Identification of REVOLUTA target genes uncovers a link between leaf patterning and shade-induced growth responses

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Abkürzungen/Abbreviations:

ABA	Abscisic acid
ABI	ABSCISIC ACID INSENSITIV
AGO	ARGONAUT
AHA	Aromatic, large Hydrophobic, Acidic context
ANT	AINTEGUMENTA
AP2	APETALA2
BRI	BRASSINOSTEROID INSENSITIVE
C	Carbon
ChIP-Seq	Chromatin-Immunoprecipitation-Sequencing
CLI	CARBON AND LIGHT INSENSITIVE
CNA	CORONA
CO2	Carbon dioxide
CPSCE	Cystein-Prolin-Serin-Cystein-Glutamin
CRY	CRYPTOCHROM
CTR	C-terminal region
DNA	Desoxyribonucleidacid
DOF	DNA BINDING WITH ONE FINGER
DR5	Synthetic auxin-inducible promoter
DRN	DORNROESCHEN
DRNL	DORNROESCHEN-Like
EAR	Ethylen-responsive element binding factor-associated Amphiphilic Repression domain
FT	FLOWERING TIME
FWA	FLOWERING OF WAGENINGEN
GA	Gibberellic acid
GA20ox1	GIBBERELLIN 20 OXIDASE 1
GARP	GOLDEN2/ARR/PSR1
GL	GLABRA
GUS	β-glucuronidase

HAT	HOMEOBOX OF ARABIDOPSIS THALIANA
HB	HOMEOBOX
H-cells	Hair cells
HD	Homeodomain
HDG	HOMEODOMAIN GLABROUS
IAA	Indole-3-acetic acid
IFL	INTERFASCICULAR FIBERLESS
IPA	Indole-3-pyruvic acid
JAP	JAIBA
KAN	KANADI1
LBD	LOB DOMAIN CONTAINING PROTEIN
miR	microRibonucleidacid
ML	MERISTEM LAYER
MP	MONOPTEROS
mRNA	messengerRibonucleidacid
N	Nitrogen
N-cells	Non-hair cells
NPA	1-N-Naphthylphthalamic acid
OCL	OUTER CELL LAYER
PAS	Period circadian-Aryl hydrocarbon receptor nuclear translocator-Single-minded protein
PDF	PROTODERMAL FACTOR2
PHB	PHABULOSA
PHV	PHAVOLUTA
PHY	PHYTOCHROME
PIF	PHYTOCHROME INTERACTION FACTOR
PIN	PIN-formed auxin transporter
REV	REVOLUTA
SAD	START-adjacent domain
SAM	Shoot apical meristem
SAS	Shade avoidance syndrom
SEU	SEUSS

START	Steroidogenic acute regulatory protein-related lipid-transfer domain
TAA1	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1
TAM	Tryptamine
tDNA	transfer DNA
TZP	TANDEM ZINC KNUCKLE/PLU3
YUC	YUCCA
ZIP	Zipper domain
ZPR	LITTLE ZIPPER

1. Zusammenfassung/Summary

1.1 Zusammenfassung

Pflanzen führen eine standortgebundene Lebensweise. Aus diesem Grund müssen sie sich permanent an wechselnde biotische und abiotische Bedingungen anpassen. Dies geschieht durch das Zusammenspiel inhärenter genetisch determinierter Programme mit Signalwegen, die auf äußere Stimuli reagieren.

Um im Kampf um Wasser, Nährstoffe und Licht in einer stark von Konkurrenz geprägten Umwelt zu überleben, haben Pflanzen komplexe regulatorische Netzwerke etabliert, bei welchem Transkriptionsfaktoren und Hormone eine entscheidende Rolle spielen. Im Rahmen dieser Dissertation zeige ich auf, dass das Zusammenspiel von Homeodomänen-Leuzin-Zipper-Transkriptionsfaktoren und Pflanzenhormonen eine „Stellschraube“ zwischen dem inhärenten Entwicklungsprogrammen und der Antwort auf äußere Reize darstellt.

REVOLUTA, ein Klasse III Homeodomänen-Leuzin-Zipper Transkriptionsfaktor (HD-ZIPIII), spielt in vielen polaritäts-assoziierten Musterbildungsprozessen eine entscheidende Rolle.

Mittels der Hochdurchsatz-Sequenzierungsmethode ChIP-Seq (Chromatin-Immunopräzipitationssequenzierung) wurden zahlreiche direkte REVOLUTA-Zielgene identifiziert, welche einerseits in Entwicklungsprozesse andererseits in die Reaktion auf abiotische Faktoren involviert sind.

Zwei dieser Zielgene sind *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1*) und *YUCCA5* (*YUC5*), welche unter Regulation von REVOLUTA das Wachstumshormon Auxin synthetisieren. Des Weiteren wurden *Klasse II Homeodomänen-Leuzin-Zipper* Transkriptionsfaktoren als Zielgene von REVOLUTA identifiziert, welche unter dessen Kontrolle nicht nur wie bisher bekannt für schatteninduzierte Hypokotylverlängerung verantwortlich sind, sondern auch in der von REVOLUTA gesteuerte Blattmusterbildung eine entscheidende Rolle spielen.

Als Gegenspieler von REVOLUTA in der Blattmusterbildung agiert KANADI1 (KAN1), ein transkriptioneller Repressor aus der GARP-Familie. Es konnte gezeigt werden, dass durch die gemeinsame Regulation von Zielgenen in der Auxinsynthese und der Schattenantwort, sowohl REVOLUTA als auch KANADI1 in beiden Prozessen eine gegensätzliche Rolle spielen.

Zusätzlich konnte anhand der ChIP-Seq-Daten eine neue positive Feedback-Schleife für REVOLUTA identifiziert und eine bereits bekannte genauer charakterisiert werden. In beiden Fällen führt eine genetische Veränderung zu Veränderungen in der Blattmusterbildung. REVOLUTA wird posttranskriptionell von den microRNA Familien *miR165* und *miR166* reguliert. Ich konnte zeigen, dass *ARGONAUTE10*, welches die miRNA165/166 bindet und in ihrer Wirkung inhibiert, durch REVOLUTA direkt positiv transkriptionell reguliert wird. Des Weiteren konnte gezeigt werden, dass REVOLUTA die Genexpression von *LITTLE ZIPPER* Mikroproteinen direkt reguliert, welche in einer negativen Feedback-Schleife REVOLUTA posttranslational inhibieren.

Zusammengefasst lässt sich sagen, dass im Rahmen dieser Dissertation das regulatorische Netzwerk um *REVOLUTA* signifikant erweitert wurde. Es konnten nicht nur neue Feedback-Schleifen identifiziert und genauer charakterisiert werden, sondern es wurde eine Funktion für REVOLUTA und KANADI1 in der Auxin-vermittelten Schattenvermeidungsantwort aufgeklärt. Darüber hinaus konnte gezeigt werden, dass die an der Schattensignaltransduktion beteiligten Klasse II Homeodomänen-Leuzin-Zipper Transkriptionsfaktoren auch eine wichtige Rolle in der Blattmusterbildung spielen.

1.2 Summary

Plants are sessile organisms and thus have to cope with unfavorable growth conditions. To survive in an ever-changing environment, they have to constantly align their growth behavior to biotic and abiotic factors.

In their struggle for water, nutrients and light in a highly competitive environment, plants have evolved gene- and hormone-regulatory networks enabling them to counteract suboptimal conditions by inducing elongation growth. In this thesis I show that the interplay of homeodomain-leucine zipper (HD-ZIP) transcription factors and plant hormones act as an adjusting screw between the inherent growth programs and the outer world.

REVOLUTA, a class III homeodomain-leucine-zipper (HD-ZIPIII) transcription factor, plays a crucial role in many polarity-associated patterning processes.

Using a ChIP-Seq (Chromatin-Immuno-Precipitation-Sequencing) approach we were able to identify a number of direct REVOLUTA target genes. Some of these targets are involved in controlling developmental processes, while a significant number is involved in responding to abiotic stimuli.

Two of the identified target genes are: *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1*) und *YUCCA5* (*YUC5*), whose gene products are involved in biosynthesis of auxin. Additionally, several *class II homeodomain-leucine-zipper* (*HD-ZIPII*) transcription factors were identified as direct REV targets. These HD-ZIPII factors are known to regulate shade-associated growth processes. We were able to show that HD-ZIPIII factors regulate *HD-ZIPII* factors, which is a prerequisite for a full shade-avoidance response. In addition, we were able to establish a new link between HD-ZIPII factors and leaf patterning.

KANADI1 (KAN1), a transcriptional repressor of the GARP family, acts antagonistically to REVOLUTA during leaf patterning. We could show that REV and KAN1 antagonistically regulate several shared target genes. Furthermore, we revealed that REV and KAN1 also control shade growth in an antagonistic manner.

Finally, we identified a new positive feedback-loop regulating REV mRNA stability. It is known, that the ARGONAUTE10 (AGO10) protein sequesters microRNAs of the *miR165/166* family that regulate HD-ZIPIII transcript stability. We were able to show, that *AGO10* expression is directly and positively controlled by REV, thereby REV established a direct and positive feedback loop.

In summary, this thesis added to the expansion of the regulatory network around REVOLUTA. In addition to the identification of a new positive feedback-loop we were able to ascribe new functions for the REV/KAN1 module in shade-induced growth promotion. Finally, we discovered that class II homeodomain-leucine-zipper proteins have a crucial role in leaf patterning.

2. Ambition of work

Unlike the situation in animals plants have a higher plasticity, allowing them to adjust their growth and development to the prevalent environmental conditions. Both biotic and abiotic factors strongly influence plant development and thus modulate final plant shape. The most important abiotic stress factor is light. It provides energy for autotrophic organisms, which is the nutritional basis for all heterotrophic species. The perception and uptake of light is mediated by the green part of plants, in general leaves.

In adaption to environmental stimuli plants evolved a complex interplay of transcription factors and hormones, which controls adaptive growth responses. Homeodomain-leucine-zipper transcription factors play a crucial role in several growth-associated processes. Based on their structure and function, they can be sub-divided into four distinct gene families. Class I HD-ZIPs are involved in ABA-signaling (abscisic acid) in response to drought and salt stress, while class II HD-ZIPs regulate hypocotyl elongation in concert with auxin under shade conditions. However, REVOLUTA, one of five *class III HD-ZIP* transcription factors, controls patterning processes in all plant organs, particular in leaves. Class IV HD-ZIP transcription factors regulate cell determination in the epidermis and control trichome development. A general function for *HD-ZIPIV* genes in hormone regulation and abiotic stimuli signaling is unknown.

Shade exposed leaves possess longer petioles and a reduced leaf lamina. We hypothesized a direct link between light perception in leaves and class III HD-ZIP mediated leaf patterning. The aim of this thesis was to elucidate the role of *REVOLUTA* in the shade avoidance syndrome SAS and how this response influences growth processes.

Not only from the scientific view, but also from the economic side it is of big interest to understand, how plants percept and response to abiotic stimuli (light, temperature or drought) and how this leads to growth responses, to identify stress tolerant sorts and ecotypes with high yield and large quantity of biomass.

3. Introduction

3.1. Homeodomain-leucine zipper transcription factors mediate between inherent and adaptive growth processes in plants

Both animals and plants are able to detect environmental changes in light, temperature or water and nutrient availability. In contrast to animals, plants are sessile and therefore have to cope with permanently changing environmental conditions. These adaptive growth responses result from a crosstalk of abiotic stress induced environmental stimuli and the inherent programs, mainly involving transcription factors and plant hormones.

The function of plant hormones such as auxin, cytokinines or abscisic acid is well understood. Their role in plant development, also in response to abiotic stress is complex. To spatially restrict hormone action, transcription factors of related signaling pathways regulate genes involved in hormone synthesis, signaling and hormone-induced transcriptional regulation. Auxin (indole-3-acetic acid IAA) is one of the most prominent and best analyzed phytohormones. IAA acidifies the cell wall by activating proton pumps. This acidification breaks hydrogen bonds and allows the integration of new cell wall components. Furthermore, IAA also induces synthesis of cell wall material by influencing gene expression.

In conclusion, hormones play important roles in inherent developmental processes as well as in adaptive growth responses. Members of the Homeodomain-Leucine-Zipper transcription factor superfamily (HD-ZIP) are well known for their function in both growth processes. Here we present that the cooperation of hormones and HD-ZIPs mediate between the inner program and environmental stress.

3.2. HD-ZIP proteins: domains and function

The Arabidopsis genome encodes for 48 *Homeodomain-Leucine*-transcription factors (*HD-ZIP*s). Based on their protein structure and function, they can be subdivided into four distinct protein families (HD-ZIPI to IV). All HD-ZIPs have an amino-terminal homeodomain (HD) (Scott et al., 1989) for DNA-binding followed by a leucine zipper (ZIP)-domain for protein-protein interactions. This general organization is conserved from basal plant species like moss and gymnosperms, to monocots and eudicots (Floyd and Bowman, 2006; Floyd et al., 2006; Hu et al., 2012). Variations within the homeodomain (Schena and Davis, 1994) results in similar but gene family-specific DNA-binding sites.

Class I: C**AAT**(A/T)**ATT**G (Palena et al., 1999)

Class II: C**AAT**(C/G)**ATT**G (Sessa et al., 1993)

Class III: GT**AAT**(G/C)**ATT**AC (Sessa et al., 1998)

Class IV: TAAA core (Nakamura et al., 2006)

In addition, class I HD-ZIP proteins contain an AHA (**A**romatic, large Hydrophobic, **A**cidic context) domain in their CTR (C-terminal region) (Arce et al., 2011), which enables these proteins to function as transcriptional activators. In contrast, class II HD-ZIP transcription factors possess an EAR domain (**e**thylen-responsive element binding factor-associated **a**mphiphilic **r**epression domain) for transcriptional repression (Kagale et al., 2010). In addition, HD-ZIPII proteins harbor two additional protein domains, a CPSCE (Cys, Pro, Ser, Cys, Glu) domain to sense changes of the cell redox status (Hu et al., 2012; Tron et al., 2002) and a ZIBEL domain, which might have a function in protein-protein interaction (Mukherjee et al., 2009)

Both families, HD-ZIPIII and IV, carry not just a HD and ZIP domain, but also an additional hormone-inducible lipid-binding START domain (**St**eroidogenic **a**cute **r**egulatory protein-related lipid-**t**ransfer domain) (Ponting and Aravind, 1999) and a SAD (**S**TART **a**djacent **d**omain) domain for transcriptional activation (Mukherjee

and Burglin, 2006). Furthermore, HD-ZIPIII proteins have a MEKHLA-type PAS (Per ARNT Sim) domain C-terminal of the HD-ZIP (Mukherjee and Burglin, 2006), which could be involved perceiving changes in light or redox potentials. This domain also prevents dimerization of REVOLUTA (REV) monomers by protein conformational changes and thus negatively regulates HD-ZIPIII protein activity (Magnani and Barton, 2011). Mutational analysis of the homeodomain cysteins at position 23, 38 and 42 also reveal a function for HD-ZIPIII in sensing redox status and DNA binding efficiency and specificity (Comelli and Gonzalez, 2007). Like HD-ZIPII transcription factors, also class IV HD-ZIP proteins possess a CPSCE domain (Mukherjee et al., 2009). In both protein families, the CPSCE domain is located C-terminal of the ZIP domain.

In addition to the distinct protein structures of the four HD-ZIP gene subgroups, they are also characterized by different function. Class I HD-ZIPs respond to water deficiency and salt stress by promoting ABA-responsiveness (Himmelbach et al., 2002; Johannesson et al., 2003). In contrast, HD-ZIPII transcription factors regulate the shade avoidance syndrome (SAS) in crosstalk with auxin (Ciarbelli et al., 2008; Sorin et al., 2009); whereas class III HD-ZIP proteins mainly act as patterning factors during developmental growth in leaves, roots and shoots (Emery et al., 2003; Hawker and Bowman, 2004; McConnell et al., 2001). Surprisingly, for HD-ZIPIV transcription factors, which are known to be involved in trichome development and cell differentiation in the leaf epidermis (Nakamura et al., 2006), no function in hormone regulated pathways has been identified.

3.3. HD-ZIP transcription factors translate environmental parameters in hormonal regulated growth

Plant hormones have a complex role during developmental processes under various environmental conditions. To manage effectiveness and to restrict the global function HD-ZIP transcription factors have crucial roles in hormone regulation.

3.3.1. Class I HD-ZIPs

Water and the availability of nutrients such as nitrogen, sodium or potassium but also their ionic form or concentration in solution is crucial for living plants. Water serves as solvent and allows the transport of these substances within the plant. Water deficiency evokes an emergency response in plants, resulting in rapid responses like closing of stomata to avoid water loss. Long-term responses include precautious flowering and premature senescence. A high salt concentration in the nutrient medium depletes the uptake of water caused by disruption of the osmotic gradient. During both water deficiency and salt stress, synthesis, distribution and activity of abscisic acid and also ethylene is of high importance and regulates both short-term and long-term responses. Class I HD-ZIP transcription factors play crucial roles in regulation of both hormones.

A wide gene expression study, comprising all class I HD-ZIPs in salt stress and in response to ABA application, reveals regulation of all genes by at last one or both treatments (Henriksson et al., 2005) resulting in either increasing gene expression or a decrease. Further, a few genes show an additional weak response to low temperature in their gene expression. In maize all HD-ZIP families have nearly the same number of genes belonging to one of the HD-ZIP families based on protein structures (Zhao et al., 2011). Like most HD-ZIPI in Arabidopsis many members of class I HD-ZIPs in maize respond to drought stress. This reflects a conservation of both class I HD-ZIP protein structure and function.

Expression of *AtHB6* and *AtHB7* is inducible by water deficiency or osmotic stress and in response to ABA expression of these genes changes within minutes (Himmelbach et al., 2002; Lechner et al., 2011; Söderman et al., 1996). In ABA insensitive mutant plants *abi1*, the response to ABA is diminished for both genes. This places both genes downstream of *ABI1*. In addition, AtHB6 controls its own gene expression and reduces ABA sensitivity (Himmelbach et al., 2002) reflecting a part of the complex interaction of HD-ZIPI transcription factors and hormones. In contrast, protein activity of HB6 seems to be reduced by ABA (Lechner et al., 2011). AtHB12 protein has a similar amino acid identity compared to *AtHB7* and is also induced by ABA application (Son et al., 2010). During germination, AtHB12 enhances the ABA sensitivity; in later stages it reduces the growth of inflorescence stems by inhibiting the synthesis of gibberellic acid (GA). The lack of GA is caused by a decreased gene expression of *GA20ox1* (*GIBBERELLIN 20-oxidase1*) in inflorescence stems (Son et al., 2010). Expression of *AtHB13* and its homologue *HaHB1* in sunflower is also inducible by water deficiency or high salt concentration (Cabello and Chan, 2012) indicating a functional conservation. In addition, both genes also respond to temperatures below 0°C in a similar way (Cabello et al., 2012). Transcriptom analysis of transgenic *HaHB1* plants uncovers glucanases and chitinases as target genes of HaHB1 during stress responses. The increase in gene expression of target genes as well as the accumulation of anti-freezing proteins prevents growth of ice crystals inside cells and thereby stabilizes cell membranes in these plants (Cabello and Chan, 2012).

In addition, stress induced changes in gene expression and physiological responses also result in adaption of growth behavior. In *Medicago HB1* is expressed in roots and induced by salt stress (Ariel et al., 2010). To minimize the root surface during water deficiency HB1 represses the transcription of *LBD1* (*LATERAL ORGAN BOUNDARY-Like* transcription factor) and thereby inhibits initiation of lateral root formation. Similar phenotypes can be observed in response to elevated ABA levels. Beside the regulation of *LBD1* by HB1, a member of the ABA signaling pathway, *LBD1* expression is also induced by auxin (Ariel et al., 2010). The cross-regulation by both hormones involving HB1 reflects a point of

adjusting between different growth processes, respectively between stress induced and inherent programs.

Class I HD-ZIPs regulate ABA signaling pathways in many ways under drought stress. In addition, to shorten generation time, the sunflower class I HD-ZIP transcription factor HaHB4, induces premature leaf senescence, by up-regulating ethylene-signaling pathway (Manavella et al., 2006). *HaHB1* gene expression is ethylene inducible within minutes; on the other hand HaHB1 not just represses synthesis of ethylene but also its signaling. In order to regulate senescence, *HaHB1* is expressed in mature and senescent leaves. Beside the regulation of leaf senescence, HD-ZIPIs are also associated with leaf patterning. *AtHB23*, a gibberellic acid inducible gene, is expressed in the adaxial domain of leaf primordia and young leaves. Its mRNA levels are reduced in plants overexpressing the adaxializing factor *PHB* (*PHABULOSA*) or lacking the abaxializing factors *KANADI1* and *KANADI2* (Kim et al., 2007). This places At*HB23* downstream of *class III HD-ZIP* and *KANADI* transcription factors. However, the expression pattern in the adaxial domain and the cross-regulation by KAN1, KAN2 and PHB do not enforce the proposed role in leaf patterning processes. Surprisingly, class I HD-ZIPs do not only respond to water deficiency, osmotic stress or low temperature. AtHB16 has been shown to be a negative regulator of cell expansion and is involved in the response to blue-light (Wang et al., 2003). In addition, AtHB16 acts as negative regulator of flowering time. Genetic analysis places *AtHB16* downstream of *CRY1* and *CRY2* (*CRYPTOCHROM1* and *2*) in blue-light dependent inhibition of hypocotyl elongation.

In conclusion, class I HD-ZIP transcription factors play crucial roles in synthesis and signaling of abscisic acid and ethylene during responses to drought or osmotic stress. Furthermore, they are also target genes of both hormones, reflecting the complex network of *HD-ZIPI* genes and ABA or ethylene. In addition, a function in response to blue-light or low temperature and synthesis and signaling of auxin and gibberellic acid was revealed.

3.3.2. Class II HD-ZIPs

Light is one of the most important abiotic factors. It not only provides energy for life, it also influences the temperature of the environment. Plants grown in the absence of light for several days will die due to the loss of energy required to maintain cellular functions. Not only light quantity, but also light quality is crucial for plant development. Plants are enabled to sense blue light as well as red and far-red light, while only blue light and red light are used as source of energy. Green light and far-red light are being reflected. Plants growing under a canopy or in the proximity of other plants are exposed to shade, defined by a high ratio of red to far-red light. To sense the red/far-red ratio, plants used the phytochrome system resulting in changes of protein conformation to activate/ suppress downstream targets. Phytochrome-interacting factors (PIFs) are important transcription factors, which play crucial roles in these light-signaling pathways.

Plants exposed to shade respond with a number of growth responses summarized as shade avoidance syndrome (SAS). SAS results in longer hypocotyls and petioles, less branched stems, smaller and dark green leaves with the aim to grow out of unprofitable light conditions. Class II HD-ZIP transcription factors are widely known to play a crucial role in shade avoidance responses.

The changes in light quality and quantity that occur naturally during the day, entrain the circadian system of plants. Hypocotyl elongation during long-term shade exposure occurs primarily in the dawn. *AtHB2/HAT4* is expressed in the hypocotyl of seedlings (Schena et al., 1993). Phytochrome-interaction factors PIF4 and PIF5 regulate *AtHB2/HAT4* expression positively during dawn resulting in hypocotyl elongation in etiolated seedlings exposed to shade (Kunihiro et al., 2011). Conversely, plants carrying a mutation in *PIF4* and *PIF5* exhibit low mRNA levels of *AtHB2* under shade conditions, similar to wild type plants grown in white light conditions. Concerning the reversibility in changes of protein conformation of phytochromes, mRNA levels of *AtHB2* decrease in shade-treated plants exposed to white light. Like *AtHB2/HAT4* also *HAT1*, *HAT3* and *AtHB4* are under direct control of the phytochrome system (Ciarbelli et al., 2008) and gene expression

increases within minutes after shade exposure (Ciarbelli et al., 2008; Sorin et al., 2009). These high mRNA levels of *class II HD-ZIPs* result in typical shade avoidance phenotypes: longer hypocotyls, less branches or small leaves by suppression of cell proliferation (Ciarbelli et al., 2008; Sawa et al., 2002; Schena et al., 1993; Sorin et al., 2009). In contrast, *HAT2* is indirectly induced in through shade induced auxin synthesis (Ciarbelli et al., 2008) and its gene expression increases within minutes to elevated auxin levels (Sawa et al., 2002). In addition, HAT2 regulates its own expression in a direct negative manner by binding to its own promoter, beside the negative transcriptional regulation of other *class II HD-ZIPs* (Ohgishi et al., 2001; Sawa et al., 2002). This might result in the identified reduction of auxin sensitivity in plants overexpressing *HAT2* (Sawa et al., 2002). Like HAT2 also HAT1, HAT4 and HB4 regulate expression of *class II HD-ZIPs* negatively (Sorin et al., 2009).

Auxin is not the only phytohormone involved in regulation of *HD-ZIPII* activity. *AtHB4* also responds to brassinosteroids (Sorin et al., 2009). While plants overexpressing *AtHB4* exhibit a slight hypocotyl elongation and *hat3 athb4* double mutant plants disclaim from responding to shade treatments, both respond with hypocotyl elongation after brassinosteroid application (Sorin et al., 2009), reflecting a role of AtHB4 and other class II HD-ZIP in brassinosteroid signaling processes.

Furthermore, *HAT2*, *HAT3* and *HAT4* are under control of a tandem zinc knuckle/PLU3 (TZP) protein encoding gene (Loudet et al., 2008). In contrast to PIFs, TZP responds to blue-light and its expression is regulated by the circadian clock, but it also controls hypocotyl elongation in dawn in a positive way. This indicates, that TZP creates a second light-dependent pathways beside the phytochrome system to regulate *class II HD-ZIP* transcription factors or that TZP acts downstream or as co-factor of PIF proteins (Loudet et al., 2008).

The strategy of plants exposed to shade, is to grow out of these unprofitable light conditions. A second earlier response is to suppress germination in light with high red/far-red ratio. AtHB2/HAT4 suppress the germination of seeds in shade, thus it is part of the light-induced germination signaling cascade (Schena et al., 1993).

Light-regulated activity of class II HD-ZIPs is not limited to the shade-avoidance syndrome. CLI86 (CARBON AND LIGHT INSENSITIVE86) is part of the PHYTOCHROME A signaling pathway and regulates the assimilation of nitrogen and carbon during the night (Thum et al., 2008). *HAT22* is a target of CLI86 and might therefore be involved in light-dependent uptake of both carbon and nitrogen, thus HAT22 potentially influences the N- and C-metabolism as well as the energy balance.

Class II HD-ZIP transcription factors play crucial roles in all stages of the vegetative phase. They regulate hypocotyl elongation in seedlings but also leaf development and stem branching in young and mature plants. In addition, they also control flower development during the reproductive phase. JAB (JAIBA/HAT1), a paralog of HAT2, regulates meristem activity in different tissues. In *jab* mutant plants male and female reproductive organs show defects in development (Zuniga-Mayo et al., 2012). Especially, the gynoeceum, responsible for seed production, development and dispersion, is affected in this mutant, resulting in smaller and thin siliques and reduced fruit size. In the stamens, the male part of flowers, pollen production is decreased compared to wild type plants. In contrast, in these mutants plants the number of flower buds increases (Zuniga-Mayo et al., 2012).

Altogether, class II HD-ZIPs are important components of light-signaling and light-independent pathways during developmental processes. They act in a complex network in concert with auxin to regulate auxin sensitivity as well as their own gene expression and expression of other HD-ZIPII members.

3.3.3. Class III HD-ZIPs

The *class III HD-ZIP* transcription factor family is the smallest gene family of all *HD-ZIPs* in Arabidopsis and consists of five members: REVOLUTA, PHABULOSA, PHAVOLUTA, ATHB8 and ATHB15/CNA. *HD-ZIPIII* genes are highly conserved during evolution and can be found in mosses, gymnosperms and flowering plants, in monocots and eudicots (Floyd et al., 2006). However, their function changed during evolution in adaption to the different lifestyles of plant clades.

In higher plants HD-ZIPIIIs are involved in meristem initiation (Gordon et al., 2007) as well as patterning and developmental processes in all organs, root, shoot and leaves but also in reproductive organs, in vasculature and other tissues (Itoh et al., 2008; Prigge et al., 2005). In spite of their close relationship within the HD-ZIPIII family, the function of the different members differs and results in redundant but also antagonistic phenotypes in Arabidopsis. While REV (REVOLUTA), PHB (PHABULOSA) and PHV (PHAVOLUTA) have overlapping functions during meristem formation and patterning processes, the function of CNA (CORONA) and ATHB8 results in opposite phenotypes (Prigge et al., 2005). Single mutant plants show either no or only subtle mutant phenotypes, whereas double or triple mutant plants exhibit strong developmental defects such as abaxialized leaves, terminal differentiation of apical stem cells (Emery et al., 2003; Prigge et al., 2005), but also a disruption in patterning of vascular bundles (Emery et al., 2003; Prigge et al., 2005), reflecting the redundancy within this gene family.

Their eminent role in the regulation of developmental processes suggests that HD-ZIPIII proteins are part of growth-promoting hormone-signaling pathways. Especially for REV/IFL1 (REVOLUTA/INTERFASCICULAR FIBERLESS1) a function in auxin flow by regulation of the PIN (PIN-formed) auxin transporter was proposed (Heisler et al., 2005; Zhong and Ye, 2001) based on overlapping expression patterns. Expression studies in *ifl1* mutant plants uncover an altered gene expression for *PIN3* and *PIN4* in seedlings and stems resulting in phenotypes similar to plants carrying defects in auxin polar transport (Zhong and Ye, 2001) with pin-like inflorescences, reduced cauline branches and

inflorescences. In addition, auxin regulates MP (MONOPTEROS), which targets *HD-ZIPIII* (Ohashi-Ito and Fukuda, 2010). This results in positive *PIN* regulation that forms a positive feedback loop with auxin. This self-energizing feedback loop is negatively controlled by the MP target gene *ATHB8* (Donner et al., 2009; Ohashi-Ito and Fukuda, 2010) during determination of the procambial cell fate.

HD-ZIPIII proteins are well-known for their role in dorso-ventral patterning of vasculature and leaves. In these processes, they negatively interact with the antagonistically acting *KANADI* genes. The correct outgrowth of leaf blades requires a defined set up of leaf polarity at the leaf margin. Both HD-ZIPIIIs and KANADI transcription factors regulate *YUCCA* genes, known for their function in auxin synthesis, to ensure correct outgrowth of the leaf margin and leaf polarity (Wang et al., 2011). Further members of both gene families control direction of auxin flow by regulation of the PIN-auxin-transporter during embryogenesis (Ilegems et al., 2010; Izhaki and Bowman, 2007).

In spite of the broad function in development and patterning in concert with auxin and other phytohormones and the intensive analysis of class III HD-ZIP transcription factors, no role in response to abiotic environmental stimuli was revealed until now.

3.3.4. Class IV HD-ZIPs

The HD-*ZIPIV* gene family is also known as “GLABRA” gene family, named after the founding member in trichome development. The *Arabidopsis* genome encodes for sixteen class IV HD-ZIP proteins. In contrast to other HD-ZIP transcription factors, proteins encoded by members of this gene family are not known for a crosstalk with hormones in general. The huge number of members of this family is an indicator for high redundancy and makes analysis of single gene function difficult.

A global analysis of all *HD-ZIPIV genes* in Arabidopsis reflects the redundancy within this gene family. The analysis of single loss-of-function mutant plants by studying T-DNA-insertion lines revealed a wild type plant growth behavior, except for *hdg11* (*HOMEODOMAIN GLABROUS11*) mutants that exhibited an excess branching of trichomes (Nakamura et al., 2006). Tissue-specific gene expression and promoter-GUS studies of *HD-ZIPIV* genes reveal an expression pattern and function of family members in developing shoot and reproductive organs, what ascribes the class IV HD-ZIPs a broad function in developmental processes (Nakamura et al., 2006). In contrast, loss-of function mutant plants of *GLABRA1*, *2* or *3* (*GL1*, *2* or *3*) are characterized by glabrous leaves or having trichomes with less branches caused by regulation of cell differentiation processes in the epidermis (Marks et al., 2009; Qing and Aoyama, 2012). In addition to its function in leaf epidermis patterning, GL2 plays a crucial role in root hair development by cell fate determination of H-cells (hair cells; trichoblast) and N-cells (non-hair cells; atrichoblast). Brassinosteroid is a positive regulator of *GL2* gene expression; *bri1* mutants (*BRASSINAZOLE INSENSITIVE1*) are insensitive for brassinosteroid and are characterized by lower *GL2* mRNA levels compared to wild-type mRNA level resulting in less branched trichomes (Kuppusamy et al., 2009). In the presence of high CO_2-levels or elevated auxin levels, gene expression of both *GL2* and *GL3* are decreased and root hair initiation is inhibited (Niu et al., 2011). The role of *HD-ZIPIV* genes in epidermal cell fate regulation is not restricted to the model plant *Arabidopsis*. Homologs in other species have similar functions. OCL4 (OUTER

CELL LAYER4), a class IV HD-ZIP transcription factor in maize, regulates trichome patterning and anthere development (Vernoud et al., 2009). *GhHD1* in cotton (*Gossypium hirsutum*), a homologue to *Arabidopsis AtGL2*, (Walford et al., 2012) is involved in fiber generation on the seed coat. Here, overexpression of *GhHD1* leads to an increase in fiber initiation, while silencing of *GhHD1* results in delayed fiber initiation and reduces the total number of fibers - conceding this pathway an agronomic impact. In *Gossypium arboretum* (cotton) a homologue to *Arabidopsis AtGL1* was identified (Desai et al., 2008). Concerning that fibres are trichomes on the coat of cotton seeds and AtGL1 regulates trichomes development in Arabidopsis, for the homolog in cotton a function in fiber initiation is proposed. In addition, AtGL2 is also involved in seed coat mucilage production and seed oil accumulation (Qing and Aoyama, 2012; Shen et al., 2006). In oilseed rape (*Brassica*) four orthologous genes were identified which might have an agronomic relevance (Chai et al., 2010).

In Arabidopsis, *PDF2* (*PROTODERMAL FACTOR2*) and its closest homolog *ML1* (*MERISTEM LAYER1*) are also involved in epidermal cell fate determination. Both are expressed in the epidermal cell layer L1 in embryos and in the shoot (Abe et al., 2003; Sessions et al., 1999; Takada and Jurgens, 2007). In mature plants *ML1* is restricted to apical cells, but it is already expressed in the one-cell stage and later its expression pattern also comprises the basal cell lineage of embryos (Takada and Jurgens, 2007).

AtFWA (*FLOWERING WAGENINGEN*) occupies a special role in plant development. In contrast to other members of the HD-ZIPIV gene family, FWA is not involved in epidermis cell fate regulation, but rather in flowering time regulation (Kawanabe and Fujimoto, 2011). FWA is a floral repressor and thus overexpression results in late flowering phenotypes, even under inductive long day conditions. Surprisingly, mutations in *FWA* lead to a similar phenotype and the effect is linked to hypomethylation of the *FWA* locus in this mutant background. It is proposed that dimerization with FT (FLOWERING TIME) regulates flowering induction negatively (Kawanabe and Fujimoto, 2011). Flowering time is regulated by both photoperiod and temperature. FWA is the only class IV HD-ZIP gene,

which has an obvious role in response to environmental stimuli but the regulation of *FWA* in this signaling pathway is still unknown.

In conclusion, the function of class IV HD-ZIPs is complex. In contrast to other HD-ZIP transcription factors, they play a minor role in signaling pathways responding to environmental stimuli or in hormonal crosstalk in spite of a hormone-inducible lipid-binding START domain (Ponting and Aravind, 1999).

3.4. Conclusion

Homeodomain-leucine transcription factors have diverse functions in plant development. In concert with phytohormones they regulate a plethora of developmental decisions in all plant tissues. While class I and class II HD-ZIPs mediate growth responses stimulated by environmental stress, HD-ZIPIII and HD-ZIPIV proteins mainly regulate patterning processes controlled by inherent programs.

Class I HD-ZIPs respond to water deficiency and salt stress and induce abscisic acid synthesis and responsiveness. In contrast, HD-ZIPII transcription factors are involved in the shade avoidance syndrome and regulate shade induced growth responses in a complex regulatory network of interdependent gene expression regulation within the class II HD-ZIP transcription factor family in concert with auxin. HD-ZIPIII and HD-ZIPIV are controlled by inherent programs. While HD-ZIPIIIs regulate patterning processes especially in the vasculature and in leaves, HD-ZIPIV transcription factors control epidermal cell fate determination in embryos and trichome development.

In comprehensive experiments we revealed new functions for class II and class III HD-ZIP transcription factors. We could show that HD-ZIPII transcription factors are regulated by HD-ZIPIIIs and thereby HD-ZIPIIIs intersect with shade-induced growth processes. Furthermore, HD-ZIPIIIs stimulate auxin synthesis which might additionally affect the auxin-inducible class II HD-ZIPs. Vice versa, we established

a function for HD-ZIPII transcription factors in leaf patterning processes. In conclusion, a network of HD-ZIPII and HD-ZIPIII transcription factors and auxin controls inherent programs of leaf patterning as well as adaptive growth responses to shade exposure.

4. Publications

4.1. **Genome-wide binding-site analysis of REVOLUTA reveals a link between leaf patterning and light-mediated growth responses**; Brandt R; Salla-Martret M; Bou-Torrent J; Musielak T; Stahl M; Lanz C; Ott F; Schmid M; Greb T; Schwarz M; Choi SB; Barton MK; Reinhart BJ; Liu T; Quint M; Palauqui JC; Martinez-Garcia JF and Wenkel S; The Plant Journal 2012

Eigenanteil: Versuchsdurchführung für Abbildungen 2g, i, 3, 4, 5, 6, 7; Analyse des freien Auxins in Zusammenarbeit mit Karl Wurster (Analytikgruppe Mark Stahl); Auswertung ChIP-Seq-Datensatzes und Manuskript erstellen in Zusammenarbeit mit Dr. Wenkel

4.2. **ATHB4 and HAT3, two class II HD-ZIP transcription factors, control leaf development in Arabidopsis**; Bou-Torrent J; Salla-Martret M; Brandt R; Musielak T; Palauqui JC; Martinez-Garcia JF and Wenkel S; Plant Signaling and Behaviour; 2012

Eigenanteil: Erstellung und Charakterisierung von p35S::FLAG::GR-HAT3 Linie als Basis für die Veröffentlichung; Charakterisierung von hat3 athb4 Pflanzen; Erstellung der Publikation fand in Kooperation der beteiligten Forschungsgruppen statt; auf Grund gleicher Anteile wurde die Erstautorenschaft geteilt

4.3. **Control of stem cell homeostasis via interlocking microRNA and microProtein feedback loops**; Brandt R; Xie Y; Musielak T; Graeff M; Stierhof YD; Huang H; Liu CM; Wenkel S; Mechanisms of Development; 2012

Eigenanteil: Datenerhebung für Abbildung 1, 2 und 4 (Fotos, Chromatin-Immunopräzipitation und Genexpression); Erstellen des Modells und Schreiben der Veröffentlichung in Zusammenarbeit mit Dr. Wenkel

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Genome-wide binding-site analysis of REVOLUTA reveals a link between leaf patterning and light-mediated growth responses

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SUMMARY

Unlike the situation in animals, the final morphology of the plant body is highly modulated by the environment. During Arabidopsis development, intrinsic factors provide the framework for basic patterning processes. CLASS III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIPIII) transcription factors are involved in embryo, shoot and root patterning. During vegetative growth HD-ZIPIII proteins control several polarity set-up processes such as in leaves and the vascular system. We have identified several direct target genes of the HD-ZIPIII transcription factor REVOLUTA (REV) using a chromatin immunoprecipitation/DNA sequencing (ChIP-Seq) approach. This analysis revealed that REV acts upstream of auxin biosynthesis and affects directly the expression of several class II HD-ZIP transcription factors that have been shown to act in the shade-avoidance response pathway. We show that, as well as involvement in basic patterning, HD-ZIPIII transcription factors have a critical role in the control of the elongation growth that is induced when plants experience shade. Leaf polarity is established by the opposed actions of HD-ZIPIII and KANADI transcription factors. Finally, our study reveals that the module that consists of HD-ZIPIII/KANADI transcription factors controls shade growth antagonistically and that this antagonism is manifested in the opposed regulation of shared target genes.

Keywords: leaf development, auxin, HD-ZIPIII, shade avoidance, HD-ZIPII, *Arabidopsis thaliana*.

INTRODUCTION

Plants are sessile organisms and, therefore, have to cope with changing environmental conditions. In nature, plants usually live in communities with other plants and are thus under a constant struggle for optimal capture of sunlight. More than two centuries ago, Johann Wolfgang von Goethe recognized in his studies on plant morphology and adaptation ('Schriften zur Morphologie', 1790) an 'inner nature' that provides the information for the body plan and the 'outside world' that shapes the final morphology of the body plan. Both the inner nature and the outside world interact, which led Goethe to propose the theory of the 'double law'.

Angiosperm plants have evolved refined mechanisms to alter their growth behavior in order to avoid life in a suboptimal light environment. An early warning of the proximity of other plants ('proximity neighbor detection') is the decrease in the ratio of red (R) to far-red (FR) light (R:FR

ratio); this decrease is caused because plant leaves selectively reflect FR light toward neighboring plants, almost without affecting the rest of the daylight spectrum (Ballaré *et al.*, 1990). By contrast, under the canopy of other plants ('canopy shade detection'), red light is absorbed by the shade-causing plants, lets the photosynthetic inactive far-red light pass, and causes a reduction in both the R:FR ratio and the amount of photosynthetic active radiation (Franklin, 2008; Martinez-Garcia *et al.*, 2010). In either case, the decrease in the R:FR ratio is detected by the plant phytochrome system (Chen *et al.*, 2004; Franklin *et al.*, 2003). In Arabidopsis, a low R:FR ratio stimulates elongation of the hypocotyl in seedlings and the petiole (leaf stalk) in older plants. Prolonged exposure to shade alters the leaf developmental program and reduces the outgrowth of side shoots (Morelli and Ruberti, 2002). Furthermore, rapid flowering and thus rapid seed set is induced in order to shorten generation time (Franklin and Whitelam, 2005).

Downstream of the phytochrome system, several genes are induced transcriptionally by shade. These genes execute a physiological response termed the 'shade avoidance syndrome' (SAS). Genes that belong to the class II homeodomain leucine zipper transcription factor (HD-ZIPII) family are among these rapidly induced genes (Ariel *et al.*, 2007; Ciarbelli *et al.*, 2008). Ectopic overexpression of those HD-ZIPII genes that use the viral *35S* promoter causes hypocotyl elongation in non-shade conditions (Ciarbelli *et al.*, 2008; Schena *et al.*, 1993; Sorin *et al.*, 2009; Steindler *et al.*, 1999), an action that supports a role as positive regulators of hypocotyl growth. However, in shade conditions, they seem to repress hypocotyl elongation (Sorin *et al.*, 2009). Loss of HD-ZIPII function can result in short hypocotyls in shade or non-shade conditions (Sorin *et al.*, 2009).

An intricate, and not well understood, hormonal signaling network underlies the regulation of SAS. The TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) is a key enzyme in tryptophan-dependent production of auxin via the intermediate indole-3-pyruvic acid (Stepanova *et al.*, 2008; Tao *et al.*, 2008). Plants that carry loss-of-function mutations in *TAA1* are defective in the shade-induced hypocotyl elongation (Tao *et al.*, 2008).

Class III homeodomain leucine zipper (HD-ZIPIII) transcription factors are key determinants in embryo, shoot and root patterning (Carlsbecker *et al.*, 2010; McConnell *et al.*, 2001; Smith and Long, 2010) and during vegetative growth regulate several polarity set-up processes such as in leaves and the vascular system (Bowman and Floyd, 2008; Juarez *et al.*, 2004). In Arabidopsis, expression of *HD-ZIPIII* genes is regulated strongly by microRNAs and an additional control at the post-translational level occurs via the formation of non-functional heterodimers with LITTLE ZIPPER (ZPR) proteins (Kim *et al.*, 2008; Wenkel *et al.*, 2007).

Here, we show that, as well as involvement in the regulation of basic patterning processes, HD-ZIPIII transcription factors also have an important function in the regulation of adaptive development. By direct control of the expression of the genes that encode the auxin biosynthetic enzymes *TAA1* and *YUCCA5* (*YUC5*), REV can influence directly the levels of free auxin, a prerequisite for shade-induced growth. In addition, four known regulators of shade signaling, the HD-ZIPII transcription factors *HAT2*, *HAT3*, *ATHB2*/*HAT4* and *ATHB4*, are regulated transcriptionally by REV. We, furthermore, show that the identified targets have overlapping patterns of expression with REV and their expression is lower in plants with reduced HD-ZIPIII activity, which implies that they are true direct positive targets. Genetic analysis supports a role for *HD-ZIPIIIs* in the regulation of the shade-avoidance response as *rev* mutant plants are affected in the promotion of elongation growth in response to shade. Finally, we provide evidence that the leaf regulatory module, which consists of HD-ZIPIII and KANADI transcription factors, is involved in the regulation of adaptive growth by oppositely regulating the expression of key shared target genes.

RESULTS AND DISCUSSION

Genome-wide identification of REVOLUTA target genes

To identify direct targets of REVOLUTA, an HD-ZIPIII protein, we used transgenic plants that expressed a FLAG-tagged ligand-binding domain of the glucocorticoid receptor, fused to a microRNA-resistant version of *REV* under control of the *35S*-promoter (*35S::FLAG-GR-REVd*). We then used chromatin immunoprecipitation/DNA sequencing (ChIP-Seq) to monitor binding sites of the fusion protein in the *A. thaliana* genome. Comparative analysis of two biologically independent ChIP-Seq experiments revealed regions that showed enrichment in both datasets (Figure 1a,b and Data S1). Among the identified putative targets is also the *LITTLE ZIPPER* (*ZPR*) gene *ZPR1*, which is known to be regulated by REV (Wenkel *et al.*, 2007), which supported the validity of our screen. Using EasyGO (Zhou and Su, 2007), we tested in an unbiased fashion for enrichments of gene ontologies (GO) for the gene loci that surround the genomic regions bound by REV. Strong enrichment was observed for genes involved in biological regulation and regulation of development (Figure 1c). Surprisingly, the second strongest GO enrichment was seen for genes that were involved in the response to abiotic stimuli. This group contained factors known to be involved in R/FR signaling. A further analysis of lower order GOs is shown in the Supplementary Table S1 and further confirms that genes involved in R/FR signaling are enriched in our ChIP-Seq dataset (Table S1). Several of these regulators of light responses belong to the class II homeodomain leucine zipper (HD-ZIPII) transcription factor family, where we find binding sites in the promoter regions in seven out of nine genes.

To identify a *cis*-regulatory motif required for regulation by REV, we used MEME (meme.sdsc.edu) and compared the

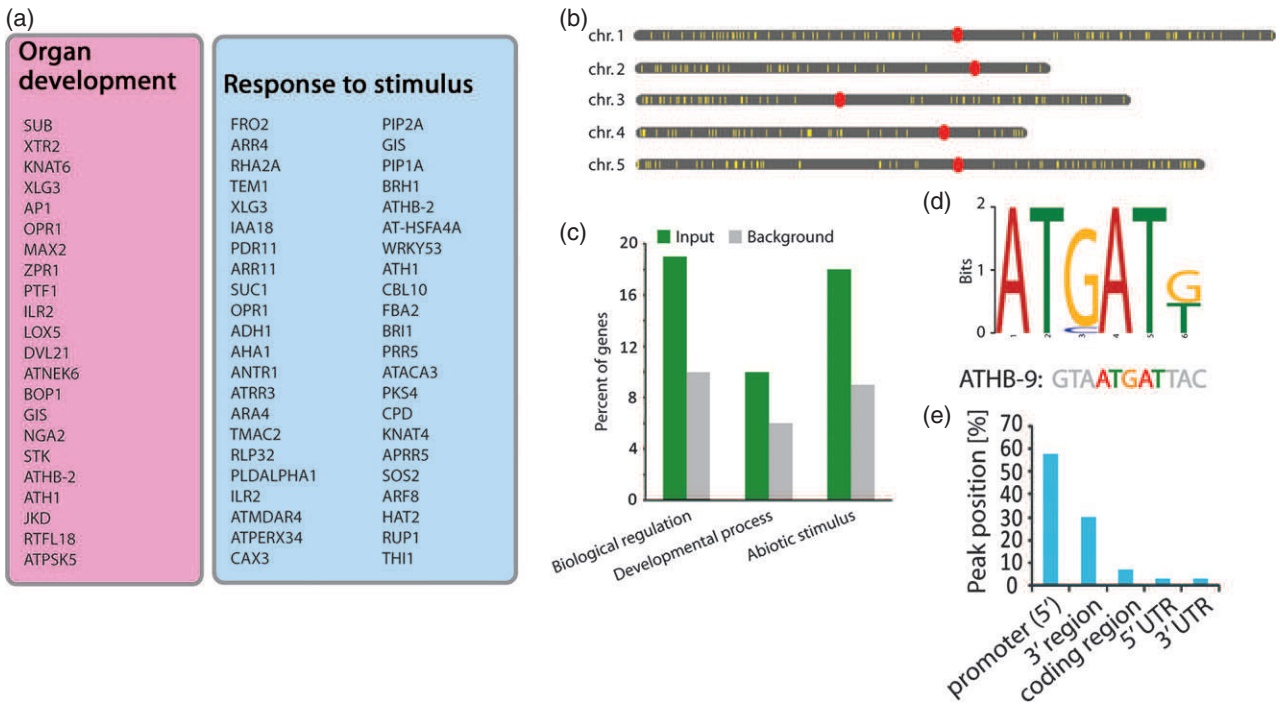


Figure 1. Regulation of REVOLUTA target genes identified by ChIP-Seq.
(a) Shown are putative target genes involved in organ development (pink) and factors involved in the regulation of adaptive responses (blue).
(b) Distribution of the 286 high confidence REV-binding regions along the five Arabidopsis chromosomes.
(c) Enrichment of selected gene ontology (GO) categories of loci that surround the REV-binding sites. Plotted is the percentage of genes (*Y*-axis) identified in the chromatin immunoprecipitation/DNA sequencing (ChIP-Seq) experiments (green bars) in comparison with the abundance in the whole genome (gray bars) for three significantly enriched GO categories.
(d) DNA sequence identified in the top 50 putative REV-target genes. This sequence represents the core of the *in vitro* determined pseudo-palindromic HD-ZIPIII-binding site (shown below).
(e) Diagram that shows the location of binding peaks identified by ChIP-Seq.

top 50 immunoprecipitated regions from both ChIP-Seq experiments. This analysis yielded the sequence motif AT[G/C]AT (Figure 1d). The AT[G/C]AT sequence represents the inner core of the inverted palindromic sequence GTAAT[G/C]ATTAC, which was identified as *in vitro* binding sequence for HD-ZIPIII proteins (Sessa *et al.*, 1998). Of the 286 high confidence peaks, identified in both ChIP-Seq experiments, we find about 60% to be located in the 5′ promoter region of putative target genes and about 30% in the 3′ region (Figure 1e). Binding in the coding sequence or in the untranslated regions (UTRs) was seldom detected (Figure 1e). Taken together, our data suggest that REV is a DNA-binding protein that regulates the expression of genes involved in basic patterning but also controls genes involved in adaptive developmental processes.

REVOLUTA and its target genes have overlapping patterns of expression

A prerequisite for true positively regulated target genes is that regulator and target are expressed in the same tissue. For this reason, we performed *in situ* hybridizations on transverse shoot apical meristem sections. As expected, a specific signal for *REV* was detected in the vasculature and in the adaxial domain of developing leaves (Figure 2a,b). Very similar patterns of expression were found for *HAT2*, *HAT3*, *ATHB4* and *TAA1* (Figure 2c–f). The strong overlap of the spatial expression pattern implies that these genes are true REV targets. The comparison of a REV-β-glucuronidase (GUS) reporter (*rev-9*) with a *TAA1* promoter–GUS reporter line (Yamada *et al.*, 2009) revealed that both *REV* and *TAA1* are expressed in both the vasculature and the shoot apical meristem region of young seedlings (Figure 2g–j). These findings support a role for REV as a direct upstream regulator of both *HD-ZIPII* and *TAA1* expression.

Systematic analysis of REVOLUTA target genes that encode class II HD-ZIP transcription factors

The class II HD-ZIP transcription factor family (*HD-ZIPII*) comprises nine genes in Arabidopsis (Figure 3a). To determine whether REV is involved in the transcriptional regulation of the seven class II HD-ZIP transcription factors identified by ChIP-Seq, we analyzed expression changes executed by induction of GR-REVd. Therefore, we grew wild-type and *35S::GR-REVd* plants (Wenkel *et al.*, 2007) in liquid culture and induced them for 30 min with dexamethasone (DEX). We then tested if the expression of REV-target genes

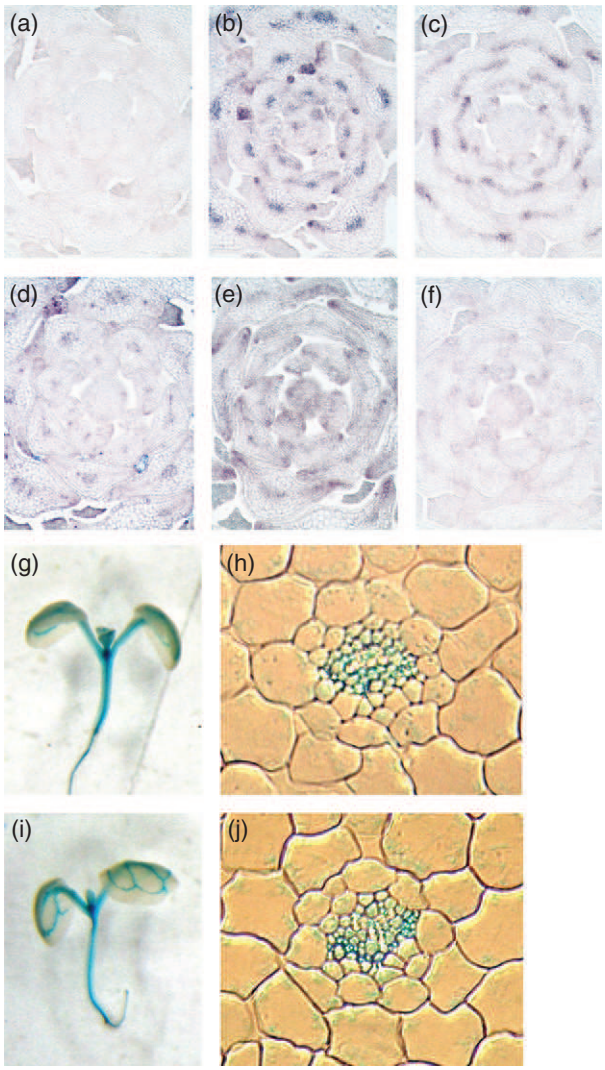


Figure 2. Spatial expression pattern of *REVOLUTA* and its targets.
In situ hybridizations that show patterns of expression of (a) *REV* sense, (b) *REV* antisense, (c) *TAA1* antisense, (d) *HAT2* antisense, (e) *HAT3* antisense and (f) *ATHB4* antisense in the vasculature and the adaxial domain of developing leaves. Expression pattern analysis using GUS reporter lines in 7-day-old Arabidopsis seedlings (g) *REV* and (i) *TAA1*, *pTAA1::GUS* (= *pTIR2::GUS*) plants (Yamada *et al.*, 2009). Hypocotyl cross-sections reveal that both *REV* (h) and *TAA1* (j) are primarily expressed in the vascular cylinder.

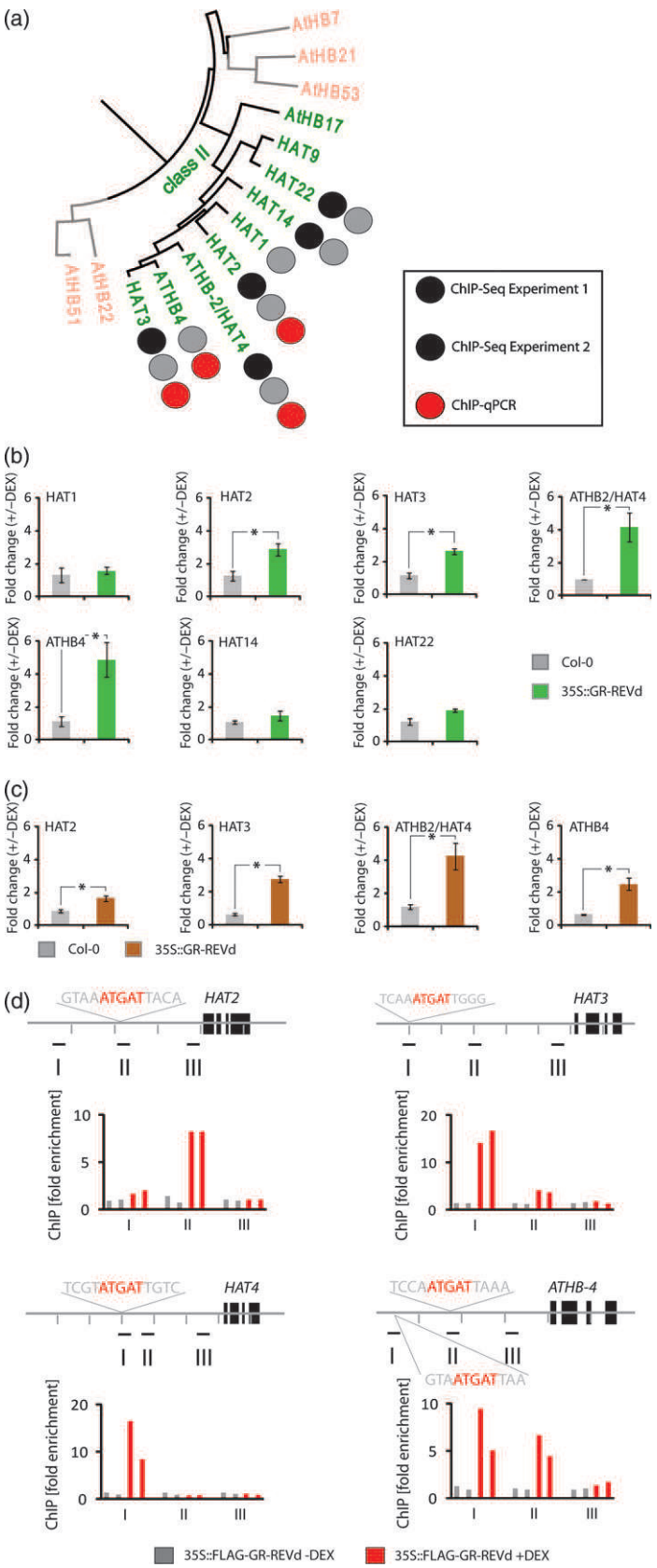
is altered in response to GR-REVd induction. Our analysis revealed a strong transcriptional upregulation of *HAT2*, *HAT3*, *ATHB2*/*HAT4* and *ATHB4*, in response to DEX induction in *35S::GR-REVd* transgenic plants (Figure 3b). Even though identified as putative targets, the expression of *HAT1*, *HAT14* and *HAT22* did not change significantly in response to DEX application, at least not in the conditions tested. To verify that the mode of regulation of these *HD-ZIPII* genes is of a direct nature, we performed the experiment in the presence of the protein biosynthesis inhibitor cycloheximide (CHX). Also in the presence of CHX, *HAT2*, *HAT3*, *ATHB2*/*HAT4* and *ATHB4* showed significant expression changes in response to DEX application, which implies that they are direct REV-target genes (Figure 3c).

To confirm our ChIP-Seq data, we performed independent ChIP experiments followed by quantitative polymerase chain reaction (qPCR). Three different positions around the investigated transcription units were examined. One primer pair was designed to amplify in the vicinity of the identified ChIP-Seq peak and two other primer pairs amplify regions for which no enrichment was seen in either ChIP-Seq experiment. For all four *HD-ZIPII* genes, we observed an enrichment of chromatin fragments precipitated from DEX-induced *35S::FLAG-GR-REVd* plants compared with non-induced transgenic plants (Figure 3d). Therefore, we can conclude that the *HD-ZIPII* genes *HAT2*, *HAT3*, *ATHB2*/*HAT4* and *ATHB4* represent direct targets that are positively regulated by REV.

REVOLUTA directly regulates genes that encode auxin biosynthetic enzymes

Both ChIP-Seq experiments also identified *TAA1* as a putative direct target gene. While the first ChIP-Seq experiment revealed a binding site in the 3′ region of *TAA1*, the second ChIP-Seq study revealed binding in the 5′ region. To find out if REV can transcriptionally regulate other genes that encode auxin biosynthetic enzymes, we analyzed systematically expression changes of all *YUCCA*-type and *TAA1*-related genes in wild-type Col-0 and transgenic *35S::GR-REVd* plants elicited by DEX induction (Figure S1). This study revealed that *YUCCA5* expression is also regulated by REV

Figure 3. Identification of class II HD-ZIP transcription factors regulated by REVOLUTA.
(a) Phylogenetic tree of Arabidopsis class II HD-ZIP proteins. Black circles indicate an enrichment detected in the first ChIP-Seq experiment, gray circles indicate that binding was identified in the second chromatin immunoprecipitation/DNA sequencing (ChIP-Seq) experiment. Red circles indicate that the binding of REV to the binding site located in the respective promoters was verified by ChIP-qPCR (quantitative polymerase chain reaction).
(b) Real-time quantitative reverse transcription (RT)-PCR experiments that show expression changes of *HAT1, HAT2, HAT3, ATHB2/HAT4, ATHB4, HAT14* and *HAT22* in Col-0 (gray) and *35S::GR-REVd* (green) in response to 30 min. Dexamethasone (DEX) induction. Average expression levels of three biological replicates are plotted, normalized to actin of the ratio +DEX versus –DEX treatments with standard error. $*p < 0.05$.
(c) Real-time quantitative RT-PCR experiments that show expression changes of *HAT2, HAT3, ATHB2/HAT4* and *ATHB4* in Col-0 (gray) and *35S::GR-REVd* (brown) in response to 60 min. DEX induction in the presence of the protein biosynthesis inhibitor cycloheximide (CHX). $*p < 0.05$
(d) ChIP experiments with two biological replicates for *35S::FLAG-GR-REVd* without DEX (gray bars) and *35S::FLAG-GR-REVd* with DEX (red bars) plants to test the class II HD-ZIP loci *HAT2, HAT3, ATHB2/HAT4* and *ATHB4*. Genomic regions were tested with three primer pairs (I–III) for each locus by qPCR. *Y*-axis shows the fold enrichment normalized to the non-induced Immunoprecipitation (IPs). Gene maps above the charts show the localization of the REV-binding site identified by ChIP-Seq and the regions that were tested. Distance between two marks along the chromosomes represents 1.0kb.



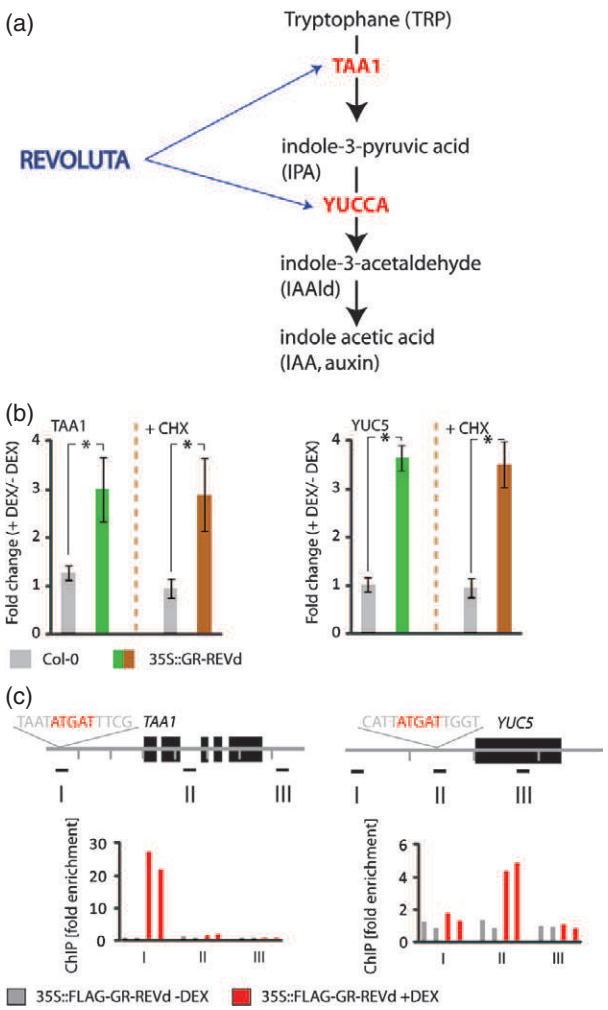


Figure 4. Identification of genes that encode auxin biosynthetic enzymes regulated by REV.
(a) Schematic diagram that depicts the proposed tryptophan-dependent auxin biosynthetic pathways (according to Stepanova *et al.* (2011) and Won *et al.* (2011)) and the role of REV in the regulation of *TAA1* and *YUC5*.
(b) Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) experiments that show expression changes of *TAA1* and *YUC5* in response to DEX induction. Fold changes in response to dexamethasone (DEX) in Col-0 (gray) and the inducible *35S::GR-REVd* transgenic line (green and brown) are plotted. Bars on the left show expression changes in the absence of the protein biosynthesis inhibitor cycloheximide, whereas bars on the right show expression changes in plants pre-treated with cycloheximide (+CHX).
(c) Chromatin-immunoprecipitation experiments with two biological replicates for *35S::FLAG-GR-REVd* without DEX (gray bars) and *35S::FLAG-GR-REVd* with DEX (red bars) plants to test the *TAA1* and *YUC5* loci. Genomic regions were tested with three primer pairs (I–III) for each locus by quantitative (q)PCR.

(Figure S1). These findings suggest that REV is able to induce auxin biosynthesis via the tryptophan-dependent Indole-3-Pyruvic acid (IPA) biosynthetic pathway (Figure 4a). To confirm that REV can upregulate both *TAA1* and *YUC5* expression, we performed quantitative RT-PCR reaction on wild-type Col-0 and transgenic *35S::GR-REVd* seedlings with and without DEX application. These experiments demonstrated that the level of transcription is increased in both *TAA1* and *YUC5* in response to DEX application in the transgenic *35S::GR-REVd* plants (Figure 4b). The induction also occurs in seedlings pre-treated with cycloheximide (CHX), which supports a direct role for REV in the regulation of these genes (Figure 4b). This direct regulation was again confirmed by qChIP-PCRs and we detected binding of REV to the 5′ promoter of both *YUC5* and *TAA1* (Figure 4c). In summary, these findings indicate that REV is a direct regulator of the auxin biosynthetic genes *YUC5* and *TAA1*.

HD-ZIPIII transcription factors play a role in shade-induced hypocotyl elongation

We have shown that REV regulates the transcription of *TAA1*, *YUC5* and four *HD-ZIPII* genes. *TAA1* and the four *HD-ZIPII* genes have all been shown to be associated with light-induced growth responses. To understand whether REV and/or other HD-ZIPIII transcription factors play a role in growth promotion in shade conditions, we analyzed various mutants grown in simulated canopy shade conditions (for ease of reading, we refer in the following paragraphs to these conditions as ‘shade’). As expected, Col-0 wild-type plants develop elongated hypocotyls when grown in shade conditions (Figure 5a,b). Conversely, hypocotyls of *taa1* mutant plants (*sav3-2*) do not elongate in response to shade (Figure 5a,b). Plants that carry a mutation in *REV* (*rev-5*) have shorter hypocotyls when grown in shade when compared with wild-type plants, indicative of a compromised shade-growth response (Figure 5a,b). Transgenic lines in which the dimerization of HD-ZIPIII proteins is inhibited by over-expression of ZPR-type microProteins (Kim *et al.*, 2008; Staudt and Wenkel, 2011; Wenkel *et al.*, 2007) and plants in which the stability of HD-ZIPIII mRNA is affected by over-expression of *miR165a* (Kim *et al.*, 2010) also display growth defects in response to shade (Figure 5a,b). The analysis of *rev10D* mutant plants and the corresponding Landsberg *erecta* wild-type revealed that rev10D mutant plants show a slight increase in hypocotyl length in response to the shade treatment (Figure 5a,b). This increase is not easy to interpret and might be due to the genetic background. Also, in response to ‘neighbor proximity detection’ conditions in which only the amount of FR light is increased, *hd-zipIII* mutant plants showed significantly shorter hypocotyls compared with wild-type plants (Figure S2). These findings demonstrate that HD-ZIPIII transcription factors are required for a full shade-avoidance response.

Next, we analyzed if gene-expression changes in response to shade are altered in plants that are depleted of HD-ZIPIII function. Gene-expression studies using quantitative RT-PCR revealed that the expression of not also the FR-induced genes *HAT2*, *HAT3*, *ATHB2*/*HAT4*, *ATHB4* but also *YUC5* is markedly induced in wild-type plants in response to shade treatment (Figure 5c). We found that the

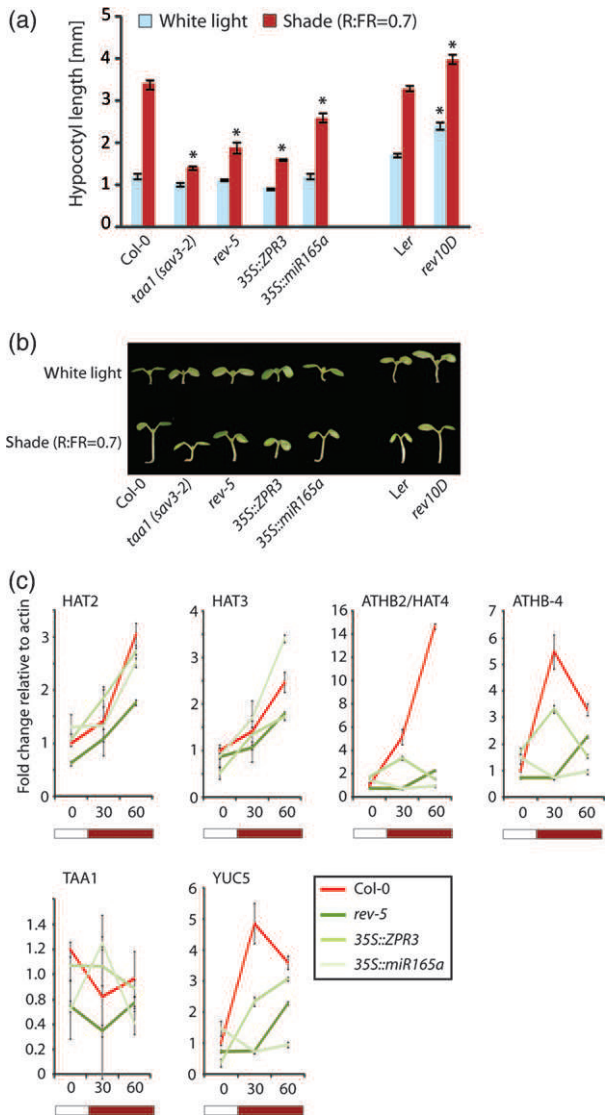


Figure 5. *hd-zipIII* mutant plants show altered shade avoidance responses.
(a) Shade avoidance measurements by determination of hypocotyl length in white light (WL) and white light supplemented with far-red light (WL+FR). Error bars represent the standard error. Asterisks indicate significant differences relative to the wild-type controls ($p < 0.01$). Both Student's *t*-test and bifactorial analysis of variance (ANOVA) were performed to calculate *P*-values.
(b) Representative seedlings.
(c) Gene-expression analysis of *HAT2*, *HAT3*, *ATHB2*/*HAT4*, *ATHB4*, *TAA1* and *YUC5* in different *hd-zipIII* mutants (*rev-5*: dark green, *35S::ZPR3*: green, *35S::miR165a*: light green) in comparison with Col-0 wild-type plants (red). Seedlings were grown in WL for 9 days and then exposed to simulated canopy shade conditions. Samples were harvested before shade treatments and 30 and 60 min after. Error bars represent the standard deviation of three biological replicates.

expression of all shade-induced genes is significantly lower in *rev-5* and *35S::ZPR3* plants, which supports a role of REV in the regulation of these genes. In the *35S::miR165a* transgenic line, expression of *ATHB2*/*HAT4*, *ATHB4* and *YUC5* is lower compared with wild-type, but the expression of *HAT3* is slightly higher than in the wild-type controls. The expression of *TAA1* does not change in response to shade and expression levels are slightly lower in *rev-5* and *35S::ZPR3* plants compared with wild-type, which implied that REV is involved in the regulation of basal *TAA1* expression, most probably with other still unknown factors. In summary, we conclude that HD-ZIPIII transcription factors promote growth in response to shade, most probably via regulation of shade-induced growth factor signaling.

Promotion of growth in far-red light is regulated by the HD-ZIPIII–HD-ZIPII–auxin module

Up to this point we have shown that REV induces transcription of a number of target genes involved in light-/shade signaling and is required for a full shade-avoidance response. To determine whether REV is able to induce auxin levels, we have introduced the *35S::GR-REVd* transgene into plants that harbor the *DR5::GUS* reporter by crossing. Col-0 wild-type plants showed DR5-GUS expression in the leaf margins and hydathodes (Figure 6a). Induction of GR-REVd by DEX application results in a strong GUS signal throughout the leaf lamina (Figure 6a). These findings suggest that REV can either interfere with auxin signaling or induce directly the production of auxin. To verify that auxin levels are upregulated by REV, we used GC-MS to determine the levels of free auxin in DEX-treated Col-0, *35S::GR-REVd*, *taa1* (*sav3-2*) and *35S::GR-REVd sav3-2* plants that had been exposed transiently to shade. These measurements confirmed that free auxin is indeed increased upon REV induction in both wild-type and *taa1* (*sav3-2*) mutant plants (Figure 6b). The increased levels of auxin mediated by REV in the *taa1* (*sav3-2*) mutant background could be YUC5 derived. It was shown that TAA1 is required for shade-induced growth promotion (Tao *et al.*, 2008), but mRNA levels of the *TAA1* gene do not change in response to shade, which suggested post-translational activation. Plants that carry loss-of-function mutations in two *YUCCA* genes (*yuc1 yuc4*) also show a compromised shade-avoidance response (Stepanova *et al.*, 2011; Won *et al.*, 2011). Interestingly, overexpression of YUCCA1 is able to rescue the *taa1* mutant phenotype (Stepanova *et al.*, 2011; Won *et al.*, 2011). These findings demonstrate that both YUCCA enzymes and TAA1 are required for a full shade-avoidance response. While *TAA1* is not upregulated in response to shade, transcription of several *YUCCA* genes is induced in response to shade (Li *et al.*, 2012). Here we show that REV is able to stimulate auxin production directly, most probably by transcriptional regulation of both *TAA1* and *YUC5*.

Because REV can upregulate both *TAA1* and *YUC5*, we investigated if induction of REV can complement the *taa1* (*sav3-2*) mutant phenotype. To test this hypothesis, we grew Col-0, *35S::GR-REVd*, *taa1* (*sav3-2*) and *35S::GR-REVd sav3-2* plants in white light and shade in the presence of 5 μM DEX. We first observed that hypocotyls of DEX-induced *35S::-GR-REVd* transgenic plants are markedly longer in white

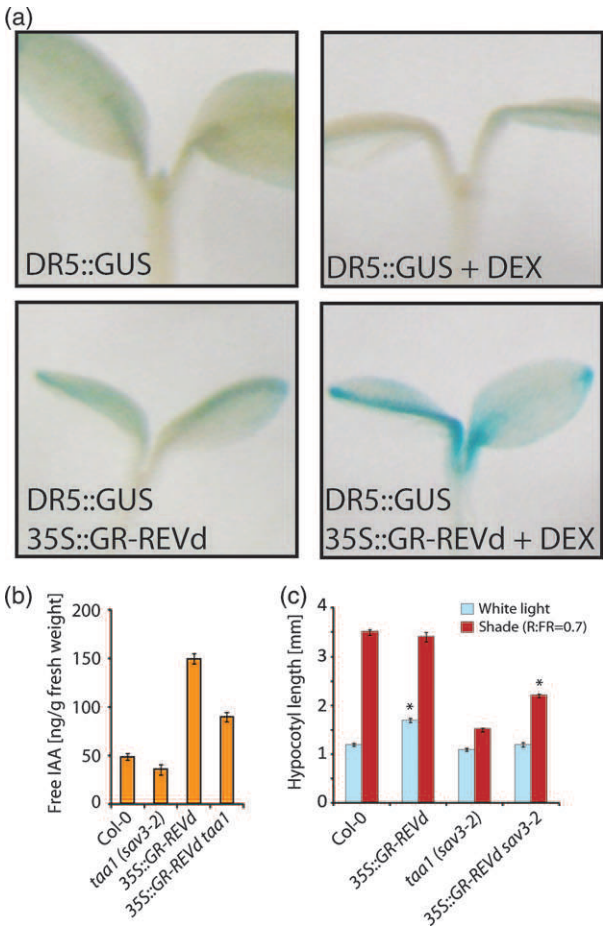


Figure 6. REV can induce the production of auxin.
(a) Analysis of the *DR5::GUS* reporter in the presence of the inducible *35S::GR-REVd* transgene. The upper panel shows *DR5::GUS* levels in wild-type in white light (control) and in the presence of dexamethasone (DEX). Below the wild-type, seedlings are shown that harbor the *35S::GR-REVd* transgene in combination with *DR5::GUS*. DEX-induced translocation of the GR-REVd chimeric protein upregulates GUS expression.
(b) Determination of free auxin levels in Col-0, *35S::GR-REVd*, *taa1* (*sav3-2*) and *35S::GR-REVd sav3-2* plants using GC-MS.
(c) Shade avoidance measurements by determination of hypocotyl length in white light (WL) and white light supplemented with far-red light (WL+FR). Error bars represent the standard error. Asterisks indicate significant differences relative to the wild-type controls ($p < 0.01$).

light, a phenotype that was also observed for plants ectopically expressing class II HD-ZIP transcription factors (Ciarbelli *et al.*, 2008; Schena *et al.*, 1993; Sorin *et al.*, 2009; Steindler *et al.*, 1999). No further elongation of the hypocotyl was observed in shade conditions. This finding is consistent with a role of REV in shade avoidance, as an additional elongation in shade would suggest that REV functions in a parallel pathway. However, ectopic induction REV partially complements the *taa1* mutant phenotype (Figure 6c). These findings suggest that either upregulation of *HD-ZIPII* factors can promote growth in the absence of TAA1-derived auxin or that *YUC5* induction can cause TAA1-independent auxin production and result in the promotion of growth. These results are in line with the auxin measurements, which revealed an increase in the levels of free auxin in shade-treated *35S::GR-REVd taa1* (*sav3-2*) plants compared with *taa1* (*sav3-2*) mutant plants (Figure 6c).

The leaf regulatory module that consists of HD-ZIPIII and KANADI transcription factors oppositely regulates shade responses

Leaf development is regulated by the concerted action of HD-ZIPIII transcription factors, expressed in the adaxial domain, and KANADI transcription factors, literally mirroring *HD-ZIPIII* expression, in the abaxial domain. Whereas HD-ZIPIIIs act as transcriptional inducers, KANADIs are thought to act mostly by transcriptional repression. To find out if KANADI transcription factors are also involved in the regulation of adaptive growth responses, we tested if ectopic KANADI (KAN1) expression affects shade-induced hypocotyl elongation. Therefore, we constructed DEX-inducible KAN1 overexpression lines (*35S::FLAG-GR-KAN1*). Wild-type and transgenic *35S::FLAG-GR-KAN1* plants were grown on DEX-containing MS plates in white light and shade. Hypocotyls of induced *35S::FLAG-GR-KAN1* plants are strongly affected in elongation in response to shade, almost resembling *taa1* mutant plants (Figure 7a–c). These findings show that KAN1 is able to repress shade growth and thus KANADIs act oppositely to HD-ZIPIIIs also in adaptive growth processes.

We next tested whether the HD-ZIPIII/KANADI antagonism is manifested in opposite regulation of potential target genes. We first checked if our *35S::FLAG-GR-KAN1* plants function in a manner similar to that of the published *35S::KAN1-GR* plants (Wu *et al.*, 2008). As shown for the published *35S::KAN1-GR* plants, our *35S::FLAG-GR-KAN1* line was also able to strongly repress expression of the *ASYMMETRIC LEAVES2* gene (Figure S3) and we were also able to detect binding of the chimeric FLAG-GR-KAN1 protein close to the first exon (Figure S3) as described before (Wu *et al.*, 2008). After confirmation of the functionality of our transgenic plants, we tested whether the identified REV-target genes are altered in expression by KAN1 induction. We found that, of the investigated REV-target genes, expression of *HAT2*, *TAA1* and *YUC5* is reduced significantly by DEX induction of *35S::FLAG-GR-KAN1* plants (Figure 7d–f). Most importantly, this effect also seems to be direct, as these changes occur in the presence of CHX (Figure 7d–f). Using chromatin immunoprecipitation, we tried to identify regions of KAN1 binding in the promoters of REV/KAN1 targets. Strong binding was observed in the *HAT2* promoter, where KAN1 interacts with a region about 1.0 kb upstream of the transcriptional start site (Figure 7d). In the *TAA1* promoter, binding of KAN1 is more complex and we identified two regions of potential KAN1 binding. The first region is about 3.0 kb upstream, whereas

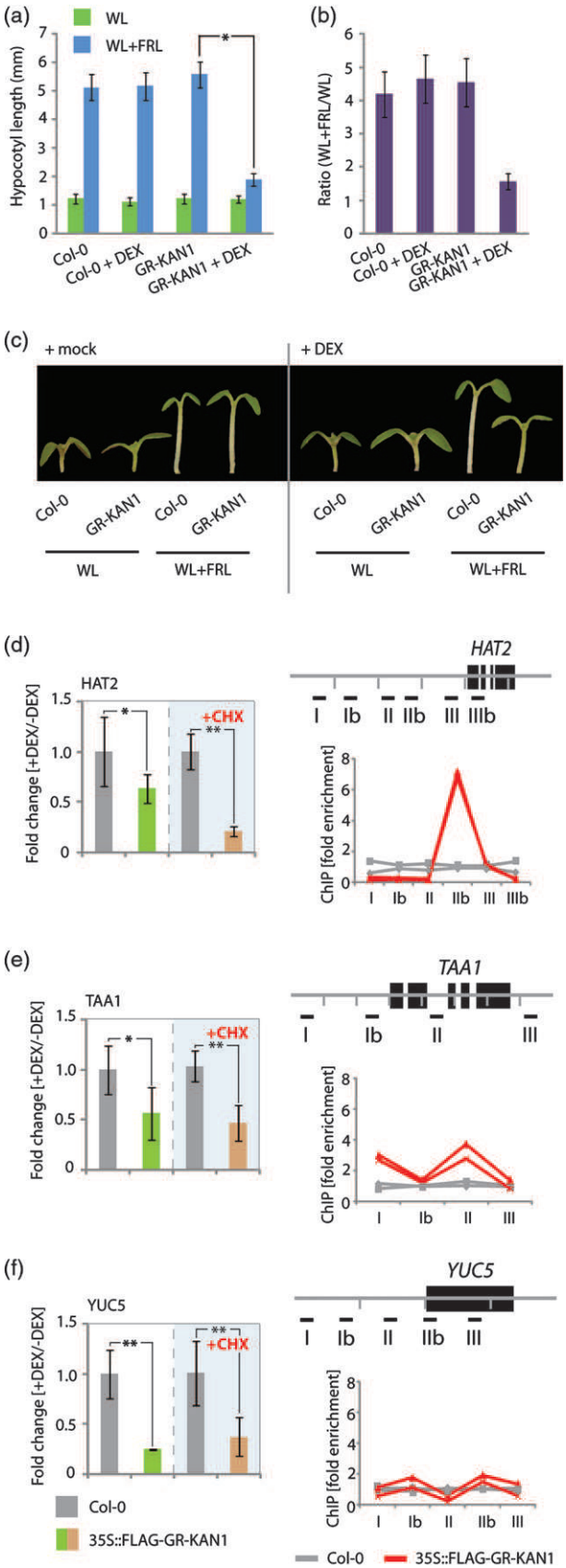


Figure 7. The leaf regulatory module that consists of HD-ZIPIII and KANADI transcription factors cross-regulates shade-response genes.
(a) Hypocotyl length of *35S::FLAG-GR-KAN1* and wild-type (WT) Col-0 w/o 5μM dexamethasone (DEX) in white light (WL) and simulated canopy shade (far-red light (FRL) enriched WL) show significant shorter hypocotyls in DEX-induced transgenic line. **t*-test, $p < 0.005$. Error bars represent standard deviation.
(b) Ratio of the lengths of hypocotyls treated with simulated canopy shade compared with WL.
(c) Representative samples. (d–f) *HAT2*, *TAA1* and *YUC5* are negatively cross-regulated by KAN1, as their expression is significantly downregulated in response to KAN1 induction. A clear enrichment of KAN1 binding was only observed for *HAT2*, which indicated that KAN1 can bind to a region around fragment IIb (d). In the *TAA1* promoter, we found two potential binding regions around fragments I and II (e). For *YUC5* we were unable to identify a binding region (f).

the second region is located in the second intron (Figure 7e). We also scanned the *YUC5* promoter for KAN1 binding, but were unable to identify a potential binding region, which indicated that the binding site might be located further up- or downstream of the regions tested (Figure 7f).

Our data reveal that the leaf regulatory network, which consisted of HD-ZIPIII and oppositely acting KANADI transcription factors, is also involved in the regulation of shade responses in Arabidopsis. We further show that the antagonistic effect is, at least partially, due to opposite regulation of gene expression. Hence it seems conceivable that shade is perceived in adaxial (HD-ZIPIII/HD-ZIPII-derived) leaf tissue. In situations of low *HD-ZIPIII* expression or ectopic *KAN1* expression, adaxial tissue might be limited, and result in a compromised shade-avoidance response. The fact that both REV and KAN1 directly regulate the expression of shade-response genes points towards a function of REV and KAN1 in adaptive development. Conversely, this finding also suggests that shade-response genes might fulfill functions in the framework of leaf development.

It remains unclear whether and how REV is activated in response to low R:FR conditions. Gene-expression studies have shown that mRNA levels of *REV* are slightly, but significantly, upregulated in response to simulated canopy shade, while expression of *PHABULOSA* and *PHAVOLUTA* remain unchanged (Figure S4). *HD-ZIPIII* gene expression is under strong regulation by microRNAs *miR165/6* (Emery *et al.*, 2003; Mallory *et al.*, 2004). Expression of the *microRNAs* mirrors *HD-ZIPIII* expression and it is thought that the gradual expression of the microRNAs establishes a gradient of *HD-ZIPIII* expression from the adaxial domain (high levels) to the abaxial domain (no expression) (Juarez *et al.*, 2004; Yao *et al.*, 2009). Our knowledge of factors that directly control *HD-ZIPIII* expression is still limited. Recently, the transcription factor DNA Binding with One Finger (DOF5.1) was identified in an activation tagging approach (Kim *et al.*, 2010). Overexpression of *DOF5.1* causes leaf adaxialization, which is due to ectopic expression of *REV* (Kim *et al.*, 2010). The analysis of publicly available microarray data (AtGenexpress light series) revealed a weak response of *DOF5.1*

expression in response to light. From these findings we can conclude that REV expression, and thus downstream developmental processes, can be altered by other transcription factors that are independent of microRNA action. It, therefore, seems plausible that other, still unknown, factors might regulate *REV* expression in response to altered environmental conditions. However, we cannot exclude that an additional regulation of REV activity might occur at the protein level. HD-ZIPIII proteins possess a carboxy-terminal MEKHLA-type PAS protein domain that has the potential to act as a sensor for light or light-induced voltage or redox changes (Mukherjee and Burglin, 2006). Recently, it has been shown that the REV-type MEKHLA domain can negatively auto-regulate HD-ZIPIII activity (Magnani and Barton, 2011). The authors propose a steric masking mechanism relieved in response to a cellular signal. Our finding that HD-ZIPIIIs are involved in light-dependent processes indicates that this signal could be light or light derived.

CONCLUDING REMARKS

More than two centuries ago, Goethe came up with the concept of the 'double law', which suggested that internal factors provide the framework for developmental processes and external factors that influence this internal framework, and result in modulation of growth and shape. Here, we provide evidence that both factors that respond to shade and factors required for normal development are partly the same, and provides a mechanistic basis for the 'double law'. HD-ZIPIIIs can induce *HD-ZIPIIs* and both factors are required for a full shade-avoidance response. The role of both transcription factor families in the framework of leaf development is to be determined. Contrary to the HD-ZIPIII/HD-ZIPII module, KAN1 represses gene expression and thereby inhibits shade growth. The role of auxin remains elusive. We observed an increase in the levels of free auxin in response to REV induction and this auxin might be important for both normal development and shade-induced hypocotyl growth.

EXPERIMENTAL PROCEDURES

Plant material, growth and phenotypic analysis

The *TAA1* mutant allele *sav3-2* (W39Stop) (Tao *et al.*, 2008) was used in this study. In addition, three different *REV* mutant alleles were used, *rev-5* (A260V) a strong Ethyl methanesulfonate (EMS) allele (Otsuga *et al.*, 2001), *rev-9* (Emery *et al.*, 2003) (introgressed into the Col-0 background) and *rev10D* (P190L) rendering *REV* mRNA microRNA resistant. To affect all HD-ZIPIIIs, *35S::ZPR3* (Wenkel *et al.*, 2007) and *35S::miR165* (Kim *et al.*, 2010) lines were used to deplete HD-ZIPIII function. REVd induction experiments were carried out using the *35S::GR-REVd* line (Wenkel *et al.*, 2007). *35S::GR-REVd sav3-2* plants were generated by crossing. For more efficient chromatin immunoprecipitations, we created transgenic *35S::FLAG-GR-REVd* plants. Therefore, the glucocorticoid receptor was cloned in frame with the FLAG epitope in the *pJAN33* vector (Weigel *et al.*, 2003) using the *Kpn*I restriction site, in the following paragraphs termed *pJAN33GR*.

For hypocotyl measurements of wild-type and mutant plants in simulated canopy shade conditions, seedlings were grown on Murashige & Skoog medium (MS) plates in long-day conditions at 22°C for 2 days in a Fi-totron 600H growth chamber (Fisons, UK) in white light [blue light (460–480 nm) = 2.34 μmol m^{-2} s^{-1}, red light (650–670) = 1.93 μmol m^{-2} s^{-1}, far-red (720–755) = 0.65 μmol m^{-2} s^{-1}, PAR (395–710 nm) = 89.3]. For shade avoidance, hypocotyl measurements plants were transferred at day 2 to a shaded compartment [using a combination of LEE filters (LEEinc.) and FR light bulbs (Narva, http://www.narva-bel.de/)] in the growth chamber and irradiated with far-red enriched light (blue light = 0.88 μmol m^{-2} s^{-1}, red light = 1.65 μmol m^{-2} s^{-1}, far-red = 2.56 μmol m^{-2} s^{-1}, PAR = 39.8). Seedlings were kept under these conditions for 4 days. Seedlings were photographed and hypocotyls were measured using IMAGEJ.

In situ hybridizations

For RNA *in situ* analyses, plants were grown for 3 weeks under short-day conditions (8 h light (10 000 LUX), 16 h dark, 21°C, 60% humidity). For probe synthesis, PCR products generated using cDNA as a template were cloned into the *pGEMT* vector (Promega, http://www.promega.com) and used as a template for transcription from the *T7* or *SP6* promoter. Primers employed for generation of PCR products for probe synthesis are listed in Supplementary Table S2. *REV* sense and antisense probes and subsequent *in situ* hybridizations were carried out according to Greb *et al.* (Greb *et al.*, 2003).

Quantification of free auxin

For analysis of indole-3-acetic acid approximately 200 mg Arabidopsis seedlings were harvested and homogenized in liquid N_2. Extraction of the free analytes was carried out at 28°C for 90 min with 1.5 ml ethyl acetate, which contained 0.1% (v/v) formic acid and the internal standards 3-hydroxybenzoeic acid and indole-5-formic acid. After centrifugation at 10 000 ***g*** and 4°C for 10 min 1.2 ml supernatant was transferred into a new vial. The ethyl acetate was removed, and the sample was dried over phosphorus pentoxide in vacuum (100 mbar) overnight. Derivatization was performed with 70 μl N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA Sigma) for 60 min at 40°C; 1 μl was injected onto the Gas chromatography (GC) column. Determination of the analytes was done by Gas chromatography (GC/MS, Agilent 6890 GC and Agilent 5973 single quad mass spectrometer; Agilent Technologies, http://www.home.agilent.com), using splitted injection mode and an SPB-50 column (30 m, 0.25 mm internal diameter; Supelco, Sigma–Aldrich, http://www.sigmaaldrich.com). The GC oven temperature was held at 70°C for 5 min, then ramped at 5°C per min to 280°C and afterwards held for an additional 10 min at 280°C. Helium was used as carrier gas with a flow rate of 1 ml min^{-1}. Detection of analytes was performed by electron impact ionization (EI) single quadrupole mass spectrometry operated in selected ion monitoring (SIM) mode.

Gene-expression analysis

To analyze gene expression, RNA was isolated from seedlings using the roboklon GeneMATRIX universal RNA purification kit following the manufacturer's recommendations. One microgram of total RNA was reverse transcribed using the Fermentas RevertAid Premium Reverse transcriptase with oligo-dT primers. cDNAs were diluted 10-fold and 3.5 ml were used for RT-PCR reactions. Quantitative measurements were performed on a Biorad CFX384 using the Fermentas SYBR Green qPCR master mix. Relative quantities were calculated using the ΔCt method. Oligonucleotide sequences are listed in Supplementary Table S2.

ChIP-Seq and ChIP analysis

For the chromatin-immunoprecipitation/DNA sequencing (ChIP-Seq) study, Col-0 and transgenic *35S::FLAG-GR-REVd* plants were grown in liquid MS medium for 10 days and induced with DEX for 90 min prior to harvesting. ChIP experiments were carried out as described by Kwon *et al.* (2005), except that anti-FLAG M2 magnetic beads (Sigma) were used and immunoprecipitations were only performed for 2 h. After immunoprecipitation of the chromatin using anti-FLAG antibody coupled beads, two ChIP-Seq libraries were generated and sequenced. This ChIP-Seq experiment resulted in the identification of 480 positions in the Arabidopsis genome at which we found an enrichment of chromatin fragments in *35S::FLAG-GR-REVd* plants compared with wild-type Columbia (Col-0) plants. To confirm the binding of REVOLUTA to these regions, a second, independent ChIP-Seq experiment was performed. This time induction with DEX was done for only 45 min and the addition of non-specific herring sperm DNA to avoid non-specific binding was omitted. This second ChIP-Seq experiment identified 8819 genomic regions that were enriched significantly.

The ChIP-Seq libraries, the Illumina sequencing and data analysis was performed as described by Yant *et al.* (2010), with the exception that the number of duplicate sequence reads was heuristically reduced prior to further analysis. ChIP-Seq data have been submitted to the Gene Expression Omnibus database (GEO accession no. GSE26722).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the on-line version of this article:

Data S1. This dataset contains the 286 binding peaks identified in two independent ChIP-Seq experiments.

Data S2. Methods.

Figure S1. Expression analysis of genes encoding auxin biosynthetic enzymes.

Figure S2. Analysis of hd-zipIII mutant plants in neighbor proximity detection shade conditions.

Figure S3. Expression of AS2, a known KAN1 target, can be altered by DEX induction of 35S::FLAG-GR-KAN1 transgenic plants.

Figure S4. REV-mediated shade response is caused by shade-induction of REV expression.

Table S1. Enrichment of selected minor gene ontologies.

Table S2. Oligonucleotides.

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REFERENCES

Ariel, F.D., Manavella, P.A., Dezar, C.A. and Chan, R.L. (2007) The true story of the HD-Zip family. *Trends Plant Sci.* **12**, 419–426.

Ballaré, C.L., Scopel, A.L. and Sánchez, R.A. (1990) Far-Red Radiation Reflected from Adjacent Leaves: An Early Signal of Competition in Plant Canopies. *Science*, **247**, 329–332.

Bowman, J.L. and Floyd, S.K. (2008) Patterning and Polarity in Seed Plant Shoots. *Annu. Rev. Plant Biol.* **59**, 67–88.

Carlsbecker, A., Lee, J.Y., Roberts, C.J. *et al.* (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature*, **465**, 316–321.

Chen, M., Chory, J. and Fankhauser, C. (2004) Light signal transduction in higher plants. *Annu. Rev. Genet.* **38**, 87–117.

Ciarbelli, A.R., Ciolfi, A., Salvucci, S., Ruzza, V., Possenti, M., Carabelli, M., Fruscalzo, A., Sessa, G., Morelli, G. and Ruberti, I. (2008) The Arabidopsis Homeodomain-leucine Zipper II gene family: diversity and redundancy. *Plant Mol. Biol.* **68**, 465–478.

Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F. and Bowman, J.L. (2003) Radial patterning of Arabidopsis shoots by class IIIHD-ZIP and KANADI genes. *Curr. Biol.* **13**, 1768–1774.

Franklin, K.A. (2008) Shade avoidance. *New Phytol.* **179**, 930–944.

Franklin, K.A. and Whitelam, G.C. (2005) Phytochromes and shade-avoidance responses in plants. *Ann. Bot.* **96**, 169–175.

Franklin, K.A., Praekelt, U., Stoddart, W.M., Billingham, O.E., Halliday, K.J. and Whitelam, G.C. (2003) Phytochromes B, D, and E act redundantly to control multiple physiological responses in Arabidopsis. *Plant Physiol.* **131**, 1340–1346.

Greb, T., Clarenz, O., Schaefer, E., Mueller, D., Herrero, R., Schmitz, G. and Theres, K. (2003) Molecular analysis of the LATERAL SUPPRESSOR gene in Arabidopsis reveals a conserved control mechanism for axillary meristem formation. *Genes Dev.* **17**, 1175–1187.

Juarez, M.T., Kui, J.S., Thomas, J., Heller, B.A. and Timmermans, M.C.P. (2004) microRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. *Nature*, **428**, 84–88.

Kim, Y.-S., Kim, S.-G., Lee, M. *et al.* (2008) HD-ZIP III Activity is modulated by competitive inhibitors via a feedback loop in Arabidopsis Shoot Apical Meristem development. *Plant Cell*, **20**, 920–933.

Kim, H.-S., Kim, S.J., Abbasi, N., Bressan, R.A., Yun, D.-J., Yoo, S.-D., Kwon, S.-Y. and Choi, S.-B. (2010) The DOF transcription factor Dof5.1 influences leaf axial patterning by promoting Revoluta transcription in Arabidopsis. *Plant J.* **64**, 524–535.

Kwon, C.S., Chen, C. and Wagner, D. (2005) WUSCHEL is a primary target for transcriptional regulation by SPLAYED in dynamic control of stem cell fate in Arabidopsis. *Genes Dev.* **19**, 992–1003.

Li, L., Ljung, K., Breton, G. *et al.* (2012) Linking photoreceptor excitation to changes in plant architecture. *Genes Dev.* **26**, 785–790.

Magnani, E. and Barton, M.K. (2011) A Per-ARNT-Sim-Like sensor domain uniquely regulates the activity of the Homeodomain Leucine Zipper Transcription Factor REVOLUTA in Arabidopsis. *Plant Cell*, **23**, 567–582.

Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., Zamore, P.D., Barton, M.K. and Bartel, D.P. (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5′ region. *EMBO J.* **23**, 3356–3364.

Martinez-Garcia, J.F., Galstyan, A., Salla-Martret, M., Cifuentes-Esquivel, N., Gallemi, M. and Bou-Torrent, J. (2010) Regulatory components of shade avoidance syndrome. *Adv. Bot. Res.* **53**, 65–116.

McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J. and Barton, M.K. (2001) Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. *Nature*, **411**, 709–713.

Morelli, G. and Ruberti, I. (2002) Light and shade in the photocontrol of Arabidopsis growth. *Trends Plant Sci.* **7**, 399–404.

Mukherjee, K. and Burglin, T.R. (2006) MEKHLA, a Novel Domain with Similarity to PAS Domains, Is Fused to Plant Homeodomain-Leucine Zipper III Proteins. *Plant Physiol.* **140**, 1142–1150.

Otsuga, D., DeGuzman, B., Prigge, M.J., Drews, G.N. and Clark, S.E. (2001) REVOLUTA regulates meristem initiation at lateral positions. *Plant J.* **25**, 223–236.

Schena, M., Lloyd, A.M. and Davis, R.W. (1993) The HAT4 gene of Arabidopsis encodes a developmental regulator. *Genes Dev.* **7**, 367–379.

Sessa, G., Steindler, C., Morelli, G. and Ruberti, I. (1998) The Arabidopsis Athb-8, -9 and -14 genes are members of a small gene family coding for highly related HD-ZIP proteins. *Plant Mol. Biol.* **38**, 609–622.

Smith, Z.R. and Long, J.A. (2010) Control of Arabidopsis apical-basal embryo polarity by antagonistic transcription factors. *Nature*, **464**, 423–U121.

Sorin, C., Salla-Martret, M., Bou-Torrent, J., Roig-Villanova, I. and Martinez-Garcia, J.F. (2009) ATHB4, a regulator of shade avoidance, modulates hormone response in Arabidopsis seedlings. *Plant J.* **59**, 266–277.

Staudt, A.-C. and Wenkel, S. (2011) Regulation of protein function by micro-Proteins. *EMBO Rep.* **12**, 35–42.

Steindler, C., Matteucci, A., Sessa, G., Weimar, T., Ohgishi, M., Aoyama, T., Morelli, G. and Ruberti, I. (1999) Shade avoidance responses are mediated by the ATHB-2 HD-Zip protein, a negative regulator of gene expression. *Development*, **126**, 4235–4245.

Stepanova, A.N., Robertson-Hoyt, J., Yun, J., Benavente, L.M., Xie, D.-Y., Dolezal, K., Schlereth, A., Jürgens, G. and Alonso, J.M. (2008) TAA1-Mediated Auxin biosynthesis is essential for h ormone crosstalk and plant development. *Cell*, **133**, 177–191.

Stepanova, A.N., Yun, J., Robles, L.M., Novak, O., He, W., Guo, H., Ljung, K. and Alonso, J.M. (2011) The Arabidopsis YUCCA1 Flavin Monooxygenase functions in the Indole-3-Pyruvic Acid branch of Auxin biosynthesis. *Plant Cell Online*, **23**, 3961–3973.

Tao, Y., Ferrer, J.L., Ljung, K. *et al.* (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. *Cell*, **133**, 164–176.

Weigel, M., Varotto, C., Pesaresi, P., Finazzi, G., Rappaport, F., Salamini, F. and Leister, D. (2003) Plastocyanin is indispensable for photosynthetic electron flow in Arabidopsis thaliana. *J. Biol. Chem.* **278**, 31286–31289.

Wenkel, S., Emery, J., Hou, B.-H., Evans, M.M.S. and Barton, M.K. (2007) A feedback regulatory module formed by LITTLE ZIPPER and HD-ZIPIII Genes. *Plant Cell*, **19**, 3379–3390.

Won, C., Shen, X., Mashiguchi, K., Zheng, Z., Dai, X., Cheng, Y., Kasahara, H., Kamiya, Y., Chory, J. and Zhao, Y. (2011) Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF ARABIDOPSIS and YUCCAs in Arabidopsis. *Proc. Natl Acad. Sci.* **108**, 18518–18523.

Wu, G., Lin, W.C., Huang, T.B., Poethig, R.S., Springer, P.S. and Kerstetter, R.A. (2008) KANADI1 regulates adaxial-abaxial polarity in Arabidopsis by directly repressing the transcription of ASYMMETRIC LEAVES2. *Proc. Natl Acad. Sci. USA,* **105**, 16392–16397.

Yamada, M., Greenham, K., Prigge, M.J., Jensen, P.J. and Estelle, M. (2009) The TRANSPORT INHIBITOR RESPONSE2 Gene is required for Auxin synthesis and diverse aspects of plant development. *Plant Physiol.* **151**, 168–179.

Yant, L., Mathieu, J., Dinh, T.T., Ott, F., Lanz, C., Wollmann, H., Chen, X. and Schmid, M. (2010) Orchestration of the floral transition and floral development in Arabidopsis by the Bifunctional transcription factor APETALA2. *Plant Cell*, **22**, 2156–2170.

Yao, X., Wang, H., Li, H., Yuan, Z., Li, F., Yang, L. and Huang, H. (2009) Two types of cis-acting elements control the abaxial epidermis-specific transcription of the MIR165a and MIR166a genes. *FEBS Lett.* **583**, 3711–3717.

Zhou, X. and Su, Z. (2007) EasyGO: Gene Ontology-based annotation and functional enrichment analysis tool for agronomical species. *BMC Genomics*, **8**, 246.

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ATHB4 and HAT3, two class II HD-ZIP transcription factors, control leaf development in Arabidopsis

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Abbreviations: HD-ZIP, homeodomain leucine-zipper; R:FR ratio, red to far-red ratio

In response to plant proximity or canopy shade, plants can react by altering elongation growth and development. Several members of the class II homeodomain-leucine zipper (HD-ZIPII) transcription factor family have been shown to play an instrumental role in the responses to shade. HD-ZIP members of the class III (HD-ZIPIII), by contrast, are involved in basic patterning processes. We recently showed that REVOLUTA (REV), a member of the HD-ZIPIII family, directly and positively regulates the expression of several genes involved in shade-induced growth, such as those encoding HD-ZIPII factors HAT2, HAT3, ATHB2/HAT4 and ATHB4, and of the components of the auxin biosynthesis pathway *YUCCA5* and *TAA1*. Furthermore, we could demonstrate a novel role for HD-ZIPIII in shade-induced promotion of growth. Here we show that besides responding to shade, *ATHB4* and *HAT3* have a critical role in establishing the dorso-ventral axis in cotyledons and developing leaves. Loss-of-function mutations in these two *HD-ZIPII* genes (*athb4 hat3*) results in severely abaxialized, entirely radialized leaves. Conversely, overexpression of *HAT3* results in adaxialized leaf development. Taken together, our findings unravel a so far unappreciated role for an HD-ZIPII/HD-ZIPIII module required for dorso-ventral patterning of leaves. The finding that HD-ZIPII/HD-ZIPIII also function in shade avoidance suggests that this module is at the nexus of patterning and growth promotion.

Introduction

Plants are sessile organisms and to maximize reproductive success, they have to adjust their growth behavior to their environment. Light is one of the most important environmental cues as it provides both energy and information. Plants have evolved refined mechanisms to detect both light quality and quantity and to measure the duration of the light period. Important growth responses and developmental decisions, such as plant architecture and the transition to flowering, are influenced by a combination of cues such as light quality and day length. In nature, plants live in communities with other plant species that might compete for resources. To avoid living under a canopy, they can detect plant proximity and canopy shade as changes in the red (R) to far-red (FR) ratio (R:FR ratio) of light and translate these changes into growth responses, collectively known as the shade avoidance syndrome (SAS) that include enhanced hypocotyl elongation, reduced leaf expansion, decreased branching and accelerated flowering.[1-3] As plant leaves reflect FR-light, neighboring plants can sense subtle decreases in R:FR ratio ('neighbor proximity detection') and react by inducing hypocotyl growth.[4] In case of true plant shade, canopy plant leaves selectively absorb light from the photosynthetic active radiation, which includes R light. Therefore, both the R:FR and the overall quantity of the photosynthetic active radiation (400–700 nm) is decreased (canopy shade conditions), which is also translated into growth-induction of the hypocotyl.[1-3] Changes in R:FR are perceived by the plant phytochrome system, which rapidly influences hormonal responses and a downstream transcriptional network to alter the mentioned aspects of plant development and architecture.[5-7]

The transcriptome of Arabidopsis changes significantly in response to shade[8,9] and numerous shade-induced genes are known.[10,11] Several of these rapidly shade induced genes belong to the class II homeodomain leucine-zipper (HD-ZIPII) family of transcription factors, that are mostly known to be involved in the regulation of adaptive responses to the environment.[12,13] The recent finding that HAT1/JAIBA, a HD-ZIPII protein, is also involved in the regulation of meristem activity[14] hints toward additional functions of HD-ZIPIIs such as regulation of plant development per se. We could recently show that the expression of

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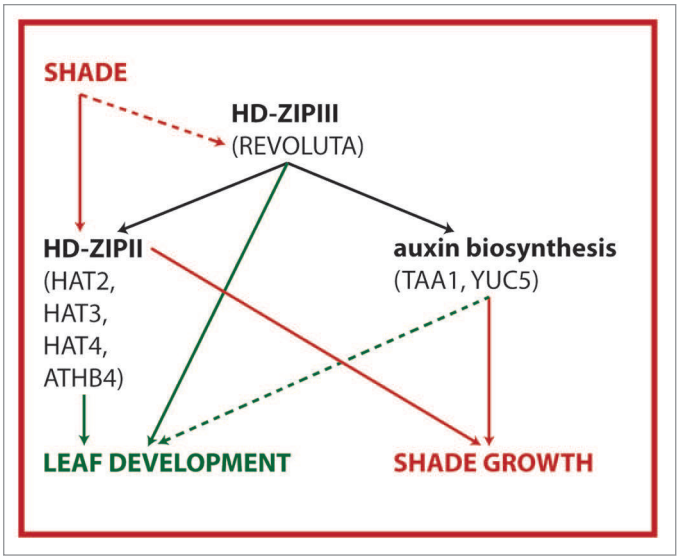

Figure 1. Model showing the role of REV targets in shade avoidance and leaf development. Using a ChIP-Seq approach we have previously shown that REV acts upstream of both HD-ZIPII and auxin biosynthesis. Thereby REV influences shade-induced growth responses. Here we show that HD-ZIPII transcription factors also have a prominent role in regulating leaf development. It is unknown whether and how HD-ZIPIIIs are activated by shade and whether TAA1/YUC5 play a role in leaf development.

several *HD-ZIPII* genes is directly controlled by the HD-ZIPIII transcription factor REVOLUTA (REV).[15] HD-ZIPIII factors have known roles in controlling embryo, shoot and root patterning[16-18] and our previous finding that they are involved in an adaptive process such as the SAS, suggested that they function at the nexus of adjusting growth to the environment. Previously it was shown that double mutant plants in two HD-ZIPII genes (*ATHB4* and *HAT3*) display strong alterations in their development.[3,10] Using a genetic approach, we have investigated whether HD-ZIPII transcription factors also have a role in the regulation of leaf development. Our data demonstrates that the combined loss of *ATHB4* and *HAT3* function results in radialized leaves with abaxial characteristics, reminiscent of *hd-zipIII* mutant plants. Conversely, the analysis of gain-of-function overexpression plants reveals that HAT3 promotes adaxial leaf development, strongly resembling HD-ZIPIII overexpression. Taken together our results support a role for these two HD-ZIPII factors downstream of HD-ZIPIII in the regulation of leaf development. Since long-term exposure to shade results in leaves with longer petioles but reduced leaf blades,[3,19] our results suggest that HD-ZIPs might be part of the mechanisms regulating this process.

Results and Discussion

Our analysis of REV target genes revealed several HD-ZIPII transcription factors that are directly and positively regulated by REV.[15] Some HD-ZIPII transcription factors are known to be involved in shade signaling[10] and our recent analysis showed that HD-ZIPIIIs are also involved in shade growth (**Fig. 1**).[15]

Using available double mutant plants in HD-ZIPII genes (*hat1 hat2* and *athb4 hat3*) and various *hd-zipIII* mutant plants (*rev-5*, *35S::miR165a* and *35S::ZPR3*), we performed comparative leaf growth studies. When grown side-by-side, *athb4 hat3* double mutant plants were severely impaired in development and retarded in growth before reaching the reproductive phase (**Fig. 2A**). The *hat1 hat2* double mutant did not display a mutant phenotype in regard to altered leaf polarity (**Fig. 2A**). Together, these studies revealed that, like *HD-ZIPIIIs*, also some *HD-ZIPIIs* play a prominent role in regulating polar leaf development in Arabidopsis. We next examined the vascular strands of petioles of different *hd-zipII*/*hd-zipIII* mutant plants to detect more subtle polarity-associated defects. Vascular strands of wild type plants, as well as *hat1 hat2* double mutant, showed a typical sandwich-structure with xylem on top (colored in blue), cambium cells in the middle (colored in red) and phloem on the bottom (green). In plants with reduced HD-ZIPIII activity (*35S::miR165a*, *rev-5*, *35S::ZPR3*), the vasculature showed different degrees of abaxialized and radialized characteristics, with phloem surrounding the xylem. Histological analyses of vascular strands of leaves of *athb4 hat3* double mutant plants showed strong abaxialization, manifested by radialization of transport elements and also a severe disruption of the overall organization (**Fig. 2A**). Thus, the *athb4 hat3* mutant phenotype somewhat resembles *hd-zipIII* mutant plants. Interestingly, in our growth conditions, the mutations caused strong leaf patterning defects in the early post-embryonic growth phase and both cotyledons and early leaves showed strong developmental defects (**Fig. 2B**). Later in development, the mutant *athb4 hat3* phenotype was alleviated and leaf development resumed to a more normal state (**Fig. 2B**, lower panel), an effect that was not observed in *hd-zipIII* mutant plants. These findings illustrate that these two HD-ZIPII transcription factors play an important role in leaf patterning, very likely downstream of HD-ZIPIII action. Because they affect more strongly the early post-embryonic growth phase, their action might be less required for the development leaves formed by older plants.

Using scanning-electron microscopy we further characterized the early growth defects of *athb4 hat3* mutant plants and could observe that both cotyledons and leaves were radialized to different degrees and lacked adaxial characteristics (**Fig. 3B, D, F, H**) compared with wild type plants (**Fig. 3A, C, E, G**). Wild type and *athb4 hat3* mutant seedlings were also compared using confocal microscopy and 3D-reconstruction (**Figs. 3I and J**, Vids. S1 and S2), displaying the alterations in leaf development at higher resolution. Optical sections through developing cotyledons revealed normal polarity of wild-type cotyledons with vascular strands vs. strongly radialized and abaxialized cotyledons with disorganized vascular strands in *athb4 hat3* mutant plants (**Fig. 3I**). The results further corroborated that ATHB4 and HAT3 transcription factors are involved in patterning the adaxial domain in the early leaf primordium.

To find out whether mis-regulation of *HD-ZIPIII* genes is a consequence of the *athb4 hat3* mutant phenotype, we analyzed the expression *PHB* and *PHV*, two adaxial marker genes of the HD-ZIPIII family. Plants carrying dominant mutations in either *PHB* or *PHV* display dramatic adaxialized phenotypes[17] and

thus behave opposite to the developmental defects observed in *athb4 hat3* mutant plants. Our expression analysis shows that both *PHB* and *PHV* expression is significantly lower in *athb4 hat3* mutant plants compared with Col-0 wild type plants (**Fig. 3K**). These findings suggest that besides acting downstream of REV, ATHB4 and HAT3 might have an additional function upstream of REV. The observation that *PHB* and *PHV* expression are reduced in *athb4 hat3* mutant plants might suggest that *ATHB4* and *HAT3* act positively on *HD-ZIPIII* expression. Based on the auto-activation capacity in a yeast two-hybrid assay, it has been suggested that HAT1/JAIBA may act as a transcriptional activator.[14] However, all HD-ZIPII proteins contain an N-terminal EAR motif, required for transcriptional repression.[20] It is furthermore known that when overexpressed in plants, several HD-ZIPIIs act as transcriptional repressors over the expression of some genes,[10,21-23] for which reason it is unlikely that they act by directly and positively regulating *HD-ZIPIII* expression. Therefore it seems plausible that the reduced expression of *PHB* and *PHV* is an indirect effect, i.e., a mere consequence of reduced adaxial tissue. We therefore conclude that the combined loss of *ATHB4* and *HAT3* causes strongly abaxialized leaf development, which is reflected by reduced expression of the adaxial identity markers *PHB* and *PHV*.

We next examined whether the ectopic expression of *HAT3*, a REV target gene, can elicit phenotypes associated with either loss- or gain-of-REV function. In order to avoid strong pleiotropic overexpression-phenotypes, we decided to employ the glucocorticoid-receptor (GR) inducible system. Using Gateway recombination (Invitrogen), transgenic *35S::FLAG-GR-HAT3* plants were constructed. Four-week old transgenic T2 plants (n = 20) were grown in short day conditions and treated once a day with DEX by spraying for one week. DEX-induced transgenic *35S::FLAG-GR-HAT3* plants showed strong upward-curling of leaf blades, largely resembling DEX-induced *35S::FLAG-GR-REVd* transgenic plants (**Fig. 4**), a phenotype caused by over-proliferation of adaxial-derived tissue in leaves.[24] These data further support our previous conclusion that HD-ZIPIIIs and these two HD-ZIPIIs (ATHB4 and HAT3) have common functions in both shade-induced growth promotion and leaf patterning.

Besides promotion of hypocotyl growth, long-term exposure to shade significantly alters the leaf developmental program.[19] A reduced leaf blade expansion has been commonly observed in plants that constitutively overexpressed some HD-ZIPII, such as ATHB2/HAT4,[25] ATHB4,[10] HAT2[21] or HAT3 (**Fig. 4**), suggesting that high levels of HD-ZIPII transcription factors might account for this SAS-related phenotype. Our results suggested that HD-ZIPIIIs might also participate in this SAS response. It remains unclear how HD-ZIPIIs execute their influence on dorso-ventral axis formation. It seems conceivable that both HD-ZIPII and HD-ZIPIII act in a common protein complex, which, based on environmental influence, switches between transcriptional activation and repression. Alternatively, HD-ZIPIIIs can activate the expression of HD-ZIPIIs-encoding genes in concert with environmental conditions, which in turn would repress expression of the actual executors involved in dorso-ventral leaf patterning.

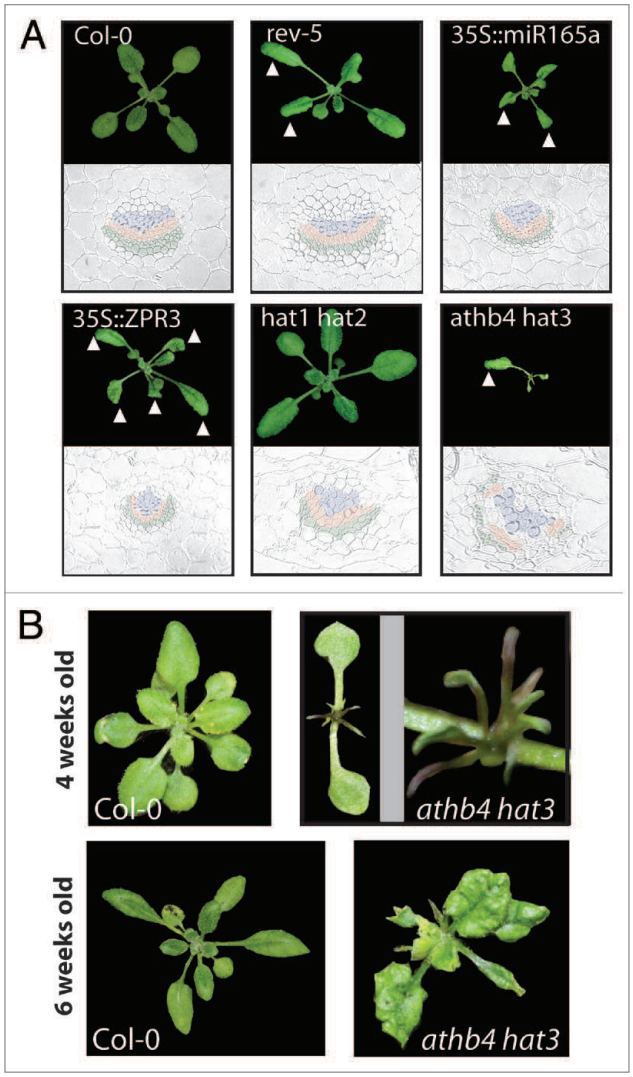

Figure 2. Mutations in both *HD-ZIPII* and *HD-ZIPIII* genes cause polarity defects in leaves and vascular organization. (A) Comparative growth analysis of different mutant plants. The triangle highlights abaxialized leaves of *rev-5*, *35S::miR165a*, *35S::ZPR3* and *athb4 hat3* plants. In wild type Col-0 and *hat1 hat2* plants no growth abnormalities were observed. Below the photographs of wild type and the different mutant plants, sections through petioles are shown. The vasculature of wild type Col-0 plants shows the typical sandwich structure tissue containing phloem cells (green), cambium cells (red) and tissue containing xylem elements (blue) on top. Both *35S::miR165a* and *35S::ZPR3* transgenic plants show abaxialized vascular strands with phloem nearly surrounding the xylem. In *athb4 hat3* mutant plants, the vascular organization is severely disturbed but is also showing abaxialized characteristics. (B) In the juvenile phase of post-embryonic growth, *athb4 hat3* double mutants produce radial leaves in comparison to Col-0 wild type plants. Later in development (around 6 weeks after germination; lower panel), leaves with weaker abaxialized characteristics, such as downward bending leaf blade, are being produced.

Materials and Methods

Fixation, clearing and staining procedure for three-dimensional imaging. For the experiments shown in **Figure 3**, seeds were germinated and grown in a growth chamber at 22°C under

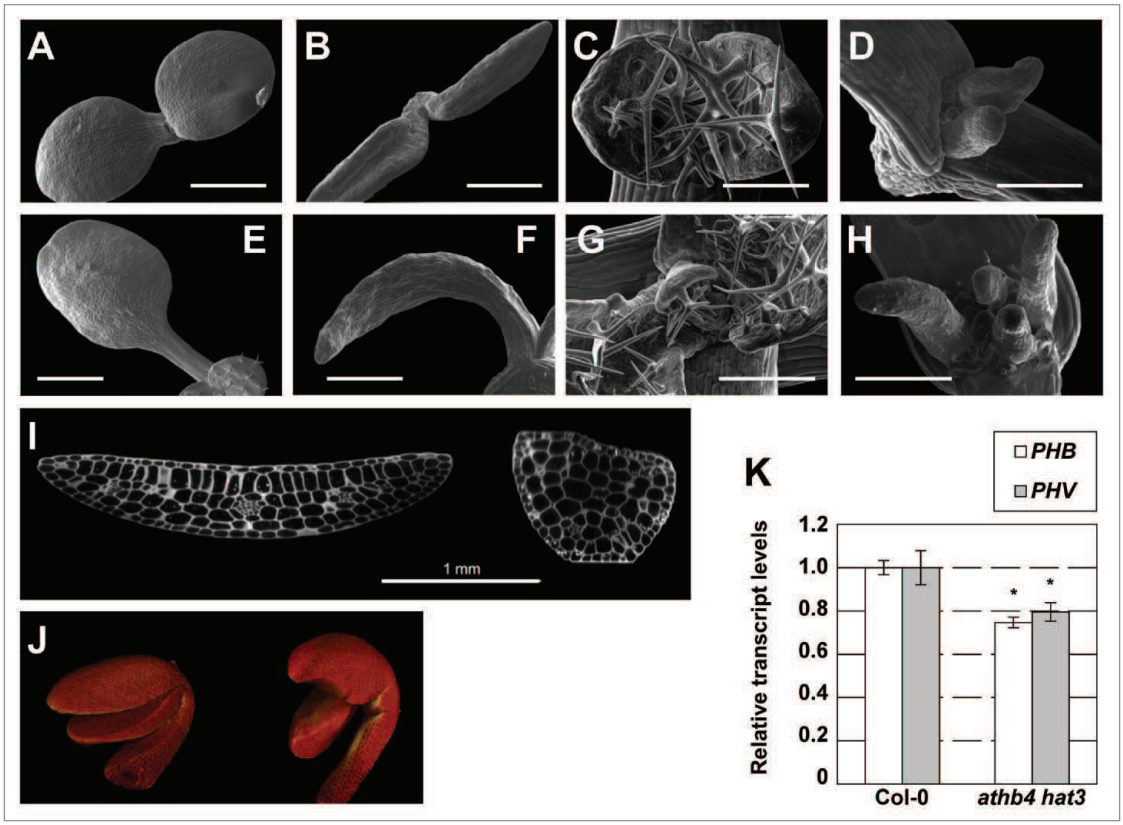

Figure 3. Mutations in HD-ZIPII genes cause abnormal growth and polarity defects in cotyledons and leaves. (A-D) Scanning Electron Microcopy images showing wild-type and *athb4 hat3* mutant seedlings on days 3 and 5. Phenotypes of wild type (A, C, E, G) and *athb4 hat3* (B, D, F, H) seedlings on day 3 (cotyledons: A, B), day 5 (primary leaves: B, D), day 7 (cotyledons: E, F) and day 10 (primary leaves: G, H). (I) Confocal microscopy images showing a transversal section of a wild-type (left) and *athb4 hat3* (right) cotyledon to visualize the different types of cells. (J) 3D-reconstruction using the Osirix software from image sections of wild-type (left) and *athb4 hat3* (right) seedlings. Images were taken from 1-d-old seedlings. Mutant seedlings lack mostly palisade cells, suggesting they are missing adaxial identity. Bar size corresponds to 0.5 (A, B), 0.2 (C, D), 1 (E, F) and 0.3 (G, H) mm. (K) Real-time quantitative PCR (qPCR) experiments showing expressionchanges of *PHB* and *PHV* in Col-0 and *athb4 hat3* mutant seedlings. Transcript abundance is measured relative to Col-0 values. Values are means ± SE of three (Col-0) or five (*athb4 hat3*) independent biological qPCR replicates normalized to *UBQ10*. Asterisk indicate significant differences ($p < 0.01$) relative to the Col-0 plants growing under the same conditions.

continuous white light (W, 80 μmol·m^{-2} s^{-1} of photosynthetic active radiation; R:FR ratio of 3.2–4.5). On the day of harvest, plant material was processed as described previously.[26-28] Briefly, seedlings of different ages were immersed in fixative solution (50% methanol and 10% acetic acid) at 4°C for at least 24 h (up to 1 mo). Plant material was then transferred to 80% (v/v) ethanol and incubated at 80°C for 5 min, briefly rinsed in ethanol dilutions (70%, 50%, 30% and 10% (v/v) ethanol) and finally rinsed twice with water. Then samples were incubated in 1% (v/v) periodic acid at room temperature with gentle agitation (about 100 rpm) for 40 min, rinsed again with water and incubated in Schiff reagent with freshly prepared 100 mg/mL propidium iodide in 100 mM sodium metabisulphite and 0.15 N HCl for 1–2 h (until plants were visibly stained). Then samples were rinsed with water and transferred onto microscope slides and covered with chloral hydrate solution (4 g chloral hydrate, 1 mL glycerol, and 2 mL water). Slides were kept overnight at room temperature in a closed environment to prevent drying out. The following day, the excess of chloral hydrate was removed, several drops of Hoyer's solution (30 g gum arabic, 200 g chloral hydrate, 20 g glycerol and 50 mL water) were added and a coverslip was placed on top. Slides were left undisturbed for a minimum of 3 d to allow the mounting solution to set.

Confocal microscopy and data processing. A Leica TCS-SP2-AOBS spectral confocal laser-scanning microscope (Leica Microsystems) was used. The excitation wavelength for PS-PI–stained samples was 488 nm, and emission was collected at 520 to 720 nm. Data were processed for some two-dimensional orthogonal sections, 3D rendering and movie exports using the open source software Osirix[29] (http://osirix.softonic.com/mac/) on a quadxeon 2.66-Ghz, 2-GB RAM Apple Mac pro workstation. RGB stacks of confocal images were imported as DICOM files into Osirix prior to surface rendering.

Scanning electron microscopy. For scanning electron microscopy, seeds were sown on growth medium containing 1% (w/v) sucrose. After stratification (3 d), plates were transferred to continuous white light. On days 0, 3, 5, 7 and 10, plant material was transferred into the microscope without any further treatment. Plant material was imaged with a MEB Hirox SH-1500 (*Hirox* Europe-Jyfel) microscope at -30°C.

RNA expression analysis by quantitative PCR. For reverse transcriptase quantitative PCR (qPCR) analyses of gene expression, seeds were sown on filter paper on top of GM- medium. Seedlings were grown under continuous W for 7 d. qPCR analyses were performed as indicated elsewhere.[10] *UBQ10* gene was used for normalization. We assayed 3–5 biological replicas for each sample. Primer sequences for qPCR were MSO40 (5'-GCT AAC AAC CCA GCA GGA CTC CT-3') and MSO41 (5'-TAA GCT CGA TCG TCC CAC CGT T-3') for *PHB* (At2g34710) and MSO42 (5'-GCT AAT CTT CTC TCG ATT GCG GAG GA-3') and MSO43 (5'-GCT CGA TAG TAC CAC CAT TTC CAG TG-3') for *PHV* (At1g30490). Primers for *UBQ10* transcript level analyses were described before.[10]

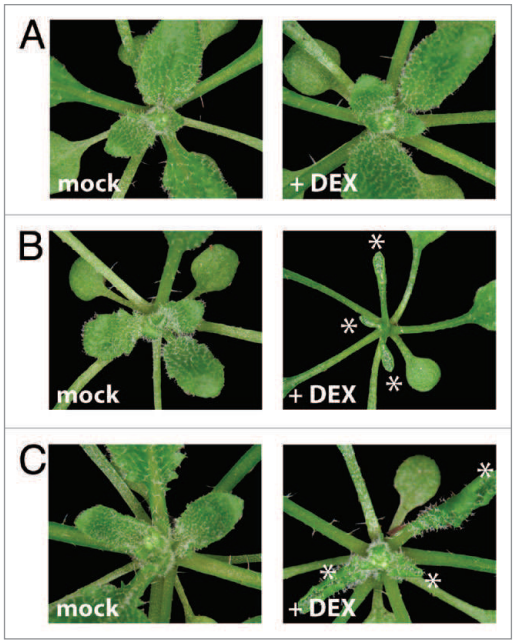

Figure 4. Inducible overexpression lines of either REVOLUTA or HAT3 cause similar developmental alterations in leaf formation. Four-week old soil-grown seedlings treated for one week with either Dexamethasone or a mock solution by spraying. Strongly adaxialized leaves (see asterisks) were observed in DEX-treated *35S::FLAG-GR-HAT3* (B) and *35S::FLAG-GR-REVd* (C) plants in comparison to Col-0 wild type plants (A).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/psb/article21824

References

1. Ballaré CL. Keeping up with the neighbours: phytochrome sensing and other signalling mechanisms. Trends Plant Sci 1999; 4:97-102; PMID:10322540; http://dx.doi.org/10.1016/S1360-1385(99)01383-7.
2. Franklin KA. Shade avoidance. New Phytol 2008; 179:930-44; PMID:18537892; http://dx.doi.org/10.1111/j.1469-8137.2008.02507.x.
3. Martinez-Garcia JF, Galstyan A, Salla-Martret M, Cifuentes-Esquivel N, Gallemi M, Bou-Torrent J. Regulatory Components of Shade Avoidance Syndrome. Advances in Botanical Research 2010; 53:65-116; http://dx.doi.org/10.1016/S0065-2296(10)53003-9.
4. Ballaré CL, Scopel AL, Sánchez RA. Far-red radiation reflected from adjacent leaves: an early signal of competition in plant canopies. Science 1990; 247:329-32; PMID:17735851; http://dx.doi.org/10.1126/science.247.4940.329.
5. Li L, Ljung K, Breton G, Schmitz RJ, Pruneda-Paz J, Cowing-Zitron C, et al. Linking photoreceptor excitation to changes in plant architecture. Genes Dev 2012; 26:785-90; PMID:22508725; http://dx.doi.org/10.1101/gad.187849.112.
6. Roig-Villanova I, Bou-Torrent J, Galstyan A, Carretero-Paulet L, Portolés S, Rodríguez-Concepción M, et al. Interaction of shade avoidance and auxin responses: a role for two novel atypical bHLH proteins. EMBO J 2007; 26:4756-67; PMID:17948056; http://dx.doi.org/10.1038/sj.emboj.7601890.
7. Hornitschek P, Kohnen MV, Lorrain S, Rougemont J, Ljung K, López-Vidriero I, et al. Phytochrome interacting factors 4 and 5 control seedling growth in changing light conditions by directly controlling auxin signaling. Plant J 2012; In Press; PMID:22536829; http://dx.doi.org/10.1111/j.1365-313X.2012.05033.x.
8. Sessa G, Carabelli M, Sassi M, Ciolfi A, Possenti M, Mittempergher F, et al. A dynamic balance between gene activation and repression regulates the shade avoidance response in Arabidopsis. Genes Dev 2005; 19:2811-5; PMID:16322556; http://dx.doi.org/10.1101/gad.364005.
9. Tao Y, Ferrer JL, Ljung K, Pojer F, Hong FX, Long JA, et al. Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell 2008; 133:164-76; PMID:18394996; http://dx.doi.org/10.1016/j.cell.2008.01.049.
10. Sorin C, Salla-Martret M, Bou-Torrent J, Roig-Villanova I, Martínez-García JF. ATHB4, a regulator of shade avoidance, modulates hormone response in Arabidopsis seedlings. Plant J 2009; 59:266-77; PMID:19392702; http://dx.doi.org/10.1111/j.1365-313X.2009.03866.x.
11. Roig-Villanova I, Bou J, Sorin C, Devlin PF, Martínez-García JF. Identification of primary target genes of phytochrome signaling. Early transcriptional control during shade avoidance responses in Arabidopsis. Plant Physiol 2006; 141:85-96; PMID:16565297; http://dx.doi.org/10.1104/pp.105.076331.
12. Ariel FD, Manavella PA, Dezar CA, Chan RL. The true story of the HD-Zip family. Trends Plant Sci 2007; 12:419-26; PMID:17698401; http://dx.doi.org/10.1016/j.tplants.2007.08.003.
13. Ciarbelli AR, Ciolfi A, Salvucci S, Ruzza V, Possenti M, Carabelli M, et al. The Arabidopsis homeodomain-leucine zipper II gene family: diversity and redundancy. Plant Mol Biol 2008; 68:465-78; PMID:18758690; http://dx.doi.org/10.1007/s11103-008-9383-8.
14. Zúñiga-Mayo VM, Marsch-Martínez N, de Folter S. JAIBA, a class-II HD-ZIP transcription factor involved in the regulation of meristematic activity, and important for correct gynoecium and fruit development in Arabidopsis. Plant J 2012; 71:314-26; PMID:22409594; http://dx.doi.org/10.1111/j.1365-313X.2012.04990.x.
15. Brandt R, Salla-Martret M, Bou-Torrent J, Musielak T, Stahl M, Lanz C, et al. Genome-wide binding-site analysis of REVOLUTA reveals a link between leaf patterning and light-mediated growth responses. Plant J 2012; In press; PMID:22578006; http://dx.doi.org/10.1111/j.1365-313X.2012.05049.x.
16. Carlsbecker A, Lee JY, Roberts CJ, Dettmer J, Lehesranta S, Zhou J, et al. Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature 2010; 465:316-21; PMID:20410882; http://dx.doi.org/10.1038/nature08977.
17. McConnell JR, Emery J, Eshed Y, Bao N, Bowman J, Barton MK. Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. Nature 2001; 411:709-13; PMID:11395776; http://dx.doi.org/10.1038/35079635.
18. Smith ZR, Long JA. Control of Arabidopsis apical-basal embryo polarity by antagonistic transcription factors. Nature 2010; 464:423-6; PMID:20190735; http://dx.doi.org/10.1038/nature08843.

19. Morelli G, Ruberti I. Light and shade in the photocontrol of Arabidopsis growth. Trends Plant Sci 2002; 7:399-404; PMID:12234731; http://dx.doi.org/10.1016/S1360-1385(02)02314-2.
20. Kagale S, Links MG, Rozwadowski K. Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in Arabidopsis. Plant Physiol 2010; 152:1109-34; PMID:20097792; http://dx.doi.org/10.1104/pp.109.151704.
21. Sawa S, Ohgishi M, Goda H, Higuchi K, Shimada Y, Yoshida S, et al. The HAT2 gene, a member of the HD-Zip gene family, isolated as an auxin inducible gene by DNA microarray screening, affects auxin response in Arabidopsis. Plant J 2002; 32:1011-22; PMID:12492842; http://dx.doi.org/10.1046/j.1365-313X.2002.01488.x.
22. Ohgishi M, Oka A, Morelli G, Ruberti I, Aoyama T. Negative autoregulation of the Arabidopsis homeobox gene ATHB-2. Plant J 2001; 25:389-98; PMID:11260495; http://dx.doi.org/10.1046/j.1365-313x.2001.00966.x.
23. Steindler C, Matteucci A, Sessa G, Weimar T, Ohgishi M, Aoyama T, et al. Shade avoidance responses are mediated by the ATHB-2 HD-zip protein, a negative regulator of gene expression. Development 1999; 126:4235-45; PMID:10477292.
24. Wenkel S, Emery J, Hou B-H, Evans MMS, Barton MK. A feedback regulatory module formed by LITTLE ZIPPER and HD-ZIPIII genes. Plant Cell 2007; 19:3379-90; PMID:18055602; http://dx.doi.org/10.1105/tpc.107.055772.
25. Schena M, Lloyd AM, Davis RW. The HAT4 gene of Arabidopsis encodes a developmental regulator. Genes Dev 1993; 7:367-79; PMID:8449400; http://dx.doi.org/10.1101/gad.7.3.367.
26. Wuyts N, Palauqui J-C, Conejero G, Verdeil J-L, Granier C, Massonnet C. High-contrast three-dimensional imaging of the Arabidopsis leaf enables the analysis of cell dimensions in the epidermis and mesophyll. Plant Methods 2010; 6:17; PMID:20598116; http://dx.doi.org/10.1186/1746-4811-6-17.
27. Truernit E, Bauby H, Dubreucq B, Grandjean O, Runions J, Barthélémy J, et al. High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of Phloem development and structure in Arabidopsis. Plant Cell 2008; 20:1494-503; PMID:18523061; http://dx.doi.org/10.1105/tpc.107.056069.
28. Truernit E, Palauqui J-C. Looking deeper: whole-mount confocal imaging of plant tissue for the accurate study of inner tissue layers. Plant Signal Behav 2009; 4:151-2; PMID:19649197; http://dx.doi.org/10.4161/psb.4.2.7683.
29. Rosset A, Spadola L, Ratib O. OsiriX: an open-source software for navigating in multidimensional DICOM images. J Digit Imaging 2004; 17:205-16; PMID:15534753; http://dx.doi.org/10.1007/s10278-004-1014-6.

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Control of stem cell homeostasis via interlocking microRNA and microProtein feedback loops

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ABSTRACT

Stem cells in the shoot apex of plants produce cells required for the formation of new leaves. Adult leaves are composed of multiple tissue layers arranged along the dorso-ventral (adaxial/abaxial) axis. Class III homeodomain leucine zipper (HD-ZIPIII) transcription factors play an important role in the set-up of leaf polarity in plants. Loss of *HD-ZIPIII* function results in strongly misshapen leaves and in severe cases fosters the consumption of the apical stem cells, thus causing a growth arrest in mutant plants. *HD-ZIPIII* mRNA is under tight control by *microRNAs 165/166*. In addition to the microRNA-action a second layer of regulation is established by LITTLE ZIPPER (ZPR)-type microProteins, which can interact with HD-ZIPIII proteins, forming attenuated protein complexes. Here we show that REVOLUTA (REV, a member of the HD-ZIPIII family) directly regulates the expression of *ARGONAUTE10* (*AGO10*), *ZPR1* and *ZPR3*. Because AGO10 was shown to dampen *microRNA165/6* function, REV establishes a positive feedback loop on its own activity. Since ZPR-type microProteins are known to reduce HD-ZIPIII protein activity, REV concomitantly establishes a negative feedback loop. We propose that the interconnection of these microRNA/microProtein feedback loops regulates polarity set-up and stem cell activity in plants.

1. Introduction

Development of eukaryotic organisms is governed by a precise control of transcription factor activities, steering differentiation processes required for tissue formation. By changing the transcriptional program, cells can change from a non-differentiated state to a highly specialized state. Stem cells are non-differentiated cells, which have the ability to adopt highly diverse cell fates. The shoot tip of plants harbors a population of stem cells, named the shoot apical meristem (SAM), which is essential for growth and development. Using forward and reverse genetic approaches, several factors involved in meristem organization and maintenance have been identified. The WUSCHEL (WUS) transcription factor plays a key role in shoot apical meristem maintenance (Mayer et al., 1998). WUS is expressed in a cell population underlying the SAM, named organizing center, and has recently been shown to act non-cell autonomously in the central zone of the SAM, where it induces expression of CLAVATA3, a negatively acting peptide ligand of the CLAVATA1 receptor kinase (Yadav et al., 2011). Besides the activities of transcriptional regulators, it was also shown that the tight balance of the

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plant hormones cytokinine and auxin influences the stem cell niche (Zhao et al., 2010).

New organs are initiated at the flanks of the SAM, thereby influencing the self-perpetuating system of stem cells. The plant-specific CLASS III HOMEODOMAIN LEUCINE-ZIPPER (HD-ZIPIII) transcription factors are involved in both stem cell maintenance and polarity set-up processes in the embryo, shoot and root as well as in cell-fate choices of developing leaves (Carlsbecker et al., 2010; McConnell et al., 2001; Smith and Long, 2010). Expression of *HD-ZIPIII* mRNA is governed by *microRNA165/166*, restricting their pattern of expression to the shoot apical meristem and the adaxial domain of developing leaf primordia (Juarez et al., 2004; Mallory et al., 2004).

Post-transcriptional gene silencing by microRNAs requires the function of several other protein factors. Most notably, DICER-like proteins which act in the processing of longer precursor RNAs and ARGONAUTE (AGO) proteins which bind the mature microRNA and guide the riboprotein complex to their target mRNAs. AGOs are essential factors for microRNA (miRNA) function in both plants and animals. Plant AGO proteins can be subdivided into five distinct clades based on their biochemical properties. AGO1 binds primarily microRNAs and directs either target cleavage or translational inhibition (Brodersen et al., 2008; Kidner and Martienssen, 2004; Vaucheret et al., 2004). AGO7 has been shown to bind miR390 and to regulate TAS RNAs which are further processed to trans-acting siRNAs and associate with AGO2/AGO3/AGO5, thus acting downstream of AGO7 (Montgomery et al., 2008). AGO4/AGO6/AGO9 bind 24nt siRNAs and are involved in guiding small RNA-mediated DNA-methylation (Eun et al., 2011; Gao et al., 2010; Havecker et al., 2010; Rowley et al., 2011). AGO10 has a high substrate specificity and predominantly associates with miR165/6 and thereby acts as a microRNA locker, sequestering miR165/6 (Zhu et al., 2011). Mutant screens in plants have yielded loss-of-function alleles of several *AGO* genes. Mutations in *AGO10/PINHEAD* (*PNH*)/*ZWILLE* (*ZLL*) disturb the self-renewal of the apical stem cells in the shoot tip, resulting in plants with arrested meristems (Lynn et al., 1999; Moussian et al., 1998). The observed phenotype of *ago10/pnh/zll* mutant plants is, *inter alia*, due to an increased expression of *miR165/166*, resulting in the down-regulation of its *HD-ZIPIII* target mRNAs (Liu et al., 2009). In flowers, the interplay of AGO1, AGO10/PNH/ZLL and *miR172* and *miR165/166* specifies temporal cell fates through the regulation of their *APETALA2* and *HD-ZIPIII* targets (Ji et al., 2011). It was shown that in the central region of the shoot tip, AGO10/PNH/ZLL sequesters *miR165/166* allowing HD-ZIPIIIs to be active, while in peripheral regions of the shoot, *miR165/166* together with AGO1 depletes *HD-ZIPIII* expression (Zhu et al., 2011).

In addition to the control by microRNAs, a second layer of HD-ZIPIII regulation occurs at the post-translational level, via the formation of non-functional heterodimeric complexes. HD-ZIPIII proteins regulate the expression of *LITTLE ZIPPER* (*ZPR*) genes encoding microProteins, which are able to form non-functional HD-ZIPIII/ZPR protein complexes (Kim et al., 2008; Staudt and Wenkel, 2011; Wenkel et al., 2007). Overexpression of ZPR-type microProteins causes in weak overexpression lines a downward curling of the leaf blade, as seen in *hd-zipIII* mutant plants (Kim et al., 2008; Prigge et al., 2005; Wenkel et al., 2007). In strong ZPR-overexpression lines the shoot apical meristem terminates with the production of one or two radialized leaves, strongly resembling *ago10/pnh/zll* mutant plants.

We have carried out a ChIP-Seq study to identify genes directly regulated by the HD-ZIPIII transcription factor REVOLUTA (REV) (Brandt et al., 2012). This screen resulted, amongst others, in the identification of *ZPR1* and *AGO10*, as putative direct targets of REV. Here we show that REV directly and positively regulates *AGO10*, *ZPR1* and *ZPR3* expression. Transgenic plants overexpressing ZPR3-type microProteins resemble an *ago10* mutant plant, which is reflected in meristem arrest and radialization of vascular bundles in cotyledons. In addition, *hd-zipIII* loss-of-function mutant plants have lower levels of *ZPR* and *AGO10* expression, indicative of positive regulation by HD-ZIPIIIs. Because AGO10 is able to capture *microRNA165/6* and thereby protect *HD-ZIPIIIs* from microRNA-dependent degradation, REV establishes a direct positive feedback loop allowing *HD-ZIPIII* transcripts to accumulate. In addition, REV regulates expression of the *LITTLE ZIPPER* genes, establishing a direct negative feedback loop via microProtein-directed protein inhibition. We propose that HD-ZIPIII transcription factors can directly influence their activity state by controlling positive and negative feedback loops, which is important for the regulation of biological processes such as meristem maintenance or polarity set up in leaves. Uncoupling these feedback loops by mutation or in transgenic overexpression approaches strongly affects developmental processes regulated by HD-ZIPIIIs emphasizing the biological importance of these feedback loops.

2. Results

2.1. *An inducible system to study REVOLUTA DNA-binding*

We previously showed that transgenic plants constitutively expressing a microRNA-resistant form of the REVOLUTA transcription factor (REVd) fused to the glucocorticoid receptor (GR), can be used to create developmental defects by inducing the translocation of the chimeric GR-REVd protein from the cytoplasm to the nucleus, by treating plants with Dexamethasone (DEX) (Wenkel et al., 2007). In transcriptome profiling experiments, using microarrays, we were able to identify the *LITTLE ZIPPER* genes being transcriptionally regulated REV (Wenkel et al., 2007). In order to being able to perform efficient chromatin-immunoprecipitations, to demonstrate binding of GR-REVd to the chromatin of potential target genes, we have constructed plants constitutively expressing the GR-REVd protein with an additional FLAG-epitope at the GR moiety. Induction of FLAG-GR-REVd by DEX results in the same developmental defects as observed for the GR-REVd inducible line (Fig. 1a). Using a ChIP-Seq approach, we were able to identify a number of direct REV target genes (Brandt et al., 2012).

2.2. *Identification of AGO10 as a direct target gene of REV*

Interestingly, the *ARGONAUTE10/PINHEAD/ZWILLE* gene (in the following referred to as *AGO10*) is among the list of putative target genes regulated by REV. To confirm binding of REV

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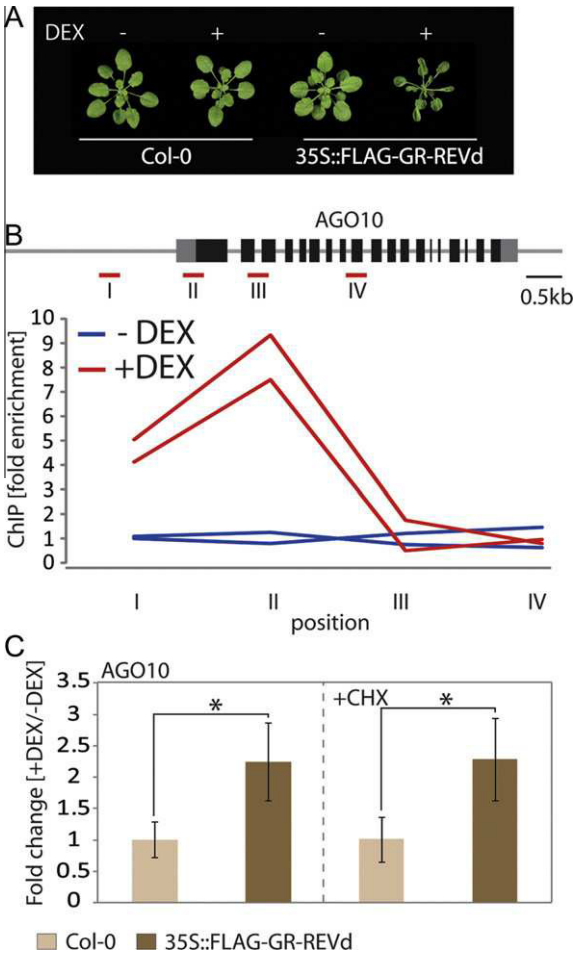

Fig. 1 – REVOLUTA directly regulates *AGO10* expression. (a) Induction of REVOLUTA causes adaxialization of leaves (Col and GR-REV +/−DEX). Plants were cultivated in long day conditions and after the production of the first true leaves sprayed daily with a 50 μM DEX solution or a mock substrate for 2 weeks. (b) REV binds to the *AGO10* promoter. The gene model depicts the organization of the AGO10 locus. Protein coding exons are in black, UTRs in grey. Chromatin-immunoprecipitations, two biological replicates, were carried out with *35S::FLAG-GR-REVd* plants either induced with DEX (red lines) or a mock substrate (blue lines). Four different genomic regions were tested (I–IV) by qPCR. Plotted is the fold enrichment normalized to the non-induced control IPs. (c) *AGO10* expression can be regulated by REV. Real-time quantitative RT-PCR experiments showing expression changes of *AGO10* in Col-0 (light brown) and 35S::FLAG-GR-REVd (dark brown) in response to DEX-induction. Plotted are average expression levels of three independent biological replicates normalized to actin of the ratio +DEX versus −DEX treatments, with standard error. Asterisk: $p < 0.01$.v Bars on the right show expression changes in plants pre-treated with Cycloheximide (CHX). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to the chromatin of *AGO10*, we carried out independent chromatin-immunoprecipitations of transgenic *35S::FLAG-GR-REVd* plants either treated with DEX or a mock substrate. Subsequent qPCR reactions confirmed our ChIP-Seq data, demonstrating that REV indeed interacts with the chromatin of *AGO10* and binds to a region located in the 5′UTR (Fig. 1b). Because from binding to the chromatin, a positive or negative regulation cannot be inferred, we performed DEX-induction experiments with Col-0 wild type plants and transgenic *35S::FLAG-GR-REVd* plants. Expression of *AGO10* is significantly increased in induced *35S::FLAG-GR-REVd* plants compared to wild type plants, revealing that REV is both a direct and positive upstream regulator of *AGO10* expression (Fig. 1c). Furthermore, the induction also occurs in the presence of the protein biosynthesis inhibitor cycloheximide (CHX), supporting the direct nature of this regulation (Fig. 1c). Taken together, we show that REV interacts with the chromatin of *AGO10* and directly and positively influences *AGO10* expression.

2.3. *REVOLUTA can directly regulate ZPR expression*

We have previously shown that REV is able to induce expression of all four *LITTLE ZIPPER* genes (Wenkel et al., 2007). It remained unclear whether the regulation of the *LITTLE ZIPPERs* by REV is of direct or indirect nature. Our ChIP-Seq study revealed that REV is able to bind the chromatin of all *ZPR* genes. Here, we exemplary demonstrate that REV is able to bind to the chromatin of the *ZPR3* gene (Fig. 2a). By using different primer pairs amplifying regions spanning the whole *ZPR3* locus, we can show that a binding maximum exists in the first intron close to the translational start site (Fig. 2a). As mentioned before, all *ZPR* genes were shown to be regulated by REV (Wenkel et al., 2007). We tested whether positive regulation of *ZPR* gene expression is also possible in our newly constructed transgenic *35S::FLAG-GR-REVd* plants. Upon DEX application, expression of *ZPR1*, *ZPR3* and *ZPR4* is strongly induced in *35S::FLAG-GR-REVd* plants compared to the wild type control, while expression of *ZPR2* is only moderately affected (Fig. 2b). Because it still remained unclear, whether regulation of the expression of the *ZPR* genes is of direct nature, we examined DEX-induced expression changes in conditions of inhibited protein biosynthesis, by pre-treating plants with cycloheximide (CHX). Even in conditions of inhibited protein biosynthesis (by CHX) REV is still able to significantly up-regulate *ZPR1*, *ZPR3* and *ZPR4* expression (Fig. 2b). It is important to note that the levels of *ZPR* induction is lower in plants pre-treated with CHX, suggesting that other factors might be required to induce *ZPR* expression to very high levels. Taken together, these findings confirm that REV is a direct and positive regulator of *ZPR1*, *ZPR3* and *ZPR4* expression.

The LITTLE ZIPPER proteins are plant specific microProteins that are able to interact with the much larger HD-ZIPIII proteins and trap these into non-functional complexes (Kim et al., 2008; Staudt and Wenkel, 2011; Wenkel et al., 2007). For ZPR3 it was shown, that the formation of ZPR3/REV heterodimers prevents REV from binding DNA (Wenkel et al., 2007). In summary, we show that REV can induce expression of all *ZPR* genes and the up-regulation of *ZPR1*, *ZPR3* and *ZPR4* seems to be of direct nature.

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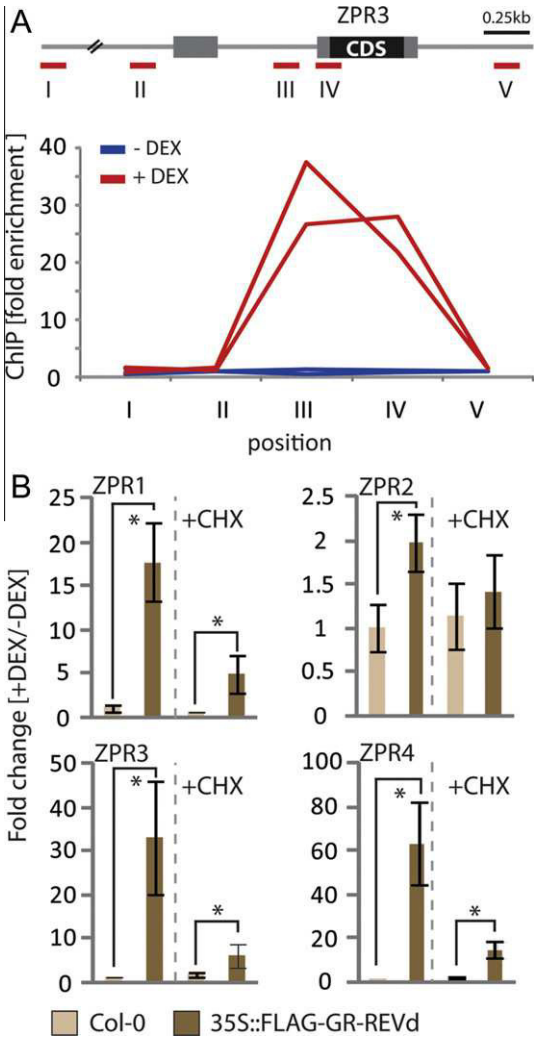

Fig. 2 – REVOLUTA directly regulates expression of *LITTLE ZIPPER* genes. (a) REV binds to the promoter of the *LITTLE ZIPPER3* gene. Chromatin-immunoprecipitation experiments with two biological replicates for *35S::FLAG-GR-REVd* without DEX (blue lines) and *35S::FLAG-GR-REVd* with DEX (red lines) plants testing the *ZPR3* locus. Genomic regions were tested with five primer pairs (I–V) by qPCR. Y-axis shows the fold enrichment normalized to the non-induced IPs. Gene maps above the charts show the location of the regions that were tested. Bar represents 0.25 kb. (b) Expression of all *LITTLE ZIPPER* genes is regulated by REV. Real-time quantitative RT-PCR experiments showing expression changes of *ZPR1*, *ZPR2*, *ZPR3* and *ZPR4* in response to DEX-induction. Plotted are fold changes in response to DEX in Col-0 (light brown) and the inducible 35S::GR-REVd transgenic line (dark brown) of the average of three independent biological replicates with standard error. Bars on the left show expression changes in the absence of the protein biosynthesis inhibitor cycloheximide, whereas bars on the right show expression changes in plants pre-treated with cycloheximide (+CHX). Asterisk: $p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. *ago10 and hd-zipIII mutant plants share phenotypic similarities*

AGO10 is required for proper organization of the shoot apical meristem. In plants harboring loss-of-function alleles of *AGO10*, stem cells in the shoot apex cannot be maintained, resulting in consumption of the apical stem cells (Lynn et al., 1999; Moussian et al., 1998). In *ago10* mutant plants, the meristem often terminates before the production of leaves, but occasionally one or two strongly radialized leaves or one terminal leaf are produced (Lynn et al., 1999; Moussian et al., 1998). The shoot meristem defect of *ago10* mutant plants is reminiscent of strong *ZPR3*-overexpression lines. When compared side-by-side, no difference between *35S::FLAG-ZPR3* and *ago10* plants can be observed (Fig. 3a and b). The same is true for high overexpression of *microRNA165*, which also causes consumption of the apical stem cells (Zhou et al., 2007).

2.5. *Polarity defects of vasculature observed in hd-zipIII and ago10 mutant plants*

Adaxialized leaves exhibit a strong downward curling of the leaf blade and vascular strands show polarity defects manifested in phloem tissue surrounding the xylem strands. The vasculature of wild type plants shows a typical sandwich-like structure composed of phloem at the bottom, cambium cells in the middle and xylem tissue on top. When compared side-by-side, both *35S::FLAG-ZPR3* transgenic plants and *ago10* mutant plants show radialized vascular strands with abaxialized characteristics (Fig. 3c). The phenotype of the *ago10* mutation is more severe and the vascular strands have no obvious organization. Overexpression of microRNA165 has been shown to also cause severe developmental defects and radialization of transport elements (Zhou et al., 2007).

2.6. *Expression of AGO10 and LITTLE ZIPPER genes are altered in hd-zipIII mutant seedlings*

We have shown that both *AGO10* and *ZPR3* are direct and positive targets of the REVOLUTA transcription factor. To further corroborate the finding that *AGO10*, *ZPR1* and *ZPR3* are *bona fide* REV target genes, we have analyzed their expression levels in different *hd-zipIII* mutant plants (Fig. 4). *AGO10* expression is significantly lower in both *rev-5* and *rev-6* mutant plants compared to wild type control plants, indicating that *AGO10* expression is mainly regulated by REV (Fig. 4). An even stronger reduction of *AGO10* mRNA levels was observed in transgenic plants expressing *35S::FLAG-ZPR3*, which points towards a redundant regulation by other HD-ZIPIII proteins. No reduction in expression was observed in transgenic plants overexpressing *miR165a* (*35S::miR165a*). It is important to note that the transgenic line overexpressing *microRNA165a* (Kim et al., 2010) shows only moderate developmental defects and also HD-ZIPIII levels are only somewhat lower. We therefore also investigated the levels of expression in plants carrying mutations in more *HD-ZIPIII* genes. Here we find that the expression of *AGO10* is slightly higher in plants carrying mutations in *PHB* and *PHV* and are heterozygote for *REV*

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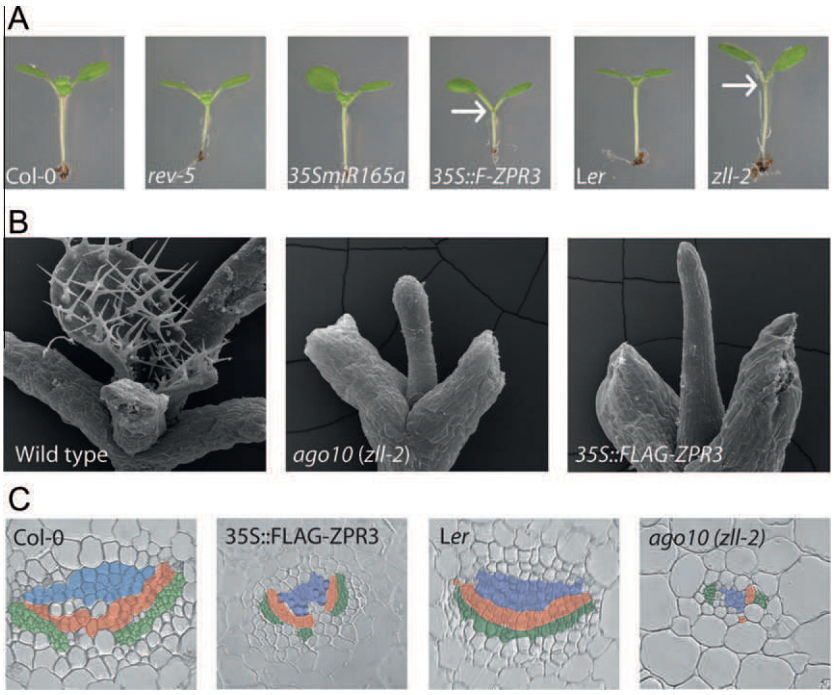

Fig. 3 – Mutations in *hd-zipIII* and *ago10* cause severe phenotypic defects. (a) Comparative growth analysis of hd-zipIII and ago10 mutant plants with corresponding wild type plants. Both *35S::FLAG-ZPR3* and *ago10* (*zll-2*) mutant plants show termination of the shoot apical meristem (arrow shows the terminated shoot apical meristems). (b) Scanning electron micrographs of apices from seedlings shown in a. Both *ago10* and *35S::FLAG-ZPR3* plants have terminated meristems and only produce one radial leaf compared to the wild type shoot apex (here: *Ler*). (c) Sections through petioles of Col-0, *35S::FLAG-ZPR3*, Ler, *ago10* (*zll-2*). The vasculature of wild type Col-0 and *Ler* plants show the typical sandwich structure: tissue containing phloem cells (green) at the bottom, cambium cells (red) in the middle and tissue containing xylem elements (blue) on top. *35S::FLAG-ZPR3* transgenic plants show abaxialized vascular strands with phloem nearly surrounding the xylem whereas the structure of *ago10* vascular is completely disorganized with abaxialized features. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(*phb phv rev*/+). In the *phb phv rev* triple mutant, *AGO10* expression is not detectable, which is most likely due to the complete loss of the apical meristem, as these seedlings develop pin-like and arrest early in development.

Endogenous *ZPR1* and *ZPR3* expression levels are reduced in transgenic plants ectopically mis-expressing *ZPR3* (*35S::FLAG-ZPR3*), indicating that in these plants HD-ZIPIII activity is more strongly depleted. Expression levels of *ZPR1* and *ZPR3* are strongly affected in *rev-6*, *phb phv rev*/+ and *phb phv rev* triple mutant plants indicating that REV is a major regulator of both *ZPR1* and *ZPR3* expression. Taken together, we can conclude that *AGO10*, *ZPR1* and *ZPR3* are *bona fide* REVOLUTA target genes because induction of REV causes an increase in expression and more importantly, their expression is lower in plants having either decreased levels of *HD-ZIPIII* mRNA or reduced HD-ZIPIII activity.

3. Discussion

3.1. *AGO10 and ZPR3 are a bona fide REVOLUTA target genes*

We find that *AGO10*, *ZPR1* and *ZPR3* expression are both positively and directly regulated by REVOLUTA. In transgenic plants expressing *35S::FLAG-GR-REVd*, expression of *AGO10* and all *ZPR* genes can be induced by the application of DEX. The induction of expression also takes place in plants pre-treated with cycloheximide, indicating that the transcriptional regulation is of direct nature (Figs. 1 and 2). It is important to note, that levels *ZPR* up-regulation is reduced in cycloheximide pre-treated plants (Fig. 2), suggesting that either REV requires other proteins for the up-regulation of these targets or that REV is modified at the post-translational level allowing high level of up-regulation. Using chromatin-immunoprecipitations, we show that REV interacts with the chromatin of both *ZPR3* and *AGO10* further supporting a direct role in the control of gene expression (Figs. 1 and 2). Finally, we see a reduction of both *AGO10* and *ZPR3* in transgenic plants overexpressing the ZPR3 microProtein implying that both genes are *bona fide* direct targets of REV (Fig. 4). Because *AGO10* expression is significantly lower in *rev*-5 mutant plants (Fig. 4), we can assume that REV is a major regulator of *AGO10* expression. In plants carrying the *rev-6* mutant allele, *AGO10* mRNA is slightly reduced while *phb phv rev*/+ plants show a slight increase of *AGO10* expression. These increased *AGO10* levels might reflect the partially antagonistic nature of HD-ZIPIII function (Prigge et al., 2005). When three *HD-ZIPIII* genes are mutated (as in *phb phv rev* triple mutant plants), these seedlings develop pin-formed and arrest soon after germination. Expression of *AGO10* is not detectable in these mutant

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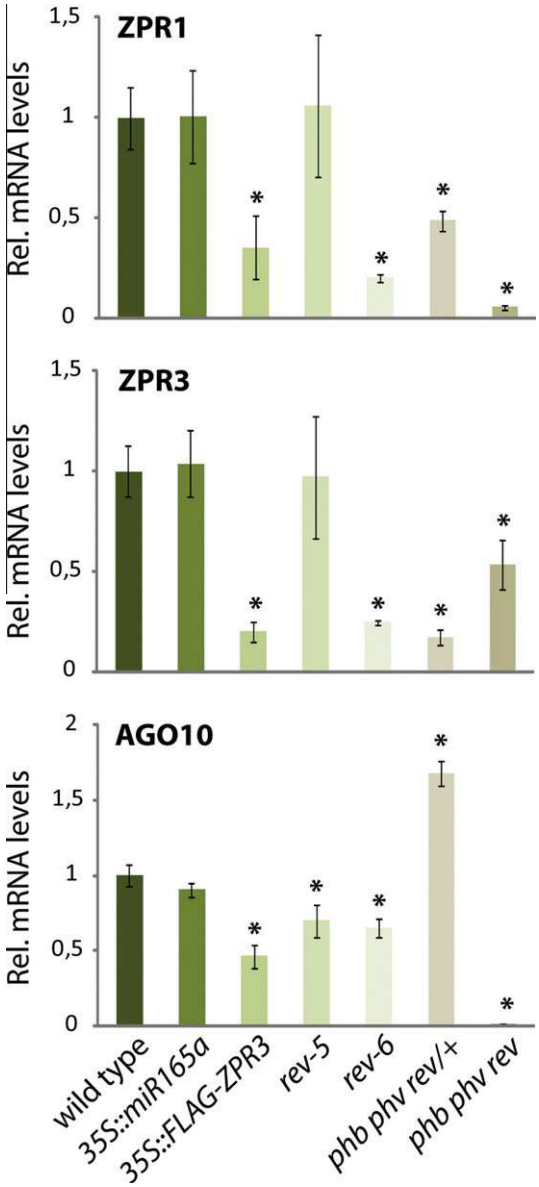

Fig. 4 – *AGO10*, ZPR1 and ZPR3 expression are altered in *hd-zipIII* mutant plants. Expression of *AGO10* and *ZPR3* was analyzed in mutants with either compromised *HD-ZIPIII* expression (*rev-5*, *rev-6*, *phb phv rev/+*, *phb phv rev* and *35S::miR165a*) or inhibited HD-ZIPIII protein activity (*35S::FLAG-ZPR3*). Plotted are expression levels relative to wild type including standard errors of the mean of three individual biological experiments. Asterisk: $p < 0.05$.

seedlings, for which the missing shoot apical meristem might be causal. No down-regulation of *AGO10*, *ZPR1* or *ZPR3* expression was observed in transgenic plants overexpressing *miR165a* (Fig. 4), which is most likely due to weak overexpression phenotype of this particular line.

3.2. *Transgenic plants overexpressing ZPR-type microProteins resemble ago10 mutant plants*

Transgenic plants overexpressing the ZPR3-type microProtein show, in weak overexpression plants, leaf polarity defects while strong overexpression plants exhibit a meristem arrest phenotype. Conversely, plants in which both *ZPR3* and *ZPR4* are mutated show an enlarged and severely disorganized shoot apical meristem (Kim et al., 2008). By growing *35S::FLAG-ZPR3* and *ago10* mutant plants side-by-side, we show that both mutant phenotypes strongly resemble each other. It is interesting to note, that the strong *ago10* mutant phenotype is only visible in the Landsberg *erecta* (*Ler*) ecotype, while in Col-0 *AGO10* appears to be expendable. Furthermore, *ago10* mutant plants have the ability to induce adventitious shoot meristems later in development and progress to the reproductive phase, while *35S::FLAG-ZPR3* plants with terminated meristems will senesce and do not reproduce. This indicates, that repressing HD-ZIPIII protein function by microProteins is, most likely, more potent than reducing *HD-ZIPIII* mRNA levels by overexpressing microRNAs.

3.3. *REVOLUTA controls HD-ZIPIII expression and protein activity via positive and negative feedback loops*

Using a chromatin-immunoprecipitation/high throughput sequencing approach, we have identified *AGO10* as a direct target of REV. Expression analysis revealed that REV can also upregulate *AGO10* expression while in *hd-zipIII* mutant plants *AGO10* expression is lower compared to wild type plants. AGO10 can tightly interact with microRNAs *miR165/6*, which are known to target *HD-ZIPIIIs* (Zhu et al., 2011). Because AGO10 keeps *miR165/6* in an inactive state, *HD-ZIPIII* mRNA levels can increase and may thus potentiate this positive feedback regulation (Fig. 5). When *AGO10* activity is lost by mutation (in the *Ler* background) the shoot meristem is severely compromised and the apical stem cell population is lost. This phenotype might be due to a strong down-regulation of *HD-ZIPIII* mRNAs, most likely by *miR165/6* and AGO1. In addition to *AGO10*, REV also directly up-regulates the expression of genes encoding the ZPR-type microProteins. In contrast to AGO10, ZPR-type microProteins establish a negative feedback loop by sequestering HD-ZIPIII proteins into non-functional heterodimeric complexes (Fig. 5). In case of *ZPR*-overexpression shoot defects similar to the *ago10* mutation are observed, indicating that HD-ZIPIII activity is required for the maintenance of the apical stem cells in plants.

Thus, REV directly establishes two different feedback mechanisms channeling back on its own activity. Positive regulation is established via microRNA inhibition and negative regulation via microProtein action. Further characterization of the interconnection of these feedback loops in the wild type plant will yield a better understanding on the role of HD-ZIPIII proteins in both stem cell maintenance and in development in general.

4. Experimental procedures

4.1. *Plant material and phenotypic analysis*

For efficient chromatin-immunoprecipitations, we have created transgenic *35S::FLAG-GR-REVd* plants. The glucocorticoid receptor was cloned in frame to the FLAG epitope in the *pJAN33* vector (Weigel et al., 2003) using the KpnI restriction site, in the following termed *pJAN33GR*. Different mutant and

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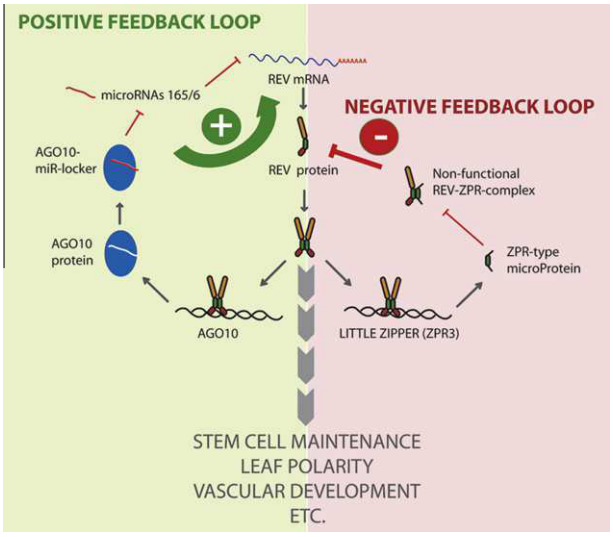

Fig. 5 – Interlocking positive and negative feedback-loops regulate stem-cell homeostasis in *Arabidopsis*. Model for the feedback loops established by AGO10 and ZPR3. Active homodimeric HD-ZIPIII proteins regulate developmental processes such as leaf polarity and stem cell maintenance. The positive feedback loop is established by up-regulation of *AGO10* gene expression. The AGO10 protein can capture microRNAs 165/6 allowing *HD-ZIPIII* transcripts to accumulate. In case of ZPR-induction, HD-ZIPIII protein function is attenuated because the protein complex consisting of REV and ZPR can no longer bind DNA.

transgenic plants were used to analyze plants with reduced or depleted REV activity: the *35S-miR165a* seeds were kindly provided by Sang-Bong Choi (Myongji University, South Korea); *rev-5* (A260 V) a strong EMS allele (Otsuga et al., 2001) and 35S::FLAG-ZPR3 plants (this line was generated by SW in Kathryn Barton's laboratory). *rev-6*, *phb phv rev/+* and *phb phv rev* were described previously (Prigge et al., 2005). The *zll-2* EMS mutant was previously characterized by Moussian et al. (1998).

4.2. *Histology and SEM microscopy*

Petioles of 3-week-old plants were prefixed with 90% ice cold acetone for 2 h following transfer into fixative (50 mM NaPh pH 7.2; 1% glutaraldehyde; 4% formaldehyde) for 2 days. Afterwards, the petioles were dehydrated in an ethanol series (30%/50%/70% each for 2 h) and finally stored in 100% ethanol prior embedding in Technovit (Heraeus). Two-micron sections were cut using a Leica microtome. Sections were stained with toluidine blue.

Scanning electron microscopy was done on 10-day old seedlings. Plants were dissected, fixed in methanol, washed with ethanol twice, critical point dried and mounted. After gold/palladium coating, plants were examined on a Hitachi S800 electron microscope.

4.3. *Gene expression analysis*

For gene expression analysis and chromatin-immunoprecipitation experiment, plants (Col-0; pJAN33-GR-REVd) were grown for 10 days in liquid culture medium [MS (4.3 g/l; Duchefa), MES (0.3 g/l; Duchefa) and Sucrose (5 g/l; Roth), pH 5.7] in continuous white light at 22 °C. To induce the translocation of the chimeric GR-REVd protein from the cytoplasm to the nucleus, plants were treated with either 50 μM dexamethasone (Sigma) or a mock solution for 60 min for gene expression analysis and for 45 min for chromatin-immunoprecipitation experiments. Altered gene expression in Col-0, *rev5*, *pJAN33 ZPR3*, *35S-miR165a*, *rev-6*, *phb phv rev/+* and *phb phv rev* was analyzed in 14 days old seedlings grown on soil under long-day condition (16 h white light, 8 h darkness) at 22 °C. Expression of *rev-6*, *phb phv rev/+* and *phb phv rev* was quantified relative to the corresponding wild type (here Col *er-2*). RNA was isolated using GeneMATRIX universal RNA purification kit [roboklon] following manufacturer's recommendation. 1 μg of purified RNA was used for reverse transcription using Fermentas Revert Aid Reverse Transcriptase with oligo-dT primers. Real-time quantitative PCRs were carried out using the Fermentas SYBR Green qPCR master mix on a Biorad CFX384. Gene expression levels were calculated using the delta-Ct method and a standard curve relative to actin. To detect endogenous levels of *ZPR3* expression in plants ectopically overexpressing the *ZPR3* coding sequence (*pJAN33-ZPR3*) we use a forward primer spanning the first intron and amplifying a part of the non-translated exon 1.

4.4. *Chromatin-immunoprecipitation*

Chromatin-immunoprecipitation experiments were carried out as described by Kwon et al. (2005), except that anti-FLAG M2 magnetic beads (Sigma) were used and immunoprecipitations were only performed for 2 h.

4.5. *Oligonucleotides*

(a) Gene expression analysis
qAGO10f:ATCACGAGAACGGGAAAGAA; qAGO10r:CATGCCTGAGACTTCACACA; qZPR1f:CGTGGAGAATCAAAACATCA; qZPR1r:CCTTGCTTGTAAAACCCAAA; qZPR2f:CTCACCAGCAGGAGGAGAAG; qZPR2r:CAGGGGAGTATTTTGGGTGA; qZPR3f:CACTCCTTCCCAAAAGCAAG; qZPR3r:TGTCCAGAAGCAGAGCTTGA; qZPR4f:GGAGAACGAGAGGTTGAGGA; qZPR4r:CCAGAAGCAGAGCTTGATGA

(b) ChIP-PCR
PNH-I-F:TTGCTGCCATAAACCAAACA; PNH-I-R:CAGGCTCTCAGCCTCATCTC; PNH-II-F:GCCAAGGAAGGGATCAGTTT; PNH-II-R:TGGTTTTTGGATTGTGGTGC; PNH-III-F:CGGTATCATCAATGGCCCTA; PNH-III-R:GACAATCTGCCCGTTTACCA; PNH-IV-F/R (qAGO10f/r); ZPR3-I-F:GGGCAAACGAACGAGTTTTA; ZPR3-I-R:GTTTGGACTTTGGAGCCGTA; ZPR3-II-F:CGATGAAGAGCCAAAGGAAG; ZPR3-II-R:GCCGCAAGAAGAGAGAGAGA; ZPR3-III-F:CAACACTCCTTCCCAAAAGG; ZPR3-III-R:GGGTTTGTCTTCACGTTAGTTG; ZPR3-IV-F:AATCATGTTCTTCTTCTCTCTTTGA; ZPR3-IV-R:ATCACACATGGGTTGTGCAG; ZPR3-V-F:TCGGAGATGGTGGGAATCTA; ZPR3-V-R:GCCCGAAACTTGCTTCTCTA

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REFERENCES

Brandt, R., Salla-Martret, M., Bou-Torrent, J., Musielak, T., Stahl, M., Lanz, C., Ott, F., Schmid, M., Greb, T., Schwarz, M., Choi, S.-B., Kathryn Barton, M., Reinhart, B.J., Liu, T., Quint, M., Palauqui, J.-C., Martínez-García, J.F. and Wenkel, S., 2012. Genome-wide binding-site analysis of REVOLUTA reveals a link between leaf patterning and light-mediated growth responses. The Plant Journal, in press.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., Voinnet, O., 2008. Widespread translational inhibition by plant miRNAs and siRNAs. Science 320, 1185–1190.

Carlsbecker, A., Lee, J.Y., Roberts, C.J., Dettmer, J., Lehesranta, S., Zhou, J., Lindgren, O., Moreno-Risueno, M.A., Vaten, A., Thitamadee, S., Campilho, A., Sebastian, J., Bowman, J.L., Helariutta, Y., Benfey, P.N., 2010. Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature 465, 316–321.

Eun, C., Lorkovic, Z.J., Naumann, U., Long, Q., Havecker, E.R., Simon, S.A., Meyers, B.C., Matzke, A.J.M., Matzke, M., 2011. AGO6 functions in RNA-mediated transcriptional gene silencing in shoot and root meristems in *Arabidopsis thaliana*. PLoS One 6, e25730.

Gao, Z., Liu, H.-L., Daxinger, L., Pontes, O., He, X., Qian, W., Lin, H., Xie, M., Lorkovic, Z.J., Zhang, S., Miki, D., Zhan, X., Pontier, D., Lagrange, T., Jin, H., Matzke, A.J.M., Matzke, M., Pikaard, C.S., Zhu, J.-K., 2010. An RNA polymerase II- and AGO4-associated protein acts in RNA-directed DNA methylation. Nature 465, 106–109.

Havecker, E.R., Wallbridge, L.M., Hardcastle, T.J., Bush, M.S., Kelly, K.A., Dunn, R.M., Schwach, F., Doonan, J.H., Baulcombe, D.C., 2010. The *Arabidopsis* RNA-directed DNA methylation argonautes functionally diverge based on their expression and interaction with target loci. The Plant Cell Online 22, 321–334.

Ji, L., Liu, X., Yan, J., Wang, W., Yumul, R.E., Kim, Y.J., Dinh, T.T., Liu, J., Cui, X., Zheng, B., Agarwal, M., Liu, C., Cao, X., Tang, G., Chen, X., 2011. ARGONAUTE10 and ARGONAUTE1 regulate the termination of floral stem cells through two microRNAs in *Arabidopsis*. PLoS Genetics 7, e1001358.

Juarez, M.T., Kui, J.S., Thomas, J., Heller, B.A., Timmermans, M.C.P., 2004. MicroRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. Nature 428, 84–88.

Kidner, C.A., Martienssen, R.A., 2004. Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. Nature 428, 81–84.

Kim, H.-S., Kim, S.J., Abbasi, N., Bressan, R.A., Yun, D.-J., Yoo, S.-D., Kwon, S.-Y., Choi, S.-B., 2010. The DOF transcription factor Dof5.1 influences leaf axial patterning by promoting Revoluta transcription in *Arabidopsis*. The Plant Journal 64, 524–535.

Kim, Y.S., Kim, S.G., Lee, M., Lee, I., Park, H.Y., Seo, P.J., Jung, J.H., Kwon, E.J., Suh, S.W., Paek, K.H., Park, C.M., 2008. HD-ZIP III activity is modulated by competitive inhibitors via a feedback loop in *Arabidopsis* shoot apical meristem development. The Plant Cell 20, 920–933.

Kwon, C.S., Chen, C., Wagner, D., 2005. WUSCHEL is a primary target for transcriptional regulation by SPLAYED in dynamic control of stem cell fate in *Arabidopsis*. Genes & Development 19, 992–1003.

Liu, Q., Yao, X., Pi, L., Wang, H., Cui, X., Huang, H., 2009. The ARGONAUTE10 gene modulates shoot apical meristem maintenance and establishment of leaf polarity by repressing miR165/166 in *Arabidopsis*. The Plant Journal 58, 27–40.

Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P., Barton, M.K., 1999. The PINHEAD/ZWILLE gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the ARGONAUTE1 gene. Development 126, 469–481.

Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., Zamore, P.D., Barton, M.K., Bartel, D.P., 2004. MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5[prime] region. EMBO Journal 23, 3356–3364.

Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G., Laux, T., 1998. Role of WUSCHEL in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell 95, 805–815.

McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., Barton, M.K., 2001. Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. Nature 411, 709–713.

Montgomery, T.A., Howell, M.D., Cuperus, J.T., Li, D., Hansen, J.E., Alexander, A.L., Chapman, E.J., Fahlgren, N., Allen, E., Carrington, J.C., 2008. Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. Cell 133, 128–141.

Moussian, B., Schoof, H., Haecker, A., Jurgens, G., Laux, T., 1998. Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. EMBO Journal 17, 1799–1809.

Otsuga, D., DeGuzman, B., Prigge, M.J., Drews, G.N., Clark, S.E., 2001. REVOLUTA regulates meristem initiation at lateral positions. The Plant Journal 25, 223–236.

Prigge, M.J., Otsuga, D., Alonso, J.M., Ecker, J.R., Drews, G.N., Clark, S.E., 2005. Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in *Arabidopsis* development. The Plant Cell 17, 61–76.

Rowley, M.J., Avrutsky, M.I., Sifuentes, C.J., Pereira, L., Wierzbicki, A.T., 2011. Independent chromatin binding of ARGONAUTE4 and SPT5L/KTF1 mediates transcriptional gene silencing. PLoS Genetics 7, e1002120.

Smith, Z.R., Long, J.A., 2010. Control of *Arabidopsis* apical-basal embryo polarity by antagonistic transcription factors. Nature 464, U121–U423.

Staudt, A.-C., Wenkel, S., 2011. Regulation of protein function by microProteins. EMBO Reports 12, 35–42.

Vaucheret, H., Vazquez, F., Crete, P., Bartel, D.P., 2004. The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes & Development 18, 1187–1197.

Weigel, M., Varotto, C., Pesaresi, P., Finazzi, G., Rappaport, F., Salamini, F., Leister, D., 2003. Plastocyanin is indispensable for photosynthetic electron flow in *Arabidopsis thaliana*. Journal of Biological Chemistry 278, 31286–31289.

Wenkel, S., Emery, J., Hou, B.H., Evans, M.M.S., Barton, M.K., 2007. A feedback regulatory module formed by LITTLE ZIPPER and HD-ZIPIII genes. Plant Cell 19, 3379–3390.

Yadav, R.K., Perales, M., Gruel, J., Girke, T., Joensson, H., Reddy, G.V., 2011. WUSCHEL protein movement mediates stem cell homeostasis in the *Arabidopsis* shoot apex. Genes & Development 25, 2025–2030.

Zhao, Z., Andersen, S.U., Ljung, K., Dolezal, K., Miotk, A., Schultheiss, S.J., Lohmann, J.U., 2010. Hormonal control of the shoot stem-cell niche. Nature 465, 1089–1092.

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Zhou, G.K., Kubo, M., Zhong, R.Q., Demura, T., Ye, Z.H., 2007. Overexpression of miR165 affects apical meristem formation, organ polarity establishment and vascular development in *Arabidopsis*. Plant and Cell Physiology 48, 391–404.

Zhu, H., Hu, F., Wang, R., Zhou, X., Sze, S.-H., Liou, Lisa W., Barefoot, A., Dickman, M., Zhang, X., 2011. *Arabidopsis* argonaute10 specifically sequesters miR166/165 to regulate shoot apical meristem development. Cell 145, 242–256.

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5. Discussion

5.1. Multiple feedback loops regulate stem cell homeostasis and leaf patterning

We identified a set of putative target genes of REVOLUTA, a representative of the *HD-ZIPII* gene family, using a ChIP-Seq approach. Previous research has mainly focused on the role of *REVOLUTA* in patterning-related processes in leaves, stems and roots (Emery et al., 2003; Ilegems et al., 2010; Prigge et al., 2005). Because in our dataset we find enrichment for genes acting in environment-controlled signaling pathways, we have focused our attention on shade-avoidance responses.

Through mutational analysis and subsequent genetic studies, the function of *REV* and other *HD-ZIPIII* genes was characterized. However, the regulation of genes downstream of HD-ZIPIII and the upstream regulation of *HD-ZIPIII* genes are still poorly understood. One of the factors known to directly control *REV* expression is DOF5.1 (DNA BINDING WITH ONE FINGER5.1). The characterization of *dof5-1D* overexpression plants revealed that DOF5.1 affects establishment of abaxial leaf polarity and transgenic plants show severely adaxialized leaves (Kim et al., 2010). DOF5.1 binds to the *REV* 5'-promotor region to induce *REV* transcription. Hence, plants overexpressing *DOF5.1* have up-ward curled leaves opposite to *rev* mutant plants (Kim et al., 2010).

Other regulators are SEUSS (SEU) and AINTEGUMENTA (ANT), which control *REV* expression positively during gynoecium development (Azhakanandam et al., 2008). However, their effect on polarity is not limited to reproductive tissues, both genes are also involved in adaxialization of vegetative tissues. Consequently, *seu ant* double mutant plants show partial loss of adaxial identity in the leaf epidermis (Azhakanandam et al., 2008). Both *REV* regulation by DOF5.1 and by SEU and ANT set these transcription factors upstream of *REV* function and support the role of *class III HD-ZIPs* as key regulators of adaxial cell fate.

In addition to the transcriptional regulation, *class III HD-ZIP* mRNA levels are under control by members of the microRNA families *165* and *166* (*miR165/166*)

(Emery et al., 2003). Both miRNA families are highly specific for *HD-ZIPIII* mRNA and bind to the complementary region encoding the START domain of HD-ZIPIII proteins. Plants overexpressing *miR165a* show decreases in mRNA levels of *HD-ZIPIII* resulting in strong abaxialized leaves and vasculature and loss of shoot apical meristem SAM (Zhou et al., 2007). ARGONAUTE proteins guide microRNAs to their targets, thereby ARGONAUTEs are involved in miRNA-mediated gene silencing. AGO1 and AGO10 have specificity for miR165/166 whereby they are involved in establishing leaf polarity. While AGO1 proteins guide *miR165/166* to their targets, AGO10 protein has a higher specificity for these miRNAs, sequesters them and prevents them from inducing *HD-ZIPIII* degradation (Zhu et al., 2011). While plants carrying mutations in *AGO1* show adaxialized leaf phenotypes, *ago10* mutant plants exhibit abaxialized characteristics (Mallory et al., 2009; Moussian et al., 1998) and defects in SAM maintenance, thereby they phenocopy transgenic plants overexpressing *miR165/166* (Yao et al., 2009) and *HD-ZIPIII* triple mutant plants (Prigge et al., 2005). We show that REV regulates *AGO10* in a direct positive manner to establish a previously unknown positive feedback loop. Besides this positive feedback loop, HD-ZIPIII proteins are regulated at the post-translational level by a negative feedback loop established by LITTLE ZIPPER microProteins (ZPR). LITTLE ZIPPER proteins form inactive heterodimers with HD-ZIPIII proteins (Wenkel et al., 2007). We could show that the positive gene expression of *ZPR* genes by active HD-ZIPIII homodimers is of direct nature and that elevated ZPR protein levels result also in phenotypes similar to plants overexpressing *miRNA165/166* (Yao et al., 2009) or *HD-ZIPIII* multiple mutant lines (Prigge et al., 2005). Other post-translational regulators of HD-ZIPIII activity include DRN and DRNL, two AP2 transcription factors which can form heterodimers with HD-ZIPIII proteins via the PAS domain (Chandler et al., 2007). Plants carrying mutations in *DRN* have cup-shaped leaves, a typical phenotype for strong adaxialization. Furthermore, *drn* mutants show additional defects in auxin flux during embryogenesis, a process strongly controlled by HD-ZIPIII proteins (Chandler et al., 2007). In conclusion, the posttranslational regulation of class III HD-ZIPs by LITTLE ZIPPERs or DRN/DRNL does not only affect leaf patterning but also auxin synthesis and transport.

5.2. Patterning factors in line with auxin synthesis control leaf development and shade avoidance

We could show that *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1*) and *YUCCA5* (*YUC5*), whose gene products encode for two enzymes involved in auxin biosynthesis, are directly and positively regulated targets of REV and that this regulation leads to an increased abundance of free auxin. Furthermore, KANADI1 controls gene expression of both enzymes in an opposite manner.

TAA1 is responsible for conversion of tryptophan to indole-3-pyruvic acid (IPA), a precursor of auxin, while the role of YUCCAs is complex and poorly understood. It is proposed that YUCCA enzymes act in the tryptamine pathway (TAM) of auxin biosynthesis (Tao et al., 2008). Recent results lead to the hypothesis that flavine monooxygenases-like YUCCA enzymes also act in the same tryptophan-dependent IPA-pathway for auxin synthesis as TAA1. Here, YUCCAs convert IPA to IAA downstream of TAA1 (Stepanova et al., 2011). The *taa1*-mutant plant phenocopies *yucca*-multiple mutant plants, supporting their role in the IPA-pathway (Won et al., 2011). For *TAA1* a role in shade-induced hypocotyl elongation was shown (Tao et al., 2008). Overexpression of *YUCCA1* partially rescues the *taa1*-mutant defect, further supporting their role in a common synthesis pathway (Won et al., 2011). We have shown that REV induces *TAA1* expression resulting in elevated levels of free auxin. Furthermore, in plants carrying a mutation in *TAA1*, overexpression of *REV* resulted in increased levels of free auxin. This increase can be explained by ectopic expression of *YUC5* in the *taa1* mutant background. Similar to overexpression of *YUC1*, YUC5 might also be able to partially complement the *taa1* mutant.

In addition, class III HD-ZIPs do not only act by inducing auxin-synthesis, they are also involved in the correct auxin-distribution by adjusting the flux direction of PIN-auxin transporters during patterning processes (Heisler et al., 2005; Ohashi-Ito and Fukuda, 2010; Zhong and Ye, 2001). This process also involves the KANADI transcription factors *KAN1*, *KAN2* and *KAN4*. Triple mutants lacking all three

genes display misexpression of *PIN1* auxin-transporter (Izhaki and Bowman, 2007). During embryogenesis *PIN1* expression is restricted to later vasculature and cotelydones in transition and heart stage in wild type plants, whereas in *kan124*-mutant plants expression of *PIN1* can be observed in cells of the future hypocotyl. As mentioned previously, REV protein can physically interact with either DORNROESCHEN (DRN) or DORNROESCHEN-LIKE (DRNL) (Chandler et al., 2007). Both DRN and DRNL strongly affect auxin transport and response. It is currently unknown how REV integrates different types of input from these auxin-mediated signaling pathways.

Auxin is known to control both initiation and outgrowth of leaf primodia. Patterns of PIN1-auxin transporter and DR5-auxin reporter overlap at points of primordia initiation (Heisler et al., 2005). The finding that the treatment of plants with the auxin transport inhibitor NPA causes a *pin*-mutant phenotype supports the theory that auxin transport is underlying primordia initiation (Reinhardt et al., 2000). These NPA-induced defects are reversible by local application of IAA. It appears that at points of auxin maxima new primordia are initiated, whereby not only the total abundance but rather the auxin gradient and efflux are crucial (Benková et al., 2003).

The cross-regulation of *TAA1* and *YUC5* by REV and KAN1 and the proposed polar transport of auxin mediated by PIN-auxin transporter under control of REV and KANADI transcription factors uncovers roles for both protein families in primordia initiation, outgrowth and patterning. Current models suggest that auxin transport and the subsequently established gradients are crucial for polarity-associated processes (Benková et al., 2003; Heisler et al., 2005). Our data supports a role for the spatial regulation of auxin-synthesis by factors involved in establishing polarity. Auxin-transport and establishment of gradients could be an after-effect. Current work involving genetic analysis in combination with genome-wide approaches is aimed at understanding the role of local auxin synthesis.

5.3. Cross-regulated target genes of the REV/KAN1 module control the shade avoidance syndrome SAS

REVOLUTA and KANADI1 play crucial roles in auxin-biosynthesis by controlling the IPA-pathway. In addition to genes encoding auxin biosynthetic enzymes, we also identified four *class II HD-ZIP* transcription factors directly and positively regulated by REVOLUTA. These *HD-ZIPII* genes (*HAT2*, *HAT3*, *ATHB2/HAT4*, *ATHB4*) were shown to be also regulated by auxin and known regulators of the shade avoidance syndrome SAS (Ciarbelli et al., 2008). Both the regulation of these *class II HD-ZIP* transcription factors as well as *TAA1* places *REV* upstream of the SAS. We performed gene expression studies to uncover the regulation of *HD-ZIPIII* by shade. In wild type plants treated with either white light or far-red enriched white light, we could observe a slight but significant increase for *REV* expression in shade-treated plants, gene expression of two other *HD-ZIPIII* transcription factors, *PHABULOSA* and *PHAVOLUTA*, remained unchanged. Whether also REV protein activity is modulated by shade requires further studies.

Two scenarios of shade-induced posttranslational regulation for REV seem plausible: 1/ Class III HD-ZIP proteins possess a MEKHLA-type PAS-domain (Mukherjee and Burglin, 2006) which might enable them to sense changes in light quality and/or redox-potential. It seems possible that REV protein itself might acts as a sensor for both.

2/ It seems conceivable that physical interactions of REV with other transcription factors involved in shade-induced signaling pathways might be affected by low red/far-red light.

In conclusion, the regulation of REV activity by shade is still unclear and has to be elucidated in future.

In addition, we could show that KAN1 represses gene-expression of both auxin-synthesizing enzymes and *HAT2*. While REV promotes hypocotyl elongation under low red/far-red conditions, KAN1 disrupts the shade response. We can

therefore conclude that the opposite regulation of *HAT2*, *TAA1* and *YUC5* by REV and KAN1 is crucial for a full shade response and patterning processes.

Future experiments should focus on the regulation of *REVOLUTA* by shade. In addition, the role of REV and KAN1 in auxin-regulated *HD-ZIPII* gene-expression should be examined for both patterning and shade-induced growth processes.

5.4. The shade-inducible HD-ZIPII/HD-ZIPIII module governs pattern formation in leaves

Class III HD-ZIPs and KANADI transcriptions factors control SAS by regulating *class II HD-ZIPs* and *TAA1* and *YUC5*. We also identified a new function for class II HD-ZIPs in leaf patterning processes. Both *HOMEOBOX PROTEIN OF ARABIDOPSIS THALIANA3* (*HAT3*) and *ARABIDOPSIS THALIANA HOMEOBOX4* (*ATHB4*) play crucial roles in establishing leaf polarity patterns. During early leaf development, *HAT3* overexpression causes phenotypic changes resembling *REV*-overexpression phenotypes with upward curled leaf-blades. Conversely, *hat3 athb4* double mutant plants display severe leaf patterning defects and have a highly reduced stem cell population in the shoot apex, similar to *HD-ZIPIII* double or triple mutant phenotypes (Prigge et al., 2005) or plants overexpressing *ZPR3* (Wenkel et al., 2007).

Like all HD-ZIP transcription factors also class II HD-ZIP proteins possess a homeodomain with an associated leucine-zipper domain. In addition, HD-ZIPII proteins carry a CPSCE (Cys, Pro, Ser, Cys, Glu) motif to perceive changes in the redox status (Hu et al., 2012; Tron et al., 2002), but also an aminoterminal EAR domain (**e**thylen-responsive element binding factor-associated **a**mphiphilic **r**epression domain) for transcriptional repression of target genes (Kagale et al., 2010). It was shown that HAT2 represses its own gene expression negatively as well as the expression of other *class II HD-ZIP*s (Sawa et al., 2002). Regarding

this repressive function an expression pattern in the abaxial domain of leaves can be assumed to suppress abaxialization, respectively in the adaxial leaf domain to suppress adaxializing factors.

The *HAT2* transcriptional repressor is a direct positive target gene of REV. So far, we were unable to elucidate a role for *HAT2* in patterning processes. However, the repressive function of HAT2 might regulate *HAT3* and *ATHB4* gene expression negatively to restrict strong adaxializing effects of both genes, resulting in a flat leaf lamina. A similar function for *HAT4* can be assumed.

Next to the proposed transcriptional regulation within the class II HD-ZIPs, a posttranslational regulation by dimerization might also be possible. Like HD-ZIPIIIs, also class II HD-ZIP proteins possess a leucine zipper domain. Heterodimerization within the HD-ZIPII family respectively with HD-ZIPIIIs might result in changes of protein activity of both protein families during leaf patterning processes. Alternatively, heterodimerization of HD-ZIPII with LITTLE ZIPPER microProtein might lead to more active class III HD-ZIP proteins, resulting in enhanced adaxialization.

HD-ZIPII gene expression is auxin-inducible (Kunihiro et al., 2011; Sawa et al., 2002). Plants overexpressing *HAT2* have elongated hypocotyl cells supporting a role for HAT2 in hypocotyl elongation in shade-avoidance. We assume a positive feedback loop for HD-ZIPIIs by suppression of either *LITTLE ZIPPER* or *miR165/166* expression, resulting in enhanced HD-ZIPIII protein activity. These HD-ZIPIII homodimers regulate *HD-ZIPII* expression directly and potentially via REV-induced auxin synthesis. This feedback loop might implicate that not HD-ZIPII itself control leaf patterning processes, but rather the indirect positive regulation of *HD-ZIPIII* activity.

In summary, a novel role for class II HD-ZIP transcription factors in shade-induced hypocotyl elongation and leaf patterning under control of REV and KAN1 was uncovered. We hypothesize that the adaxializing function of class II HD-ZIPs might result from suppression of abaxializing factors or by establishing a positive feedback-loop by repressing either the LITTLE ZIPPER or the miR165/166 to enhance HD-ZIPIII activity. HD-ZIPII downstream processes have to be identified

to elucidate their role during either patterning processes in leaves or elongation growth in the hypocotyl.

5.5 Concluding remarks

Plants are able to sense their environment and respond to biotic and abiotic stimuli by promoting growth in line with inherent developmental programs. The autotroph lifestyle requires sufficient perception of light. To survive in a highly competitive environment, plants have to continuously sense light quantity and quality to outgrow competitors.

We could show that the REV/KAN1 module is crucial for shade responses and proper patterning of leaves. In addition, REV activity is regulated by two direct feedback loops: a positive one involving AGO10 and a negative one involving ZPR-type microProteins. It is conceivable that the activity of HD-ZIPIII factors could be modulated by factors responding strongly to environmental changes.

Future projects should focus on shade-induced regulation of these modules and the identification of *class II HD-ZIP* downstream processes to reveal how the leaf patterning modules influence adaptive growth processes.

6. Literature

Abe, M., Katsumata, H., Komeda, Y., and Takahashi, T. (2003). Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in Arabidopsis. Development *130*, 635-643.

Arce, A.L., Raineri, J., Capella, M., Cabello, J.V., and Chan, R.L. (2011). Uncharacterized conserved motifs outside the HD-Zip domain in HD-Zip subfamily I transcription factors; a potential source of functional diversity. BMC plant biology *11*, 42.

Ariel, F., Diet, A., Verdenaud, M., Gruber, V., Frugier, F., Chan, R., and Crespi, M. (2010). Environmental regulation of lateral root emergence in Medicago truncatula requires the HD-Zip I transcription factor HB1. The Plant cell *22*, 2171-2183.

Azhakanandam, S., Nole-Wilson, S., Bao, F., and Franks, R.G. (2008). SEUSS and AINTEGUMENTA mediate patterning and ovule initiation during gynoecium medial domain development. Plant physiology *146*, 1165-1181.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant Organ Formation. Cell *115*, 591-602.

Cabello, J.V., Arce, A.L., and Chan, R.L. (2012). The homologous HD-Zip I transcription factors HaHB1 and AtHB13 confer cold tolerance via the induction of pathogenesis-related and glucanase proteins. The Plant journal : for cell and molecular biology *69*, 141-153.

Cabello, J.V., and Chan, R.L. (2012). The homologous homeodomain-leucine zipper transcription factors HaHB1 and AtHB13 confer tolerance to drought and salinity stresses via the induction of proteins that stabilize membranes. Plant biotechnology journal *10*, 815-825.

Chai, G., Bai, Z., Wei, F., King, G.J., Wang, C., Shi, L., Dong, C., Chen, H., and Liu, S. (2010). Brassica GLABRA2 genes: analysis of function related to seed oil content and development of functional markers. TAG Theoretical and applied genetics Theoretische und angewandte Genetik *120*, 1597-1610.

Chandler, J.W., Cole, M., Flier, A., Grewe, B., and Werr, W. (2007). The AP2 transcription factors DORNROSCHEN and DORNROSCHEN-LIKE redundantly control Arabidopsis embryo patterning via interaction with PHAVOLUTA. Development *134*, 1653-1662.

Ciarbelli, A.R., Ciolfi, A., Salvucci, S., Ruzza, V., Possenti, M., Carabelli, M., Fruscalzo, A., Sessa, G., Morelli, G., and Ruberti, I. (2008). The Arabidopsis homeodomain-leucine zipper II gene family: diversity and redundancy. Plant molecular biology *68*, 465-478.

Comelli, R.N., and Gonzalez, D.H. (2007). Conserved homeodomain cysteines confer redox sensitivity and influence the DNA binding properties of plant class III HD-Zip proteins. Arch Biochem Biophys *467*, 41-47.

Desai, A., Chee, P.W., May, O.L., and Paterson, A.H. (2008). Correspondence of trichome mutations in diploid and tetraploid cottons. The Journal of heredity *99*, 182-186.

Donner, T.J., Sherr, I., and Scarpella, E. (2009). Regulation of preprocambial cell state acquisition by auxin signaling in Arabidopsis leaves. Development *136*, 3235-3246.

Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F., and Bowman, J.L. (2003). Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. Current biology : CB *13*, 1768-1774.

Floyd, S.K., and Bowman, J.L. (2006). Distinct developmental mechanisms reflect the independent origins of leaves in vascular plants. Current biology : CB *16*, 1911-1917.

Floyd, S.K., Zalewski, C.S., and Bowman, J.L. (2006). Evolution of class III homeodomain-leucine zipper genes in streptophytes. Genetics *173*, 373-388.

Gordon, S.P., Heisler, M.G., Reddy, G.V., Ohno, C., Das, P., and Meyerowitz, E.M. (2007). Pattern formation during de novo assembly of the Arabidopsis shoot meristem. Development *134*, 3539-3548.

Hawker, N.P., and Bowman, J.L. (2004). Roles for Class III HD-Zip and KANADI genes in Arabidopsis root development. Plant physiology *135*, 2261-2270.

Heisler, M.G., Ohno, C., Das, P., Sieber, P., Reddy, G.V., Long, J.A., and Meyerowitz, E.M. (2005). Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. Current biology : CB *15*, 1899-1911.

Henriksson, E., Olsson, A.S., Johannesson, H., Johansson, H., Hanson, J., Engstrom, P., and Soderman, E. (2005). Homeodomain leucine zipper class I genes in Arabidopsis. Expression patterns and phylogenetic relationships. Plant physiology *139*, 509-518.

Himmelbach, A., Hoffmann, T., Leube, M., Hohener, B., and Grill, E. (2002). Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis. EMBO J *21*, 3029-3038.

Hu, R., Chi, X., Chai, G., Kong, Y., He, G., Wang, X., Shi, D., Zhang, D., and Zhou, G. (2012). Genome-wide identification, evolutionary expansion, and expression profile of homeodomain-leucine zipper gene family in poplar (Populus trichocarpa). PloS one *7*, e31149.

Ilegems, M., Douet, V., Meylan-Bettex, M., Uyttewaal, M., Brand, L., Bowman, J.L., and Stieger, P.A. (2010). Interplay of auxin, KANADI and Class III HD-ZIP transcription factors in vascular tissue formation. Development *137*, 975-984.

Itoh, J., Hibara, K., Sato, Y., and Nagato, Y. (2008). Developmental role and auxin responsiveness of Class III homeodomain leucine zipper gene family members in rice. Plant physiology *147*, 1960-1975.

Izhaki, A., and Bowman, J.L. (2007). KANADI and class III HD-Zip gene families regulate embryo patterning and modulate auxin flow during embryogenesis in Arabidopsis. The Plant cell *19*, 495-508.

Johannesson, H., Wang, Y., Hanson, J., and Engström, P. (2003). The Arabidopsis thaliana homeobox gene ATHB5 is a potential regulator of abscisic acid responsiveness in developing seedlings. Plant molecular biology *51*, 719-729.

Kagale, S., Links, M.G., and Rozwadowski, K. (2010). Genome-wide analysis of ethylene-responsive element binding factor-associated amphiphilic repression motif-containing transcriptional regulators in Arabidopsis. Plant physiology *152*, 1109-1134.

Kawanabe, T., and Fujimoto, R. (2011). Inflorescence abnormalities occur with overexpression of Arabidopsis lyrata FT in the fwa mutant of Arabidopsis thaliana. Plant science : an international journal of experimental plant biology *181*, 496-503.

Kim, Y.K., Son, O., Kim, M.R., Nam, K.H., Kim, G.T., Lee, M.S., Choi, S.Y., and Cheon, C.I. (2007). ATHB23, an Arabidopsis class I homeodomain-leucine zipper gene, is expressed in the adaxial region of young leaves. Plant cell reports *26*, 1179-1185.

Kim, H.S., Kim, S.J., Abbasi, N., Bressan, R.A., Yun, D.J., Yoo, S.D., Kwon, S.Y., and Choi, S.B. (2010). The DOF transcription factor Dof5.1 influences leaf axial patterning by promoting Revoluta transcription in Arabidopsis. The Plant journal : for cell and molecular biology *64*, 524-535.

Kunihiro, A., Yamashino, T., Nakamichi, N., Niwa, Y., Nakanishi, H., and Mizuno, T. (2011). Phytochrome-interacting factor 4 and 5 (PIF4 and PIF5) activate the homeobox ATHB2 and auxin-inducible IAA29 genes in the coincidence mechanism underlying photoperiodic control of plant growth of Arabidopsis thaliana. Plant & cell physiology *52*, 1315-1329.

Kuppusamy, K.T., Chen, A.Y., and Nemhauser, J.L. (2009). Steroids are required for epidermal cell fate establishment in Arabidopsis roots. Proceedings of the National Academy of Sciences of the United States of America *106*, 8073-8076.

Lechner, E., Leonhardt, N., Eisler, H., Parmentier, Y., Alioua, M., Jacquet, H., Leung, J., and Genschik, P. (2011). MATH/BTB CRL3 receptors target the homeodomain-leucine zipper ATHB6 to modulate abscisic acid signaling. Developmental cell *21*, 1116-1128.

Loudet, O., Michael, T.P., Burger, B.T., Le Mette, C., Mockler, T.C., Weigel, D., and Chory, J. (2008). A zinc knuckle protein that negatively controls morning-specific growth in Arabidopsis thaliana. Proceedings of the National Academy of Sciences of the United States of America *105*, 17193-17198.

Magnani, E., and Barton, M.K. (2011). A per-ARNT-sim-like sensor domain uniquely regulates the activity of the homeodomain leucine zipper transcription factor REVOLUTA in Arabidopsis. The Plant cell *23*, 567-582.

Mallory, A.C., Hinze, A., Tucker, M.R., Bouche, N., Gasciolli, V., Elmayan, T., Lauressergues, D., Jauvion, V., Vaucheret, H., and Laux, T. (2009). Redundant and specific roles of the ARGONAUTE proteins AGO1 and ZLL in development and small RNA-directed gene silencing. PLoS genetics *5*, e1000646.

Manavella, P.A., Arce, A.L., Dezar, C.A., Bitton, F., Renou, J.P., Crespi, M., and Chan, R.L. (2006). Cross-talk between ethylene and drought signalling pathways is mediated by the sunflower Hahb-4 transcription factor. The Plant journal : for cell and molecular biology *48*, 125-137.

Marks, M.D., Wenger, J.P., Gilding, E., Jilk, R., and Dixon, R.A. (2009). Transcriptome analysis of Arabidopsis wild-type and gl3-sst sim trichomes identifies four additional genes required for trichome development. Molecular plant *2*, 803-822.

McConnell, J.R., Emery, J., Eshed, Y., Bao, N., Bowman, J., and Barton, M.K. (2001). Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. Nature *411*, 709-713.

Moussian, B., Schoof, H., Haecker, A., Jurgens, G., and Laux, T. (1998). Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during Arabidopsis embryogenesis. EMBO J *17*, 1799-1809.

Mukherjee, K., and Burglin, T.R. (2006). MEKHLA, a novel domain with similarity to PAS domains, is fused to plant homeodomain-leucine zipper III proteins. Plant physiology *140*, 1142-1150.

Mukherjee, K., Brocchieri, L., and Burglin, T.R. (2009). A comprehensive classification and evolutionary analysis of plant homeobox genes. Molecular biology and evolution *26*, 2775-2794.

Nakamura, M., Katsumata, H., Abe, M., Yabe, N., Komeda, Y., Yamamoto, K.T., and Takahashi, T. (2006). Characterization of the class IV homeodomain-Leucine Zipper gene family in Arabidopsis. Plant physiology *141*, 1363-1375.

Niu, Y., Jin, C., Jin, G., Zhou, Q., Lin, X., Tang, C., and Zhang, Y. (2011). Auxin modulates the enhanced development of root hairs in Arabidopsis thaliana (L.) Heynh. under elevated CO(2). Plant, cell & environment *34*, 1304-1317.

Ohashi-Ito, K., and Fukuda, H. (2010). Transcriptional regulation of vascular cell fates. Current opinion in plant biology *13*, 670-676.

Ohgishi, M., Oka, A., Morelli, G., Ruberti, I., and Aoyama, T. (2001). Negative autoregulation of the Arabidopsis homeobox gene ATHB-2. The Plant journal : for cell and molecular biology *25*, 389-398.

Palena, C.M., Gonzalez, D.H., and Chan, R.L. (1999). A monomer-dimer equilibrium modulates the interaction of the sunflower homeodomain leucine-zipper protein Hahb-4 with DNA. Biochem J *341 (Pt 1)*, 81-87.

Ponting, C.P., and Aravind, L. (1999). START: a lipid-binding domain in StAR, HD-ZIP and signalling proteins. Trends Biochem Sci *24*, 130-132.

Prigge, M.J., Otsuga, D., Alonso, J.M., Ecker, J.R., Drews, G.N., and Clark, S.E. (2005). Class III homeodomain-leucine zipper gene family members have overlapping, antagonistic, and distinct roles in Arabidopsis development. The Plant cell *17*, 61-76.

Qing, L., and Aoyama, T. (2012). Pathways for epidermal cell differentiation via the homeobox gene GLABRA2: update on the roles of the classic regulator. Journal of integrative plant biology *54*, 729-737.

Reinhardt, D., Mandel, T., and Kuhlemeier, C. (2000). Auxin regulates the initiation and radial position of plant lateral organs. The Plant cell *12*, 507-518.

Sawa, S., Ohgishi, M., Goda, H., Higuchi, K., Shimada, Y., Yoshida, S., and Koshiba, T. (2002). The HAT2 gene, a member of the HD-Zip gene family, isolated as an auxin inducible gene by DNA microarray screening, affects auxin response in Arabidopsis. The Plant Journal *32*, 1011-1022.

Schena, M., Lloyd, A.M., and Davis, R.W. (1993). The HAT4 gene of Arabidopsis encodes a developmental regulator. Genes & development *7*, 367-379.

Schena, M., and Davis, R.W. (1994). Structure of homeobox-leucine zipper genes suggests a model for the evolution of gene families. Proceedings of the National Academy of Sciences of the United States of America *91*, 8393-8397.

Scott, M.P., Tamkun, J.W., and Hartzell, G.W., 3rd (1989). The structure and function of the homeodomain. Biochimica et biophysica acta *989*, 25-48.

Sessa, G., Morelli, G., and Ruberti, I. (1993). The Athb-1 and -2 HD-Zip domains homodimerize forming complexes of different DNA binding specificities. EMBO J *12*, 3507-3517.

Sessa, G., Steindler, C., Morelli, G., and Ruberti, I. (1998). The Arabidopsis Athb-8, -9 and -14 genes are members of a small gene family coding for highly related HD-ZIP proteins. Plant molecular biology *38*, 609-622.

Sessions, A., Weigel, D., and Yanofski, M.F. (1999). The Arabidopsis thaliana MERISTEM LAYER1 promoter specifies epidermal expression in meristems and young primordia. The Plant journal : for cell and molecular biology *20*, 259-263.

Shen, B., Sinkevicius, K.W., Selinger, D.A., and Tarczynski, M.C. (2006). The homeobox gene GLABRA2 affects seed oil content in Arabidopsis. Plant molecular biology *60*, 377-387.

Söderman, E., Mattsson, J., and Engström, P. (1996). The Arabidopsis homeobox gene ATHB-7 is induced by water deficit and abscisic acid. The Plant journal : for cell and molecular biology *10*, 375-381.

Son, O., Hur, Y.S., Kim, Y.K., Lee, H.J., Kim, S., Kim, M.R., Nam, K.H., Lee, M.S., Kim, B.Y., Park, J.*, et al.* (2010). ATHB12, an ABA-inducible homeodomain-leucine zipper (HD-Zip) protein of Arabidopsis, negatively regulates the growth of the inflorescence stem by decreasing the expression of a gibberellin 20-oxidase gene. Plant & cell physiology *51*, 1537-1547.

Sorin, C., Salla-Martret, M., Bou-Torrent, J., Roig-Villanova, I., and Martinez-Garcia, J.F. (2009). ATHB4, a regulator of shade avoidance, modulates hormone response in Arabidopsis seedlings. The Plant journal : for cell and molecular biology *59*, 266-277.

Stepanova, A.N., Yun, J., Robles, L.M., Novak, O., He, W., Guo, H., Ljung, K., and Alonso, J.M. (2011). The Arabidopsis YUCCA1 flavin monooxygenase functions in the indole-3-pyruvic acid branch of auxin biosynthesis. The Plant cell *23*, 3961-3973.

Takada, S., and Jurgens, G. (2007). Transcriptional regulation of epidermal cell fate in the Arabidopsis embryo. Development *134*, 1141-1150.

Tao, Y., Ferrer, J.L., Ljung, K., Pojer, F., Hong, F., Long, J.A., Li, L., Moreno, J.E., Bowman, M.E., Ivans, L.J.*, et al.* (2008). Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell *133*, 164-176.

Thum, K.E., Shin, M.J., Gutierrez, R.A., Mukherjee, I., Katari, M.S., Nero, D., Shasha, D., and Coruzzi, G.M. (2008). An integrated genetic, genomic and systems approach defines gene networks regulated by the interaction of light and carbon signaling pathways in Arabidopsis. BMC systems biology *2*, 31.

Tron, A.E., Bertoncini, C.W., Chan, R.L., and Gonzalez, D.H. (2002). Redox regulation of plant homeodomain transcription factors. The Journal of biological chemistry *277*, 34800-34807.

Vernoud, V., Laigle, G., Rozier, F., Meeley, R.B., Perez, P., and Rogowsky, P.M. (2009). The HD-ZIP IV transcription factor OCL4 is necessary for trichome patterning and anther development in maize. The Plant journal : for cell and molecular biology *59*, 883-894.

Walford, S.A., Wu, Y., Llewellyn, D.J., and Dennis, E.S. (2012). Epidermal cell differentiation in cotton mediated by the homeodomain leucine zipper gene, GhHD-1. The Plant journal : for cell and molecular biology *71*, 464-478.

Wang, Y., Henriksson, E., Soderman, E., Henriksson, K.N., Sundberg, E., and Engstrom, P. (2003). The Arabidopsis homeobox gene, ATHB16, regulates leaf development and the sensitivity to photoperiod in Arabidopsis. Developmental biology *264*, 228-239.

Wang, W., Xu, B., Wang, H., Li, J., Huang, H., and Xu, L. (2011). YUCCA genes are expressed in response to leaf adaxial-abaxial juxtaposition and are required for leaf margin development. Plant physiology *157*, 1805-1819.

Wenkel, S., Emery, J., Hou, B.H., Evans, M.M., and Barton, M.K. (2007). A feedback regulatory module formed by LITTLE ZIPPER and HD-ZIPIII genes. The Plant cell *19*, 3379-3390.

Won, C., Shen, X., Mashiguchi, K., Zheng, Z., Dai, X., Cheng, Y., Kasahara, H., Kamiya, Y., Chory, J., and Zhao, Y. (2011). Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES OF ARABIDOPSIS and YUCCAs in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America *108*, 18518-18523.

Yao, X., Wang, H., Li, H., Yuan, Z., Li, F., Yang, L., and Huang, H. (2009). Two types of cis-acting elements control the abaxial epidermis-specific transcription of the MIR165a and MIR166a genes. FEBS letters *583*, 3711-3717.

Zhao, Y., Zhou, Y., Jiang, H., Li, X., Gan, D., Peng, X., Zhu, S., and Cheng, B. (2011). Systematic analysis of sequences and expression patterns of drought-responsive members of the HD-Zip gene family in maize. PloS one *6*, e28488.

Zhong, R., and Ye, Z.H. (2001). Alteration of auxin polar transport in the Arabidopsis ifl1 mutants. Plant physiology *126*, 549-563.

Zhou, G.K., Kubo, M., Zhong, R., Demura, T., and Ye, Z.H. (2007). Overexpression of miR165 affects apical meristem formation, organ polarity establishment and vascular development in Arabidopsis. Plant & cell physiology *48*, 391-404.

Zhu, H., Hu, F., Wang, R., Zhou, X., Sze, S.H., Liou, L.W., Barefoot, A., Dickman, M., and Zhang, X. (2011). Arabidopsis Argonaute10 specifically sequesters miR166/165 to regulate shoot apical meristem development. Cell *145*, 242-256.

Zuniga-Mayo, V.M., Marsch-Martinez, N., and de Folter, S. (2012). JAIBA, a class-II HD-ZIP transcription factor involved in the regulation of meristematic activity, and important for correct gynoecium and fruit development in Arabidopsis. The Plant journal : for cell and molecular biology *71*, 314-326.

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