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Drought Stress Signal Transduction by the HD-Zip Transcription Factors ATHB6 and ATHB7

BY

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Abstract

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This work describes the regulation of drought stress responses in *Arabidopsis thaliana* and addresses the roles of the homeodomain-leucine zipper (HD-Zip) transcription factors in this regulation. The characteristics of *ATHB6* and *ATHB7*, two genes encoding class I HD-Zip transcription factors were analyzed.

Expression of *ATHB6* and *ATHB7* was transcriptionally activated in plants subjected to water deficit or exogenous treatment with abscisic acid (ABA).

Transgenic plants constitutively expressing the *ATHB7* gene displayed a delayed elongation growth of the main inflorescence stem after transition to reproductive development. This phenotype is consistent with ATHB7 acting as a negative regulator of growth and development of the elongating stem in response to water availability.

Transgenic *abi1-1* mutant plants constitutively expressing the *ATHB7* gene displayed a reduced wiltiness as compared to monogenic *abi1-1* mutants. These data are consistent with the ATHB7 protein having a central role in the drought stress response, regulating the water balance of the plant, and acting downstream to *ABI1*. Furthermore, the data is consistent with ATHB7 acting as a positive regulator of the drought stress response.

The ABA-induced expression of the *ATHB7* gene displayed a dependence on the phytochrome system, suggesting an interplay between light and osmotic stress signaling in the regulation of the *ATHB7* gene.

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To Inga-Stina Holmberg-Almby

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

I. Söderman, E. Hjellström, M. Fahleson, J. Engström, P. (1999) The HD-Zip gene *ATHB6* in Arabidopsis is expressed in developing leaves, roots and carpels and up-regulated by water deficit conditions. Plant Molecular Biology **40**: 1073-1083

II. Hjellström, M. Olsson, A. Engström, P. Söderman, E. Constitutive expression of the drought inducible homeobox gene *ATHB7* in transgenic Arabidopsis causes a suppression of stem elongation growth (in manuscript)

III. Hjellström, M. Olsson, A. Söderman, E. Engström, P. The *abi1-1* mutant phenotype is partially suppressed by constitutive expression of the drought inducible homeobox gene *ATHB7* in transgenic Arabidopsis (in manuscript)

IV. Hjellström, M. Fahleson, J. Söderman, E. Engström, P. Constitutive expression of the ABA-inducible homeobox gene *ATHB7* partially complements phytochrome deficiency in transgenic Arabidopsis (in manuscript)

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ABBREVIATIONS

ABA	abscisic acid
aba	ABA-deficient
abi	ABA-insensitive
ABRE	ABA-responsive element
ATHB	*Arabidopsis thaliana* homeobox gene
bp	base pairs
b-ZIP	basic leucine zipper
cDNA	complementary DNA
DRE	dehydration-responsive element
DREB	DRE-binding protein
cry	cryptochrome
GA	gibberellic acid
HD-Zip	homeodomain-leucine zipper
I_{ca}	hyperpolarization-activated Ca^{2+} current
kb	kilo base pair
LD	long day
PP	protein phosphatase
Rd	responsive to dehydration
R/FR	red/far-red ratio
SD	short day

The following nomenclature conventions have been used in this thesis:

Names of genes are written in italicised upper-case letters, e.g. *ATHB7*.
Names of proteins are written in non-italicised upper-case letters, e.g. ATHB7.
Names of mutants are written in italicised lower-case letters, e.g. *abi1-1*.
Names of photoreceptor holoproteins are written in non-italicised lower-cease letters, e.g. phyB.
Names of photoreceptor apoproteins are written in non-italicised upper-case lettters, e.g. PHYB

INTRODUCTION

Plant growth

Plant growth can be studied at different levels ranging from growth of cells to whole plant growth. Growth is determined, at cell level, by cell division, cell expansion and cell differentiation. Cell division, however, cannot result in an increase in volume and does not drive growth by itself. Rather, it provides the material for subsequent cell expansion (Green 1976). The formation of organs is an important growth process. Likewise, the progression of stages in a life cycle could also be thought of as growth. Key factors driving whole plant growth are photosynthetic area and net assimilation rate. One could also think of the plants nutrient concentration as an important variable (Lambers et al. 1998).

Lengthening of internodes (bolting) in Arabidopsis thaliana

One example of plant growth is the lengthening of internodes in many plants at initiation of reproductive development. The primary plant body is formed by the root and shoot apical meristems. During the vegetative stage of rosette plants, like *Arabidopsis thaliana*, cell division and development within the shoot apical meristem form leaf primordia in such rapid succession that the stem (shoot axis) cannot form nodes and internodes. Later, internodes begin to develop between the nodes by intercalary growth. The timing and duration of internodal elongation depends on the plant species, environmental conditions, and the type of stem (for review see Steeves and Sussex 1989). In *Arabidopsis thaliana* the internodes are undeveloped between leaf-bearing nodes in the rosette. However, at floral induction, leaf production is inhibited, lateral primordia develop into cauline leaves, side branches and flowers, and the main stem elongates to give rise to an inflorescence (bolting). The internodal elongation then occurs between the nodes that bear flowers or paraclades (axillary flowering shoots).

This implies the existence of a specific developmental program of stem elongation at the reproductive stage in rosette type of plants. In wild-type Arabidopsis plants, the initiation of internodal elongation is followed by continued flower production from the apical inflorescence meristem. Thus, the number of flower-bearing nodes is an important factor in determining the height of Arabidopsis plants. According to Shannon and Meeks-Wagner (1991), the primary inflorescence meristem ceases proliferative activity after the production of 30 to 50 flowers. The late stages of the reproductive growth are accompanied by the progressive senescence of existing somatic tissues (Hensel et al. 1994). Therefore, the rate of internodal elongation is gradually decreased.

Plant growth in relation to environment

How plant growth responds to various changes in the environment is an important aspect of plant biology. Often these responses constitute important adaptations to an adverse environment. A growth decline could be directly due to a reduction in resource supply and acquisition or to an anticipation and response to specific signals before any single resource becomes strictly limiting. In other words, growth could be source-controlled or controlled by specific signals that modulate sink activity (growth) that then govern rates of resource acquisition (feedforward control). Examples of regulation of growth in response to environmental stresses are limitations of shoot expansion (Saab et al. 1990) and the acceleration of flowering (for review see Smith 1995). In this thesis regulation of *Arabidopsis thaliana* growth in response to drought and shading is analyzed.

Environmental water potential influences plant growth

When water deficit develops slowly enough to allow changes in developmental processes, water stress has several effects on growth, two of which are limitations in leaf and stem expansion. At a low soil water potential, the rate of shoot expansion decreases, whereas the rate of root elongation is much less affected (Saab et al. 1990). The effect of water stress on leaf growth is mediated by the phytohormone abscisic acid (ABA) (Munns and Cramer 1996). Soil drying and salinity enhance the concentration of this hormone in the leaves (Tardieu et al. 1992; He and Cramer 1996). Although leaf area is important because photosynthesis is usually proportional to it, rapid leaf expansion can adversely affect water availability. Accelerated early growth can lead to large leaf areas, rapid early water depletion, and too little residual soil moisture for the plant to complete its life cycle. For maximum reproductive success in an erratic environment, then, a plant must be able to respond rapidly to both stress and relief of stress (Taiz and Zeiger 1998). Because leaf and stem growth depends mostly on cell expansion, inhibition of cell expansion results in a slowing of leaf and stem growth early in the development of water deficits. Limitation of shoot area can be considered a first line of defense against drought. Water-deficient plants tend to be become rehydrated at night, and as a result substantial leaf and stem growth occurs at night. The process of stem growth has been studied less than that of leaf expansion, but it is probably affected by the same forces that limit leaf growth during stress.

Mild water deficits also affect the development of the root system (for a review see Lambers et al. 1998). Root-shoot relations appear to be governed by a functional balance between water uptake by the root and photosynthesis by the shoot. Although root-shoot relations depend on complex developmental and nutritional processes, the concept of functional balance can be simply stated: a shoot will grow until it is so large that water uptake by the roots becomes limiting too further growth; conversely, roots will grow until their demand for

photosynthate from the shoot equals the supply. Deeper root growth into wet soil can be considered as second line of defense against drought.

Light quality influences plant elongation growth

The angiosperms have evolved impressive capacity to avoid shade by stimulation of extension growth. Several lines of evidence indicate that the shade avoidance reactions are all initiated by a single environmental signal, the reduction in the ratio of R to FR radiation, perceived by light-stable phytochromes (Smith and Whitelam 1997 and refs therein). Many plants react within 5-10 minutes of exposure to reduced R/FR ratios by accelerating extension up to 3- or 4- fold. Stem and petiole elongation of shade-avoiding plants growing in the shade are greatly enhanced whereas branching is reduced (increased apical dominance). There is also an acceleration of flowering (Smith 1995).

The photoperiod influences flowering

The progression of stages in the plant life cycle is an important growth variable. Floral initiation is to a large extent regulated by light. As mentioned above there is an acceleration of flowering in shade. Flowering time of many plants is determined by the daily duration of light and/or darkness-the photoperiod. Plants in which flowering requires or is accelerated by short-day (SD) or long-day (LD) photoperiods are referred to as short-day plants or long-day plants, respectively. Plants that flower at about the same time regardless of LD or SD conditions are known as day-neutral plants. Arabidopsis thaliana is a facultative long-day plant, meaning that flowering is promoted by LD and delayed by SD (for review see Koornneef et al. 1998).

Plant cell wall properties influence cell expansion and plant growth

Once the polarity is established, plant cells typically expand 10- to 100-fold in volume before reaching maturity. Cell expansion is coordinately regulated at the whole organ level by external stimuli such as light, temperature, gravity and water availability as well as internal factors such as gibberellins, auxins and other hormones (Cosgrove 1998). The critical physical event required for cell enlargement is wall stress relaxation (Cosgrove 1993). When hormones and other agents modulate the rate of cell expansion, they usually do so by influencing cell wall properties. These internal and external factors most likely modify cell expansion by influencing relaxation and yielding properties of the cell wall. According to one model, the acid-growth hypothesis, stress relaxation is induced by cell wall acidification resulting from proton extrusion across the plasma membrane. Indeed, the essential difference between growing and non-growing cells resides in the cell wall's ability to undergo stress relaxation and polymer creep. Creep refers to a time-dependent irreversible extension, typically due to slippage of the wall polymers relative to one another (Cosgrove 1998).

The effects of red light and ABA signaling on elongation growth of plants is an important topic of this thesis and will be described below. Red light inhibits elongation mainly by lowering the cell-wall yield coefficient, whereas blue light predominantly acts by enhancing the yield threshold (Kigel and Cosgrove 1991). ABA likely hardens the cell wall of leaf cells by increasing yield threshold, and decreasing wall extensibility. Both the carbohydrate and the protein component of cell walls are affected (Munns and Cramer 1996).

Mechanisms of ABA-dependent drought signaling

Abscisic acid (ABA) is an essential hormone that has profound and diverse effects on plant growth and development. The phytohormone ABA regulates seed maturation and maintains seed dormancy. In adult plants, ABA acts as a negative regulator of growth by controlling the adaptive responses to environmental stresses such as cold, drought and high salinity (reviewed in Giraudat 1995; Leung and Giraudat 1998). Analyses of the expression of drought-inducible genes in Arabidopsis have indicated that at least four independent signal pathways function in the induction of stress-inducible genes in response to dehydration (Shinozaki and Yamaguchi-Shinozaki 1997). Two of these signal pathways are abscisic acid (ABA)-dependent and two are ABA-independent.

At the physiological level, this stress hormone is responsible for decreasing the turgor of the guard cells that surround the stomatal pore, resulting in a reduction of pore size and an increase in water retention (MacRobbie 1998). Calcium ions have been shown to mediate transduction of ABA signaling for gene expression (Wu et al. 1997). Further, stomatal closure (Gilroy et al. 1990; McAinsh et al. 1990; Schroeder and Hagiwara 1990) has been shown to require an increase in the cytosolic Ca^{2+} concentration. Cytosolic [Ca^{2+}] elevation activates slow (S-type) anion channels and downregulates inward K^+ channels in guard cells (Shroeder and Hagiwara 1989), resulting in net ion release and turgor reduction leading to stomatal closure. ABA triggers Ca^{2+} influx via nonselective Ca^{2+}-permeable channels in Vicia guard cells (Schroeder and Hagiwara 1990). In Arabidopsis guard cells, hydrogen peroxide (H_2O_2) and ABA stimulated hyperpolarization-activated Ca^{2+}-permeable (I_{ca}) channels (Pei et al. 2000). ABA was shown to induce the production of reactive oxygen species (ROS) in Arabidopsis guard cells (Pei et al. 2000). Furthermore, H_2O_2 activation of I_{ca} channels and H_2O_2-induced stomatal closing were abolished in the ABA-insensitive mutant *gca2* (Himmelbach et al. 1998), providing genetic evidence for roles of ROS and I_{ca} channels in ABA signaling (Pei et al. 2000).

Interestingly, in maize embryos and Vicia guard cells, ABA was shown recently to increase H_2O_2 levels (Guan et al. 2000; Zhang et al. 2001).

Genetic studies, especially those with Arabidopsis plants, have identified a large number of loci involved in the responses to ABA. To date, eight genes required for wild-type ABA response have been reported cloned. These genes represent five classes of proteins; Two orthologous transcriptional regulators (Viviparous1(VP1) of Maize and ABA insensitive 3 (ABI3) of Arabidopsis) (McCarty et al. 1991; Giraudat et al. 1992), three highly homologous members of the protein serine/threonine phosphatase 2C family, the PP2Cs (ABI1, ABI2 and AtPP2CA of Arabidopsis) (Leung et al. 1994; Meyer et al. 1994; Bertauche et al. 1996; Leung et al. 1997; Leube et al. 1998; Rodriguez et al. 1998; Tahtiharju and Palva 2001) a member of the Apetala2 domain family (ABI4 of Arabidopsis) (Finkelstein et al. 1998), a farnesyl transferase (Enhanced response to ABA (ERA1) of Arabidopsis)(Cutler et al. 1996) and a basic leucine zipper transcription factor (ABI5 of Arabidopsis) (Finkelstein and Lynch 2000). The *abi3*, *abi4* and *abi5* mutants exhibit defects in various aspects of seed maturation but do not seem to be altered in the adult plant responses to ABA (Koornneef et al. 1984; Finkelstein and Somerville 1990; Ooms et al. 1993; Finkelstein 1994; Parcy et al. 1994; Nambara et al. 1995). In contrast, the *abi1-1*, *abi2-1* and *era1* mutations are more pleiotropic in that they affect ABA sensitivity in both seeds and vegetative tissues.

The *abi1-1* and *abi2-1* mutations are both dominant with strongly reduced protein phosphatase (PP) activity. This impaired activation characteristic of *abi1-1* and *abi2-1* is consistent with the substitution at equivalent positions in the ABI1 and ABI2 proteins of an aspartic acid residue for glycine at a conserved metal co-ordination site within the catalytic domain, respectively (Bertauche et al. 1996; Leung et al. 1997; Leube et al. 1998; Rodriguez et al. 1998). Comparative studies on the mutant and the wild-type form of ABI1 revealed a linear activation of the enzymes by the free Mg^{2+} concentration. In addition, ABI1 and ABI2 are regulated by proton concentration and to some extent by ionic strength of the cytosol. Further, in a recent study ABI1 was shown to be

inactivated reversibly by H_2O_2 (Meinhard and Grill 2001). The *abi1-1* and *abi2-1* mutations lead to largely overlapping sets of phenotypic alterations including ABA-resistant seed germination and seedling growth, including root growth, reduced seed dormancy, abnormal stomatal regulation, and defects in various responses to drought stress (Koornneef et al. 1984; Finkelstein and Somerville 1990; Vartanian et al. 1994; Gosti et al. 1995; Leung et al. 1997). The *abi1-1* and *abi2-1* mutations reduce ABA-induced cytosolic [Ca^{2+}] increases in guard cells (Allen et al. 1999). Furthermore, experimentally imposing cytosolic [Ca^{2+}] elevations bypasses these mutants and restores S-type anion channel activation and stomatal closure (Allen et al. 1999), demonstrating that *abi1-1* and *abi2-1* disrupt early ABA signaling at the level of, or upstream of, ABA-induced cytosolic [Ca^{2+}] increases (Allen et al. 1999). Recently, it was shown that ABA activation of plasma membrane Ca^{2+} channels in guard cells is differentially disrupted upstream and downstream of ROS production in the *abi1-1* and *abi2-1* mutants (Murata et al. 2001).

Several distinct loss-of–function alleles of the ABI1 gene as intragenic revertants of the *abi1-1* mutant recently was isolated by Gosti et al, (1999). In marked contrast to the ABA-resistant *abi1-1* mutant, all of these intragenic revertants were more responsive to ABA than the wild-type plants, and this ABA supersensitivity phenotype was recessive. Moreover, each of these revertant alleles encodes an ABI1 protein that lacked any detectable phosphatase activity in an in vitro enzymatic assay. These results thus provide genetic evidence that a loss of ABI1 PP2C activity leads to an enhanced responsiveness to ABA, and hence that the wild-type ABI1 phosphatase is a negative regulator of ABA responses (Gosti et al. 1999).

Many ABA-inducible genes contain a conserved, ABA-responsive, cis-acting element named ABRE (ABA-responsive element; PyACGTGGC) in their promoter regions (Bonetta and McCourt 1998; Grill and Himmelbach 1998; Leung and Giraudat 1998). ABRES were first identified in the wheat Em gene, which functions mainly in seed during late embryogenesis (Guiltinan et al. 1990) and in the rice rab16 gene, which is expressed in both dehydrated vegetative

tissues and in maturating seeds (Mundy et al. 1990). A cDNA for the ABRE-binding protein EmBP-1 was shown to encode a basic leucine zipper (bZIP) protein containing a basic DNA-binding domain linked to a leucine zipper domain (Guiltinan et al. 1990). A single copy of ABRE, however, is not sufficient for ABA-responsive transcription. Further, a coupling element is required to specify the function of ABRE. ABRE and a coupling element (CE; CACC) constitute an ABA-responsive complex in the regulation of the wheat gene HVA22 (Shen and Ho 1995). Further, nucleotides around the ACGT core motif are important for determining the binding specificity of bZIP proteins. The G-box resembles the ABRE motif and function in the regulation of plant genes in a variety of environmental conditions, such as red light, UV light, anaerobiosis, and wounding (Menkens et al. 1995). Recently, three different cDNAs encoding ABRE-binding proteins (AREB1, AREB2 and AREB3) were reported cloned. Each AREB protein contained a single bZIP type DNA-binding domain, and the corresponding transcripts were up-regulated by ABA (Choi et al. 2000; Uno et al. 2000).

ABA- regulated transcription factors expressed during the vegetative phase of plants have been identified such as the homeodomain-leucine zipper proteins ATHB6, ATHB7, and ATHB12 (Söderman et al. 1996; Lee and Chun 1998; Söderman et al. 1999) and the basic helix-loop helix leucine zipper motif containing rd22BP1 and the Arabidopsis transcription factor ATMYB2 that is structurally related to the mammalian proto-onco-gene *myb* (Abe et al. 1997).

Mechanisms of light signaling

Cues from the light environment are involved in the regulation of seed germination, the establishment of seedlings, the determination of growth habit, and the transition to flowering. Light can induce leaf formation, leaf expansion and inhibit stem elongation. To perceive information about their light environment, plants have evolved at least four families of photoreceptors that recognize different wavelengths of light: the red (R)/ far-red (FR)-sensing phytochrome family, the blue/UV-A photoreceptors called cryptochromes, the blue/UV-A photoreceptors NPH1 and NPL1 and the UV-B photoreceptors. In Arabidopsis, which has been the subject of the most extensive study, there are five phytochromes; they are known as phyA through phyE (Clack et al. 1994). The phytochromes exist in two distinct but interconvertible forms, R-absorbing P_R and FR-absorbing P_{FR}, and interconversion between the forms initiates phytochrome signaling (Quail et al. 1995). Photoconversion of cytoplasmic P_R to P_{FR} causes translocation of phytochrome into the nucleus (Sakamoto and Nagatani 1996; Kircher et al. 1999; Yamaguchi et al. 1999). Thus, activation of phytochrome signaling brings phytochrome into the vicinity of the genes that it regulates. The P_{FR} form interacts with transcriptional regulators such as PIF3 (Ni et al. 1998; Halliday et al. 1999; Ni et al. 1999; Martinez-Garcia et al. 2000; Zhu et al. 2000). Together, the advances have resulted in an emerging general model for phytochrome action, whereby phytochromes perceive light, enter the nucleus, interact with transcriptional regulators, and thus activate or repress gene activity (Martinez-Garcia et al. 2000).

Among the transcription factors implicated in light-regulated transcription in plants, most have been isolated by their ability to bind to specific promoter sequences. These include the MYB protein CCA1, several leucine zipper proteins (ATHB2/4, CPRF and GBF1) and GT-1 (Weisshaar et al. 1991; Gilmartin et al. 1992; Schindler et al. 1992; Carabelli et al. 1993; Wang et al. 1997). A set of transcription factors have been analyzed by the study of mutants disrupted in genes encoding these proteins and the characterization of the light

responses in these mutants. Examples are the bZIP HY5, whose deficiency in plants resulted in elongated hypocotyls under all light conditions, the MYB protein LHY1, which is involved in circadian rhythm, the MYB protein LAF1 involved in phyA signaling, and the bHLH proteins PIF3 and RSF1/HFR1/REP1 (Oyama et al. 1997; Schaffer et al. 1998; Halliday et al. 1999; Fairchild et al. 2000; Soh et al. 2000; Spiegelman et al. 2000; Ballesteros et al. 2001).

The various phytochromes and cryptochromes share some functions, but are also specialized to some degree. For example, different photoreceptors contribute to inhibition of hypocotyl elongation under different light conditions. In white light, phyB, and cry1 play the largest roles and phyA, phyD, and cry2 are of reduced importance under these conditions (Reed and Chory 1994; Aukerman et al. 1997; Smith et al. 1997; Lin et al. 1998). Signal transduction pathways downstream of these photoreceptors probably interact. PHYA, the product of the *PHYA* gene, is light labile and predominates in etiolated seedlings, where it accumulates to relatively high levels. PhyB and phyC are more light stable, with phyB predominating in light –grown tissues (Somers et al. 1991). Light- grown *phyA* mutant seedlings display a more or less wild-type phenotype, although they are unable to detect a far-red-rich, low-fluency, incandescent day extension that accelerates flowering in wild-type seedlings (Johnson et al. 1994). Etiolated seedlings of the *phyB-1* mutant are deficient in several responses to red light (Koorneef et al. 1980; Reed et al. 1993). Light-grown seedlings of *phyB-1* have an elongated, early flowering phenotype characteristic of the shade-avoidance syndrome of wild-type seedlings grown under a low R/FR or in EOD far-red-light treatments.

The HD-Zip transcription factor ATHB7 in relation to ABA signaling

The *ATHB7* transcript, in the *abi1-1* mutant plants, have a reduced ABA and/or stress induction similar to multiple mRNAs including the *Rab18* transcript (Lång et al. 1994; Gosti et al. 1995; Söderman et al. 1996). The recorded induction of *ATHB7* in *abi1-1* was approximately 30% of the wild-type response. In the *abi2-1* mutant plants, however, there is no similar reduction of *ATHB7* transcript (Söderman et al. 1996). The dependence of *ATHB7* on ABI1 implies protein phosphorylation to be involved in the transcriptional control of the *ATHB7* gene. The drought-induced increase of *ATHB7* transcription requires endogenous ABA-synthesis, as shown by its absence in the ABA-deficient *aba1-3* mutant (Söderman et al. 1996).

AIMS OF MY THESIS

My thesis is an attempt to make a systematic analysis of the presence and relationship of different components involved in homeodomain-leucine zipper transcription factor mediated drought stress signaling in Arabidopsis. I started out in this project by studying the promoters of the *ATHB6* and *ATHB7* genes. These genes are known to be induced by drought stress and abscisic acid.

I aimed to establish the extent to which the *ATHB7* gene is responsible for the drought response and acclimation of the plant. To address this question I investigated phenotypic properties of transgenic plants constitutively expressing the *ATHB7* mRNA. Another means to establish the possible function of ATHB7 was to describe the spatial expression of an *ATHB7* promoter-reporter gene construct in transgenic Arabidopsis plants grown under different conditions of water availability.

Further, plants mutated in the *ABI1* locus and transgenic with a *35S::ATHB7* construct were used to address the question to what extent changed gene activity of *ATHB7* is the cause of the phenotype of the *abi1-1* mutation and to what extent constitutive *ATHB7* expression could bypass the phenotypic changes caused by the *abi1-1* mutation.

In an attempt to unravel connections between the ABA-induced *ATHB7* gene and the light signaling pathway I measured the ABA-driven induction of the *ATHB7* gene in the two light regulatory mutants, *phyA-201* and *phyB-1* respectively.

METHODS, IN BRIEF

Loss-of-function alleles are important tools in assessing gene function and has been used successfully in many species. A problem with the use of loss-of-function alleles to determine gene function is that the function of redundantly acting genes cannot be easily characterized. In those circumstances, the standard routine has been to generate transgenic plants in which those genes are constitutively expressed. There are many recent instances in which this approach has provided a useful means for revealing genetic function, especially when the function of the gene was already partially understood. Despite such successes, gain-of-function approaches are subject to a number of difficulties. Most importantly, it can be unclear whether a phenotype reflects the true function of a gene or whether it is simply caused by interference with unrelated processes. Such problems could be particularly prevalent for transcription factors (Riechmann and Ratcliffe 2000). The phenonomen that genes, when knocked out, result in no obvious phenotype is often ascribed to redundancy in regulatory networks, caused by duplicated genes. An alternative explanation suggests that genes might evolve by very weak selection, which would mean that their true function cannot be studied in normal laboratory experiments (Tautz 2000).

Another reverse genetics approach used in this study is the promoter-reporter method to analyze gene expression, a method widely used for the easiness by which a wealth of expression data is obtained. This method is based on the isolation of a DNA fragment constituting the transcriptional control region of the gene under study. The isolated gene fragment is fused to a reporter gene and this construct is then introduced into the plant under study. Expression of the wild-type gene is inferred from the activity of the reporter enzyme. In this study the ß-Glucuronidase enzyme was chosen primarily because of a low corresponding background activity in Arabidopsis but also

because of its high stability and that it is easily detected. The possible drawbacks with the reporter-promoter methods are several. The method is open for criticism regarding whether the expression of the reporter gene really reflects the expression of the wild-type gene. It is not possible to determine if all the regulatory elements of a given gene has been incorporated in the promoter-reporter construct. In many reports on the expression of genes, however, it has been shown that the endogenous transcript levels and the activities of the promoter-reporter genes are highly correlated.

The plant species Arabidopsis thaliana

Like many plant geneticists, I have done all my work on Arabidopsis thaliana, a frail weed in the mustard family ideal for research because it is very small, reproduces quickly and it is easy to manipulate through genetic engineering. This plant species has been in the focus for large scale genetic and reverse genetic efforts within the plant scientific community.

RESULTS AND DISCUSSION

ABA-induced expression of ATHB6 is dependent on abi1-1 and abi2-1 (I)

ATHB6 encodes a class I homeodomain-leucine zipper protein (Söderman et al. 1994). This class of transcription factors, among other members, also includes; *ATHB7, 12* and *16.*

Northern blot data showed that the *ATHB6* gene is constitutively expressed in seedlings, but also that expression levels are dependent on the growth conditions of the plant (paper I). The transcript levels are increased by a factor of approximately 3 in plants subjected to drought or to high levels of salt in the medium. Transcript levels are also increased by a factor of 5 in plants grown in the presence of ABA at 10 μM. Transcription of *ATHB6* in response to desiccation and osmotic stress was analyzed in the Arabidopsis mutant *aba-1-3.* This mutant is deficient in the ABA synthesis and therefore unable to respond to drought by an increase in ABA. The result of the northern analysis showed an increase in *ATHB6* transcription in the *aba1-3* mutant after treatment with exogenous ABA but not after desiccation or high salt treatment. Thus, endogenous synthesis of ABA is required for the enhanced expression of *ATHB6* in response to desiccation and osmotic stress. The ABA induction of *ATHB6* displayed a distinct reduction in the *abi1-1* and *abi2-1* mutant backgrounds as compared to that of wild-type. Thus, the *ABI1* and *ABI2* gene products are required for the *ATHB6* transcription in response to ABA. The degree of induction was quantitatively determined in several independent experiments and found to range between 10-60 % of the induction level in the wild-type.

From these data, we conclude that the water deficit response of *ATHB6* depends on the endogenous synthesis in the plant of abscisic acid, and that the gene is active in a drought signaling pathway down-stream to both the *ABI1* and *ABI2* genes.

ATHB6::GUS gene expression in transgenic Arabidopsis plants (I)

The sequence of a 2.4 kb BglII *ATHB6* genomic fragment was determined. The fragment includes 1.4 kb of upstream sequences as well as 1 kb of coding sequence including the entire homeodomain, the leucine zipper domain and two introns. At position -324 from the putative site of transcription initiation, a sequence similar to the consensus sequence of an abscisic acid responsive element (ABRE) (CACGTA) is located. The expression of an *ATHB6::*GUS chimeric gene including the 2.4 kb BglII fragment was studied in transgenic Arabidopsis plants. In seedlings, GUS staining was detected in the developing stomatal guard cells of the cotyledons, in leaf primordia, the cell division zone of the primary root as well as in the first lateral root primordia. The expression pattern of the *ATHB6* promoter-GUS fusion in the different organs, suggests a possible role for ATHB6 related to cell division and/or differentiation. As shown by microscopic sections of two days old seedlings germinated in the dark, GUS expression in the cotyledons was restricted to the epidermal cell layer, and high in the stomatal guard cells and the cells surrounding the stomatal pore. This may indicate a specific role for ATHB6 in the development of the stomata, but may also be due to a delayed development of the stomatal cells, as compared to the epidermal cells. Sections of a two days old seedling also showed high levels of expression in leaf primordia. In fully expanded leaves the overall staining was lower and restricted to the vascular tissue. A newly formed expanding leaf taken from a 16 days old plant showed *ATHB6* expression to be distributed transiently as a gradient along the apical-basal axis of the leaf with the strongest expression in the latest formed tissue. GUS staining in the basal parts of expanding leaves was detected in the stomata but not in the developing trichomes. In experiments where plants were drought-treated or treated by exogenous application of ABA or salt, the intensity of staining increased but the cellular pattern of staining did not differ from that of non-ABA-treated plants.

Two other members of the class I homeodomain-leucine zipper protein, *ATHB7* (Söderman et al. 1996) and *12* (Lee and Chun 1998), have been shown to be transcriptionally activated by conditions similar to those that induce *ATHB6* transcription; by water limitation and by exogenous abscisic acid. *ATHB7*, like *ATHB6*, requires ABA-biosynthesis and the *ABI1* gene product, but, in contrast to *ATHB6*, is independent of the *ABI2* gene product for its transcriptional activation. Whether *ATHB12* activation is dependent on the function of ABI1 or ABI2 gene products is not known. *ATHB6* and *7* also differ in their expression levels. The basal, non-induced, level of expression of *ATHB6* is relatively high and inductive conditions only moderately increase the level, but does not affect the pattern of expression. In contrast, the basal level of expression of *ATHB7* is low, and the expression level at inductive conditions high, and induction requiring only quite mild inducing conditions (Söderman et al. 1996). Taken together, these data show that *ATHB6* and *7* are activated transcriptionally by water deficit by similar but non-identical mechanisms. Since other HD-Zip proteins are known to act as activators (Aoyama et al. 1995) or repressors (Meijer et al. 1997) of downstream genes and to bind DNA as dimers (Sessa et al. 1993) it is possible that *ATHB6, 7* and *12* may interact at the protein level to form active heterodimers with activities different from the respective homodimers. If this is the case, the plant would be equipped with a mechanism for the activation of a potentially complex response by partially, but in the case of *ATHB6* and *7* not entirely overlapping signaling pathways. The GUS expression patterns of the *ATHB6* and the *ATHB7* GUS-promoter constructs coincide to a large degree, indicating that the respective proteins may occur simultaneously in the cells.

ATHB7::GUS gene expression in transgenic Arabidopsis plants (II)

Histochemical staining of transgenic plants carrying the *ATHB7*::GUS gene construct was performed after plant drought treatment. The promoter activity from the *ATHB7* gene was highly dependent on drought stress (Paper II). Plants grown under optimal water conditions showed no or very low GUS activity in all organs and tissues analyzed with an exception for the axillary buds that showed strong GUS activity. In contrast, the GUS activity was strongly induced in plants exposed to limiting water supply. This induction, however, varied between experiments. In adult plants exposed to extended water limitation, high promoter activity could be detected in young leaves, in the main inflorescence stem and in flower buds. The GUS activity was most intense in the young parts of the inflorescence and the stem. Drought induced promoter activity was also detected in the siliques and occasionally in the developing embryos. In young leaves exposed to limiting water supply, strong GUS activity was detected but at later stages of leaf development, expression was maintained at a moderate level only in the vascular tissue.

Phenotypic effects of alterations in ATHB7 expression levels (II)

A reverse genetics approach was used to study the possible function of the *ATHB7* gene. For this purpose the 35S promoter was fused to sense and antisense constructs of the *ATHB7* cDNA (paper II)(Figure 1, Table 1). These constructs were transferred into wild-type Arabidopsis plants (ecotype Wassilevskija and Landsberg erecta). Five antisense transformant lines were generated showing a 3:1 segregation of the resistance marker. Three of these lines showed approximately wild-type levels of the *ATHB7* transcript, whereas two lines showed a reduced transcript level as compared to the wild-type. The two antisense lines showing a 30 to 60% reduction in transcript levels were investigated in their overall morphology and flowering time in LD as well as in SD conditions. No changes from wild-type phenotype were observed.

In the reverse genetics approach using sense constructs five independent lines were analyzed by northern blot experiments. The transcript levels ranged from two-fold to 12 times the wild-type level. For a comparison, the increase in the *ATHB7* transcript level caused by treatment with 10μM exogenous ABA for four hours of 12 days old wild-type Arabidopsis plants is approximately eight-fold, as compared to the non-induced level (Söderman et al. 1996).

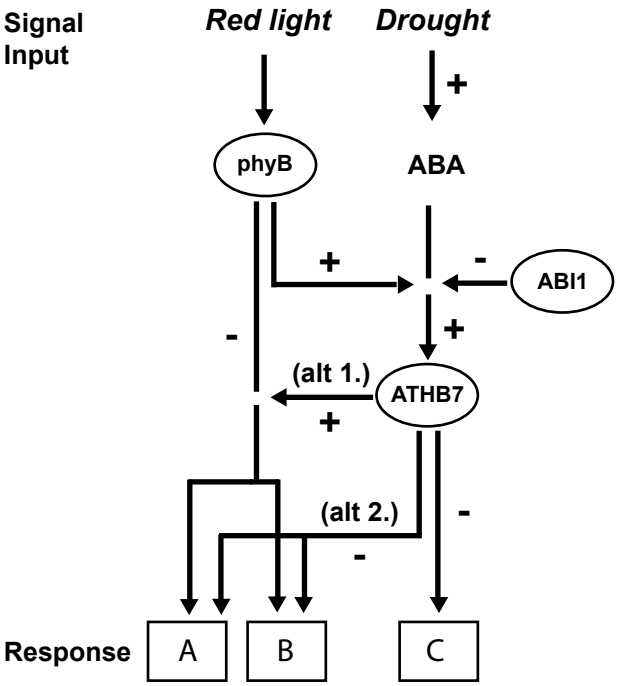
The constitutive expression of the *ATHB7* mRNA (expression corresponding to nine times the wild-type level) led to a suppression of shoot elongation growth after transition to reproductive development constituting a phase shift by retarding the initiation of bolting by approximately eight days. The time point of flower bud initiation, in contrast, was not altered. The difference in flower bud initiation between the two lines were no more than a day in LD conditions, the wild-type (Ws) plants initiating a flower bud at 15 days after vernalization and the transformant line 15-16 days after vernalization. The difference in time-point of bolting can therefore not be explained by difference in timing of transition to reproductive development. Further, the number of leaves at initiation of reproductive development were six to seven in both the wild-type plant and the transformant line, indicating

that the timing of transition to reproductive development in long day conditions as measured on a developmental time-scale was not different.

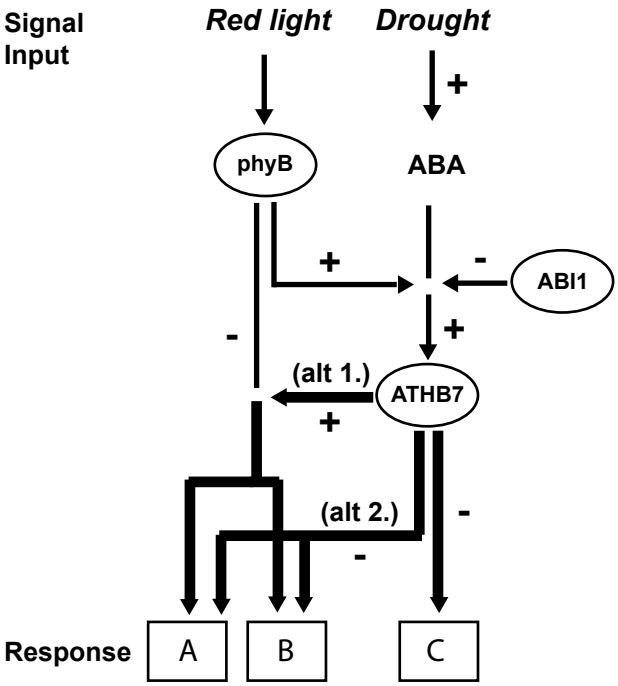
The observed phenotypic changes in the elongation growth zone of the main inflorescence stem is consistent with the detected promoter activity in that zone. The fact that the constitutive expression of the *ATHB7* mRNA under the control of the 35S promoter leads to a phenotype relatively restricted to the stem and leaf elongation growth zones and not to obvious alterations in phenotype at other developmental stages might be explained by a dependency on other components of the signal system limiting the phenotypic output. The suppression of elongation growth could in principle be explained by a decrease in source strength in the rosette leaves and thus have a phenotypic origin other than the place of the observable effect. This however is less likely since the timing of initiation of reproductive development was about the same in the plants constitutively expressing *ATHB7* as compared to wild–type. Further, there was no difference in leaf area between the transgenic plants and the wild-type in this experiment.

Our data, derived from constitutively expressing *ATHB7*, taken together with the promoter GUS data indicate that ATHB7 may act as a regulator of growth and development of the elongating stem in response to water availability. As GUS expression was detected also in other tissues it is likely that ATHB7 additionally may have other functions.

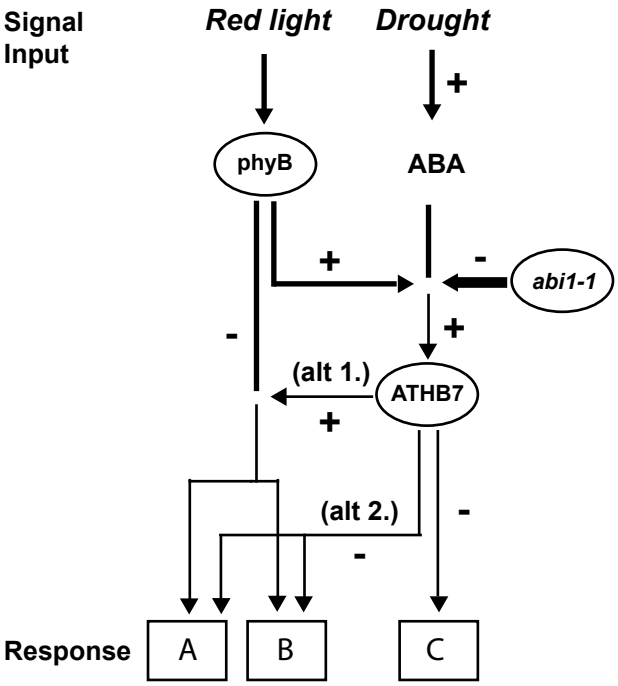
Fig. 1 (a), Wild-type



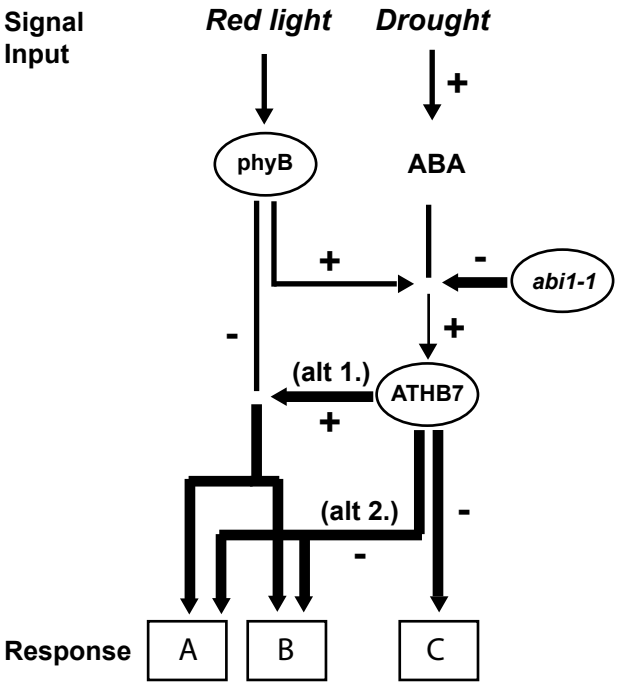
(b), *35S::ATHB7*



(c), *abi1-1*



(d), *abi1-1X35S::ATHB7*



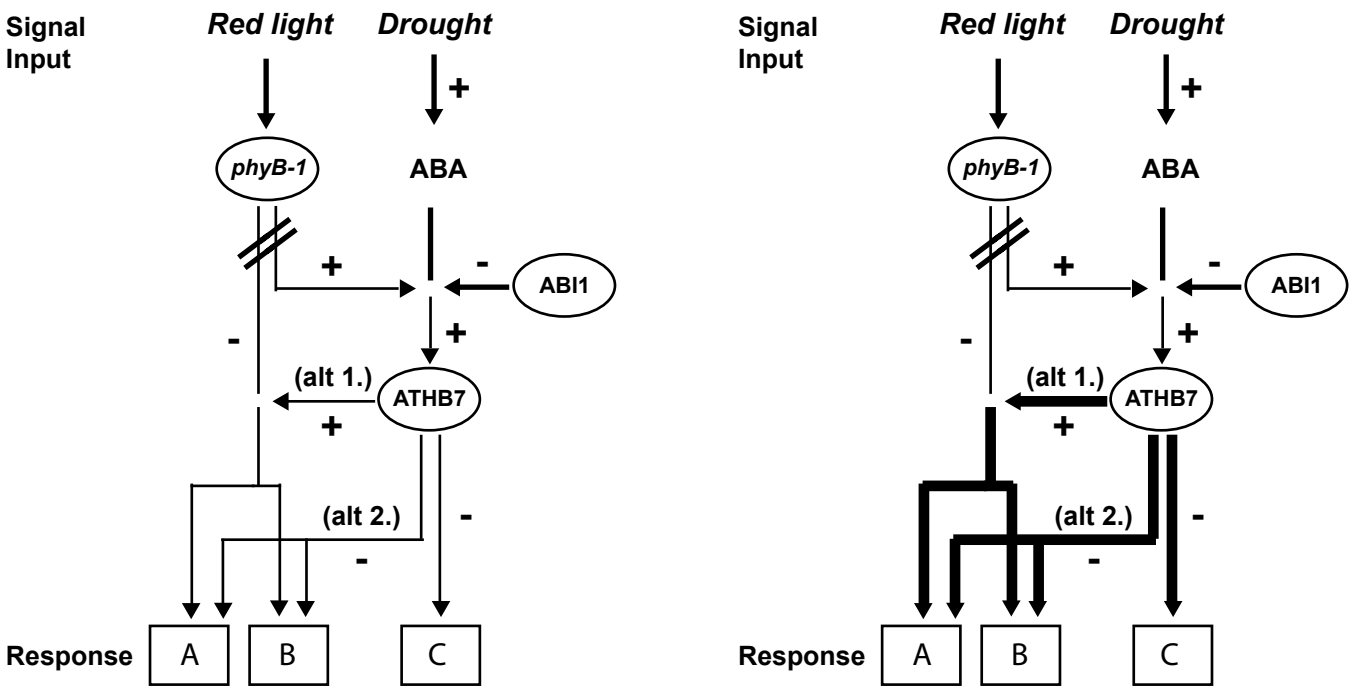


Fig 1. The model represents the dynamic behavior of a drought and light signaling complex in genotypes differing in ABA- or red light-sensitivity and/or expression levels of *ATHB7*. The genotypes are used in experiments in paper I, II and III. Signal input identity is indicated at the top and responses at the bottom; **A** Initiation of reproductive development, **B** Elongation growth, **C** Stomata closure. Arrows indicate regulatory interaction with the causal link in the direction of the arrowhead. Plus and minus signs denotes a positive or negative regulation, respectively. Circles enclose gene products and squares enclose responses. Thick arrows indicate a strong signal, whereas narrow arrows indicate a weaker signal.

(a) Ler or Ws wild-type plants (**La-er, Ws**),
(b) Transgenic plants constitutively expressing the *ATHB7* mRNA (***35S::ATHB7***),
(c) *abi1-1* mutant plants (***abi1-1***),
(d) *abi1-1* mutant plants constitutively expressing the *ATHB7* mRNA (***abi1-1X35S::ATHB7***),
(e) *phyB-1* mutant plants (***phyB-1***),
(f) *phyB-1* mutant plants constitutively expressing the *ATHB7* mRNA (***phyB-1 X35S::ATHB7***).

Table 1. Characteristics of plants in paper II, III and IV

Ler wild-type plants (La-er), transgenic plants constitutively expressing the *ATHB7* gene (*35S::ATHB7*), *abi1-1* mutant plants (*abi1-1*), *abi1-1* mutant plants constitutively expressing the *ATHB7* gene (*abi1-1X35S::ATHB7), phyB-1* mutant plants (*phyB-1*) and *phyB-1* mutant plants constitutively expressing the *ATHB7* gene (*phyB-1 X35S::ATHB7*) Phenotypes as compared to wild-type if not indicated otherwise.

	35S::ATHB7	***abi1-1***	***abi1-1 X35S::ATHB7***
Wiltiness in Drought Conditions	Plant of Similar Size as wt have a Similar Resistance to Drought	Wilty	Wiltiness Intermediate Between *abi1-1* and wt
Fv/Fm in Drought Conditions	Enhanced	Similar to wt	Enhanced
Stomatal Conductance	Reduced	Enhanced	Reduced Stomatal Conductance as Compared to *abi1-1*
Elongation Growth	Delayed	Slightly Delayed	Delayed
ABA- Sensitivity of Root Growth	Similar to wt	Less Sensitive	Less Sensitive

	35S::ATHB7	***phyB-1***	***phyB-1X35S::ATHB7***
Hypocotyl Length	Reduced	Increased	Hypocotyl Length Intermediate Between *phyB-1* and wt
Petiole Length	Reduced	Increased	Petiole Length Intermediate Between *phyB-1* and wt
Flowering in LD and SD	Late	Early	Late
Branching	Increased	Reduced Branching (Reed, 1993)	No data
Colour	Similar to wt	Pale	Pale
Elongation Growth	Delayed	Rapid	Delayed

The 35S::ATHB7 transgene partially suppressed the wilty phenotype of abi1-1 (III)

Previous data indicate that ATHB7 acts downstream of ABI1 in an ABA signaling pathway (Söderman et al. 1996). This suggestion is based on the reduced transcriptional response of *ATHB7* to induction conditions in the *abi1-1* mutant. Accumulation of the transcript provoked by water stress, is affected in *abi1-1* but not in *abi2-1* (Söderman et al. 1996). The recorded induction in *abi1-1* was approximately 30% of the wild-type response. Our previous study has shown that transgenic plants constitutively expressing the *ATHB7* gene, controlled by the 35S promoter, exhibit a delay in bolting after transition to reproductive development. This is consistent with the possible function of ATHB7 acting as a growth repressor in response to drought stress. However, when plants with similar size were analyzed no difference in frequency of survival to drought conditions was observed in the transgenic plants constitutively expressing *ATHB7* as compared to wild-type (paper II). This fact prompted us to examine the role of ATHB7 in the *abi1-1* and *abi2-1* mutant backgrounds, respectively.

We thus quantified, side by side, the relative impact of the *35S:ATHB7* transgene in the *abi1-1* and *abi2-1* mutation backgrounds on several characteristic drought and ABA responses (Paper III). The material analyzed was double homozygous with respect to the *abi1-1* or *abi2-1* mutations and the *35S:ATHB7* transgene as confirmed by PCR detection of the mutant alleles and by selection on kanamycin, respectively.

The expression of the *35S::ATHB7* transgene was able to partially suppress the wilty phenotype of the *abi1-1* mutation. Also, the frequency of survival in lack-of-water conditions was restored in the *abi1-1* mutant plants expressing the *35S::ATHB7* transgene. This is consistent with the ATHB7 protein having a central role in the drought stress response and acting downstream to the ABI protein. Furthermore, the data is consistent with the role of ATHB7 acting as a positive regulator of the drought stress response.

In lack-of-water conditions the low Fv/Fm value of the abi1-1 mutants was restored by expression of the 35S::ATHB7 transgene (III)

To further analyze plant stress performance, in different water conditions, we measured the chlorophyll fluorescence emission from leaves (Fv/Fm). Plants expressing the *35S::ATHB7* transgene, cultivated in well-watered conditions, exhibited a similar Fv/Fm value as did the wild-type, indicating that the *35S::ATHB7* transgene by itself did not confer a strain to the plants. Under conditions characterized by lack-of-water the monogenic plants expressing the *35S::ATHB7* transgene showed a higher Fv/Fm value than did the wild-type, indicating that the photosystem II suffered less damage in the plants expressing the *35S::ATHB7* transgene. The *abi1-1* mutant plants exhibited a similar Fv/Fm value as compared to the wild-type in lack-of-water conditions. The Fv/Fm value, however, was enhanced in the *abi1-1* mutant expressing the *35S::ATHB7* transgene, indicating that the expression of the *35S::ATHB7* transgene resulted in less damage of the photosystem II in these plants. These data confirm the effect detected in the observed differences in wiltiness and frequency of survival in lack-of-water conditions.

abi2-1 mutant plants exhibited a lower Fv/Fm value than the wild-type in lack-of-water conditions. The Fv/Fm value, however, was similar in the *abi2-1* mutant plants expressing the *35S::ATHB7* as compared to that of the monogenic *abi2-1* mutant plants, indicating that the expression of the *35S::ATHB7* transgene did not restore the photosynthesis efficiency in these plants.

To conclude, in conditions characterized by a lack-of-water, in two out of three genetic backgrounds, expression of the *35S::ATHB7* transgene mediates a higher maximal photochemical efficiency of PSII, consistent with ATHB7 having a role in the regulation of water-balance in the plant.

The 35S::ATHB7 transgenic plants exhibited a lower stomatal conductance (III)

Excessive water loss displayed by the Arabidopsis *abi1-1* mutant is a consequence of abnormal stomatal regulation by ABA (Roelfsema and Prins 1995; Pei et al. 1997). The stomatal conductance to loss of water vapour was significantly lower in plants expressing the *35S::ATHB7* transgene as compared to the wild-type plants. This is consistent with the ATHB7 protein acting through a reduction of stomatal conductance. Similarly, in *abi1-1* mutant plants expressing the *35S::ATHB7* transgene stomatal conductance was reduced as compared to the monogenic *abi1-1* mutant plants, indicating that the expression of the *35S::ATHB7* transgene could to some extent suppress the phenotype of the *abi1-1* mutation. However, the stomatal conductance of the *abi1-1* mutant plants was not fully restored to wild-type level, indicating that expression of ATHB7 not is sufficient to relay a wild-type ABA-signal downstream to ABI1.

In *abi2-1* mutant plants expressing the *35S::ATHB7* transgene stomatal conductance also was reduced as compared to the monogenic *abi2-1* mutant plants.

Root growth response to ABA was not to a large extent altered (III)

The different water balance in the plants could possibly be due to different root properties, such as root biomass and sensitivity to ABA. Root growth, as affected by 10 μM ABA, however did not statistically differ between plants expressing the *35S::ATHB7* transgene and wild-type or between the *abi1-1* mutant plants expressing the *35S::ATHB7* transgene and the monogenic *abi1-1* mutant plants. This indicates that a different root function does not constitute the main mechanism leading to altered drought resistance in the *35S::ATHB7* transgenic plants.

The shading syndrome and ATHB7-driven suppression of elongation growth (IV)

In a previous study we have shown that constitutive expression of the *ATHB7* gene, controlled by the 35S promoter, in transgenic Arabidopsis delays elongation growth of the main inflorescence stem after initiation of reproductive development (paper II). These data together with expression data, showing a strong ABA induction of the *ATHB7* gene, is consistent with the hypothesis that ATHB7 downregulates elongation growth of the stem in response to drought.

When shading occurs there is a shift in the spectral composition of the light reducing the red/far-red ratio (R/FR). Shade-avoiding species respond to the spectral composition in the shade primarily with enhanced stem elongation (Smith 1991). We here attempt to explore how this signal (low R/FR ratio) relates to the ATHB7-driven drought signal in regulating elongation growth (Figure 2, Table 1).

Bearing in mind the importance of optimal elongation growth one might hypothesize that there is a down-regulation of the drought feed-forward growth repression when shading occurs. This would equip the plant with the possibility to modulate the drought repression of elongation growth when competing for light. In an analyses of *ATHB7* gene expression in different light regulatory mutant backgrounds we addressed this issue (Paper IV).

ATHB7 expression was reduced in phytochrome deficient seedlings (IV)

phyB-1 mutant seedlings display attenuated responses to a low R/FR or in end-of-day far-red light, leading to the proposal that *PHYB* plays a key role in the shade-avoidance response (Nagatani et al. 1991; Whitelam and Smith 1991a).

The recorded reduction in ABA induced *ATHB7* expression in the *phyB-1* and *phyA-201* mutant seedlings clearly shows that there is a dependence of the ABA-induced *ATHB7* expression on the phytochrome system, suggesting that there is an interplay between light and osmotic stress signaling in the regulation of the *ATHB7* gene. The *phyA-201* mutation reduced, to a similar

extent as that of *phyB-1*, the induction of the *ATHB7* gene. This implies that the PHYA and the PHYB proteins are equally important for the ABA-inductive expression of *ATHB7* in seedlings of this age. There was no big difference in the basal levels of *ATHB7* expression between the *phyA-201* and *phyB-1* mutants and the wild-type. Thus, the basal expression of the *ATHB7* gene at the seedling stage seems not to be dependent on the phytochrome system.

One implication of the phyB modulated ABA-induction of *ATHB7* would be that shade via the inactive phyB FR-absorbing P_{FR} form downregulates the ABA-driven expression of ATHB7, thus allowing the plant to elongate and compete for light although drought signaling propose a repression of elongation growth. The question then arises what would happen in a hypotethical plant if low R/FR and drought would result in simultaneous signaling i.e. the light signal promoting elongation growth and the drought signal repressing elongation growth. In order to investigate this we crossed transgenic plants constitutively expressing the *ATHB7* gene, controlled by the 35S promoter, and plants carrying a mutation in the *PHYB* locus.

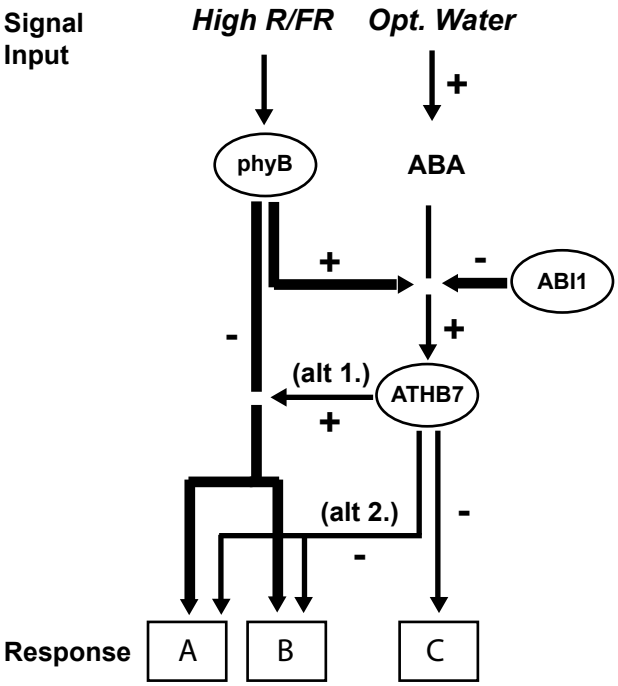
The 35S::ATHB7 transgene partially suppressed the hypocotyl phenotype of phyB-1 (IV)

Expression of the *35S::ATHB7* transgene suppressed the elongated hypocotyl phenotype of the *phyB-1* mutation indicating that an expression of ATHB7 forces the suppression of the elongation growth independently of other factors of the shade avoidance pathway.

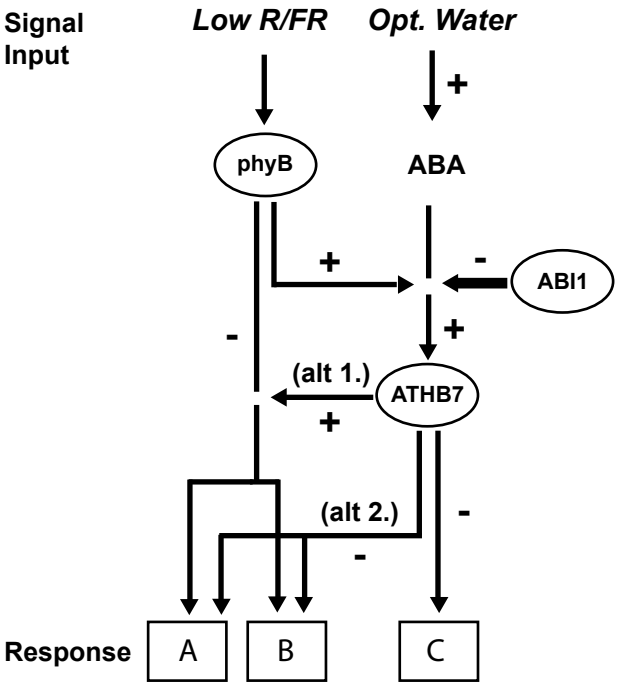
Rosette leaves were more rounded in phyB-1X35S::ATHB7 (IV)

It is a well known phenomenon that light affects the onset of leaf development and expansion (Cosgrove and Durachko 1994). The rosette leaves in the *phyB-1* mutant are slender with long petioles as previously described by Reed et al. (1993). Rosette leaves of wild-type plants expressing the *35S::ATHB7* transgene were more rounded and petioles shorter as compared to wild-type, suggesting that the phenotypic effect of the constitutive expression of ATHB7

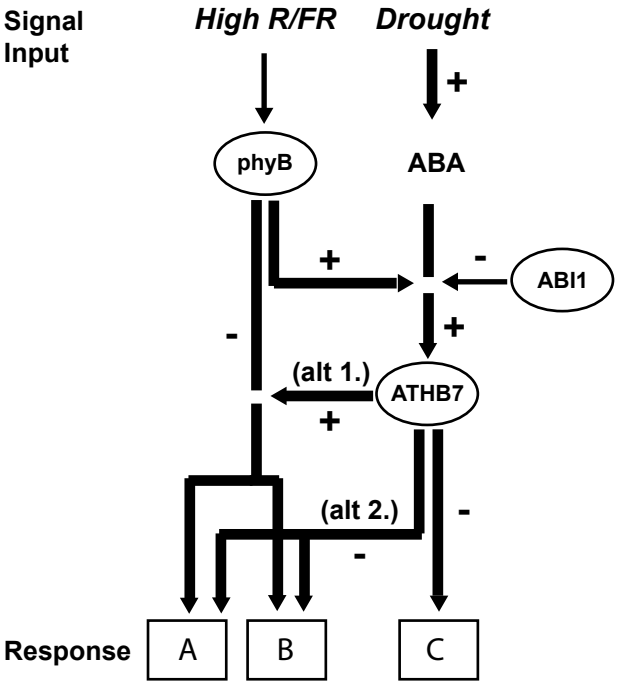
Fig. 2 (a), ***High R/FR, Opt. Water***



(b), ***Low R/FR, Opt. Water***



(c), ***High R/FR, Drought***



(d), ***Low R/FR, Drought***

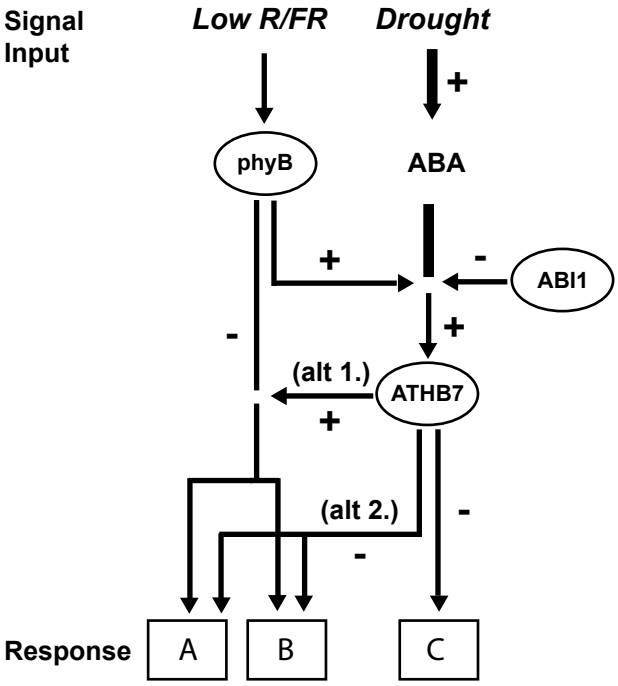


Fig 2. The model represents the dynamic behavior of a drought and light signaling complex in wild-type plants subjected to different R/FR ratio and/or drought stimuli. Signal input identity is indicated at the top and responses at the bottom; [A] Initiation of reproductive development, [B] Elongation growth, [C] Stomata closure.

Arrows indicate regulatory interaction with the causal link in the direction of the arrowhead. Plus and minus signs denotes the positive or negative regulation. Circles enclose gene products and squares encloses responses. Thick arrows indicate a strong signal whereas narrow arrows indicate a weak signal.

(a) High R/FR and well watered conditions (Opt. Water).

(b) Low R/FR and well watered conditions (Opt. Water).

(c) High R/FR and lack-of-water conditions (Drought).

(d) Low R/FR and lack-of-water conditions (Drought).

is expressive in a plant background that has wild-type alleles at the *PHYB* locus. Rosette leaves of the *phyB-1* mutants expressing the *35S::ATHB7* transgene, were more rounded and the petioles shorter as compared to the monogenic *phyB-1* plants. The *35S::ATHB7* transgene thus reverted the constitutive shade-avoidance phenotype of the *phyB-1* mutation back to a phenotype more reminiscent of wild-type. This suggests that a constitutive drought signal, acting through ATHB7, is sufficient to partially downregulate the shade-avoidance reactions of a plant, thus further underlining the possible importance of combinatorial control of the ATHB7 expression.

Early Initiation of reproductive development and bolting was suppressed in phyB-1X35S::ATHB7 (IV)

Phytochrome B plays a major role in the regulation of flowering time in Arabidopsis and *phyB-1* mutants display a characteristic early-flowering phenotype under a wide range of light conditions (Whitelam and Smith 1991b; Halliday et al. 1994; Bagnall et al. 1995). The early transition to reproductive development phenotype of the *phyB-1* mutation was suppressed in *phyB-1* mutants expressing the *35S::ATHB7* transgene, whereas the timing of transition to reproductive development in the wild-type plants expressing the *35S::ATHB7* transgene was only slightly altered as compared to wild-type. This suggests that the *35S::ATHB7* transgene is able to couple with the endogenous pathway controlling floral induction in Arabidopsis. Further, the *phyB-1* mutants expressing the *35S::ATHB7* transgene displayed a delay in the onset of bolting as compared to the monogenic *phyB-1* mutants.

The major mechanism was not a change in ABA biosynthesis (IV)

Weatherwax et al, (1996) found that the ABA-responsive Em promoter of wheat can be negatively regulated by phytochrome action. They state that this regulation is mediated at least in part by phytochrome-induced changes in ABA levels. One might speculate that the differences in ABA induction of the *ATHB7* transcript level in the *phyA-201* and *phyB-1* mutant seedlings compared

to wild-type in our experiment is due to altered ABA degradation or distribution in the phytochrome mutants. We argue that this could not explain the whole variation in *ATHB7* transcript levels between the phytochrome mutants and wild-type. We have not, however, measured the ABA content in the *phyA-201* and *phyB-1* mutant seedlings after treatment by exogenous ABA.

No data on the position of the ATHB7 gene in the light signal transduction pathway (IV)

The phenotypic data presented here together with the expression data strongly indicate that part of the *phyB-1* mutant phenotype is due to a reduced expression of *ATHB7* or of a gene with similar function. The data, presented here, do not allow us, however, to address the position of the *ATHB7* gene in the light signal transduction pathway. Further genetic analysis of the interaction between light regulatory mutants and ABA response mutants will be necessary to clarify the action mechanism of the *ATHB7* constitutive expression effect.

MAJOR CONCLUSIONS FROM THIS WORK

The Arabidopsis thaliana transcription factor ATHB7 regulates plant elongation growth in response to drought stress, indicated by the delayed onset of bolting in transgenic plants expressing a *35S::ATHB7* transgene and the drought-stimulated GUS-reporter expression in the stem (paper II). The ATHB7 mediated delay in elongation growth is consistent with a strategy of minimizing water transpiratory area at low water availability.

Further, in this report ATHB7 has been shown to be a positive regulator of Arabidopsis thaliana drought stress (paper III). This is indicated by the reduced wiltiness of *abi1-1* mutant plants expressing the *35S::ATHB7* transgene as compared to monogenic *abi1-1* mutant plants.

In conditions characterized by a lack-of-water, in several genetic backgrounds, expression of the *35S::ATHB7* transgene mediates a higher maximal photochemical efficiency of PSII, consistent with ATHB7 having a role in the regulation of water-balance in the plant. Further, constitutive expression of a *35S::ATHB7* transgene reduces stomatal conductance to loss-of-water vapour in transgenic Arabidopsis plants.

ABA-induction of the *ATHB7* gene is positively regulated by phytochrome signaling, providing evidence for an integrative role of ATHB7 in drought- and light-signaling leading to regulation of plant elongation growth (paper IV). Further strengthening the case, constitutive expression of the *35S::ATHB7* transgene restores the elongated hypocotyl, stem and petiole growth and early initiation of reproductive development of transgenic phytochrome deficient plants, indicating that a shade avoiding syndrome of wild-type Arabidopsis plants occurring in low R/FR not could act in parallel with ATHB7-driven drought signaling.

FURTHER REMARKS

It is easy to reconcile that the importance of connecting the signaling events in the plant and the corresponding phenotypic output is paramount to plant biology. The models so far generated constitute a framework for future models that take into account the strength and duration of the different signals. Such models that are in a respect more quantitative are generated by studies based on statistical methods. So far little work has been published that extend into that direction. The driving force of research enterprises in the field of plant physiology is often that of a possible biotechnological application. In contrast with traditional breeding and marker-assisted selection programs, the direct introduction of a small number of genes by genetic engineering seems to be a more attractive and rapid approach to improve stress tolerance. Present engineering strategies rely on the transfer of one or several genes that encode either biochemical pathways or endpoints of signaling pathways that are controlled by a constitutively active promoter. Both the *ATHB6* and *ATHB7* genes are good candidates for an application in improving the plants drought resistance by such engineering strategies.

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