylem transport of ABA derived from the roots causes the differences in *ATHB7* expression in the shoots (Zhang and Davies 1991). However, the *ATHB7* induction was not totally impaired in the *aba3-1* mutant indicating that either the low levels of ABA present in the mutant (Léon-Kloosterziel *et al.* 1996) contribute to *ATHB7* induction or that components independent of ABA are involved.

Changes in free $[Ca^{2+}]_{cyt}$, as monitored using Arabidopsis seedlings expressing the calcium sensitive apoaequorin, have been detected in response to salt and mannitol (II; Knight *et al.* 1997). However, evidence for the importance of this calcium signal for the downstream responses is largely missing. To date, only one gene, *P5CS* encoding for Δ^1-pyrroline-5-carboxylase synthase, has been shown to be dependent on a calcium signal

for induction by salt stress (Knight *et al.* 1997). To study whether calcium is an essential component for *ATHB7* induction we blocked the salt induced $[Ca^{2+}]_{cyt}$ by calcium release inhibitors and analysed *ATHB7* expression by northern blot analyses (II). We found the induction of *ATHB7* to be independent of the calcium signal evoked by salt. In accordance with this result, treatments that resulted in strong induction of *ATHB7* expression did not result in detectable $[Ca^{2+}]_{cyt}$ changes. Thus a salt stress signaling pathway, inducing the expression of *ATHB7*, appears to exist which does not involve calcium signaling. Instead, ABA or an ABA dependent component that functions as a systemic signal for induction of *ATHB7* expression is involved.

Blue light regulated expression of HDZip I genes (IV)

Northern blot experiments showed that the transcript levels of *ATHB6* are up-regulated also by blue light enriched conditions (IV). Consistent with the northern blot analyses the promoter reporter gene (GUS) analyses revealed *ATHB6* promoter activity to be redistributed and enhanced in the cotyledons and in the hypocotyl of blue light grown seedlings compared to white light grown seedlings (IV). Furthermore, under blue light conditions the *ATHB6* expression levels were found to be increased in plants mutant for the *CRY1* and *CRY2* genes and decreased in plants with elevated levels of *CRY2* (IV). Thus, *ATHB6* transcript levels are dependent on light quality and CRY1 and CRY2.

In northern blot analyses no differences were found in the transcript levels of *ATHB16* in plants grown in blue light, white light or darkness (III). However, *ATHB16::GUS* analyses revealed that in blue light grown seedlings the *ATHB16* promoter activity was expanded to the vasculature of the cotyledons, whereas in the white light grown seedlings no promoter activity was detected in these tissues (data not shown). *ATHB16* was also found to be dependent on CRY1 and CRY2 for its transcription as detected with northern blot analyses (data not shown). In these analyses the expression of *ATHB16* was enhanced in *cry1 cry2* mutant and impaired in plants with increased level of *CRY2*, in a similar way as *ATHB6*.

HDZip I proteins are activators of gene expression (I)

HDZip I proteins from different plant species have been shown to interact with the binding site CAATNATTG (Johannesson *et al*. 2001; Meijer *et al.* 2000; Sessa *et al.* 1993) and three HDZip I proteins, one from Arabidopsis and two from rice, to activate transcription (Aoyama *et al.* 1995; Sessa *et al.* 1998a; Meijer *et al.* 2000). To test whether ATHB5, -6 and -16 can regulate transcription in a binding site (CAAT(A/T)ATTG) specific manner, transient expression assays on Arabidopsis leaves were performed (I). Arabidopsis leaves were co-bombarded with reporter and transacting plasmids and relative transactivation values for ATHB5, -6 and -16 were determined in relation to the previously known activator, ATHB1 (Aoyama *et al.* 1995, Sessa *et al.* 1998a). ATHB5, -6 and -16 were all found to activate gene expression in Arabidopsis leaves in a binding site dependent manner.

In previous *in vitro* DNA-binding assays ATHB7 and -12 were found not to bind to the CAATNATTG sequence recognised by the other HDZip I proteins (Johannesson *et al.* 2001). We tested whether in *in vivo* assays ATHB7 and -12 can regulate transcription in a binding site (CAATNATTG) dependent manner. We found, in transient expression assays on Arabidopsis leaves (I), both ATHB7 and -12 to activate gene expression and to be able to activate transcription from CAAT(A/T)ATTG as well as CAAT(G/C)ATTG binding sites. Differences in the *in vivo* and the *in vitro* results suggest that the DNA binding of ATHB7 and -12 is dependent on conditions or components present *in vivo* but missing from the *in vitro* assays.

Taken together, these data show that the closely related HDZip I proteins (subclass β and γ members) bind to DNA *in vivo* and regulate transcription in a similar fashion. All tested HDZip I proteins have been found to function as transcriptional activators in plants. This is in concordance with the proposal that activation is a common property of all HDZip I proteins, whereas members of the HDZip II have the ability to repress transcription (Meijer *et al.* 2000). However, a transcriptional repressor in rice, the Oshox1, was found to activate transcription in yeast indicating that dependent on the context, a HDZip II protein can function as a repressor or an activator of gene expression (Meijer *et al.* 1997; Meijer *et*

al. 2000). At present there is no evidence for a dual function of HDZip I proteins as ATHB7 and -12 were found to activate transcription in a binding site dependent manner also in yeast (our unpublished data).

A reverse genetics approach to study the function of HDZip I genes (III and IV)

To assess the biological functions of *ATHB6* and *-16*, plants with altered levels of these gene products were generated. For elevation of *ATHB6* or *-16* levels and reduction of *ATHB16* levels, Arabidopsis plants were transformed with constructs containing the *ATHB6* or *-16* gene in sense or antisense (only *ATHB16*) orientation under the control of the cauliflower mosaic virus (CaMV) 35S promoter (III; IV). Available T-DNA mutant collections were searched in order to find loss-of-function mutants for *ATHB6* and *-16*. These searches resulted in the isolation of *athb6-1*, *athb6-2* and *athb16-1*. *athb6-1* and *athb6-2* produced no detectable transcripts of *ATHB6* and were thus deduced to be *ATHB6* loss-of-function mutants (IV), whereas *athb16-1* produced wild-type levels of *ATHB16* transcripts and was not used for further studies (data not shown). The *athb6-1* and *athb6-2* mutants displayed wild-type like phenotypes under normal growth conditions.

ATHB16 regulates leaf development, sensitivity to photoperiod and hypocotyl elongation in response to light (III)

Transgenic plants with altered levels of *ATHB16* expression showed phenotypical deviations in leaf expansion, shoot elongation and flowering time (III). Furthermore, plants with decreased levels of *ATHB16* expression had reduced sensitivity specifically to the blue light mediated inhibition of hypocotyl elongation, whereas a reciprocal phenotype was recorded for plants with elevated levels of *ATHB16* expression (III). The phenotypical analyses of these transgenic plants indicated that ATHB16 is a negative regulator of leaf cell expansion, a suppressor of the flowering time sensitivity to photoperiod and that ATHB16 acts as a negative regulator of hypocotyl elongation, in response to blue light.

ATHB6 affects hypocotyl elongation in response to light (IV)

In hypocotyl elongation assays plants with elevated levels of *ATHB6* expression showed an enhanced inhibition of hypocotyl elongation in response to white and blue light (IV). The severity of the phenotype correlated with the *ATHB6* expression levels, since lines with higher *ATHB6* expression levels also had more severe phenotypes. The loss of *ATHB6* function caused a subtle, but reciprocal, effect on the hypocotyl elongation in response to light (IV). The phenotypes recorded by altering the *ATHB6* expression levels resembled the phenotypes of *cry1*, *cry2* or plants with elevated levels of *CRY1* or *CRY2* (Koorneef *et al.* 1980; Ahmad and Cashmore 1993; Lin *et al*. 1995; Guo *et al*. 1998; Lin *et al*. 1998). Elevated levels of *ATHB6, CRY1* or *CRY2* expression resulted in hypersensitivity to white and blue light in hypocotyl elongation assays, whereas the reverse phenotypes were scored for *athb6*, *cry1* or *cry2* plants. The phenotypic analyses, thus, suggested ATHB6 to act as a negative regulator of hypocotyl elongation in response to light.

Partial conservation of function between ATHB6 *and* -16 (III, IV)

The paralogous genes *ATHB6* and *-16* encode proteins with very similar molecular functions, as interpreted from the phenotypes of plants with elevated levels of either gene. The phenotypic changes observed in *35S::ATHB6* plants, in addition to the altered light sensitivity in the hypocotyl, were alterations in leaf expansion, shoot elongation and flowering time (data not shown). These phenotypic traits resemble the changes observed in *35S::ATHB16* plants (III). However, the two proteins also differ in function since the effect of *ATHB16* on hypocotyl elongation was restricted to blue light conditions, whereas *ATHB6* had an effect also in white light (III; IV). Even though the interpretation of these phenotypes caused by the elevated levels of these genes in itself is ambiguous, our findings that the phenotypes of the *athb6* or *35S::antiATHB16* plants were reciprocal to those of the *35S::ATHB6* or *-16* plants confirmed that at least part of the overexpression phenotypes reflects the function of these genes in wild-type. In the case of *ATHB16,* reciprocal phenotypes were detected in

leaf cell expansion, photoperiodic control of flowering and blue light mediated inhibition of hypocotyl elongation (III), whereas the *athb6* plants, so far, have only confirmed the altered hypocotyl elongation phenotype displayed by the *35S::ATHB6* plants (IV).

The hypocotyl measurements under white light conditions showed that differences in the function of ATHB6 and ATHB16 exist. Thus, even though plants with altered levels of *ATHB16* did not show phenotypical deviations under the red or far-red conditions used (III), *ATHB6* might be involved in the mediation of red or far-red light signals on hypocotyl elongation. Therefore, an important issue for the future is to study hypocotyl elongation of *athb6* and *35S::ATHB6* plants under red and far-red light conditions.

Redundant functions of HDZip I genes in the regulation of hypocotyl elongation in response to light (IV)

The subtle phenotypical changes recorded for the *athb6* and *35S::antiATHB16* plants and the lack of discernible phenotype of *athb5-1*, in previous studies (Johannesson *et al.* 2003), raised the possibility that *ATHB5, -6* and *-16* can functionally compensate for each other and, thus, the single mutants might not fully reveal the function of each gene. To address these potential functional relationships, double and triple loss-of-function mutants, *athb6-1 athb5-1* and *athb6-1 athb5-1 35S::antiATHB16*, were generated (IV). The *athb5-1* enhanced the *athb6-1* mutant phenotype and the combination of *35S::antiATHB16* to *athb6-1 athb5-1* further enhanced the double mutant phenotype. These data demonstrate that at least partial functional redundancy exists between *ATHB5, -6* and *-16* in the light dependent inhibition of hypocotyl elongation. This provides evidence, for the first time, for functional redundancy in HDZip I. Previously, mutant analyses have revealed substantial functional redundancy to exist between the HDZip III genes (Zhong and Ye 1999; McConnell *et al.* 2001; Otsuga *et al.* 2001; Emery *et al.* 2003).

Importantly, the phenotypic analyses of *athb6-1 athb5-1* plants also revealed a new function in light signaling for ATHB5, which has previously been implicated in the mediation of ABA signals (Johannesson *et al.* 2003). This function was undetected in *athb5-1* plants, which display a wild-type like light response in hypocotyl elongation. Apparently, the loss of *ATHB5* in the *athb5-1* plants is compensated by its paralog, *ATHB6,* and potentially by the paralog of *ATHB6, ATHB16*, as regards the light response.

Co-operations of light and hormones in responses involving HDZip I genes?

The available data suggest a set of HDZip I genes to be involved in the regulation of growth in response to ABA signaling as well as in response to altered light conditions. Thus these genes integrate several environmental cues in the regulation of growth. However, interactions between light and hormones exist and ABA itself has been suggested to act in the integration of light signals in the control of plant development (reviewed by Kraepiel and Miginiac 1997). At present the information of such interactions is sparse. Studies with heterologous systems have provided evidence that blue and red light modify the level of endogenous ABA, although the physiological importance of this connection is not clear (reviewed by Kraepiel and Miginiac 1997). Contradictory reports of the involvement of ABA in the blue light responses in different species have been presented. Lin and Yang (1999) argued that blue light signals are not mediated by ABA in the waterclover, *Marsilea quadrifolia*, whereas Fellner and Sawhney (2002) suggested a defect in blue light perception or signal transduction to cause resistance to osmotic stress and ABA in a tomato mutant *7B-1*.

The transcription factor ABI3 has been suggested to function downstream or in cooperation with both light and ABA signals (Rohde *et al.* 2000). Interestingly, *ATHB5* is dependent on ABI3 for its transcription and has roles in both ABA and light signaling (IV; Johannesson *et al.* 2003). Thus an interesting issue, to be studied in the future, is in what respect the light and ABA signaling co-operate or overlap in the regulation of *ATHB5, -6* and *-16*.

CONCLUDING REMARKS

The HDZip I genes of Arabidopsis have a common evolutionary origin and multiple rounds of genome duplications have contributed to the expansion of the gene family. Paralogous HDZip I genes originating from duplication events have been retained in the Arabidopsis genome at high frequency suggesting a selective advantage for the retention of HDZip I genes. The *cis* regulatory regions of the HDZip I genes appear to have undergone vast functional diversification, whereas the gene products are suggested to be more conserved.

The present data show that the HDZip I genes are involved in the integration of environmental cues to the regulation of plant growth. *ATHB5, -6* and *-16*, previously suggested to function in ABA related responses, were also found to be mediators of light effects on growth. In what respect the light and ABA signaling co-operate or overlap in the regulation of *ATHB5, -6* and *-16* function is an interesting issue for future studies. The mutant analyses revealed also that the closely related HDZip I genes, *ATHB5, -6* and *-16*, have overlapping functions in the regulation of light dependent hypocotyl elongation. This evokes the question whether these genes may overlap in function also with other HDZip I genes and, thus, may have additional functions that are not yet discovered. Furthermore, since the HDZip I genes are involved in the mediation of several environmental signals it is also possible that the loss-of-function mutants display more severe and/or even additional phenotypes in conditions with optimal combinations of environmental parameters.

Retention of as many as 17 HDZip I genes in the Arabidopsis genome provides a basis for an expanding complexity in the regulatory networks the HDZip I genes are involved in. The closely related, but differentially expressed HDZip I genes, might regulate the same set of target genes or alternatively function in duplicated diverged pathways. The identification of the downstream genes is of importance for revealing the functional networks

that the HDZip I proteins are involved in as well as the target genes shared by related HDZip I proteins. Since the HDZip I genes are involved in the integration of external signals to growth regulation, they are likely to play an important role in the adaptability of the plant to the prevailing environmental conditions.

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SUMMARY IN SWEDISH

Den grupp transkriptionsfaktorer som kallas homeodomän-leucinzipper-proteiner (HDZip) karaktäriseras av att de innehåller en homeodomän (HD) med en DNA-bindande funktion samt en leucinzipper (Zip) som medierar proteindimerisering. Denna kombination av HD- och Zip-domäner intill varandra har inte hittats in några proteiner från djur eller jäst varför denna typ av transkriptionsfaktorer troligen är specifik för växter. Hos *Arabidopsis thaliana* har man funnit 47 gener som kodar för HDZip-proteiner. Dessa gener har delats in i fyra olika klasser; HDZip I-IV (Sessa *et al.* 1994). Denna avhandling beskriver karaktäriseringen och den fylogenetiska analysen av generna i HDZip klass I samt den funktionella karaktäriseringen av några av generna i HDZip I.

HDZip I består av 17 gener som i fylogenetiska analyser bildar sex olika undergrupper. Placeringen av generna i undergrupper stöds även av mönstret för var introner/exoner är lokaliserade i de regioner som kodar för HDZip-domänerna, samt av HDZip-genernas dupliceringshistoria. Arabidopsisgenomet har genomgått multipla dupliceringar vilket har bidragit till en expansion av HDZip I. Märkvärdigt nog är i många fall båda kopiorna av ett HDZip I gen-par kvar i arabidopsisgenomet.

Analyser av HDZip-genernas uttrycksmönster visade att generna uttrycks i de flesta organ som undersökts; rötter, blad, stam, blommor, fröskidor samt i unga groddplantor. Vi kunde dock se att närbesläktade gener inte alltid hade samma uttycksmönster. Detta tyder på att de regioner hos generna som styr deras uttryck har förändrats med tiden. Däremot verkar de proteiner som kodas av närbesläktade gener i de flesta fall vara väldigt lika och ha en liknande funktion. Alla testade HDZip I-proteiner binder till likartade DNA-sekvenser (CAATNATTG) och fungerar genom att aktivera transkription. Dessutom har vi sett att växter med förstärkt uttryck av närbesläktede HDZip-I gener har mycket likartade fenotyper.

Uttrycket av flera av generna inom HDZip I regleras av externa faktorer. Resultat från tidigare undersökningar har antytt att några av HDZip I-generna har en roll i regleringen av växtens tillväxt som ett svar på vattentillgången. I denna avhandling ingår en studie som visar att den induktion av HDZip I-genen *ATHB7* som erhålls i bladen hos groddplantor som utsatts för osmotisk stress, till stor del är beroende av en systemisk signal som transporteras via xylemet från roten till bladen. Plantor vars rötter behandlades med salt eller mannitol fick ett starkare uttryck av *ATHB7* i bladen än de plantor vars blad behandlades med en salt- eller mannitollösning. Motsvarande analyser av en mutant som inte producerar växthormonet abskisinsyra (ABA) visade att ABA, eller en ABA-beroende signal, verkar vara, åtminstone delvis, nödvändig för den både den lokala och den systemiska induktionen av *ATHB7* under osmotisk stress.

HDZip-generna verkar också vara inblandade i överföringen av andra yttre signaler som reglerar växtens tillväxt. ATHB16 visade sig negativt reglera bladexpansion, minska blomningstidens känslighet för fotoperioden, samt positivt reglera den av ljus påverkade inhiberingen av hypokotylelongering. Studier av växter med förstärkt uttryck av *ATHB6*, den andra genen i gen-paret, visade att ATHB6 har en funktion till stor del liknar ATHB16. Analyser av växter med mutationer i *ATHB6* visade att *ATHB6* medierar ljusets effekt på hypokotylelongeringen. *ATHB6*-mutanterna visade en minskad känslighet för den ljusreglerade inhiberingen av hypokotylelongering medan växter med förstärkt uttryck av *ATHB6* visade en motsatt fenotyp. I växter där även uttrycket av *ATHB5*, en annan närbesläktad gen till *ATHB6*, slagits ut blev hypokotylfenotypen förstärkt. Resultaten från analyserna av dubbelmutanterna visade alltså att också *ATHB5* är inblandat i medieringen av ljusets effekt på hypokotylelongering, något som inte kunnat detekteras i växter med en mutation i endast *ATHB5*. Växter med reducerad uttryck av *ATHB16* i kombination med *ATHB5*- och *ATHB6*-mutationerna visade en ytterligare förstärkning av den ljusberoende hypokotylfenotypen. Detta visar att de närbesläktade generna *ATHB5*, *-6* och *-16* kodar för proteiner med överlappande och delvis överflödiga funktioner.

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