The role of *REVOLUTA* and *KANADI1* in plant development and environmental responses

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Abbreviations:

ABA	Abscisic acid
AGO10	ARGONAUT10
ARF	AUXIN RESPONSE FACTOR
ARP	AS1/RS2/Phantastica
AS1	ASSYMETRIC LEAVES1
BP	BREVIPEDICELLUS
ChIP-Seq	Chromatin-Immunoprecipitation-Sequencing
CNA	CORONA
CZ	Center zone
DCL4	Dicer-like 4
DPI-ELISA	DNA-protein-Interaction-ELISA
GA	Gibberellic acid
GARP	GOLDEN2/ARR/PSR1
GUS	glucuronidase
HB	HOMEOBOX
HD	Homeodomain
HD-ZIP III	class III HOMEODOMAIN-LEUCINE ZIPPER
IAA	Indole-3-acetic acid
JA	Jasmonic acid
KAN1	KANADI1
KNOX	KNOTTED1-like homeobox
mRNA	messenger Ribonucleidacid
PCD	Programmed cell death
PHAN	PHANTASTICA
PHB	PHABULOSA
PHV	PHAVOLUTA
PID	PINOID
PIN	PIN-formed auxin transporter

PIN1	PIN-FORMED1
RDR6	the RNA-dependent RNA polymerase
REV	REVOLUTA
RS2	ROUGH SHEATH2
SA	salicylic acid
SAM	Shoot apical meristem
SAUR	SMALL AUXIN UP RNA
SAGs	Senescence-associated genes
SGS3	SUPPRESSOR OF GENE SILENCING 3
START	Steroidogenic acute regulatory protein-related lipid-transfer domain
STM	SHOOT MERISTEMLESS
YUC	YUCCA
ZIP	Zipper domain
ZPR	LITTLE ZIPPER

1. Zusammenfassung/Summary

1.1. Zusammenfassung

Die Etablierung und Aufrechterhaltung der polaren Blattachse wird durch ein komplexes Netzwerk von Transkriptionsfaktoren bewerkstelligt. Mitglieder der KANADI und HD-ZIPIII Transkriptionsfaktorfamilien sind an der Festlegung der Organpolarität beteiligt und agieren in diesem Prozess antagonistisch. In unseren Studien haben wir vergleichende Chromatin-Immunopraezipitationsexperimente durchgeführt und haben die direkten Zielgene von KANADI1 identifiziert. Die Analyse dieser Zielgene legt nahe dass KAN1 Musterbildungsprozess in Blättern durch Kontrolle der Auxinantwort kontrolliert. Des Weitern haben unsere Studie ergeben dass REVOLUTA (REV), ein Mitglied der HD-ZIPIII Familie und KAN1 viele Gene gegensätzlich regulieren.

Da Pflanzen sessil sind müssen sie ihr Wachstum kontinuierlich mit der Umweltsituation synchronisieren. Das Auslösen der Blattseneszenz leitet die letzte Phase der Blattentwicklung ein. In diesem Stadium werden energiereiche Substanzen von den Blättern zu den Wachstumszonen transportiert. Dieser Vorgang erhöht der reproduktiven Erfolg und ist eng an das Entwicklungsalter der Pflanze gekoppelt.

Wir konnten in unseren Studien zeigen dass HD-ZIPIII eine wichtige Rolle in der Regulation der Blattseneszenz spielen. So kontrolliert REV direkt die Expression von WRKY53, einem wichtigen Seneszenzregulator. Des Weiteren haben wir gezeigt dass diese Regulation abhängig vom Redox-Status von REV erfolgt. Die weitere Analyse anderer REV Zielgene legt nahe dass REV verschieden Seneszenzfaktoren stadium-spezifisch reguliert. REV kontrolliert demnach die frühen und späten Stadien der Blattentwicklung.

1.2. Summary

In plants, the establishment of organ patterning and polarity is mediated by the action of several transcription factors. Among them, *KANADI*s and *HD-ZIPIII*s act antagonistically by which they play crucial roles in organ polarity. Using a combination of chromatin immunoprecipitation (ChIP-Seq) approach and tiling arrays, we could identify a set of potential direct target genes of *KAN1*. Further analysis showed that a number of *KAN1* targets appear to regulate organ patterning or response to auxin. In addition, *KAN1* shares a set of common direct targets with *REV*, implying that the *REV/KAN1* module acts in organ patterning through opposite regulation of shared targets.

As sessile organisms, plants have to continuously adjust growth and development to changing environmental conditions. During the final stage of growth, plants induce leaf senescence to reallocate nutrients and energy-rich substances from mature leaves to reproductive seeds, leading to increased reproductive success. Therefore leaf senescence is tightly coupled to the developmental age of the plant.

In this study, we show that class III *HD-ZIP* transcription factors have an additional role in controlling the onset of leaf senescence in Arabidopsis. We report that acting as a redox-sensitive transcription factor, *REV* directly and positively regulates the expression of *WRKY53*, a senescence-related transcription factor. *REV* is required for the induction of *WRKY53* in response to oxidative stress, and reducing the activity of *HD-ZIP III* genes strongly delays the onset of leaf senescence. Besides *WRKY53*, we also identified nine direct *REV* targets which are differentially expressed during senescence. Thus, a crosstalk between early and late stages of leaf development appears to contribute to reproductive success.

2. Aim of the work

Adaxial-abaxial polarity is the developmental basis of leaf shape diversity. For instance, most leaves are flat with two distinctive surfaces and the formation of the leaf lamina requires the establishment of dorsiventrality. Previous genetic studies have showed that *Class III HD-ZIP*s and *KANADI*s are critically involved in mediating leaf polarity establishment. Interestingly, these two types of transcription factors act antagonistically to regulate organ patterning and polarity. This study is focusing on investigating the downstream genes of *REV* and *KAN1* to better elucidate how these two patterning factors act to control developmental processes.

In the first part, we could identify a number of target genes of *KAN1* by using a combination of next-generation sequencing and genetic analysis. Further analysis of these potential targets will indirectly contribute to examine the roles of *KAN1* in plant development.

Based on our previous finding that the senescence-related *WRKY53* factor is regulated by *REV*, in the second part we wanted to understand how *REV* plays a role in controlling leaf senescence via the regulation of the *WRKY53* transcription factor.

3. Introduction

3.1. Leaf primordium initiation and the establishment of adaxial-abaxial polarity

Leaves are the main photosynthetic organs of plants. Energy-rich sun-light is captured in the leaves and converted into chemical energy through photosynthesis. In order to adapt to a variety of habitats, plants have evolved leaves displaying a wide range of shapes and sizes. Leaves vary tremendously in size, shape and color among different plant species. Even in the same plant, leaf shapes may be different at the different stages. During the past decades, the molecular mechanisms underlying leaf shape diversity have been started to be addressed. Although many details remain unclear, much progress has been made in understanding the developmental mechanisms.

3.1.1 Leaf primordium initiation

Initiation of the leaf primordium is one of the three main processes in early leaf development. In higher plants, leaves and flowers are referred to as lateral organs, which arise, from individual groups of founder cells on the meristem periphery. The center zone (CZ) of the shoot apical meristem (SAM) harbors a population of stem cells that divide slowly. These cells are always in a non-differentiated and proliferative state having the ability to continually divide. Owing to the continuous cell production in the meristem, some daughter cells are displaced toward the periphery of the meristem where they organize into organ primordia such as leaf primordia. Leaf primordia initiate from the flank of the shoot apical meristem and the simultaneously specification depends on the proper gradient of auxin distribution. Due to the presence of auxin, the cells keep the competence for the initiation of leaf primordia (Reinhardt et al., 2000).

Two *Arabidopsis* genes, *PIN-FORMED1 (PIN1)* and *PINOID (PID)* are thought to regulate boundary formation though both of them are not key genes

essential for SAM formation and/or maintenance. The *PIN1* gene is the first identified PIN family member associated with auxin transport. *PIN1* encodes a transporter-like membrane protein acting in the efflux of auxin from cells. Polar transport of auxin is believed to control the formation of leaf primordia. Moreover polar auxin transport is regulated by the putative auxin efflux carrier *PIN1*, resulting in leaf separation and delimitation. *PIN1* is shown to promote polar auxin transport in *Arabidopsis* inflorescence axes (Gälweiler et al., 1998). The *pin formed 1* (*pin1*) mutant of *Arabidopsis* shows structural abnormalities in leaves, including fused or deformed cotyledons together with much wider leaves as well as abnormal phyllotaxis on the inflorescence axes (Okada et al, 1991). The auxin efflux carrier also plays an essential role in promoting organ formation by regulating auxin distribution (Benková et al., 2003; Reinhardt et al., 2003). After treating young *pin1* plants with indole-3-acetic acid (IAA), normal leaves were formed. Additionally, ring-shaped flower primordia were induced when IAA was applied to the tip of the meristem of *pin1* mutants suggesting the role of *PIN1* in organ formation (Reinhardt et al. 2003).

Encoding a protein serine/threonine kinase, *PINOID (PID)* is an important molecular determinant in PIN polar targeting and is mainly expressed in the boundaries of cotyledon primordia. Moreover, its transcription is induced by exogenous auxin application (Christensen et al., 2000, Friml et al., 2004). The *PID* gene is involved in positive regulation of polar auxin transport indicated by both *PID* loss- and gain-of-function mutant phenotypes (Benjamins et al., 2001). Overexpression of *PID* alters auxin distribution by inducing a basal-to-apical shift in PIN polarity, resulting in developmental defects in embryo and seedling roots (Friml et al., 2004). Loss-of-function *pinoid (pid)* mutants display inflorescence and flower phenotypes similar to those of the *pin1* mutant, but different in cotyledons and leaves (Bennett et al., 1995). Mutations in the *PIN1* and *PID* genes all disrupt the patterning of cotyledons. The *pin1 pid* double mutant has a severe phenotype with completely lacked

cotyledons displaying radial symmetry due to improper auxin flux. However the double mutant develops a normal SAM that can produce leaf primordia, although these primordia display abnormal phyllotaxis (Furutani et al., 2004).

Another parallel mechanism required for leaf primordium initiation is the antagonistic interaction between class I *KNOX (KNOTTED1*-like homeobox) gene family and *ARP* (for *AS1/RS2/Phantastica*) genes, which contribute to the regulation of meristem maintenance. In *Arabidopsis thaliana*, there are four class-I *KNOX* transcription factors, including *SHOOT MERISTEMLESS (STM)*, *BREVIPEDICELLUS* (*BP* or *KNAT1*), *KNAT2*, and *KNAT6* (Hake et al., 2004). The class 1 *KNOX* genes are expressed throughout the shoot meristems but not in P0 cells which are designated for the cells in the incipient leaf primordium that will become the next leaf (Jackson et al., 1994). These genes were reported to play an important role in maintenance of meristematic cell identity during embryogenesis. Some *knox* loss-of-function phenotypes are highly infromative for understanding their functions. The first recessive *knox* mutant line discovered in plants is the *stm* mutant, which fails to form a functional shoot apical meristem. The fact that *stm* is able to produce cotyledons suggests that *STM* is required to maintain the SAM (Barton and Poethig, 1993; Long et al., 1996). *BP* and *KNAT6* contribute redundantly with *STM* to SAM maintenance, as the examination of *bp stm-11* and *knat6-2 stm-2* double mutants reveals that loss-of-function mutations in *BP/KNAT6* all reduce the residual meristematic activity of the *stm* mutants (Byrne et al., 2002; Belles-Boix et al., 2006). *KNAT2* has a similar expression pattern to *STM* mainly expressed in domains of the SAM, but its role is still unclear. In maize, the *knotted1* (*kn1*) gene is the first described gene whose expression is related to the early events in leaf initiation. *Kn1* is expressed throughout the shoot meristem but is absent in leaves. A *kn1* loss-of-function mutant, *kn1-e1* fails to establish a SAM suggesting its function in formation of organ primordia (Smith et al., 1992; Jackson et al., 1994; Vollbrecht et al., 2000).

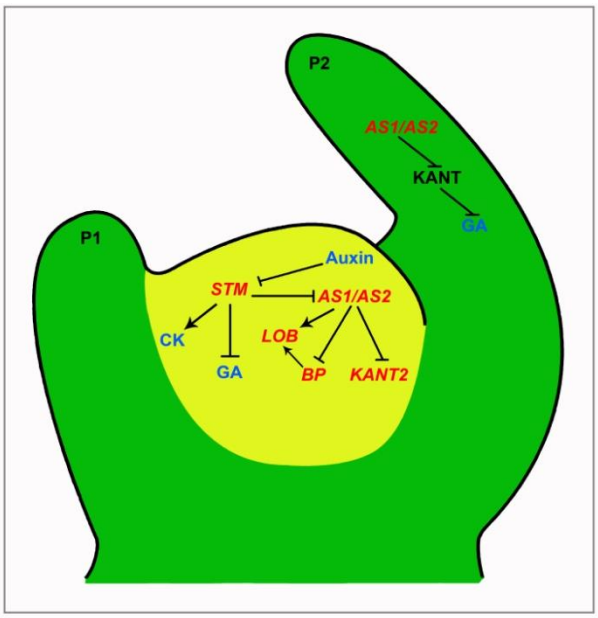

Figure 1. Genetic regulatory network between class-I *KNOX* in SAM. Arrows indicate positive regulation and lines with blunt ends indicate negative regulation. P1 and P2, leaf primordia; CK, cytokinin; GA, gibberellic acid.

Previous research has shown that cells in the leaf primordium loose indeterminacy and become determinate by completely switching off *KNOX* genes which are necessary for stem cell specification (Long et al., 1996). Then these cells begin to express *ARP* genes which are involved in negative regulation of class 1 *KNOX* genes in leaf primordia, subsequently promoting growth and differentiation. The *ARP* genes are three MYB transcription factors respectively found in *Arabidopsis* (*ASSYMETRIC LEAVES1* (*AS1*)), maize (*ROUGH SHEATH2* (*RS2*)) and *Antirrhinum* (*PHANTASTICA* (*PHAN*)). Those three negative regulators of *KNOX* genes are closely related to each other (Byrne et al. 2000; Timmermans et al. 1999; Tsiantis et al. 1999b; Waites et al. 1998). *AS1* negatively regulates the homeobox genes *KNAT1* and *KNAT2*, in turn, repressed by *STM* (Byrne et al. 2000). In maize, the *rs2* mutations induce misexpression of *KNOX* genes and resulting in a range of developmental phenotypes, such as twisted leaves and vascular patterning aberrations (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999). Briefly, whether cells switch from determinate to indeterminate is depend on the antagonistic interactions of *KNOX1* and *ARP* genes.

3.1.2. Genetic framework and molecular regulation of leaf polarity establishment

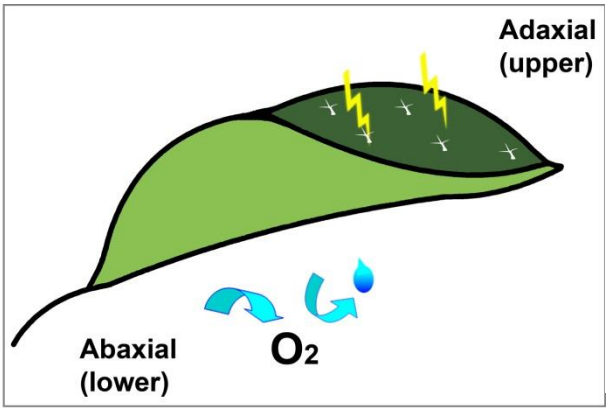

Figure 2. A cartoon showing two distinctive surfaces of leaf. The adaxial leaf side is specialized for light-harvesting while the abaxial zone is specialized for gas exchange and water loss.

Most leaves are flat with distinctive adaxial (upper leaf side specialized in photosynthesis) and abaxial (lower leaf side specialized in gas exchange) zones. The establishment of dorsoventrality (the ad-/abaxial axes) is required for the formation of the leaf lamina. In the classic microsurgical experiments in potato (*Solanum tuberosum L*.) over 60 years ago, Sussex first proposed the mechanisms that establish the adaxial-abaxial patterning in the leaf. When incipient primordia were isolated from the meristem by incision, the primordia developed centric and abaxilized leaves, suggesting that a signal from the SAM is required for specifying adaxial cell fate in leaf development, since the adaxial cells of leaf primordia are adjacent to the SAM (Sussex, 1951; Sussex, 1954). Generally, the adaxial-abaxial polarity is established after primordia initiation from the shoot apical meristem. The *MYB* transcription factor *PHAN* is the first gene recognized to be involved in the control of adaxial-abaxial patterning (Waites and Hudson, 1995). Loss of *PHAN* displays a range of leaf defect phenotypes, including radialized leaves with abaxial cell types, indicating that *PHAN* plays a role in in adaxial identity for flat lamina growth in *Antirrhinum*.

To date, several families of transcription factors and two types of small RNAs

are known to modulate establishment of adaxial-abaxial polarity. In *Arabidopsis*, one of the master players in the establishment of leaf polarity is the class III *HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIPIII*) gene family. The *HD-ZIP III* family is comprised of five members including *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *CORONA* (*CAN or ATHB15*) and *ATHB8*, which have distinct but overlapping patterns of expression (McConnell and Barton, 1998; McConnell et al., 2001; Zhong and Ye. 1999; Zhong et al., 1999). All members encode plant-specific transcription factors sharing homeodomain-leucine zipper motifs, a steroidogenic acute regulatory protein (StAR) lipid transfer domain (START), a homeodomain-START associated domain (HD-SAD) and a Per-ARNT-Sim-like (PAS-like) MEKHLA domain at the C terminus (Sessa et al., 1998; Pontig and Aravind, 1999; Schrick et al., 2004). *PHB*, *PHV*, and *REV* are expressed in the adaxial domain of lateral organs and vasculature (McConnell et al., 2001; Otsuga et al., 2001; Emery et al., 2003), while the expressions of *ATHB8* and *ATHB15* appears to be confined to the vascular tissues (Baima et al., 1995; Ohashi-Ito and Fukuda, 2003).

In the *HD-ZIPIII* family, *PHB*, *PHV* and *REV* are much closer related to one another and are the only members that contribute to leaf polarity. Recent data suggest that the class III HD-ZIP proteins PHB, PHV and REV are likely to mediate an adaxial-promoting signal produced by the SAM. Gain-of-function mutants of *PHV*, *PHB* and *REV* display polarity defects with leaves curled upward (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003). Of the three members, only *rev* single mutants have an observable mutant phenotype, whereas *phb* or *phv* single mutants show no mutant phenotypes (Talbert et al., 1995; Zhong and Ye, 1999; Otsuga et al., 2001). The loss-of-function *rev* mutants (e.g. *rev-1*) show a failure in polarity establishment results in abaxialized leaves (Talbert et al., 1995). When *rev* mutations are combined with *phb* and /or *phv* mutations, double or triple

mutant plants display obvious developmental defects. For instance, the triple mutant *phb phv rev* has a seriously defective SAM with a pin-like cotyledon (Emery et al., 2003; Prigge et al., 2005). Thus, *REV* is more important in promoting adaxial development than *PHB* and *PHV* though the latter of two provide redundant activities for REV activity.

The small regulatory microRNAs, *miR165* and *miR166* (that have only one nucleotide difference in mature RNA sequences) are able to regulate the functions of the *HD-ZIPIII* genes by cleaving their target mRNAs (Juarez et al., 2004; Kidner and Martienssen, 2004; Kim et al., 2005; Williams et al., 2005a). The negative regulation is supported by genetic analysis of dominant mutations of *HD-ZIP III* genes. Molecular characterization of these gain-of-function mutants revealed that the microRNA complementary site in the class III *HD-ZIP* genes is disrupted because of base changes, making the mRNA resistant to miRNA. Overexpression of *miR165/miR166* (except for miR166g) causes dramatically reduced transcript levels of all five *HD-ZIP III* genes in *Arabidopsis*. As a result, the *miR165* overexpressors exhibit a variety of phenotypes reminiscent of loss-of-function mutants of rev and *phb phv rev*, including abnormal SAM, downward curled leaves and disorganized vascular tissues (Kim et al. 2005; Williams et al. 2005a; Zhou et al., 2007). It is well known that *PHB*, *PHV* and *REV* are expressed in the adaxial domain of leaf primordia inducing adaxialization while *miR165/166* is expressed on the abaxial side of leaves and contributes to repressing Class III HD-Zip activity. Interestingly, *miR165*/*166* are genetically repressed by a miRNA effector, the *Arabidopsis ARGONAUTE10* (*AGO10*), expression of which is also directly regulated by *REV* (Liu et al., 2009; Ji et al., 2011; Brandt et al., 2013). Thus, these regulators (*REV*, *AGO10* and *miR165/166*) are likely to form a feedback loop, mediating the establishment of leaf adaxial-abaxial polarity.

Recent studies have shown that *LITTLE ZIPPER* (*ZPR*) proteins play a role in leaf polarity determination via interacting with HD-ZIPIII proteins at the

post-translational level in *Arabidopsis*. *ZPR* is a small gene family containing four functionally redundant members from *ZPR1* to *ZPR4* (Wenkel et al., 2007). All of the ZPR proteins contain a ZIP motif that is quite similar to the one found in HD-ZIP III proteins. Given the similar leucine-zipper domain, the ZPR proteins repress the HD-ZIPIII activity by dimerizing with HD-ZIPIII proteins and hence prevent them from forming homodimers (Kim et al, 2008; Wenkel et al, 2007). Interestingly, HD-ZIPIII proteins transcriptionally activate *ZPR* expression suggesting a feedback loop modulating *HD-ZIPIII* function in meristem regulation (Wenkel et al, 2007). *ZPR3* overexpression line displays an abaxialized leaf phenotype reminiscent of *HD-ZIPIII* loss-of-function mutant plants, such as *rev-6* mutant. In contrast, *ZPR* mutants (e.g. *zpr3-2*) show disrupted activities of the SAM similar to *HD-ZIPIII* gain-of-function plants (Kim et al, 2008; Wenkel et al, 2007). *ZPR* genes encode small proteins (consisting of 67 to 105 residues) also called microProteins (miPs) which can modulate transcription factor activities (Staudt and Wenkel, 2011; Wenkel et al., 2007).

Unlike the *HD-ZIPIII* family specifying leaf adaxial identity, abaxial cell fate is promoted by the *KANADI* and *YABBY* genes (Eshed et al., 2001; Kerstetter et al., 2001). The *KANADI* genes encode four members of the GARP transcription factors (*KAN1* to *KAN4*) which have expression pattern complementary to that of the Class III *HD-Zip* genes (Kerstetter et al., 2001; Emery et al., 2003; Eshed et al., 2004). Single *kan1* or *kan2* mutants have slight or no effects on leaf polarity. However, the *kan1 kan2* double mutant plants exhibit polarity defects in all lateral organs which are enhanced in *kan1 kan2 kan3* and *kan1 kan2 kan3 kan4* mutants implying the redundancy between the four *KANADI* genes (Eshed et al., 2001; Emery et al., 2003; Izhaki and Bowman, 2007). Surprisingly, the adaxialized leaves and radialized vasculature bundles in *kan1 kan2 kan3* plants are similar to those of the *phb-1d* and *rev-10d* gain-of-function mutants (McConnell and Barton, 1998; Emery et al., 2003; Eshed et al., 2004). Together with the complementary

expression patterns between the Class III *HD-Zip* genes and *KANADI* genes, the two gene families may act antagonistically in leaf polarity establishment.

The *YABBY* family consists of six genes in *Arabidopsis* which are also considered as abaxial determinants. Three family members, *FIL*, *YAB2*, and *YAB3* are expressed in the abaxial domains of leaf primordia and cotyledons (Sawa et al., 1999a; Siegfried et al., 1999; Watanabe and Okada, 2003; Golz et al., 2004). Ectopic expression of some *YABBY* genes can promote differentiation of abaxial cells (Sawa et al., 1999; Siegfried et al., 1999). Furthermore, the expression patterns of the *YABBY* genes are altered in *kan1 kan2* double mutants suggesting that the *YABBY* genes act downstream of *KANADI* genes (Siegfried et al., 1999; Eshed et al., 2001). However, the *YABBY* gene family has different expression pattern within the angiosperms. For example, *YAB2* was found expressed adaxially in *Amborella trichopoda* in contrast to the abaxial expression in *Arabidopsis thaliana* (Yamada et al., 2004). Additionally two maize homologs of the *Arabidopsis FIL* and *YAB3* genes are expressed on the adaxial side of leaf primordia (Juarez et al., 2004). The contradictory evidences indicate that the *YABBY* gene family is involved in establishing abaxial identity of leaf primordia in *Arabidopsis* but may not be a key regulator.

In addition to *KAN* and *YABBY* genes, further determinants of abaxial fate are the *AUXIN RESPONSE FACTOR* genes *ARF3* (also known as *ETT*) and *ARF4* which are plant-specific transcription factors (Pekker et al., 2005). *ett arf4* double mutants have adaxialized leaves resemble the phenotypes of *kan1 kan2* double mutants. Besides, mutations in *ETT* and *ARF4* were found to suppress the ectopic *KAN1* activity. Additionally, both of *ETT* and *ARF4* are expressed in the abaxial domain of leaf primordia supporting their role in the specification of abaxial identity (Alvarez et al., 2006; Pekker et al., 2005).

ETT and *ARF4* mRNAs are targeted by a *TAS3*-derived trans-acting short interfering RNA (tasiR-ARF) through cleavage, indicating that tasiR-ARF is an

important contributor to adaxial fate in *Arabidopsis* (Allen et al., 2005; Williams et al., 2005a). ta-siRNAs are 21 nt endogenous small interfering RNAs derived from non-coding transcripts which initially undergo miRNA-guided cleavage. The following conversion of cleavage products into dsRNA is mediated by the activities of SUPPRESSOR OF GENE SILENCING 3 (SGS3) and the RNA-dependent RNA polymerase RDR6 proteins, respectively. Subsequently the Dicer-like 4 (DCL4) protein cleave dsRNA into siRNAs (Peragine et al., 2004; Gasciolli et al., 2005; Xie et al., 2005; Yoshikawa et al., 2005).

Given the role of tasiR-ARF in adaxial identity, mutants defective in ta-siRNA biogenesis are expected to show an abaxialized phenotype. However, the reduced tasiR-ARF activity (such as mutations in *RDR6*, *SGS3* and *DCL4*) causes no obvious leaf polarity phenotypes in *Arabidopsis* (Peragine et al., 2004; Yoshikawa et al., 2005; Xie et al., 2005b). The *rdr6* loss-of-function mutant displays only minor phenotypes, whereas *rdr6 as2* double mutant shows enhanced defects in adaxial-abaxial polarity even stronger than that of the *as2* single mutant plant, indirectly suggesting the contribution of the ta-siRNA to adaxial- abaxial patterning (Li et al., 2005).

In conclusion, antagonistic interactions between the adaxial and abaxial determinants form complex genetic networks in coordination with auxin by which leaf polarity is established for leaf growth and morphogenesis.

3.2. A brief summary of leaf senescence

Plant senescence is an age-related disintegration process of plants that occurs at all levels of organisms from cells to individuals, ultimately leading to death. It is a strictly organized process that is governed by the actual age of the organ and/or the developmental age of the whole organism. At the cellular level, senescence can be considered as a special form of programmed cell death (PCD). One difference is that after cell degradation the final breakdown products of cellular components are recycled for developing organs of the

plant. Moreover, senescence is reversible until very late stages of its progression which was not observed for any other form of PCD in plants or animals.

Leaf senescence is a crucial means for plants to reallocate nutrients and valuable substances from senescing leaves to reproducing seeds, eventually maximizing reproductive success (Himelblau and Amasino, 2001). Generally, leaf senescence is visible by leaf color changes during its progression in most plant species. Biochemically, it can be characterized by degradation of various types of macromolecules including proteins, lipids and nucleic acids. Developmental age is an important determinant for the induction of leaf senescence. Besides, various internal and external factors can have a strong impact onthis process (Xu et al., 2011; Guo and Gan, 2012). The internal factors that affect leaf senescence include developmental cues and reproductive development as well as phytohormones (Gan and Amasino, 1995; Pic et al., 2002; Riefler et al., 2006). The environmental cues include various stresses such as extreme temperatures, nutrient deficiency, drought, radiations, and pathogen infection.

Phytohormones are key players in long-distance communication in plants and have been shown to play crucial roles in senescence regulatory networks. Several phytohormones promote senescence in leaves including ethylene, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) (Grbic and Bleecker, 1995; Park et al., 1998; Morris et al., 2000; He et al., 2002; Guo and Gan, 2005; Jing et al., 2005). For instance, *etr1* and *ein2* mutants are insensitive to ethylene and exhibit a delay in the onset of leaf senescence (Grbic and Bleecker, 1995). By contrast, others like cytokinins, gibberellins and auxin prevent this process. For example, decreased level of cytokinin during leaf development leads to accelerated senescence in leaves (Masferrer et al., 2002).

Interestingly, the leaf transcriptome varies immensely accompanying the onset and progression of leaf senescence. For instance, a comparative transcriptome analysis revealed that 827 genes show at least three-fold increase in transcript levels during senescence in Arabidopsis (Buchanan-Wollaston et al., 2005). In particular, genome-level studies have revealed that thousands of senescence-associated genes (SAGs) are differentially expressed during leaf senescence (van der Graaff et al., 2006; Breeze et al., 2011). It was previously reported that 20 different families of transcription factors are transcriptionally up-regulated in senescing leaves, remarkably contain several large groups such as NAC, WRKY, C2H2-type zinc finger, AP2/EREBP, and MYB proteins (Guo and Gan, 2005). Among these large groups, NAC and WRKY proteins are plant specific transcription factors which are believed to especially play central roles in regulating senescence.

In Arabidopsis, approximately 20 *NAC* genes exhibit increased expression levels during senescence (Buchanan-Wollaston et al., 2005; Balazadeh et al., 2008). *AtNAP* has been shown to control leaf senescence, while *atnap* knockout mutant plants show delayed silique senescence, overexpression of *AtNAP* triggers early senescence (Guo and Gan, 2006; Kou et al., 2012). *ORE1*, *ORS1* and *JUB1* have also been characterized to regulate senescence being involved in the crosstalk between stress and senescence (Balazadeh et al., 2010, 2011; Wu et al., 2012).

Besides *NAC* factors, the WRKY family has been shown to play a role in regulating leaf senescence (Eulgem and Somssich, 2007). All WRKY proteins contain at least one WRKY domain which is composed of a zinc finger structure and a 60 amino acid region with WRKYGQK at N-terminal end. The WRKY domain is a DNA-binding domain that binds directly to various W-box variants (Eulgem et al. 2000; Yu et al. 2001). Some WRKY members such as *WRKY6* and *WRKY75*, are highly induced during leaf senescence (Robatzek

and Somssich, 2001; Guo and Gan, 2006). Another well-known WRKY member, *WRKY53* plays a regulatory role in the early events of leaf senescence. It was reported that *WRKY53* is up-regulated at a very early time point of leaf senescence. Additionally, *wrky53* knockout plants display delayed leaf senescence phenotypes, suggesting that *WRKY53* acts as a positive regulator of leaf senescence (Hinderhofer and Zentgraf, 2001; Miao et al., 2004). WRKY53 activity is regulated in a very complex way by phosphorylation, protein-protein interaction as well as by protein degradation (Zentgraf et al., 2010)

In conclusion, various internal and external factors induce multiple pathways that are possibly interconnected to form regulatory networks. Subsequently, distinct sets of senescence-associated genes are activated in response to these regulatory networks, and execute degradation processes to cause cell death.

4. Publications

4.1. **Xie Y**, Huhn K, Brandt R, Potschin M, Bieker S, Straub D, Doll J, Drechsler T, Zentgraf U, Wenkel S. REVOLUTA and WRKY53 connect early and late leaf development in Arabidopsis. ***Development*** 141(24):4772-83, 2014.

The author's contribution: Participated in the design of the experiments; Carried out the molecular analysis; Data collection for Figure 2, 3, 4 (D, E), 6 (C, D) and 7; Contributed to the writing of the manuscript with Stephan Wenkel and Ulrike Zentgraf

4.2. Merelo P, **Xie Y***, Brandt L, Ott F, Weigel D, Bowman JL, Heisler MG, Wenkel S. Genome-wide identification of KANADI1 target genes. ***PLoS one*** 8(10): e77341. doi:10.1371/ journal.pone.0077341, 2013.

The author's contribution: Participated in the design of the experiments; performed the experiments (chromatin immunoprecipitation and gene expression); Took part in the revision of the article.

*shared first authors

4.3. Brandt R, **Xie Y**, Musielak T, Graeff M, Stierhof YD, Huang H, Liu CM and Wenkel S. Control of stem cell homeostasis via interlocking microRNA and microProtein feedback loops. ***Mechanisms of Development*** 130 (1), 25-33, 2013.

The author's contribution: Contributed to qRT-PCR and qChIP-PCRs.

4.4. Brandt R, Cabedo M, **Xie Y** and Wenkel S. Homeodomain leucine-zipper proteins and their role in synchronizing growth and development with the environment. ***Journal of Integrative Plant Biology*** 56(6):518-26, 2014.

The author's contribution: Contributed to the writing of the manuscript.

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RESEARCH ARTICLE

REVOLUTA and WRKY53 connect early and late leaf development in *Arabidopsis*

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ABSTRACT

As sessile organisms, plants have to continuously adjust growth and development to ever-changing environmental conditions. At the end of the growing season, annual plants induce leaf senescence to reallocate nutrients and energy-rich substances from the leaves to the maturing seeds. Thus, leaf senescence is a means with which to increase reproductive success and is therefore tightly coupled to the developmental age of the plant. However, senescence can also be induced in response to sub-optimal growth conditions as an exit strategy, which is accompanied by severely reduced yield. Here, we show that class III homeodomain leucine zipper (HD-ZIPIII) transcription factors, which are known to be involved in basic pattern formation, have an additional role in controlling the onset of leaf senescence in *Arabidopsis*. Several potential direct downstream genes of the HD-ZIPIII protein REVOLUTA (REV) have known roles in environment-controlled physiological processes. We report that REV acts as a redox-sensitive transcription factor, and directly and positively regulates the expression of *WRKY53*, a master regulator of age-induced leaf senescence. HD-ZIPIII proteins are required for the full induction of *WRKY53* in response to oxidative stress, and mutations in *HD-ZIPIII* genes strongly delay the onset of senescence. Thus, a crosstalk between early and late stages of leaf development appears to contribute to reproductive success.

KEY WORDS: REVOLUTA, HD-ZIPIII, WRKY53, Leaf senescence, Hydrogen peroxide signaling

INTRODUCTION

Senescence is the final stage of leaf development and involves the concerted reallocation of nutrients from the leaves to developing parts of the plant, especially fruits and seeds. Thus, leaf senescence has a major impact on yield quantity and quality, e.g. salvaged nitrogen (N) from wheat leaves accounts for up to 90% of the total grain N content (Kichey et al., 2007). In order to minimize loss of nutrients, plants induce leaf senescence in response to endogenous cues such as plant age and altered hormone homeostasis. However, external factors, such as the availability of water or light quality can also induce senescence, referred to as premature senescence (Ballaré, 1999). Although age-induced senescence tends to maximize seed production, premature senescence describes an exit strategy that is induced in response to sub-optimal growth conditions and is often correlated with severely decreased yields.

The onset and progression of leaf senescence is accompanied by immense changes in the leaf transcriptome. It is estimated that about 20% of all genes are altered in expression upon induction of senescence, implying an important role for transcriptional regulators (Balazadeh et al., 2008; Breeze et al., 2011; Buchanan-Wollaston et al., 2005; Zentgraf et al., 2004). NAC and WRKY transcription factors are over-represented in the senescence transcriptome (Guo et al., 2004) and some members of these two transcription factor families have been shown to play central roles in regulating senescence (Balazadeh et al., 2010, 2011; Besseau et al., 2012; Breeze et al., 2011; Miao et al., 2004; Uauy et al., 2006; Ülker et al., 2007; Yang et al., 2011). WRKY proteins are plant-specific transcriptional regulators that contain a DNA-binding domain of ~60 amino acids. This domain contains a WRKYGQK motif at the N terminus and a zinc-finger structure at the C terminus, and is called the WRKY domain. Diverse processes, such as the response to pathogens or wounding but also leaf senescence, are controlled by WRKY transcription factors (Rushton et al., 2010). WRKY53, a key player in age-induced leaf senescence, regulates a complex network of downstream targets that promote vast physiological changes associated with the reallocation of nutrients and the induction of cell death (Lin and Wu, 2004; Miao et al., 2004). Owing to its important function, *WRKY53* expression, activity and protein stability are tightly controlled (Zentgraf et al., 2010). When leaf senescence is induced, the *WRKY53* locus is activated by histone modifications H3K4me2 and H3K4me3 (Ay et al., 2009; Brusslan et al., 2012), whereas DNA methylation remains low and unchanged (Zentgraf et al., 2010). Several promoter-binding proteins have already been characterized for *WRKY53* regulation, including WRKY53 itself, other WRKYs and the activation domain protein (AD protein), which has some similarity to HPT kinases and works as an activator of *WRKY53* expression (Miao et al., 2008; Potschin et al., 2014). In addition, a mitogen-activated protein kinase kinase kinase (MEKK1) was characterized to bind directly to the DNA of the *WRKY53* promoter. The binding region of MEKK1 appears to be involved in the switch from leaf age-dependent to plant age-dependent expression of *WRKY53* (Hinderhofer and Zentgraf, 2001; Miao and Zentgraf, 2007). MEKK1 can directly phosphorylate the WRKY53 protein, thereby increasing its DNA-binding activity (Miao and Zentgraf, 2007). As almost all WRKY factors contain WRKY factor-binding sites (W-boxes) in their proximal promoter regions, a complex regulatory WRKY network exists. Besides the transcriptional regulation, WRKY53 protein stability is strongly controlled by a HECT E3-ubiquitin ligase (Miao and Zentgraf, 2010). Moreover, gene expression changes are accompanied by hormonal changes. Although the plant hormones cytokinin and auxin act to delay senescence (Kim et al., 2011;

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Li et al., 2012), ethylene, abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) strongly promote leaf senescence (Li et al., 2012). Besides hormone homeostasis, elevated hydrogen peroxide levels also trigger senescence (Bieker et al., 2012; Smykowski et al., 2010).

Here, we identify REVOLUTA (REV), a transcription factor known to regulate polarity-associated growth processes in embryos, leaves, stems, vasculature and roots (Carlsbecker et al., 2010; McConnell et al., 2001; Smith and Long, 2010), as a direct regulator of *WRKY53* expression. During early leaf development, REV is involved in establishing the dorsoventral axis of leaves by specifying the domain that will later develop into the upper side of the leaf (Byrne, 2006). REV, also known as INTERFASCICULAR FIBERLESS (IFL), has been shown to play multiple roles in meristem organization, leaf polarity set-up and vascular development (Otsuga et al., 2001; Talbert et al., 1995; Zhong and Ye, 1999). Using a ChIP-Seq approach, we identified REV-binding sites in the *WRKY53* promoter and by qRT-PCR demonstrate that REV promotes *WRKY53* expression. Conversely, plants that carry loss-of-function mutations in *REV* and other *HD-ZIPIII* genes show lower levels of *WRKY53* expression, confirming that HD-ZIPIIIs are also required for *WRKY53* expression. By performing a detailed expression analysis using both *REV* and *WRKY53* GUS-reporter lines, we reveal that both genes have partially overlapping patterns of expression. In wild-type plants, *WRKY53* expression is strongly induced in response to hydrogen peroxide. However, in *rev* mutant plants and in transgenic plants with reduced *HD-ZIPIII* activity, this response is significantly dampened. Furthermore, the ability of REV to bind to the *WRKY53* promoter is also dependent on the redox environment and, under oxidative conditions, less binding is observed. In line with the lower *WRKY53* expression levels, *rev* mutant plants are considerably delayed in age-induced leaf senescence, suggesting a role for HD-ZIPIIIs in this physiological process. Taken together, we conclude that REV is a positive regulator of *WRKY53* expression, which influences the onset of leaf senescence in response to changes in the cellular redox state. Obviously, early and late leaf development are tightly linked by transcriptional networks between HD-ZIPIII and WRKY factors, in which disturbed early development is coupled to extended life span of leaves and delayed senescence.

RESULTS

REVOLUTA is a positive regulator of *WRKY53* expression, a major factor controlling age-induced leaf senescence

REVOLUTA is a member of the class III homeodomain leucine zipper (HD-ZIPIII) transcription factor family that regulates various polarity-associated growth processes during development (Carlsbecker et al., 2010; McConnell et al., 2001; Smith and Long, 2010), but plays an additional role in shade-induced growth promotion (Bou-Torrent et al., 2012; Brandt et al., 2012). REVOLUTA expression is controlled by the microRNAs *miR165* and *miR166* at the post-transcriptional level (Rhoades et al., 2002), and by the association with small leucine-zipper-type microProteins at the post-translational level (Kim et al., 2008; Staudt and Wenkel, 2011; Wenkel et al., 2007). Using a genome-wide chromatin-immunoprecipitation sequencing approach (ChIP-Seq), we recently identified binding regions for REV across the *Arabidopsis* genome (Brandt et al., 2012). This analysis revealed binding of REV to the promoter of the *WRKY53* transcription factor (Fig. 1A). Transient promoter-GUS experiments in *Arabidopsis* protoplasts revealed an induction of *WRKY53* expression after co-transformation of *35S::REVd*, a dominant microRNA-resistant version of REV (Fig. 1B). Quantitative ChIP-PCRs confirmed the binding of REV to the ChIP-Seq identified binding motifs (Fig. 1C). For better control of REV activity, we constructed transgenic plants expressing REVd fused to the rat glucocorticoid receptor carrying an N terminal FLAG epitope. In response to dexamethasone (DEX) induction, the chimeric FLAG-GR-REVd fusion protein translocates to the nucleus, where it can associate with DNA and alter the expression of target genes. In response to DEX induction, REV can significantly upregulate *WRKY53* expression (Fig. 1D), while seedlings carrying mutations in *REV* and plants with globally reduced HD-ZIPIII activity show reduced levels of *WRKY53* mRNA (Fig. 1E), thus supporting a new role for REV as a direct and positive regulator of *WRKY53*.

REVOLUTA and *WRKY53* have overlapping patterns of expression

REVOLUTA, as well as the other class III HD-ZIP transcription factors of *Arabidopsis*, have a distinct expression pattern, confining their expression to the adaxial domain of developing leaves, the xylem part of the vasculature, the pro-vasculature and the shoot apical meristem. Both *WRKY53* and *REV* are expressed in young seedlings (Fig. 2A,B). Even though REV function was initially described for polarity-associated growth processes during early leaf development, REV is still expressed at later stages of development (supplementary material Fig. S1) and an additional function in shade avoidance has recently been assigned to REV (Brandt et al., 2012). In comparison with the vascular expression pattern of *REV*, *WRKY53* shows a broader less-specific pattern of expression and is most highly expressed in old leaves (Miao and Zentgraf, 2007). In genetic backgrounds with reduced *REV* mRNA [*rev-5* (Fig. 2C), *35S::miR165a* (Fig. 2D)] or with reduced REV protein activity (*35S::ZPR3*; Fig. 2E), the spatial expression of *WRKY53* is more restricted to hydatodes and overall expression levels appear to be much lower in leaf tissue. In older seedlings, expression of both genes is found in vascular strands (Fig. 2F-M). Surprisingly, high co-expression is observed in the root vasculature at all investigated stages of development. It is not known whether WRKY53 has an additional function in root development but it might be important to note that the expression in the root vascular appears to be independent of HD-ZIPIII function (Fig. 2B-E).

Using publicly available microarray data (http://bar.utoronto.ca), we also analyzed at which stages of development and in response to which treatments *REV* and *WRKY53* are co-expressed (supplementary material Fig. S2). We find evidence for co-expression during early developmental stages but not during the later stages of leaf development. This discrepancy suggests that *REV* mRNA is not upregulated at late stages of leaf development but residual protein could respond to a cellular signal and induce the expression of REV-regulated senescence targets. However, our GUS expression analyses using *REV::GUS* plants indicate that REV is still expressed to certain extends in older leaves (supplementary material Fig. S1).

In order to identify other direct REV targets that show an expression pattern resembling *WRKY53*, we surveyed recently published timecourse microarray datasets (Reinhart et al., 2013) that revealed 119 genes to be upregulated in response to REVOLUTA induction. Our ChIP-Seq datasets resulted in the identification of 286 high confidence REV-binding sites (corresponding to 552 potentially regulated genes) across the entire *Arabidopsis* genome (Brandt et al., 2012). By comparing both datasets, we could identify 18 of the 119 REV-regulated genes (15% of the REV upregulated set) to have REV-binding sites in their respective promoters (Table 1). *WRKY53* is among these 18 genes and we investigated whether other senescence-related genes could be identified in this dataset. A

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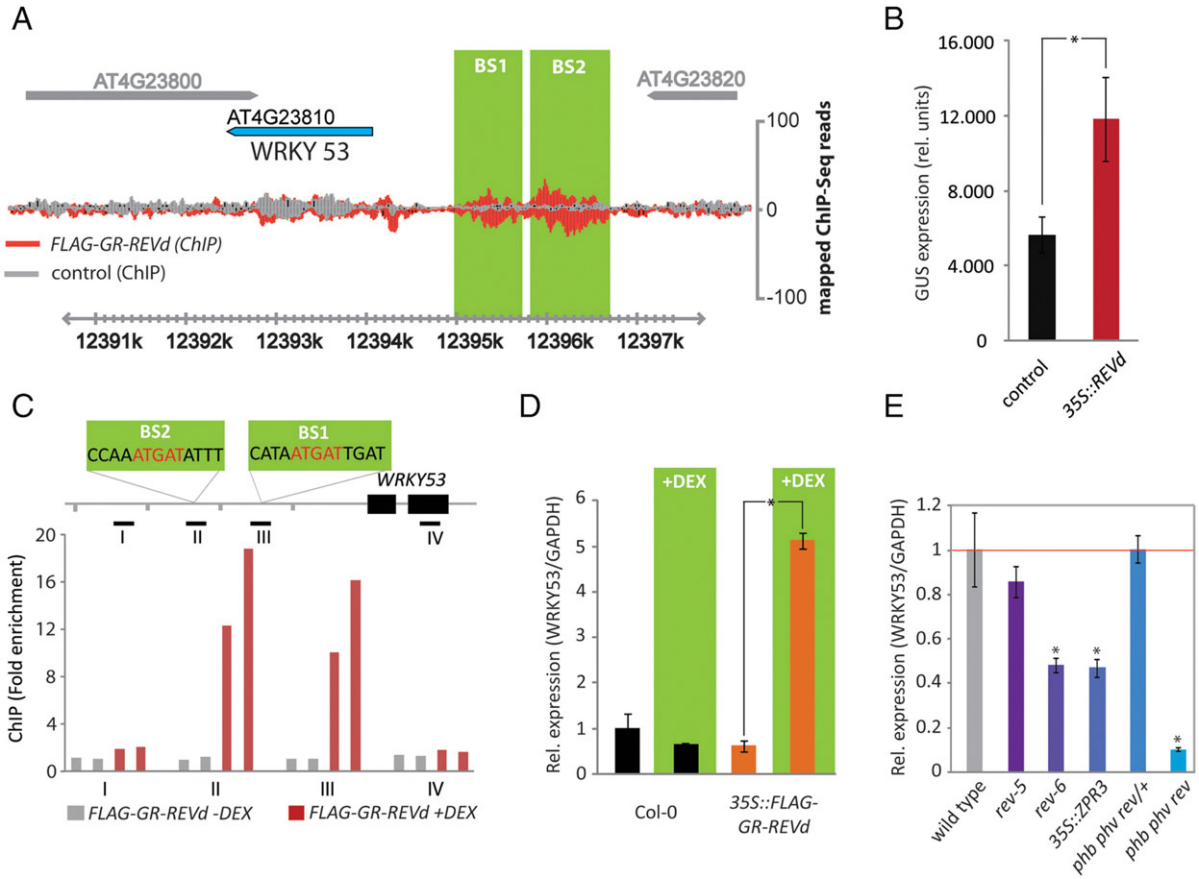

Fig. 1. REVOLUTA binds to the *WRKY53* promoter and is a direct and positive regulator of *WRKY53* expression. (A) ChIP-Seq results for the binding of REV to the *WRKY53* promoter. Two binding sites (BS) were identified, located −1.3 kb and −2.1 kb upstream of the transcriptional start site. Traces in gray are sequence reads derived from sequencing ChIP DNA from Col-0 wild-type plants; red plots ChIP DNA from dexamethasone-induced *35S::FLAG-GR-REVd* transgenic plants. (B) Transient expression assay in *Arabidopsis* protoplasts. A plasmid with a 2.8 kb *WRKY53* promoter fragment fused to the *GUS* gene was transformed along with a second plasmid containing a *CaMV35S*-promoter (control) or the *CaMV35S*-promoter driving expression of *REVd*. GUS activity was determined ~15 h after transformation. Data are mean±s.d. **P*<0.05. (C) Chromatin-immunoprecipitation qPCR experiments with two biological replicates for *35S::FLAG-GR-REVd* without DEX (gray bars) and *35S::FLAG-GR-REVd* with DEX (red bars) plants testing four positions in the *WRKY53* promoter. Y-axis shows the fold enrichment normalized to the non-induced IPs. Gene map above the chart shows 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 1.0 kb. (D) Real-time quantitative PCR experiments showing expression changes of *WRKY53* in Col-0 (black) and *35S::GR-REVd* (orange) in response to 60 min DEX induction in the presence of the protein biosynthesis inhibitor cycloheximide (CHX). Data are mean±s.d. **P*<0.05. (E) Expression of *WRKY53* was analyzed in different *rev* mutant plants (*rev-5*, *rev-6*, *phb phv rev/+* and *phb phv rev*) and in plants with reduced activity of HD-ZIPIII proteins (*35S::ZPR3*). The bars indicate expression levels relative to wild type, including standard errors of the mean of three individual biological experiments. **P*<0.05.

genome-wide survey with a high temporal resolution classified thousands of genes as differentially expressed senescence genes (DESGs) (Breeze et al., 2011). Interestingly, REV was also classified as a DESG, showing a dip of expression at the onset of leaf senescence. Furthermore, nine out of the 18 potential direct REV targets (Table 1) were also classified as DESGs, implying that REV might have an additional function in late developmental stages.

WRKY53 expression is modulated in response to oxidative stress in a REVOLUTA-dependent manner

WRKY53 expression is strongly upregulated in response to hydrogen peroxide as part of the age-induced senescence-promotion pathway (Miao et al., 2004). Because REV is a novel upstream regulator of *WRKY53* expression and possesses a domain that is suggestive of sensing changes in the redox state of the cell, we investigated whether REV is required for the induction of *WRKY53* expression in response to oxidative stress. Therefore, we grew Col-0 wild-type plants and mutant plants with reduced HD-ZIPIII activity (*rev5*, *35S::miR165a* and *35S::ZPR3*) on soil for 3 weeks in long-day conditions. In order to elicit oxidative stress, plants were sprayed with hydrogen peroxide solutions of different concentrations (0.01%, 0.1% and 1%) and plant material was harvested before and after spraying. Subsequent RNA isolation, cDNA synthesis and quantitative PCR analysis revealed a strong induction of *WRKY53* in response to H_2O_2 application in Col-0 wild-type plants. These changes of *WRKY53* mRNA levels were significantly dampened in *rev* mutant plants (*rev-5*) and *35S::miR165a*, and in plants with reduced HD-ZIPIII activity (*35S::ZPR3*), indicating that REV activity is required for high-level *WRKY53* induction in response to oxidative stress signaling (Fig. 3). To assess which externally applied hydrogen peroxide concentration is able to elicit redox changes that would occur under natural conditions, we measured intracellular hydrogen peroxide levels after applying heat stress and compared them with the intracellular levels reached after external application of H_2O_2 by spraying. To be sure that only intracellular H_2O_2 is measured, we used non-fluorescent H_2DCFDA (2′,7′-dichlorodihydrofluorescein diacetate), which is converted to the highly fluorescent 2′,7′-dichlorofluorescein upon cleavage of the acetate groups by intracellular esterases and

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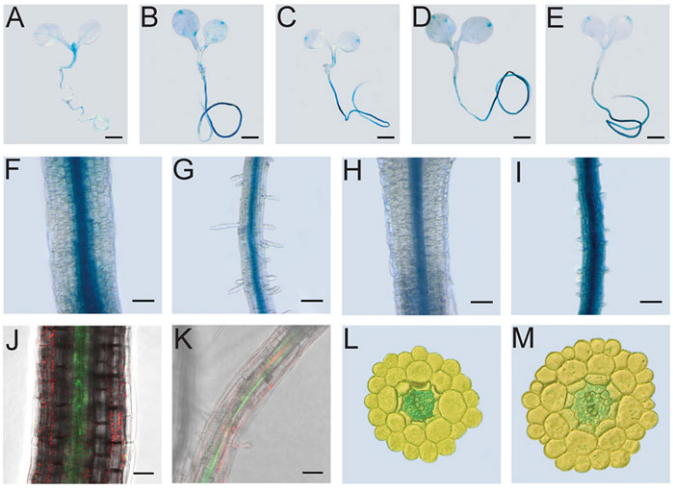

Fig. 2. Expression analysis of *REV* and *WRKY53*. (A-I) Spatial patterns of expression of *REV* (A,F,G) and *WRKY53* (B-E,H,I) in 8-day-old *Arabidopsis* seedlings. GUS staining of *REV::GUS* (A), *WRKY53::GUS* (B) in the Col-0 ecotype and *WRKY53::GUS, rev5* (C), *WRKY53::GUS, 35S::miR165* (D), *WRKY53::GUS, 35S::ZPR3* (E) seedlings. Scale bars: 1 mm. (F-I) Hypocotyls (F,H) and roots (G,I). (J,K) The pattern of GFP accumulation in the hypocotyl (J) and root (K) vascular tissue of 8-day-old plants carrying the *REV::REV-GFP* transgene. Scale bars: 50 µm. (L,M) Cross-sections of roots of 10-day-old seedlings reveal *REV* (L) and *WRKY53* (M) expression in the vascular cylinder.

subsequent oxidation. The increase in intracellular H_2O_2 was similar 1 h after heat treatment and 1 h after spraying 0.1% H_2O_2 but dropped more rapidly in the H_2O_2-treated samples. This indicates that external application of 0.1% H_2O_2 leads to intracellular changes in the range of an oxidative burst in stress response (supplementary material Fig. S3).

REVOLUTA is a redox-sensitive transcription factor

REV is a positive regulator of *WRKY53* expression and is required for high level of *WRKY53* induction in response to oxidative stress. This could be either due to an upregulation of *REV* mRNA in response to oxidative stress or to a response of the REV protein to altered redox conditions. To test whether *REV* mRNA is upregulated in response to hydrogen peroxide treatment, we treated Col-0 wild-type plants with H_2O_2 and performed quantitative RT-PCRs. We detected no induction of *REV* mRNA but a slight decrease in response to high levels of hydrogen peroxide (supplementary material Fig. S4), excluding the idea that *REV* is transcriptionally upregulated in response to oxidative stress.

It has been shown that proteins of the class II homeodomain leucine-zipper (HD-ZIPII) family from sunflower interact with DNA in a redox-sensitive manner (Tron et al., 2002). To test whether REV shows also redox-dependent DNA binding, we performed redox-sensitive DPI-ELISA experiments. Therefore, crude lysate of *E. coli* cells expressing HIS-tagged REV protein were prepared and incubated with streptavidin plates pre-loaded with biotinylated oligonucleotides containing the REV-binding site 1 of the *WRKY53* promoter (W53-BS1). ELISA plates were then washed and subsequently incubated with HRP-tagged anti-HIS antibodies. Enhanced signal was detected in the control binding reaction (HIS-REV lysate versus a lysate from BL21 cells expressing the empty vector control), indicating that HIS-REV binds to the W53-BS1 element (Fig. 4A). As observed for the sunflower HD-ZIPII proteins (Tron et al., 2002), REV also showed enhanced binding in response to reducing conditions (10 mM DTT), whereas in response to oxidative conditions (10 mM H_2O_2) DNA-binding was reduced (Fig. 4A). This negative effect is reversible as the subsequent addition of 10 mM DTT was able to restore REV DNA binding.

We examined the possibility of whether the C-terminal PAS-domain of REV might act as a redox sensor domain. Redox-DPI-ELISA experiments with HIS-REV lacking the PAS-domain (HIS-REVΔPAS) showed the same redox-sensitive behavior as observed for HIS-REV (Fig. 4B). However, without the PAS-domain, REV-DNA binding was strongly enhanced, supporting the idea that the PAS-domain regulates REV activity via a steric masking mechanism, as proposed by Magnani and Barton (2011). It is conceivable that the observed redox effects in the ELISA system

Table 1. Identification of potentially direct REV target genes by comparing ChIP-Seq and microarray experiments with an inducible version of REV

		Microarray	ChIP-Seq				
AGI	Name	Fold change	q_rank	Enrichment	Distance	Location	DESG*
AT2G41940	ZFP8	2.0	469	7.5	1691	Down	Yes
AT5G47370	HAT2	3.1	253	8.0	1548	Up	No
AT2G39705	DVL11/RTFL8	2.8	1626	7.6	2509	Down	No
AT5G06710	HAT14	2.7	272	9.3	5364	Up	No
AT5G47180	Plant VAMP protein	1.7	35	15.2	168	Up	Yes
AT5G19590	DUF538 protein	1.3	465	9.4	2810	Up	Yes
AT4G18700	CIPK12	3.0	169	13.2	282	Down	No
AT4G27730	OPT6	2.7	30	16.1	1305	Up	No
AT4G03510	RMA1	7.0	33	14.9	1989	Up	No
AT1G17970	RING/U-Box protein	5.1	1173	9.7	8	Up	Yes
AT5G14730	DUF1645	5.8	726	6.6	2299	Up	No
AT2G45450	ZPR1	13.1	400	9.2		5′UTR	No
AT5G05690	CPD	1.9	202	8.0	4847	Up	No
AT1G74940	DUF581	2.2	106	17.5	81	Up	Yes
AT3G60390	HAT3	2.9	115	8.7	5597	Up	Yes
AT4G23810	WRKY53	3.9	450	8.7	2132	Up	Yes‡
AT5G16030	Unknown protein	2.8	789	9.6	2193	Up	Yes
AT1G49200	RING/U-Box protein	14.4	18	14.2	187	Up	No
AT2G02080	IDD4	0.5	528	8.2	8334	Down	No
AT3G13810	IDD11	0.4	1643	6.7	1422	Down	Yes

*Differentially expressed genes during senescence (Breeze et al., 2011).
‡Senescence-associated gene not included in the Breeze et al. (2011) analysis.

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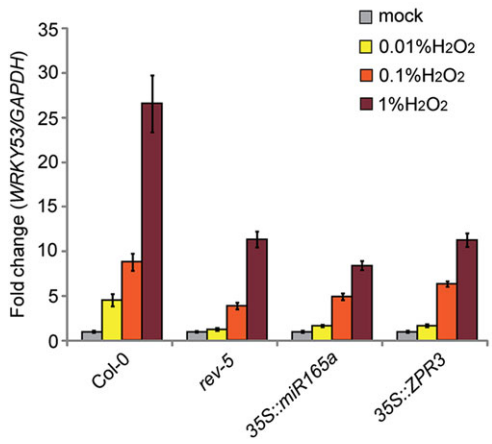

Fig. 3 HD-ZIPIII activity is required for H_2O_2-mediated upregulation of *WRKY53*. Real-time qPCR experiment showing *WRKY53* induction in response to hydrogen peroxide treatment in wild-type and *rev* mutant plants. Three-week-old plants were treated with different concentrations of H_2O_2 [0% (mock; gray bars), 0.01% (yellow bars), 0.1% (orange bars) and 1% (red bars)] for 40 min. Data are representative relative expression changes (fold change) of the mean of four technical replicates±s.d. Similar expression changes have been observed in at least two independent biological experiments.

are due to an influence of *E. coli* proteins on the activity of REV. To exclude such effects, we purified GST-REV protein from *E. coli* and performed *in vitro* gel retardation assays in the presence of reducing agents (DTT) and oxidizing agents (H_2O_2) (Fig. 4C). These gel-shift experiments largely confirm the results obtained by redox-DPI-ELISA and confirm that REV activity can be modulated by the intracellular redox state.

To validate redox-sensitive DNA binding *in planta*, we treated *35S::FLAG-GR-REVd* transgenic plants with either a mock substrate (0.1% ethanol), dexamethasone (DEX) or DEX+0.1% H_2O_2. In 12-day-old seedlings, we detected REV binding to binding site 2 (fragment II) and no binding was observed to binding site 1 (fragment III). When treated with hydrogen peroxide prior DEX induction, binding to binding site 2 was significantly affected (Fig. 4D), indicating that REV DNA binding is indeed redox sensitive. The same experiment with 7-week-old plants revealed that, at later developmental stages, both binding sites are occupied by REV and the binding seems to be enhanced but exhibits the same redox sensitivity (Fig. 4E). Taken together, we demonstrate that REV shows a stage-specific redox-dependent DNA-binding behavior and that oxidizing conditions decrease the ability to bind DNA *in vitro* and *in vivo*.

Mutations in the *REVOLUTA* gene or the overall reduction of HD-ZIPIII activity delay the onset of leaf senescence

One function of the WRKY53 protein is the regulation of the onset of senescence, documented by the phenotype of the *wrky53* mutant showing delayed senescence. As REV is an activator of *WRKY53* expression, we expected *rev* mutant plants to also display a delayed senescence phenotype. Our analysis revealed that plants carrying mutations in REV or plants with greatly reduced HD-ZIPIII activity are significantly delayed in senescence, while overall development is not retarded, which clearly confirms a role of HD-ZIPIII proteins in this process (Fig. 5; supplementary material Figs S5, S6). Furthermore, the phenotype of *rev5* was even stronger than that of *wrky53*, indicating that *WRKY53* might not be the only senescence-associated gene regulated by REV.

Overexpression of the small leucine-zipper-type microProtein ZPR3, which largely reduces the activity of HD-ZIPIIIs, led to a further enhancement of the senescence phenotype, which was ameliorated in the *wrky53* mutant background (supplementary material Fig. S3). This confirms that the senescence phenotype is

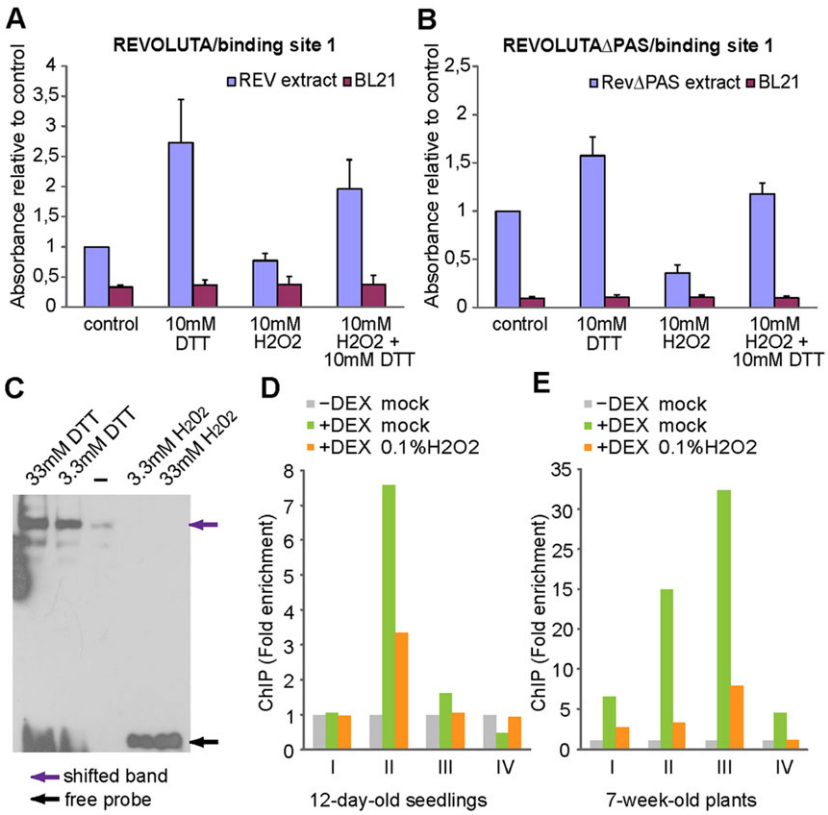

Fig. 4. Redox-mediated regulation of REVOLUTA-DNA-binding capability and influence of the PAS domain. (A,B) Redox-DPI-ELISAs. The DNA-protein interaction assays were performed by using 5′ biotinylated complementary annealed oligonucleotides coupled to a streptavidin-coated ELISA plate. Crude *E. coli* extracts (25 µg) expressing recombinant REV or REVΔPAS were pre-incubated with different concentrations of DTT and H_2O_2 to examine a redox state-dependent binding of REV. In order to test the reversibility of the redox effect, high concentrations of H_2O_2 were added first and then oxidizing conditions were reversed by addition of DTT. After binding, biotinylated DNA-protein complexes were detected using anti His-HRP conjugated antibodies. Results for REV binding site 1 of the *WRKY53* promoter are shown. *E. coli BL21* cells transformed with the empty vector were used as background control. (C) Non-radioactive electrophoretic mobility shift assays. Purified GST-REV protein was incubated with a biotinylated oligonucleotide containing the HB9-binding motif (Wenkel et al., 2007) in the presence of different redox conditions. After gel electrophoresis and subsequent blotting, the biotinylated DNA probe was detected with a HRP-strepatividin substrate. (D,E) Chromatin-immunoprecipitation qPCR assays of *35S::FLAG-GR-REVd* plants. Twelve-day-old seedlings (D) and 7-week-old transgenic plants (E) were treated with mock substrate (0.1% ethanol), DEX or 0.1% H_2O_2 and DEX. H_2O_2 was given 15 min prior to 45 min of DEX induction. Fold enrichment for the same primer sets as in Fig. 1 is shown.

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mediated by deregulation of *WRKY53* expression through HD-ZIPIIIs but also suggests that additional HD-ZIPIIIs are involved, as the senescence phenotype of *35S::ZPR3* plants is much stronger compared with *rev5* mutants (Fig. 5; supplementary material Figs S5,S6). Consistent with the phenotype, two typical senescence-related physiological parameters, the decrease in chlorophyll content and the increase in lipid peroxidation, were also delayed in *wrky53*, *rev5* and *rev5 wrky53* mutants (Fig. 6A,B). Furthermore, the mRNA expression levels of *SENESCENCE ASSOCIATED GENE* 12 (*SAG12*) and *SAG13*, which are commonly used as senescence marker genes, were significantly reduced at the late developmental stages in *wrky53*, *rev5* and *rev5 wrky53* mutants compared with Col-0 wild-type plants (Fig. 6C,D). Taken together, these results confirm that REV acts upstream of *WRKY53* in the control of age-induced senescence.

Depletion of *REV* delays the onset of leaf senescence more efficiently than depletion of *WRKY53*. To further investigate the possibility that REV acts upstream of several senescence-associated genes, we focused our attention on the potential direct REV targets classified as DESGs (Table 1). Here, we decided to investigate three groups of genes: (1) genes whose expression decreases with age (*HAT3* and *AT1G49200*); (2) genes whose expression increases with age (*AT1G74940* and *IDD11*); and (3) genes whose expression decreases with age but rises during senescence (*AT5G47180* and *ZFP8*). In the first group of genes, we found that expression in *wrky53*, *rev5* and *rev5 wrky53* mutants is maintained at a higher level towards the onset of senescence (weeks 5 and 6), whereas expression levels are dropping rapidly in wild-type plants (Fig. 7A,B). For the second group of genes whose expression increases with age in wild-type plants, we detected elevated levels in *wrky53*, *rev5* and *rev5*

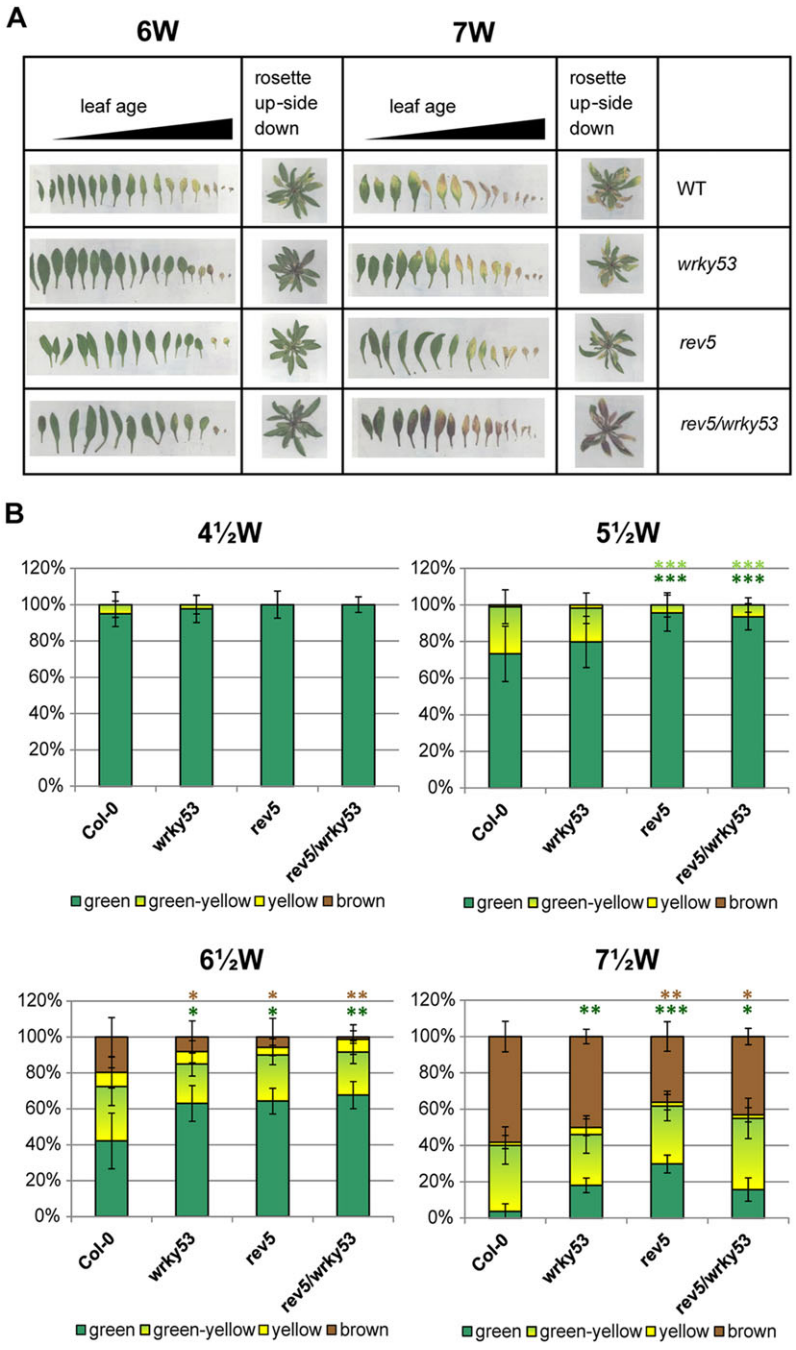

Fig. 5. Genetic interaction of *REV* with *WRKY53*. (A) Rosette leaves of 6- and 7-week-old representative plants were sorted according to their age; whole rosettes were also photographed upside down to visualize the older leaves. (B) For a quantitative evaluation of leaf senescence, plants were harvested in a weekly rhythm and leaves of at least six plants were categorized into four groups according to their leaf color: (1) 'green'; (2) leaves starting to become yellow from the tip as 'yellow-green'; (3) completely yellow leaves as 'yellow'; and (4) dry and/or brown leaves as 'brown/dry'. The percentages of each group with respect to total leaf numbers are presented. Error bars indicate s. d. Student's *t*-test was performed comparing leaf counts of *wrky53*, *rev5* and *rev5wrky53* with Col-0 numbers, $*P<0.05$, $**P<0.005$, $***P<0.0005$. n=7-15.

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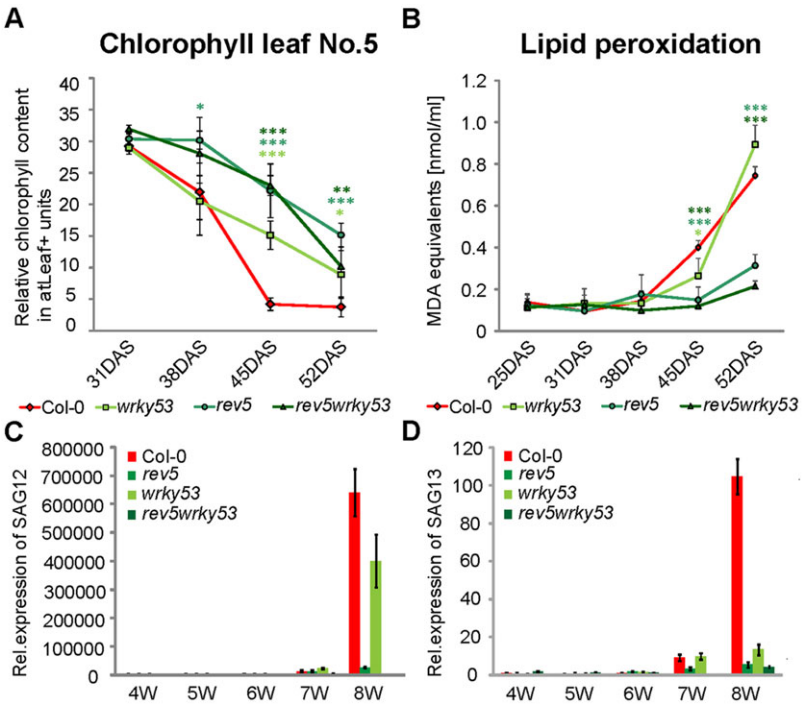

Fig. 6. Molecular senescence parameters. (A) Chlorophyll contents of number 5 leaves from *Arabidopsis* Col-0, *wrky53*, *rev5* and *rev5wrky53* plants. Left axis indicates atLeaf+ values. Plant age is indicated in days after seeding (DAS). (B) Lipid peroxidation in Col-0, *wrky53*, *rev5* and *rev5wrky53* plants. Values represent mean of at least three biological replicate±s.d. Comparison of means and the determination of statistical differences was carried out using Student's *t*-test (**P*<0.05, ***P*<0.005 and ****P*<0.0005). (C,D) qRT-PCR expression analysis of the senescence marker genes *SAG12* and *SAG13*. All values were normalized to *GAPDH* expression. Error bars indicate s.d. of four technical replicates.

wrky53 mutants at early developmental stages (weeks 4 and 5) and decreased levels at the late stages (Fig. 7C,D). Expression of the third group of genes is also altered at various time points in *wrky53*, *rev5* and *rev5 wrky53* mutants compared with Col-0, but in all lines the transcriptional increase during senescence is diminished (Fig. 7E,F), further corroborating the idea that loss of *REV* function profoundly alters the senescence transcriptome, which might be causative for the strong senescence phenotype of *rev* mutant plants.

Loss-of-function *wrky53* mutant plants do not show obvious developmental defects during early leaf development, indicating that WRKY53 is not required for REV function at these stages of development. However, the severe *35S::ZRP3*-induced leaf phenotype is ameliorated in the *wrky53* mutant background, suggesting that the action of other HD-ZIPIIIs involves WRKY53 also at early stages (supplementary material Fig. S7). Nonetheless, WRKY53 protein levels are most likely very low during these early

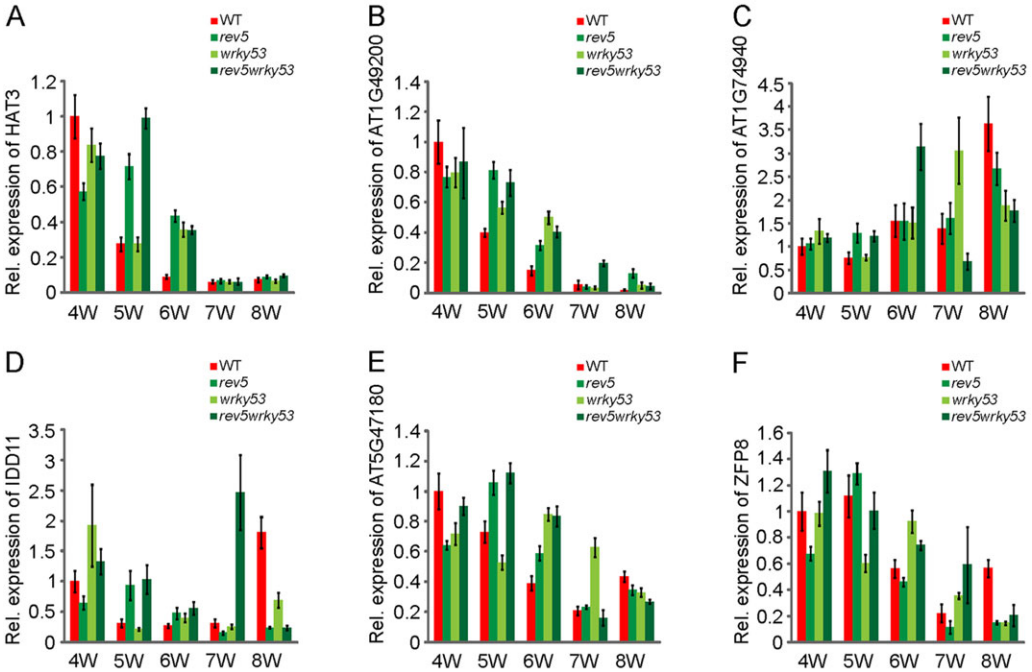

Fig. 7. qRT-PCR of other REV target genes differentially expressed during senescence. Quantitative real-time PCR profiling of putative REV target genes at late developmental stages in wild-type and mutant plants (4-, 5-, 6-, 7- and 8-week-old plants). (A-F) Expression changes over time of *HAT3*, *AT1G49200*, *AT1G74940*, *IDD11*, *AT5G47180* and *ZFP8*. The Y-axis represents the relative expression level normalized to *GAPDH*. Error bars indicate s.d. of four technical replicates.

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stages of development due to the degradation of WRKY53 by the HECT domain ubiquitin ligase UPL5, which is highly expressed in young leaves (Miao and Zentgraf, 2010). Taken together, we discovered that HD-ZIPIIIs interact with *WRKY53* genetically to promote age-induced leaf senescence, and disruption of early leaf development correlates with delayed senescence and extended life span of leaves.

Functional analyses of root-specific co-expression patterns of *REV* and *WRKY53*

It is unknown which tissues are involved in the perception of senescence signals and conversion of these into the senescence triggers. We find co-expression of *REV* and *WRKY53* during the early stages of leaf development. Later in development, co-expression was very obvious in the vasculature of the leaves and in the root vascular cylinder (Fig. 2L,M), although both *REV* and *WRKY53* are expressed throughout development (supplementary material Fig. S1). This is in agreement with the finding that REV is involved in the induction of *WRKY53* expression by hydrogen peroxide and that very high levels of hydrogen peroxide were observed in vascular tissue indicated by DAB staining of leaf sections (Zimmermann et al., 2006). Moreover, it remains tempting to speculate that the root might also act as a senescence sensor; however, whether roots play a role during onset and progression of senescence has not yet been determined and whether and to what extent hydrogen peroxide is transported through the vasculature over long distances is also not known so far. Auto-propagating waves of reactive oxygen species (ROS) that rapidly spread from the initial site of exposure to abiotic stress to the entire plant are involved in conferring systemic acquired acclimation, also allowing a much faster transcriptome and metabolome reprogramming of systemic tissues in response to abiotic stress (Mittler et al., 2011; Suzuki et al., 2013).

To further investigate the spatial aspects of *REV* and *WRKY53* expression, we decided to perform grafting experiments with Col-0 wild-type, *rev5* and *wrky53* mutant plants. When the aerial parts of Col-0 were grafted onto either *wrky53* or *rev5* rootstocks, no significant delays in the onset of senescence were observed. However, the converse grafting of the aerial parts of either *wrky53* or *rev5* to Col-0 rootstocks significantly delayed the onset of senescence where the latter again showed a much stronger effect (Fig. 8A,B). The grafting experiments revealed that the root seems not to be involved in the REV/WRKY53-mediated senescence pathway and that depletion of *REV* and *WRKY53* in only aerial tissue strongly affects senescence.

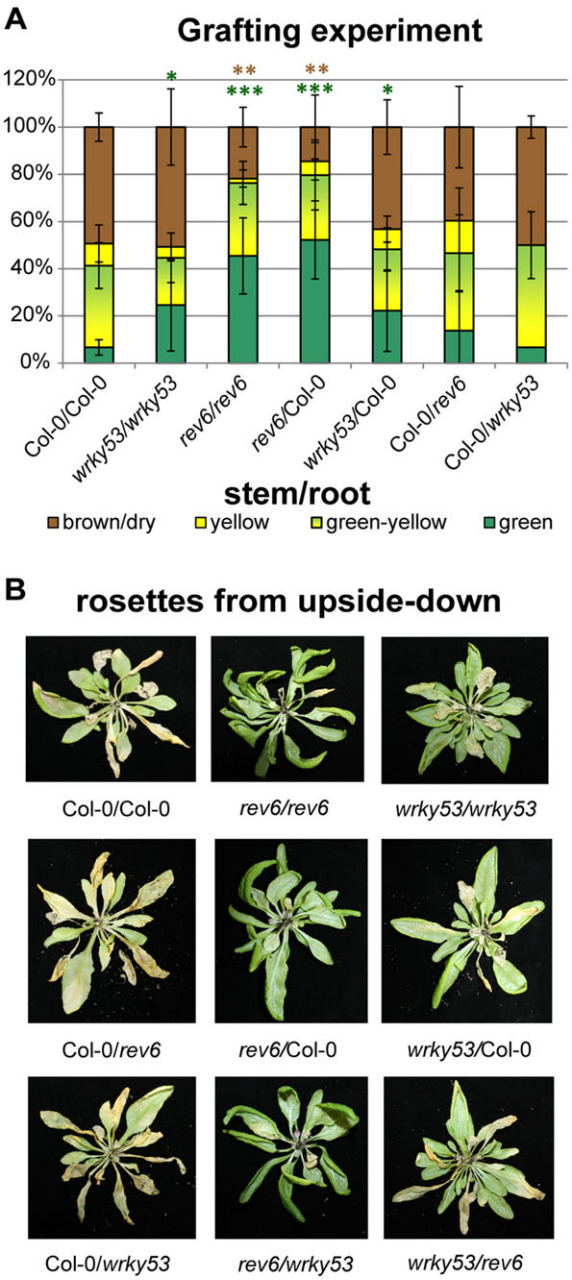

Fig. 8. Grafting experiments and senescence phenotype. (A) Nine combinations of grafted plants were generated between the wild-type and mutant plants (*rev6* and *wrky53*), including three self-grafted controls, e.g. wild type to wild type (Col-0/Col-0; scion/root). Error bars indicate s.d. (n=4-6 independent grafted plants with the exception of Col-0/wrky53, where we achieved only two successful grafts). The quantitative evaluation of leaf senescence of the non-grafted plants is shown in Fig. 5. Asterisks represent significant differences from the Col-0/Col-0 graft, as determined using Student's t-test (*P<0.05, **P<0.005, ***P<0.0005). (B) The leaf-senescence phenotypes of grafts. Photographs were taken 7 weeks after grafting.

DISCUSSION

Plants induce leaf senescence to provide carbon, nitrogen and mineral resources to the developing fruits or seeds. Senescence is induced in response to plant age but environmental signals such as light, the availability of water and temperature strongly influence this process. A high-resolution temporal transcript profiling of senescing *Arabidopsis* leaves gives insight into the temporal order of gene activation and repression (Breeze et al., 2011). Approximately 6500 genes are up- or downregulated during the course of leaf senescence, implying an important role for transcription factors in this process. Transcription factors themselves are transcriptionally upregulated in senescing leaves the largest groups being NAC, WRKY, C2H2-type zinc-finger, AP2/EREBP and MYB proteins (Guo and Gan, 2005). Here, we now show that HD-ZIPIII factors, which are known to be involved in basic patterning processes, have an additional role in the latest step of leaf development, the regulation of senescence. REV is a direct and positive regulator of *WRKY53* expression and mutations in *REV* and other *HD-ZIPIII* genes delay the onset of leaf senescence. Interestingly, the delay of the onset of leaf senescence in plants lacking *REV* is stronger compared with plants lacking only *WRKY53*, implying that REV acts also upstream of other senescence-associated genes. In plant lines with even more reduced *HD-ZIPIII* activity, achieved by overexpression of

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miRNA165a (*35S::miR165a*), rosette leaves were so strongly downward curled that it was impossible to determine the onset of senescence. The loss of several *HD-ZIPIII* genes, as in the case of the *phb phv rev* triple mutant, causes severe developmental defects, including consumption of the apical stem cells (Emery et al., 2003; Prigge et al., 2005). The severity of these developmental defects largely precludes a thorough analysis of the general role of HD-ZIPIII proteins at later stages of development. Nevertheless, our findings clearly suggest that the role of HD-ZIPIIIs in promoting senescence is more complex and involves regulation of several senescence-associated target genes. In the *rev5/wrky53* double mutant, leaf yellowing and chlorophyll loss were less severe at later stages than in the *rev* single mutant, whereas senescence-associated gene expression was more severely affected for some senescence-related genes. This clearly points towards a complex network that is altered in different aspects if one or more components are depleted from the system. It was already shown that WRKY53 acts as an upstream regulator, downstream target and protein-interaction partner of WRKY18, which is a negative regulator of leaf senescence, illustrating the complexity of the network and possibly explaining the partially intermediate phenotype of the double mutant (Potschin et al., 2014).

The mechanism by which REV promotes senescence appears to involve transcriptional regulation of direct target genes. Here, we have identified nine genes that are potential direct REV targets that are also differentially expressed during senescence. One of these target genes is *HAT3*, which has been shown to play an important role downstream of REV in the process of setting up polarity in the young leaf primordium (Bou-Torrent et al., 2012; Brandt et al., 2012; Turchi et al., 2013). In young seedlings, *HAT3* expression depends partly on the presence of REV, which is supported by lower levels of *HAT3* mRNA in *rev* mutant seedlings (Brandt et al., 2012). During senescence, *HAT3* mRNA levels decrease with plant age (Fig. 7A). In *rev* mutant seedlings, however, *HAT3* mRNA is more abundant compared with wild type (Fig. 7A). Moreover, the expression levels of several other senescence-related target REV genes changed in a complex way (Fig. 7B-F). These findings suggest that the transcriptome of *rev* mutant plants is profoundly altered, resulting in stage-dependent mis-expression of many differentially expressed senescence-associated genes.

It still remains unclear to which endogenous or exogenous signals HD-ZIPIIIs respond in order to promote senescence. The finding that *WRKY53* expression is strongly upregulated in response to hydrogen peroxide treatment and that this induction is dampened in *hd-zipIII* mutant plants implies that HD-ZIPIIIs might be involved in signal transduction processes in response to changes in the intracellular redox state. Many senescence-associated genes, especially transcription factors of the WRKY and the NAC family, transcriptionally respond to elevated levels of hydrogen peroxide but the mechanism by which the hydrogen peroxide signal is perceived and transmitted is still unclear. Remarkably, the subcellular compartment of hydrogen peroxide production appears to play a role in senescence signaling in which the cytoplasmic H_2O_2 is more effective in senescence induction than peroxisomal or mitochondrial H_2O_2 (Bieker et al., 2012; Zentgraf et al., 2012). Thus, sensors and mediators of hydrogen peroxide-induced senescence are most likely cytoplasmic and/or nuclear proteins or molecules. During bolting, intracellular hydrogen peroxide levels increase in leaf tissue. This increase is thought to be mediated by a complex regulation of the hydrogen peroxide scavenging enzymes and promotes the onset of senescence (Bieker et al., 2012; Smykowski et al., 2010).

Analysis of the redox sensitivity of the REV protein revealed a reduced DNA-binding ability of REV in response to oxidative conditions, which appears to be a direct effect on the REV protein and does not involve accessory proteins. These results contradict the finding that upregulation of *WRKY53* partially requires HD-ZIPIIIs and indicate a more complex regulatory mechanism. Most likely, DNA-binding of REV is affected by redox changes and also the transactivation activity or protein-protein interfaces, which will be further dissected in the future. However, two of the direct REV target genes encode EAR-domain proteins that are part of transcriptional repressor complexes (Causier et al., 2012). Among these transcriptional repressors are HAT3 and ZFP8, the mRNA levels of which are altered in the senescence process. Therefore, it seems plausible to conclude that REV is a redox-sensitive transcription factor, which among other targets, regulates genes encoding transcriptional repressors. Decreasing REV DNA-binding activity will result in lower expression levels of these transcriptional repressors, alleviating the repressive activity on their targets. Thus, modulation of REV activity in response to alterations of the intracellular redox state will profoundly affect the REV-regulated transcriptome. It is tempting to speculate that also within the shoot apical meristem, domains with different cellular redox states might exist that could serve as positional signals affecting HD-ZIPIII activity.

Developmental age is a major determinant for the induction of leaf senescence in an optimal growth environment. However, when plants are exposed to situations that strongly permit normal growth, senescence is accelerated in order to bypass these adverse conditions and produce seeds that can withstand these adverse conditions. We have tried to depict the complex interplay between REV and WRKY during early and late development in a model (Fig. 9) in which the regulatory cues of REV involving miRNA-dependent regulation through *miR165*, *miR166* and the LITTLE ZIPPER microProteins ZRP1-4 is connected to the MAP kinase-triggered WRKY transcriptional network. Several intersections can be detected between the formerly independently described players in early and late leaf development in which hydrogen peroxide might play a central role.

Shade causes profound developmental changes in shade-sensitive plants aimed at outgrowing competitor plants. We have previously shown that the leaf regulatory module consisting of HD-ZIPIII and KANADI transcription factors is involved in modulating

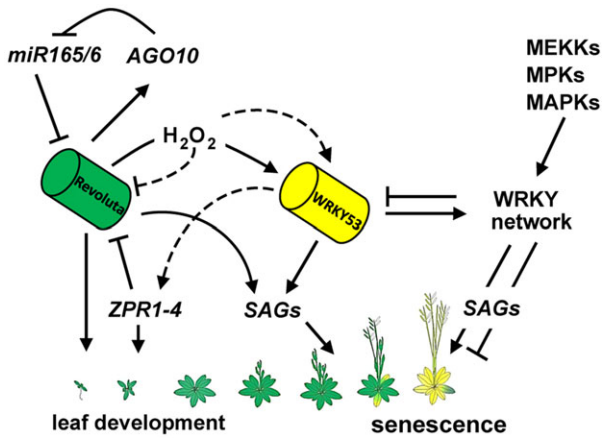

Fig. 9. Model HD-ZIPIII/senescence. A model summarizing our findings and showing the relationship between early leaf development processes and senescence. Both REV and WRKY53 intersect to regulate the late stages of leaf development.

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growth in response to shade (Brandt et al., 2012). Consistent with this, shade can also trigger leaf senescence (Brouwer et al., 2012), suggesting that leaf patterning, shade avoidance and leaf senescence are interconnected by differential activity of HD-ZIPIII proteins, thus linking early and late leaf development, and adjusting plant growth and development to changing external conditions.

Perspectives

It was recently shown that embryonic growth and patterning of mammals largely depends on cellular senescence as a developmental mechanism to shape organ growth (Muñoz-Espín et al., 2013; Storer et al., 2013). This mechanism partly relies on macrophages, which are mobile cells that invade the tissue to remove senescent cells. In this context, senescent cells also produce secreted compounds that can act as positional signals triggering pattern formation and proliferation in adjacent tissue (Storer et al., 2013). The immune system of plants is substantially different from animals and does not involve macrophage-mediated cell clearing. However, it is conceivable that local cellular senescence could provide positional information to direct growth responses. Our finding that HD-ZIPIIIs, which are known basic patterning factors, can influence senescence processes, suggest not only that early and late leaf development are coupled and processes that influence patterning in the early organ control the concerted degradation of tissue during the late phase of development, but also that physiological processes related to senescence, such as nutrient mobilization or lipid peroxidation, might be part of early leaf patterning processes. Furthermore, the puzzling reduction of DNA-binding activity under oxidizing conditions that contradicts the finding that upregulation of *WRKY53* expression by hydrogen peroxide partially requires REVOLUTA prompts us to decipher the redox-dependent changes in the REVOLUTA protein outside the DNA-binding domain in more detail. This, however, will be the subject of further investigations.

MATERIALS AND METHODS

Plant material and growth conditions

The following *rev/hd-zipIII* mutant lines were used in this study: *rev-5* (A260V) and *rev-6* (R346STOP), two strong ethyl-methylsulfonate (EMS) alleles (Otsuga et al., 2001), *phb phv rev* triple mutants introgressed in Col-0 (Prigge et al., 2005), *35S::ZPR3* (Wenkel et al., 2007) and *35S::miR165* (Kim et al., 2010). For senescence phenotyping, *Arabidopsis thaliana* plants were grown in a climatic chamber at 20°C under long-day conditions (16 h of light) with only moderate light intensity (60-100 µmol s^{-1} m^{-2}) to slow down development for better analyses. Under these conditions, the plants developed bolts and flowers within 5-6 weeks. During growth and development of the leaves, the respective positions within the rosette were color coded with different colored threads, so that even at very late stages of development, individual leaves could be analyzed according to their age. Plants were harvested in a weekly rhythm and samples were always taken at the same time in the morning to avoid circadian effects. For the evaluation of leaf senescence phenotypes, leaves of at least six plants were categorized in four groups according to their leaf color: (1) 'green'; (2) leaves starting to get yellow from the tip as 'yellow-green'; (3) completely yellow leaves as 'yellow'; and (4) dry and/or brown leaves as 'brown/dry'. Exogenous hydrogen peroxide treatment was conducted by spraying 1%, 0.1% or 0.01% hydrogen peroxide solution including 0.1% Tween20. Grafting experiments were carried out according to Marsch-Martínez et al. (2013).

Intracellular hydrogen peroxide measurements

After stress treatment, leaf 7 (0.1% H_2O_2 treatment) and leaf 8 (heat stress, 2 h at 39°C) were harvested and incubated for exactly 45 min in DCFDA working-solution (2′,7′-dichlorodihydrofluorescein diacetate, 200 µg in 40 ml MS-Medium, pH 5.7-5.8). Leaves were then rinsed with water and frozen in liquid nitrogen. After homogenization on ice, 500 µl 40 mM Tris (pH 7.0) were added and the samples were centrifuged at 4°C for 30 min. Fluorescence (480 nm excitation, 525 nm emission) of the supernatant was measured in a Berthold TriStar LB941 plate reader.

Chromatin-immunoprecipitation and quantitative PCRs

ChIP and ChIP-qPCRs were carried out as described by Brandt et al. (2012). To quantify gene expression changes, RNA was isolated from seedlings using the roboklon GeneMATRIX universal RNA purification kit following 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 µl were used for RT-PCR reactions. Quantitative measurements were performed on a Bio-Rad CFX384 using the Fermentas SYBR Green qPCR master mix. Relative quantities were calculated using the delta Ct method and normalized relative to a standard curve. Oligonucleotide sequences are listed in supplementary material Table S1. Further descriptions of the methods can be found in the supplementary material. The ChIP-Seq dataset has been published in the Gene Expression Omnibus database (accession number GSE26722).

Redox-DPI-ELISA

Recombinant 6xHis-tagged REV protein with and without the PAS domain was expressed in *E. coli* and DNA-protein interaction ELISA was basically performed as described previously (Brand et al., 2010). Crude extracts were pre-incubated with different concentrations of DTT and H_2O_2 to examine a redox state-dependent binding of REV (for a detailed description, see methods in the supplementary material).

Transformation of *Arabidopsis* protoplasts and transient promoter-GUS expression

Protoplasts were derived from a cell culture of *Arabidopsis thaliana* var. Columbia 0 and were transformed with effector and reporter plasmids following roughly the protocol of Negrutiu et al. (1987). The GUS activity assays were carried out as described by Jefferson et al. (1987). A detailed description is presented in the methods in the supplementary material.

Chlorophyll measurements and phenotypic analysis

For assessment of the leaf senescence state, chlorophyll content of leaf 5 was measured using an atLeaf+ chlorophyll meter (http://www.atleaf.com), lipid peroxidation of leaf 6 was measured using the improved thiobarbituric acid/reactive substances assay, as described previously (Hodges and Forney, 2000), and expression of the senescence-associated marker genes SAG12 (At5g45890) and SAG13 (At2g29350) was analyzed by qRT-PCR. A detailed description is presented in the methods in the supplementary material.

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Competing interests

The authors declare no competing financial interests.

Author contributions

K.H., M.P. and J.D. performed senescence phenotyping experiments and redox-ELISA; R.B., T.D. and Y.X. carried out the molecular analysis; D.S. did the gel shift experiment; S.B. measured hydrogen peroxide levels; U.Z. and S.W. designed research, analyzed the data and wrote the article.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.117689/-/DC1

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Genome-Wide Identification of KANADI1 Target Genes

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Abstract

Plant organ development and polarity establishment is mediated by the action of several transcription factors. Among these, the KANADI (KAN) subclade of the GARP protein family plays important roles in polarity-associated processes during embryo, shoot and root patterning. In this study, we have identified a set of potential direct target genes of KAN1 through a combination of chromatin immunoprecipitation/DNA sequencing (ChIP-Seq) and genome-wide transcriptional profiling using tiling arrays. Target genes are over-represented for genes involved in the regulation of organ development as well as in the response to auxin. KAN1 affects directly the expression of several genes previously shown to be important in the establishment of polarity during lateral organ and vascular tissue development. We also show that KAN1 controls through its target genes auxin effects on organ development at different levels: transport and its regulation, and signaling. In addition, KAN1 regulates genes involved in the response to abscisic acid, jasmonic acid, brassinosteroids, ethylene, cytokinins and gibberellins. The role of KAN1 in organ polarity is antagonized by HD-ZIPIII transcription factors, including REVOLUTA (REV). A comparison of their target genes reveals that the REV/KAN1 module acts in organ patterning through opposite regulation of shared targets. Evidence of mutual repression between closely related family members is also shown.

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Introduction

Plants achieve their final shoot architecture through the proper positioning of lateral organs such as leaves and flowers. In part this is mediated by the polar transport of the plant hormone auxin to specific locations, which then triggers organ initiation at these sites. The subsequent differentiation of organ progenitor cells into more specialized cell types results in highly organized tissues made up of many distinct cell types. The KAN subclade of the GARP family of transcription factors, as well as the set of class III homeodomain leucine zipper (HD-ZIPIII) transcription factors, play important roles in polarity-associated patterning processes. These transcription factors are key determinants in embryo, shoot and root patterning and during vegetative growth regulate several organ polarity processes [1-15]. In particular, during leaf development these two gene families have been shown to act antagonistically to maintain a stable abaxial/adaxial boundary (the boundary between the lower and upper side of the leaf) that is necessary for proper leaf blade growth. Here, the four members of the KAN group (*KAN1-4*) are required for abaxial cell fate, whereas the HD-ZIPIII genes, including *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REV*, promote adaxial cell identity in organ primordia [1,3,4,7,12-14].

Genetic studies have identified additional regulatory factors specifying the abaxial/adaxial sides of the leaf. The *ASYMMETRIC LEAVES2* (*AS2*) gene, a LOB domain–containing plant-specific protein, and the *ASYMMETRIC LEAVES1* (*AS1*) gene, a MYB domain transcription factor, are involved in the development of a symmetrical expanded lamina, and act to promote adaxial (upper leaf) fate in this regulatory network [16-18]. On the opposite side, members of the YABBY (YAB) gene family, such as *FILAMENTOUS FLOWER* (*FIL*), *YAB3*, *YAB5* and *YAB2*, and two *AUXIN*

RESPONSE FACTOR genes (ETTIN (ETT)/*ARF3* and *ARF4*), specify abaxial (lower leaf) cell fate [19-23]. In addition to this set of transcription factors, small RNAs have also been found to play crucial roles in the establishment of organ polarity. *HD-ZIPIII* factors are targeted by *microRNAs 165/166*, which therefore act as abaxial determinants [24-27]. The *ARF3* and *ARF4* genes are controlled by the ta-siRNAs *ta-siR2141* and *ta-siR2142* (also referred as *ta-siR-ARFs*), thus implicating *tasiR-ARFs* as important adaxial regulators [27,28].

Genetic analysis indicates that some of these genes act antagonistically: loss-of-function mutations in genes promoting adaxial development typically produce an abaxialized phenotype that is accompanied by the expanded expression of abaxial genes, whereas loss-of-function mutations in abaxial genes produce an adaxialized phenotype that correlates with the expanded expression of adaxial genes. Transgenes or mutations that cause ectopic expression of these genes, usually lead to phenotypes opposite to that of the loss-of-function mutations. The antagonistic relationship between the adaxial and abaxial transcription factors could be mediated by direct cross regulation of each other's expression, or alternatively but not mutually exclusively, via opposite effects on common downstream targets of biochemical processes, both of which have been postulated [7,10,29-31]. One strategy to understand how transcription factors mediate their developmental functions is to identify the genes they directly regulate. In this study, we focus mainly on identifying KAN1 targets and, in addition, define potential shared targets between the abaxial factor KAN1 and the adaxial-fate promoting factor REV.

Up to now, only a small number of REV and KAN1 target genes have been reported. For instance, the *LITTLE ZIPPER* (*ZPR*) genes have been proposed as direct REV targets since they are transcriptionally up-regulated by REV and other HD-ZIPIII transcription factors. Furthermore, ZPR proteins interact with and repress HD-ZIPIII activity, forming a negative feedback loop [32,33]. Recently, we demonstrated that REV acts upstream of several class II HD-ZIP transcription factors (*HAT2*, *HAT3*, *ATHB2/HAT4* and *ATHB4*) involved in shade signaling and leaf development [29,34], and the auxin biosynthetic enzymes TAA1 and YUCCA5 (YUC5). Expression of *HAT2*, *TAA1* and *YUC5* is reduced significantly by dexamethasone (DEX) in inducible KAN1 overexpression lines (*35S::FLAG-GR-KAN1*), indicating that at least one way to establish the leaf adaxial-abaxial pattern by the REV/KAN module is through the opposite regulation of shared target genes [29]. In addition, KAN activity has been proposed to negatively regulate PIN expression, and hence auxin movement, based on the ectopic expression of PIN1 in *kan* loss-of-function alleles, and the rapid down-regulation of *PIN1* expression in response to induction of ectopic KAN1 activity [7,10]. It is not known whether KAN regulation is direct or indirect, but also suggests opposing actions of KAN and HD-ZIPIII on regulation of auxin biology. The adaxial factor *AS2* is the best characterized target gene of KAN1, which represses the transcription of *AS2* in abaxial tissue [17,18,31]. Mutation of a single nucleotide in a KAN1 binding site in the *AS2* promoter causes ectopic *AS2* expression in the abaxial domain, resulting in an adaxial phenotype. Furthermore, it has been shown that the abaxial expression of *KAN1* is mediated directly by AS2 [31]. Based on these results, it has been proposed that KAN1 acts as a transcriptional repressor, and that mutual repression between KAN1 and AS2 contributes to the proper establishment of abaxial/adaxial polarity in plants.

Here, we provide a set of potential target genes of the KAN1 transcription factor identified through a combination of chromatin immunoprecipitation/deep sequencing (ChIP-Seq) and genome-wide transcriptional profiling using tiling arrays. Our dataset shows a strong over-representation of genes involved in the regulation of organ development as well as in the response to hormonal stimuli. In addition, the *cis*-element 'VGAATAW' has been identified to be enriched in the ChIP-seq dataset providing the first information about the KAN1-binding site. This *cis*-element is also present in the promoter of the KAN1 target gene *AS2* and it has been shown to be recognized by KAN1 [31], validating our ChIP-seq analysis. Finally, the identification of genes potentially dually regulated by the REV/KAN1 module enables future elucidation of different genetic networks underlying the action of these antagonistic factors.

Materials and Methods

Plant material and treatments

For efficient chromatin immunoprecipitation, transgenic *35S::FLAG-GR-KAN1* plants were used [29]. The glucocorticoid receptor (GR) was cloned in frame with the FLAG epitope in the pJAN33 vector using the KpnI restriction site [35]. Therefore, these transgenic plants can be treated with dexamethasone (DEX), inducing the transition of the chimeric FLAG-GR-KAN1 protein from the cytoplasm to the nucleus, where it can bind to DNA to regulate its downstream targets. In order to achieve equal distribution and uptake of DEX, *35S:FLAG-GR-KAN1* plants were grown in liquid culture for 10 days and induced with 25μM DEX for 45 minutes prior to chromatin-immunoprecipitation. As a control, we used wild type Columbia (Col-0) plants.

ChIP-sequencing and ChIP analysis

Chromatin extraction and immunoprecipitation (ChIP) were carried out as described by Brandt et al. (2012) [29]. In total, we constructed one control library (Col-0) and two ChIP-Seq libraries for *35S:FLAG-GR-KAN1* using the Illumina® TruSeq® ChIP Sample Preparation Kit, according to the manufacturer's protocol. For library preparation indexing adapters were ligated to the ends of the DNA fragments (AR003 for Col-0 library and AR011 and AR027 for *35S:FLAG-GR-KAN1* libraries). Indexed libraries were subsequently subjected to deep sequencing using the Illumina HiSeq instrument. The Illumina sequencing and data analysis were performed as described by Yant and colleagues (2010) [36], with the exception that the number of duplicate sequence reads was heuristically reduced prior to further analysis. This ChIP-Seq experiment resulted in the identification of 17402 positions in the *Arabidopsis* genome being enriched in *35S::FLAG-GR-KAN1* plants compared with Col-0 plants. ChIP-Seq raw data obtained in this study are

available at the Gene Expression Omnibus database under series accession number GSE48081.

Tiling arrays

To examine genome-wide effects of high levels of KAN1 activity, we used ubiqutious expression of a steroid-dependent KAN1 variant, *35S:KAN1-GR* [25]. KAN1 protein activity was induced by growing plants on 0.5x MS plates and submerging seedlings in 10 μM dexamethasone 21-acetate solution for 5 minutes. RNA was collected at three time points: 0 minutes (pre-induction) and 80, and 160 minutes post-induction. A total of 20-30 μg total RNA per sample using the RNeasy® Plant mini Kit (Qiagen, Valencia, CA, USA) was converted into a labeled probe for hybridization to *Arabidopsis* Tiling 1.0 Arrays (Affymetrix) at the Australian Genome Research Facility (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). The results were then calibrated and pooled per time point (2-3 biological replicates per time point) according to the tiling 1.0 array manual, and the resulting .chp files where loaded versus control into the Integrated Genome Browser (version 6.7) software for analyses [37]. The transcriptional changes from baseline were graphically assessed using selected threshold values and candidates with consistent up/down regulation along the full ORF/length of the predicted expressed sequence were identified.

Semi-quantitative PCRs (sqPCR) and quantitative real-time PCRs (qPCRs)

To test the *35S:KAN1-GR* line used for the tiling array experiment, *AS2* and cyclophilin were assessed as positive and negative controls, respectively, by sqPCR.

RNA was extracted from 15 day old seedlings grown on MS medium and after 80 minutes of dexamethasone treatment using the RNeasy® Plant mini Kit (Qiagen, Valencia, CA, USA). 1 μg of purified RNA was treated with DNAse RQ1 (Promega, Madison, WI, USA) and reverse transcribed using PrimeScript™ Reverse Transcriptase (TaKaRa Biotech) for sqPCR. The sqPCR was performed with three biological replicates and visualized on 1.5% agarose gels using electrophoresis [10].

To analyze the gene expression of *ATHB8*, RNA was isolated from 10 day old Col-0 and transgenic *35S:FLAG-GR-KAN1* seedlings after 4 hours of DEX induction. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as a reference gene to evaluate the amounts of mRNA (Figure S2). Real-time PCR experiments were performed as described by Brandt et al. (2012) [29].

Results

Identification of direct KAN1 target genes using ChIP-Seq

To better understand processes downstream of KAN1 action, we constructed transgenic plants over-expressing KAN1 fused to the rat glucocorticoid receptor carrying an additional FLAG-epitope (*35S:FLAG-GR-KAN1*). After growing these plants on soil until the first true leaves were visible, the plants were sprayed once a day for one week with 25μM DEX solution. This treatment resulted in the development of partially abaxialized leaves with drastically reduced petioles (Figure 1A), whereas untreated control plants showed no mutant phenotype. In order to achieve equal distribution and uptake of DEX, *35S:FLAG-GR-KAN1* plants were grown in liquid culture for 10 days and induced with 25μM DEX for 45 minutes prior to chromatin-immunoprecipitation. As a control, we isolated chromatin from Col-0 wild-type plants. One Illumina control library and two ChIP-Seq libraries for *35S:FLAG-GR-KAN1* were sequenced. After filtering for read quality, sequencing reads were mapped to the *Arabidopsis* genome (TAIR10), resulting in the identification of 17402 peaks that were enriched in two independent ChIP-Seq experiments over the control sample. We subsequently limited our analysis to peaks showing at least three-fold enrichment. This dataset contains 4183 KAN1 bound regions. From a MEME-ChIP analysis (http://www.meme.sdsc.org) a VGAATAW motif was identified in 1802 of the 4183 regions (Figure 1B), corresponding to 3151 genes potentially regulated by KAN1 (see Dataset S1). These loci were equally distributed over the five *Arabidopsis* chromosomes, with a lack of enriched peaks in the centromeric regions (Figure 1C). A further analysis of the distribution of the peaks relative to the gene models revealed that the majority of binding sites were located within 1.0 kb upstream of the transcriptional start site (about 24%) or 1.0 kb downstream of the coding region (about 11%). Peaks were underrepresented in gene coding regions (Figure 1D).

Next we examined whether our identified binding site is consistent with previous findings. The recently identified *as2-5d* mutation carries a G to A change in the promoter of *AS2*, causing ectopic *AS2* expression due to uncoupling from KAN1 regulation [31]. Our analysis revealed enrichment at three positions in the *AS2* promoter region previously identified to be recognized by KAN1. The sequence underlying the peak in the 5' UTR of *AS2* contains the VGAATAW motif, with the G being exchanged for A in *as2-5d* (Figure 2A). This finding supports the idea that the 1802 binding regions containing the VGAATAW motif are recognized by KAN1 and represent genuine binding regions. Regions for which we can detect enrichment in our ChIP-Seq dataset which do not contain the VGAATAW motif might represent regions where KAN1 is associated to, maybe in complex with other DNA-binding proteins.

Taken together, we have developed an inducible system for KAN1 expression and used it to identify KAN1 binding sites across the *Arabidopsis* genome. Furthermore, we identified a *cis*-regulatory motif common to many of these targets that may represent a sequence directly recognized by KAN1.

Promoters bound by KAN1

Having identified 1802 binding regions, we were interested in investigating whether genes encoding proteins with specific functions are enriched in this dataset. We therefore performed gene ontology studies using the Agrigo tool (http://bioinfo.cau.edu.cn/agriGO/). This analysis revealed that genes with a function in multicellular organismal development are strongly over-represented in our dataset with further

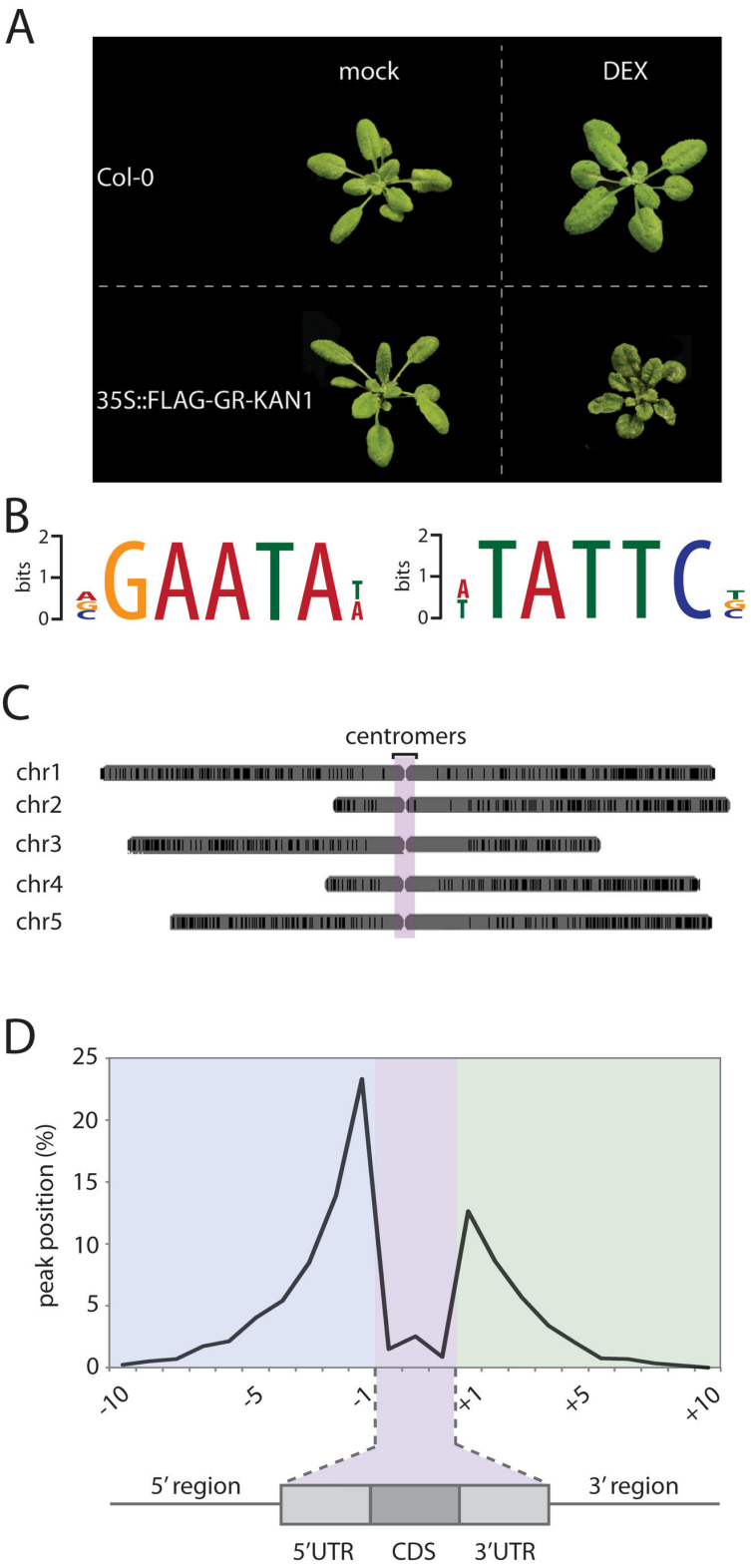

Figure 1. Identification of KAN1 target genes. **A**) Constructing an inducible KAN1 expression system. **B**) Sequence logos for the *cis*-element, forward and reverse orientation, enriched in the ChIP-Seq dataset **C**) Distribution of KAN1 binding sites across the five *Arabidopsis* chromosomes. **D**) Location of peaks identified by ChIP-Seq. About 25% of all peaks are located in the first 1000bp upstream of the transcriptional start site.

doi: 10.1371/journal.pone.0077341.g001

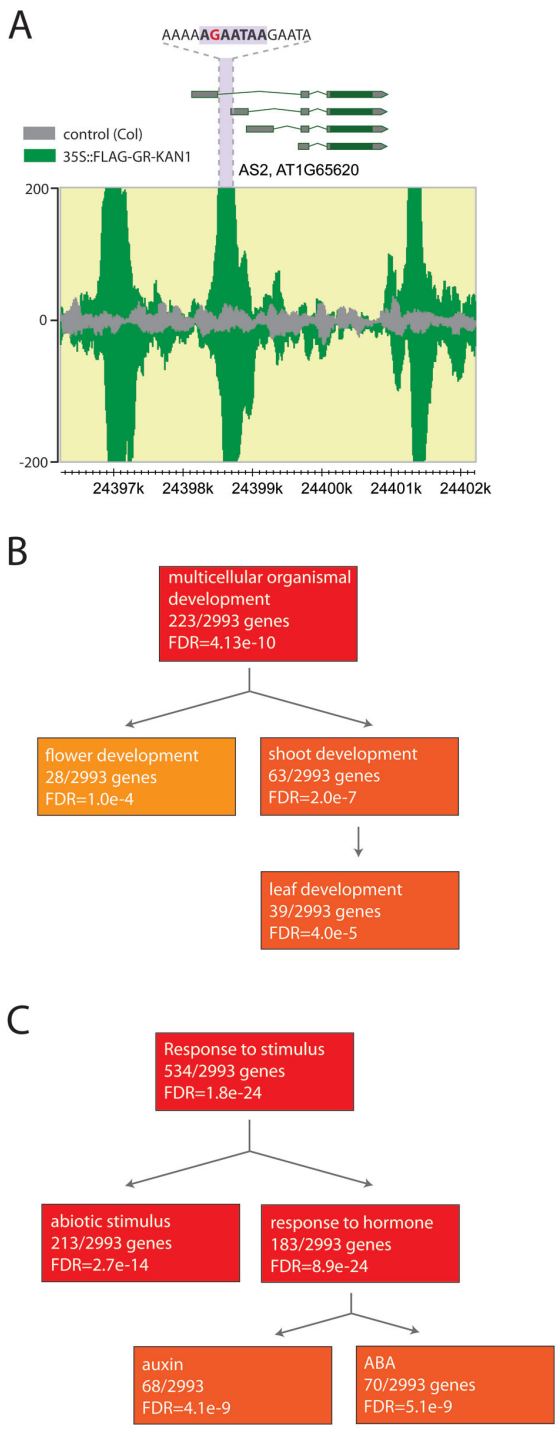

Figure 2. Gene-ontology analysis of KAN1 targets. A) KAN1 binds to the *ASYMMETRIC LEAVES2* (AS2) promoter. Three distinct binding regions were identified but only the second peak contains the VGAATAW motif. The guanine depicted in red is mutated to adenine in the *as2-5d* mutant. **B**) and **C**) Enrichment of GO terms identified in the set of genes located downstream of the KAN1-binding site. Over-representation of genes involved in multicellular organismal development and in the response to stimuli targeted by KAN1.

doi: 10.1371/journal.pone.0077341.g002

enrichment in the sub-categories flower development and shoot/leaf patterning (Figure 2B). Since KAN1 is a major patterning factor and our target gene analysis revealed an enrichment of other genes involved in patterning, this dataset contains genes with a high probability to be regulated by KAN1 (Dataset S2). In addition to genes with a role in development, we also identified genes whose products have known roles in responding to stimuli (Figure 2C). Of the hormonal signaling pathways, enrichment is found for genes involved in auxin and abscisic acid signaling supporting previous findings [30].

Identification of genes transcriptionally regulated by KAN1

Having identified putative promoter regions bound by KAN1 using ChIP-seq, we next attempted to identify genes that respond transcriptionally to KAN1 activity. To this end, we utilized a line harboring a transgene resulting in widespread expression of a hormone inducible KAN1 protein, *35S:KAN1-GR* [6]. When seeds homozygous for the *35S:KAN1-GR* transgene were germinated in the presence of dexamethasone both shoot and root meristems were arrested, no leaf primordia were produced, and seedlings die a few weeks post germination, mimicking the phenotype of *35S:KAN1* plants [3]. As positive and negative controls we followed the expression of *AS2* and cyclophilin, respectively. When assayed 80 minutes after dexamethasone treatment, expression of *AS2* was reduced in hormone treated plants relative to controls, whereas cyclophilin expression was unchanged [10]. We next assayed genome-wide gene expression levels at two time points (80 minutes and 160 minutes) post-induction and identified 500 genes and 9 unannotated genomic regions in which gene expression was down-regulated at least at one of the time points (Dataset S3). In most instances down-regulation was observed at both time points, with 43 genes down-regulated only at 160 minutes and 4 genes down-regulated only at 80 minutes. Of the down-regulated genes, 42 are known to have a role in auxin biology (Dataset S4), including auxin transport or its regulation (*PIN1*, *PIN3*, *PIN4*, *PIN7*, *AUX1*, *PGP4*, *PGP19*, *PID*, *BIG*), auxin response (*IAA2*, *IAA3*, *IAA13*, *IAA14*, *IAA16*, *ARF4*, *ARF19*, *HAT2*), and auxin regulated genes (11 SAUR and 3 GH3 genes). Also down-regulated were 102 genes implicated in transcriptional regulation (Dataset S5), including some previously implicated in regulation of leaf polarity (e.g. *PHB*, *YABBY5*, *ARF4*). Some examples of each of these classes are shown in Figure S1. In contrast, up-regulation was detected at only 30 genes and 1 un-annotated region (Dataset S6). Since most potential target genes exhibited down-regulation, and KAN1 has been shown to interact with TOPLESS [38], a transcriptional co-repressor, we next identified genes that were both down-regulated and possessed local KAN1 binding sites.

A set of putative KAN1 target genes identified through ChIP-seq are also transcriptionally regulated by KAN1

Among the 3151 putative KAN1 target genes selected from the ChIP-seq data analysis, a set of 211 genes was also regulated by KAN1 at 80 and/or 160 minutes post-induction (Figure 3A and Dataset S7) in the tiling array experiments. In

addition, gene ontology classification of these ChIP-seq/tiling array overlapping genes revealed again a strong over-representation of genes involved in multicellular organismal development and response to stimulus, with a significant enrichment of genes involved in shoot development and auxin response, respectively (Figure 3B). Interestingly, among 19 genes related to organ development and shoot patterning (Table 1), four genes were previously shown to be important factors in the genetic network controlling organ patterning: *PHABULOSA* (*PHB*) and *ATHB8* (see also Figure S2), two class III HD-ZIP genes involved in the control of adaxial cell identity [1] and provascular patterning [1,39], respectively, *MIR166F*, which targets several *HD-ZIPIII* family members including *PHV*, *PHB*, *REV*, *ATHB-8* and *ATHB-15* [1,24,40], and *PIN-FORMED 1* (*PIN1*), an auxin efflux carrier required for organ formation and positioning [41-43]. Moreover, several genes such as *LONGIFOLIA 1* (*LNG1*) and *LNG2* [44], the BEL1-like homeodomain protein SAW2 [45], associated with leaf shape establishment, the receptor-like kinase PXY/TDR (PHLOEM INTERCALATED WITH XYLEM/TDIF RECEPTOR), involved in the proliferation of procambial cells as well as in the maintenance of polarity during vascular tissue development [46,47] , *NPY3*, *NPY5* (*naked pins in yuc mutants*) and the *PINOID* homolog *WAG2*, related to auxin-mediated organogenesis [48], were identified in both studies. Additional genes with a role in general aspects of shoot growth and development are listed in the Table 1.

Out of 211 genes identified as putative KAN1 targets by both the ChIP-seq and the tiling array approaches, 21 are involved in auxin response. Table 2 shows a set of KAN1 target genes encoding proteins involved in auxin signaling as well as in auxin transport. This set of genes includes several early auxin-regulated genes with a role in auxin signaling pathways such as two *Aux/IAA* genes (*IAA2* and *IAA13*), which encode short-lived transcription factors that function as repressors of auxin response genes [49], three *GH3* genes (*DFL1*, *DFL2* and *WES1*), encoding acyl adenylate–forming isozymes that covalently modify indole-3-acetic acid (IAA) [50], and three *SAUR-like* genes (*AT1G19840*, *AT1G75590* and *AT2G21210*), which encode short-lived nuclear proteins involved in auxin signaling by interacting with calmodulin [51,52]. Furthermore, an *AUXIN RESPONSE FACTOR* gene, *ARF4*, was identified in both experiments while *ARF3/ETT* was identified only in our ChIP-seq data. *ARF4*, together with the redundant gene *ARF3/ETT* (*ETTIN*), act to promote abaxial identity in association with KAN or its downstream targets [20]. In addition, it has been shown that the negative transcriptional, post-transcriptional and epigenetic regulation of these *ARFs* by AS1 and AS2 is important for the establishment of early leaf adaxial/abaxial polarity [53]. Among this set of genes, we also found the class II HD-ZIP gene *HAT2*, which is an early auxin-inducible gene with opposite functions in regulating auxin-mediated morphogenesis in the shoot and root tissues [54]. In a previous study, we also showed that *HAT2* acts downstream of REV in the shade avoidance response [29]. Regarding those genes involved in auxin transport, two *PIN* genes, *PIN3* and *PIN4*, which are important for tropic growth of the root [55] and root patterning [56], respectively, as well as for creating local auxin gradients required for the establishment of primordia and organ development [41], were found in both studies. Furthermore, a phospholipase required for PIN protein trafficking to the plasma membrane in the root (phospholipase A2; PLA2A) [57], and the PINOID protein kinase (PID), which controls PIN polarity and mediates changes in auxin flow to create local gradients for patterning processes [58], were identified. Additionally, the auxin influx transporter AUX1 and the ATP-binding cassette transporter AtMDR1 found in both studies regulate root gravitropism, and photomorphogenesis and root development, respectively, by mediating auxin polar transport [59,60].

Finally, several genes previously described as being involved in adaxial/abaxial patterning of the leaf and the vascular tissues such as *MIR166A* and *AS2* [1,17,18,24,40] were identified as KAN1 targets exclusively through the ChIP-seq approach. Moreover, *KAN1* itself and *KAN2* were isolated as putative targets suggesting that KAN1 may control its own expression as well as the expression of other KAN gene family members.

Genes oppositely regulated by the REV/KAN1 module

REV and KAN1 have opposite functions in early leaf patterning. In order to determine whether the antagonistic roles can be attributed to an opposite regulation of common downstream target genes, we compared potential downstream REV target genes identified by ChIP-Seq [29] with the list of genes bound and regulated by KAN1 (Dataset S7). This analysis resulted in the identification of 26 genes, which are candidates for dual regulation (Table 3). Interestingly, five genes are bound by REV and KAN1 in a region less than 100bp apart, suggesting that, besides dual regulation, REV and KAN1 might also compete for chromatin accessibility. All five genes (TEM, ZFP4, SUC1, a receptor protein kinase and a NPH3-like protein) seem to be involved in the control of development corroborating the idea that they act downstream of developmental regulators.

Discussion

In this study, we utilize inducible overexpression of *KAN1* to identify KAN1 responsive genes and direct targets. Although such an approach may lead to artifacts because of the ectopic and artificially high expression levels used, the set of genes we have identified shows enrichment for genes involved in development and auxin biology, suggesting our experiments have identified genes that are biologically relevant.

Our results show that the VGAATAW motif may be a common *cis*-regulatory element recognized by KAN1, which includes the motif affected by the *as2-5d* point mutation that causes ectopic *AS2* expression due to its regulation being uncoupled from KAN1 [31]. We have focused our attention on the 1802 binding regions containing this motif (corresponding to 3151 genes potentially regulated by KAN1) and, especially, on those genes that exhibit gene expression changes in response to induction of KAN1 activity. Several of the identified downstream targets have a role in organ development, shoot patterning or auxin response and transport. In addition, we present a set of genes that are potentially controlled by both

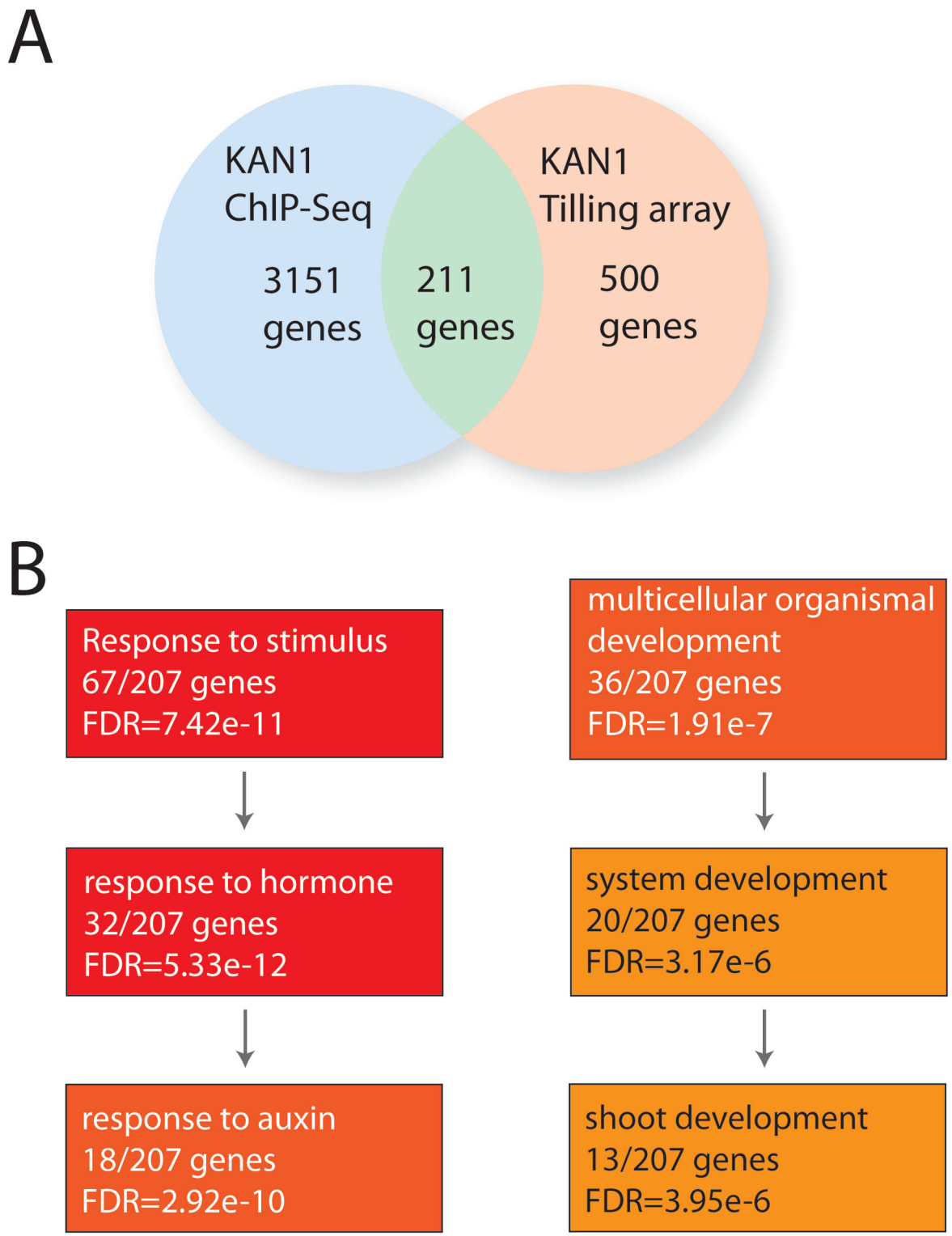

Figure 3. Genome-wide comparison of genes bound and regulated by KAN1. **A**) Venn-diagram showing numbers of genes bound by KAN1 and regulated by KAN1. The overlap contains 211 genes that are both bound and also regulated by KAN1. **B**) Gene ontology analysis of 211 potential direct KAN1 targets reveals a strong enrichment for genes involved in shoot patterning and the auxin response. Tables 1 and 2 contain these genes including the binding site information.

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Table 1. Potential KAN1 target genes with a role in organ or shoot development.

		ChIP-seq data							Tiling array data	
AGI	Gene Symbol	ORP-rank	Distance	Location	Enrichment replicate 1	Enrichment replicate 2	FDR replicate 1	FDR replicate 2	80 min	160 min
AT1G13245	RTFL17	4140	2414	DOWN	4,3	2,7	8,20E-04	3,93E-04	yes	yes
AT1G13260	RAV1	1215	519	DOWN	5,5	2,7	2,66E-39	2,20E-23	yes	yes
AT1G13260	RAV1	1003	9835	UP	7,8	3,4	6,93E-49	1,03E-23		
AT1G13260	RAV1	882	6034	UP	5,1	2,8	1,54E-43	1,79E-33		
AT1G27320	AHK3	5004	630	DOWN	4,3	2,1	8,20E-04	1,29E+02	yes	yes
AT1G56010	NAC1	7940	1500	UP	4,1	2,0	2,30E+04	1,80E+07	yes	yes
AT1G73590	PIN1	1344	1049	DOWN	6,9	3,6	3,37E-34	8,69E-23	-	yes
AT1G78240	TSD2	3471	2653	UP	5,0	2,5	2,67E-11	4,39E-04	yes	yes
AT1G78240	TSD2	6421	3459	UP	6,0	2,0	4,37E-03	1,80E+07		
AT1G78240	TSD2	866	5964	UP	12,8	6,1	3,27E-49	1,04E-29		
AT1G78240	TSD2	3094	7048	UP	6,7	2,6	1,98E-19	1,28E-02		
AT2G23760	SAW2	1267	436	UP	7,2	3,8	9,78E-36	5,35E-25	-	yes
AT2G23760	SAW2	1145	2496	DOWN	6,0	3,2	7,38E-38	5,57E-27		
AT2G31070	TCP10	344	76	UP	8,0	4,3	6,40E-73	1,79E-54	yes	yes
AT2G34710	PHB	231	937	UP	10,2	4,2	1,91E-106	2,01E-52	yes	yes
AT3G14370	WAG2	3554	1208	UP	7,9	3,5	1,08E-12	1,58E-02	yes	yes
AT5G60970	TCP5	1164	2282	DOWN	7,3	4,4	1,15E-32	4,35E-30	yes	yes
AT5G60970	TCP5	1019	3044	DOWN	7,8	4,9	2,90E-34	6,91E-34		
AT5G43603	MIR166F	2366	658	UP	3,8	2,7	2,25E-12	6,47E-18	yes	yes
AT5G61480	PXY	2404		in CDS	5,7	2,6	5,67E-22	7,40E-09	-	yes
AT5G67440	NPY3	434	413	UP	6,4	3,5	2,32E-65	1,24E-49	yes	yes
AT5G67440	NPY3	1623	184	DOWN	5,5	2,5	1,19E-32	2,38E-16		
AT4G37590	NPY5	979	1452	UP	9,9	5,0	2,37E-43	3,80E-29	yes	yes
AT3G02170	LNG2	3204	3955	UP	5,3	2,9	3,08E-11	6,67E-07	yes	yes
AT5G15580	LNG1	4944		in CDS	10,7	2,6	1,57E-12	3,27E+06	yes	yes
AT5G61960	AML1	2123	2369	UP	5,6	1,4	3,33E-34	4,59E-06	yes	yes
AT5G61960	AML1	2265		in CDS	6,8	3,1	5,15E-23	3,05E-10		
AT4G32880	ATHB8	1402	561	UP	1,8	6,5	1,69E-30	3,33E-23	yes	yes
AT2G46685	MIR166A	4624	3218	UP	2,3	4,8	4,90E-07	1,49E+02	-	-
AT1G65620	AS2	1750		in CDS	2,4	5,1	1,50E-34	6,53E-12	-	-
AT1G65620	AS2	2506	424	DOWN	2,4	5,9	1,72E-23	2,74E-06	-	-
AT5G16560	KAN1	1996	173	UP	1,8	4,5	2,88E-21	5,12E-16	-	-
AT5G16560	KAN1	2689	707	UP	2,6	9,3	8,92E-23	1,02E-04	-	-
AT5G16560	KAN1	2930	5046	UP	2,9	7,8	8,53E-23	4,06E-03	-	-
AT1G32240	KAN2	468	4442	DOWN	8,8	5	4,95E-61	4,17E-50	-	-

Notes: By analyzing the ChIP-seq and the tiling array datasets and based on gene ontology (GO) analysis and literature contrast, we identified 23 genes involved in multicellular organismal development and shoot development. These genes are listed with the AGI (Arabidopsis Genome Initiative) gene code, the Gene Symbol, the ORP-rank, the distance from the binding site to the CDS, the location (UP=upstream of a gene, DOWN=downstream of a gene, in CDS), the enrichment of ChIP-seq replicates 1 and 2 (ratio of number of reads for a binding site in KAN1+DEX versus Col0+DEX), the false discovery rate (FDR) of ChIP-seq replicates 1 and 2, and the down-regulation at 80 and/or 160 min after KANADI1 activity induction (yes=the entire length of the predicted transcript was down-regulated; - no significant down-regulation).

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KAN1 and REV. The potential regulation of the selected genes by KAN1 and its link with patterning processes and auxin-related events as well as the gene regulation by the module KAN1/REV are discussed below.

KAN1 regulates many genes related to organ patterning

In our study, we find that KAN1 binds to the promoter of two *HD-ZIPIII* genes, *PHABULOSA* and *ATHB8* (Table 1) and represses their expression, suggesting that both *HD-ZIPIII* genes are direct targets of KAN1 during organ polarity establishment. In previous studies, it was proposed that the antagonistic role between KAN and HD-ZIPIII activities in vascular tissue formation is mediated by affecting the canalization of auxin flow rather than through a direct interaction between both families of transcription factors [10]. However, our results suggest that there may be contexts in which KAN1 acts directly on *PHB* and *ATHB8*.

We also find that KAN1 binds directly to the promoters of *MIR166A* and *MIR166F* and down-regulates the expression of *MIR166F* (Table 1), suggesting that at least KAN1 may directly

Table 2. Potential KAN1 target genes involved in auxin response.

		ChIP-seq data							Tiling array data	
AGI	Gene Symbol	ORP-rank	Distance	Location	Enrichment replicate 1	Enrichment replicate 2	FDR replicate 1	FDR replicate 2	80 min	160 min
AT1G19840	SAUR-like	2341	6906	DOWN	5,1	2,8	6,76E-18	1,05E-12	yes	yes
AT1G75590	SAUR-like	3037	1915	UP	5,8	2,5	2,21E-17	1,86E-04	yes	yes
AT2G21210	SAUR-like	157	149	UP	8,3	3,4	4,47E-119	9,21E-58	yes	yes
AT2G33310	IAA13	1242		in CDS	7,4	4,1	1,00E-33	1,71E-27	yes	yes
AT2G34650	PID	2130	3923	UP	5,7	2,8	6,51E-23	1,53E-12	yes	yes
AT2G38120	AUX1	158	10052	UP	7,3	4,2	3,04E-94	1,24E-79	yes	yes
AT3G23030	IAA2	165	171	UP	9,0	4,4	4,11E-103	3,88E-67	yes	yes
AT3G28860	ATMDR1	3763	3456	UP	5,0	2,2	1,49E-11	1,45E-01	yes	yes
AT3G28860	ATMDR1	3075	8677	DOWN	4,4	2,0	3,47E-16	8,40E-05		
AT4G03400	DFL2	2385	2241	DOWN	7,4	3,1	9,71E-24	9,10E-08	yes	yes
AT4G03400	DFL2	1583	2654	DOWN	5,6	2,6	2,76E-33	1,08E-16		
AT4G27260	GH3.5, WES1	2996	1074	UP	4,0	2,2	6,37E-13	1,14E-07	yes	yes
AT4G27260	GH3.5, WES1	5906	971	DOWN	5,0	1,3	4,55E-09	1,63E+07		
AT5G47370	HAT2	3245	86	UP	4,6	2,3	1,64E-12	1,76E-05	yes	yes
AT5G54510	GH3.6, DFL1	2785	2222	DOWN	4,8	1,9	1,29E-22	1,16E-04	yes	yes
AT5G59780	MYB59	1240	5918	UP	5,6	3,3	2,16E-32	7,10E-28	yes	yes
AT5G61420	MYB28	1759	769	UP	6,2	2,7	5,59E-32	2,11E-13	yes	yes
AT5G61420	MYB28	1753	2117	UP	4,4	2,7	4,69E-22	6,78E-21		
AT5G63160	BT1	16	1273	UP	9,1	4,7	2,98E-185	8,71E-136	-	yes
AT5G67300	ATMYB44	2070	381	UP	4,5	2,4	1,11E-21	1,87E-14	yes	yes
AT5G67300	ATMYB44	53	2438	UP	11,3	5,4	9,41E-140	1,06E-92		
AT1G70940	PIN3	5237	20	UP	6,4	3,2	2,54E-03	2,51E+02	yes	yes
AT2G01420	PIN4	1622	367	UP	6,3	2,8	3,70E-35	3,35E-15	yes	yes
AT2G01420	PIN4	1630	973	DOWN	5,5	3,0	1,65E-27	7,06E-20		
AT2G26560	PLA2A	2249	1401	UP	6,1	3,1	1,59E-20	5,88E-12	yes	yes
AT5G60450	ARF4	294	2112	UP	6,7	4,0	6,92E-70	1,80E-64	yes	yes
AT2G33860	ARF3/ETT	4041	1151	UP	3,9	3,1	6,30E-01	2,50E-07	-	-
AT1G15690	AVP1	3612	2238	UP	8,1	3,4	4,21E-13	8,87E-02	yes	yes

Notes: By analyzing the ChIP-seq and the tiling array datasets and based on gene ontology (GO) analysis and literature contrast, we identified 22 genes involved in response to auxin. These genes are listed with the AGI (Arabidopsis Genome Initiative) gene code, the Gene Symbol, the ORP-rank, the distance from the binding site to the CDS, the location (UP=upstream of a gene, DOWN=downstream of a gene, in CDS), the enrichment of ChIP-seq replicates 1 and 2 (ratio of number of reads for a binding site in KAN1+DEX versus Col0+DEX), the false discovery rate (FDR) of ChIP-seq replicates 1 and 2, and the down-regulation at 80 and/or 160 min after KANADI1 activity induction (yes=the entire length of the predicted transcript was down-regulated; - no significant down-regulation).

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regulate *MIR166F*. In addition, our results indicate that KAN1 binds to its own promoter and *KAN2* via the VGAATAW motif (Table 1) but also potentially KAN3 (Dataset S1), although no VGAATAW motif was found for this binding event. Taken together, these results suggest that in some contexts KAN1 may direct a negative feedback loop that limits the levels of several abaxial factors including KAN1 itself.

KAN1 binds to the proximal promoters and represses the expression of genes involved in different aspects of organ development such as PXY/TDR, *LNG1/2* and *SAW2* (Table 1). Like *KAN1*, *PXY* is a key gene in vasculature polarity establishment. In particular, *PXY* is required for the proper orientation of cell divisions in the vascular meristem, which gives rise to specialized and spatially separated xylem and phloem cells [46,47]. The homologous genes *LNG1* and *LNG2* regulate leaf morphology by positively promoting longitudinal polar cell elongation [44]. The adaxial epidermal cells of the midveins and the leaf blade are longitudinally elongated in the *lng1-1D* mutant plants compared with wild type. *SAW2* controls leaf shape and exhibits adaxial expression in developing lateral organs [45]. Therefore, our results suggest that KAN1 may directly regulate genes involved in the development of lateral organs and vascular tissue, known sites of KAN1 activity.

KAN1 regulates auxin-related genes

Organ patterning is in part modulated by the polar transport of auxin to specific locations, generating auxin maxima that promote organ initiation and growth. PIN proteins play an important role in the regulation of auxin distribution. Loss of proper PIN polarity establishment, as in PIN multiple mutants and *gn* mutants, leads to embryo patterning defects [61-65]. Previous studies have shown a negative effect of KAN1 on PIN1 activity. Thus, ectopic expression of *PIN1* is observed in *kan1 kan2 kan4* embryos, suggesting that KAN genes may act to restrict auxin flow during embryogenesis by regulating *PIN1*

Table 3. Genes potentially cross-regulated by the REV/KAN1 module.

		REV ChIP-seq		KAN1 ChIP-seq						
AGI	Gene symbol	Distance	Location	FDR replicate 1	FDR replicate 2	Enrichment replicate 1	Enrichment replicate 2	Distance	Location	Distance REV/ KAN1 binding
AT1G22570	Major facilitator protein	1129	DOWN	1,19E-112	3,31E-68	13,5	6,2	920	UP	4085
AT1G25560	TEM1	2926	UP	2,15E-64	2,70E-31	11,7	4,9	3024	UP	61
AT1G51940	LysM-domain protein	1525	UP	1,22E-10	3,24E+02	4,5	1,8	5999	UP	4504
AT1G61660	bHLH transcription factor		in CDS	2,26E-37	1,47E-22	6,8	3,4	1365	UP	3405
AT1G66140	ZFP4		in CDS	3,75E-31	4,10E-17	6,1	2,9		in CDS	26
AT1G66140	ZFP4			1,49E-01	1,35E+02	4,0	2,2		in CDS	434
AT1G67710	ARR11	1466	UP	2,20E-54	6,92E-40	6,3	3,4	1375	UP	113
AT1G68130	IDD14	3051	UP	1,08E-12	9,72E-11	3,8	2,2	3357	DOWN	8739
AT1G68520	B-BOX zinc finger protein	741	DOWN	4,34E-26	3,09E-09	3,8	2,3	248	UP	2757
AT1G68520	B-BOX zinc finger protein			5,97E-14	2,76E-06	5,9	2,8	829	UP	3408
AT1G71880	SUC1	5858	UP	2,80E-74	1,99E-51	8,1	4,1	5878	UP	41
AT1G72300	Leucine-rich receptor protein	3145	UP	4,42E-22	4,03E-12	8,3	4,1	691	UP	2424
AT1G72300	Leucine-rich receptor protein			6,72E-09	1,69E-09	4,0	2,4	1465	UP	1630
AT3G02140	TMAC2	893	UP	2,04E-69	5,94E-57	10,5	5,9	201	UP	653
AT3G02140	TMAC2			1,01E-48	5,34E-34	7,4	3,7	2737	UP	1863
AT3G02140	TMAC2			4,50E-08	5,09E-03	4,8	2,5	3049	UP	2231
AT3G12920	BRG3	1579	DOWN	1,73E-04	8,92E-01	4,3	2,3	1782	UP	5019
AT3G12920	BRG3			1,22E-181	7,71E-141	13,2	7,0	4565	UP	7652
AT3G12920	BRG3			6,75E-90	8,25E-62	11,6	5,8	983	DOWN	568
AT3G12920	BRG3			1,13E-63	9,51E-49	9,9	5,4	2338	DOWN	797
AT3G15570	NPH3 family protein	1009	UP	6,12E-93	1,02E-63	8,9	4,4	1036	UP	14
AT3G54400	Aspartyl protease protein	602	UP	4,51E+02	8,26E+04	4,6	2,4	120	UP	402
AT3G56050	Protein kinase family protein	208	UP	4,29E-87	5,13E-48	8,8	3,9	274	UP	42
AT3G61460	BRH1	2196	UP	1,85E-17	4,61E-12	5,9	3,2	314	UP	1826
AT4G18700	CIPK12	282	DOWN	3,22E+00	3,57E+03	7,5	3,9	26	UP	2468
AT4G18700	CIPK12			4,36E-101	1,53E-92	8,4	5,0	133	DOWN	146
AT4G22190	unknown protein	2827	UP	1,45E-38	9,85E-30	8,1	4,5	1709	UP	1166
AT4G26540	Leucine rich repeat receptor	2234	UP	3,06E-86	1,40E-45	8,5	3,7	2160	UP	129
AT4G26540	Leucine rich repeat receptor			8,31E-78	1,31E-53	7,1	3,6	896	DOWN	6893
AT4G27260	GH3.5, WES1	2494	DOWN	6,37E-13	1,14E-07	4,0	2,2	1074	UP	6295
AT4G27260	GH3.5, WES1			4,55E-09	1,63E+07	5,0	1,3	971	DOWN	1591
AT5G05690	CPD	4847	UP	1,81E-06	1,67E-02	5,1	2,7	5642	UP	894
AT5G47370	HAT2	1548	UP	1,64E-12	1,76E-05	4,6	2,3	86	UP	1403
AT5G51550	EXL3	2573	UP	2,22E-174	1,08E-139	14,1	7,7	687	UP	1892
AT5G51550	EXL3			9,95E-19	1,35E-11	4,0	2,1	2133	UP	480
AT5G52060	ATBAG1, BAG1	739	UP	1,28E-20	2,48E-03	5,4	2,1	8	UP	745
AT5G64570	XYL4	2389	UP	1,84E-39	5,08E-19	4,9	2,2		in CDS	5597
AT5G64570	XYL4			5,70E-50	5,28E-46	7,7	4,6	331	UP	2090
AT5G67190	DEAR2	2710	UP	1,46E-33	1,01E-22	4,9	2,6	276	UP	2557
AT5G67190	DEAR2			5,02E-178	3,45E-151	14,2	8,1	1541	UP	1248
AT5G67190	DEAR2			1,28E-61	2,35E-45	6,9	3,6	1319	UP	6884

Table 3 (continued).

Notes: By comparing the REV target genes identified by ChIP-Seq [29] with the list of genes bound and regulated by KAN1 (Dataset S7), we identified 26 genes which are candidates for dual regulation. These genes are listed with the AGI (Arabidopsis Genome Initiative) gene code, the Gene Symbol, the false discovery rate (FDR) of ChIP-seq replicates 1 and 2, the enrichment of ChIP-seq replicates 1 and 2 (ratio of number of reads for a binding site in KAN1+DEX versus Col0+DEX), the distance from the binding site to the CDS and the location (UP=upstream of a gene, DOWN=downstream of a gene, in CDS), and the distance between REV and KAN1 binding sites.

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gene expression [7]. *PIN1* gene expression alterations have also been observed at the ectopic abaxial leaf outgrowths of *kan1 kan2* plants. In particular, *PIN1* expression was higher in the outgrowths than in the surrounding leaf tissue, suggesting that the outgrowths may be due to ectopic auxin maxima forming in the lamina [3,4]. In addition, it has been shown that polar auxin flow is essential to form procambium cells in vascular tissues, and KAN genes play a role in the distribution of this auxin flow by restricting PIN1 activity [10]. In agreement with these findings, we have identified a binding site for KAN1 downstream from *PIN1* that likely mediates direct repression of *PIN1* by KAN1 (Table 1). Motifs adjacent to other *PIN* genes such as *PIN3* and *PIN4* were also bound by KAN1, and their expression was repressed by KAN1 as well (Table 2). Therefore, KAN1 may directly regulate several PIN family members supporting previous findings that showed that, at least in some contexts, KAN proteins may act in patterning processes through auxin transport modulation. Additionally, and reinforcing this hypothesis, KAN1 bound and repressed several genes involved in the regulation of PIN activity and trafficking such as *PINOID* and *PLA2A* [57,58,66], respectively. *NPY3*, *NPY5* and *WAG2*, which are thought to act together to determine what side of the cell PIN accumulates at [48,67-69], and genes involved in auxin polar transport such as the auxin influx transporter *AUX1* and the ATP-binding cassette transporter *AtMDR1* [59,60] were also bound and repressed by KAN1 (Table 2).

Our data also point to a direct effect of KAN1 on auxin signaling pathways. For instance KAN1 bound near and repressed early auxin response genes including three *GH3* genes (*DFL1*, *DFL2* and *WES1*), three *SAUR-like* genes (*AT1G19840*, *AT1G75590* and *AT2G21210*) as well as two *Aux/IAA* genes (*IAA2* and *IAA13*) [49,70] (Table 2).

A connection of auxin signaling comes in addition from direct repression of *ARF4* and binding of *ARF3* by KAN1 (Table 2). The phenotype of *ett arf4* leaves resembles the phenotype of *kan1 kan2* leaves, leading to the proposal that ARF proteins act together with KAN proteins or its downstream targets to regulate transcription [20]. While this previous study suggested a positive interaction between these transcription factors, our findings suggest that there may also be negative feedback between *KAN1* and *ARF4* and *ARF3/ETT*, potentially again (see above), as a mechanism to maintain homeostasis among factors controlling abaxial identity.

We have identified several additional genes involved in auxin transport and its regulation or auxin signaling as being repressed after *KAN1* induction and, in some cases, also bound by KAN1 (Dataset S1 and Figure S1). This set of genes includes *PIN7*, which is involved in apical–basal axis formation of the embryo [62], *YABBY5*, a transcription factor involved in abaxial cell fate specification and auxin distribution [21,23], different early auxin-responsive genes such as *GH3.3*, the SMALL AUXIN UP RNAs (SAUR) *SAUR19*, *SAUR20* and *SAUR63*, which regulate auxin polar transport and promote auxin-mediated organ elongation [71,72], three SAUR-like genes (*AT1G19840*, *AT4G38840* and *AT5G18030*), *ARF19*, *IAA3*, *IAA16*, *IAA14*, and an auxin receptor belonging to the TIR1 subfamily (*AFB1*) that interacts with Aux/IAA proteins [73,74]. In addition, in a previous study [29], we demonstrated that the expression of *HAT2*, which was also bound and repressed by KAN1 in the current study, and two genes that encode auxin biosynthetic enzymes, *TAA1* and *YUC5*, is reduced significantly after KAN1 induction. These results together with our findings reflect that, certainly, KAN1 may control the influence of auxin on organ development through complex interactions and at different levels: biosynthesis, transport and its regulation, and signaling.

Finally, our results suggest that KAN1 may act on other hormone pathways through the regulation of genes involved in the response to abscisic acid, jasmonic acid, brassinosteroids, ethylene, cytokinins and gibberellins (Dataset S2 and Figure S1).

Regulation by KAN1 and REV of common downstream target genes

Genetic analysis has indicated that the HD-ZIPIII and KAN factors act oppositely in organ patterning [1,3,7]. However, it remains unclear whether this interaction occurs by direct mutually antagonistic regulation, through opposing regulation of a set of common direct targets or through opposing regulation of indirect targets. With respect to direct antagonistic regulation, in the current study, we did not find evidence of direct regulation of *REV* by KAN1, although KAN1 appears to bind other HD-ZIPIII genes such as *PHB* and *ATHB8* and to repress their expression. On the other hand, published work identifying *HAT2*, *TAA1* and *YUC5* as genes oppositely regulated by REV and KAN [29] supports the shared common targets hypothesis. To further investigate whether REV and KAN1 act on additional common target genes, we compared the ChIP-Seq data for KAN1 with those recently obtained for REV [29] and found an additional set of overlapping putative target genes that bring the total to 26 genes (Table 3). Among these, we found genes encoding transcription factors and proteins involved in hormone-associated processes. Finally, several genes involved in auxin transcriptional response and auxin transport are repressed by KAN1, whereas auxin biosynthesis and transport are positively regulated by HD-ZIPIII activity. Thus, another mechanism by which KAN1 and HD-

ZIPIII activities have opposing effects is via antagonistic regulation of auxin biology, which does not necessarily occur at the level of the same transcriptional targets but will create steep auxin gradients that could function as positional signals.

The vast majority of KAN1 targets identified were down-regulated. Together with the observations that KAN1 directly represses the expression of the adaxial factor *AS2* [31] and that TOPLESS, a co-repressor protein, directly interacts with KAN1 [38], our data suggests that KAN1 primarily acts as a repressor. According to the opposite regulation of common targets hypothesis, if KAN1 acts as a repressor, the HD-ZIPIII proteins should act as activators of those genes that are common targets. Consistent with HD-ZIPIII proteins acting as activators, expression of REV translationally fused with a repressor domain (REV-SRDX) phenocopies *phb phv rev* plants (Dyani Lewis and J. L. Bowman, unpublished data). Thus, our findings together with published work [1,3,7,29,31,38] indicate that HD-ZIPIII and KAN genes function antagonistically both through mutual regulation as well as through the opposite regulation of common direct targets and indirect targets. Mutual regulation may ensure the proper partitioning of adaxial and abaxial tissues while the opposite regulation of common targets may help set up contrasting transcriptional activities that distinguish adaxial and abaxial cell types.

Supporting Information

Dataset S1. All ChIP-Seq identified regions containing the VGAATAW element.
(XLS)

Dataset S2. Putative KAN1 targets with roles in system and organ development and hormone signaling.
(XLS)

Dataset S3. All genes down-regulated by KAN1.
(XLS)

Dataset S4. Genes involved in auxin biology down-regulated by KAN1.
(XLS)

Dataset S5. Genes involved in transcriptional regulation down-regulated by KAN1.
(XLS)

Dataset S6. All genes up-regulated by KAN1.
(XLS)

Dataset S7. Overlap ChIP-Seq/tiling array.
(XLS)

Figure S1. Examples of raw tiling array data. The lower two lines in each figure represent the 80 minute time point and the upper two lines represent the 160 minute time point. The upper of the lines in each time point are from a single biological experiment, whereas the lower are the average from two biological replicates. Genes are identified by their AtNg and common names, and those genes that were detected as also bound by KAN1 are denoted by an *.
(TIF)

Figure S2. Genes bound by KAN1 are also regulated by KAN1 at the transcriptional level. A) ChIP-Seq graphs show enrichment for KAN1 binding in the 3' region of the ATHB8 gene. The enriched region contains the VGAATAW motif. **B**) *ATHB8* expression is strongly repressed in DEX-treated *35S::FLAG-GR-KAN1* transgenic plants. Plotted are relative qRT-PCR expression values of two independent biological replicates. Each biological experiment was carried out with four technical replicates and average values with standard deviation were calculated. *p≤0.01; **p≤1.0E-06.
(TIF)

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Author Contributions

Conceived and designed the experiments: PM YX SW MGH LB JLB. Performed the experiments: PM YX SW LB. Analyzed the data: PM SW MGH LB FO JLB. Contributed reagents/ materials/analysis tools: SW MGH JLB DW. Wrote the manuscript: PM SW MGH DW JLB.

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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

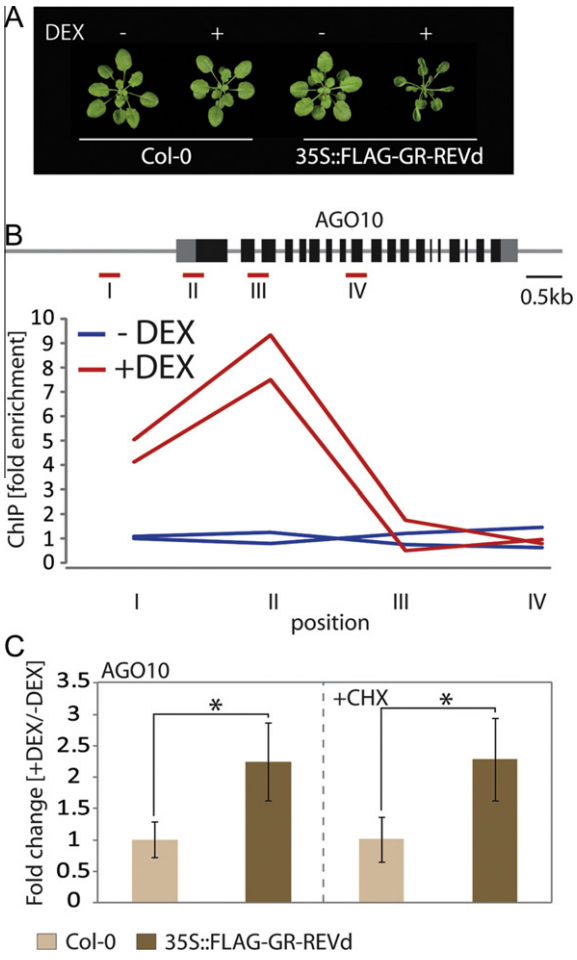

Fig. 1 – REVOLUTA directly regulates *AGO10* expression. (a) Induction of REVOLUTA causes adaxialization of leaves (Col and GR-REV +/−DEX). Plants were cultivated in long day conditions and after the production of the first true leaves sprayed daily with a 50 μM DEX solution or a mock substrate for 2 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.

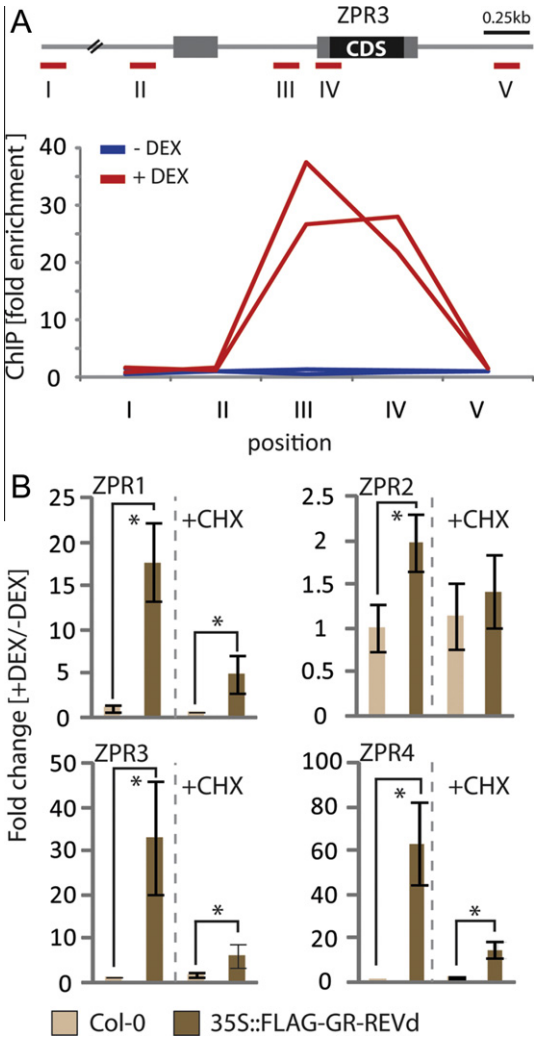

Fig. 2 – REVOLUTA directly regulates expression of *LITTLE ZIPPER* genes. (a) REV binds to the promoter of the *LITTLE ZIPPER3* gene. Chromatin-immunoprecipitation experiments with two biological replicates for *35S::FLAG-GR-REVd* without DEX (blue lines) and *35S::FLAG-GR-REVd* with DEX (red lines) 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*

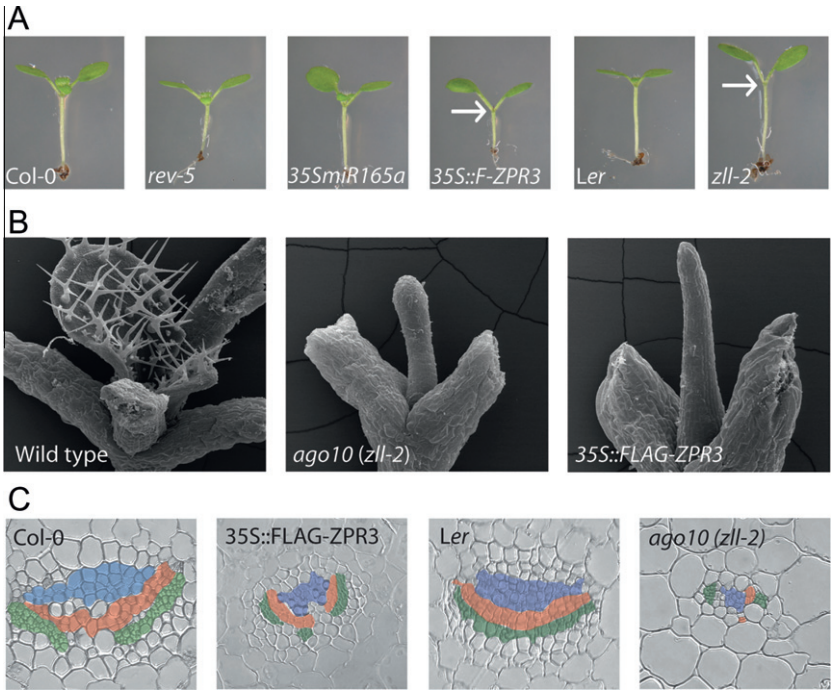

Fig. 3 – Mutations in *hd-zipIII* and *ago10* cause severe phenotypic defects. (a) Comparative growth analysis of hd-zipIII and ago10 mutant plants with corresponding wild type plants. Both *35S::FLAG-ZPR3* and *ago10* (*zll-2*) mutant plants show termination of the shoot apical meristem (arrow shows 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

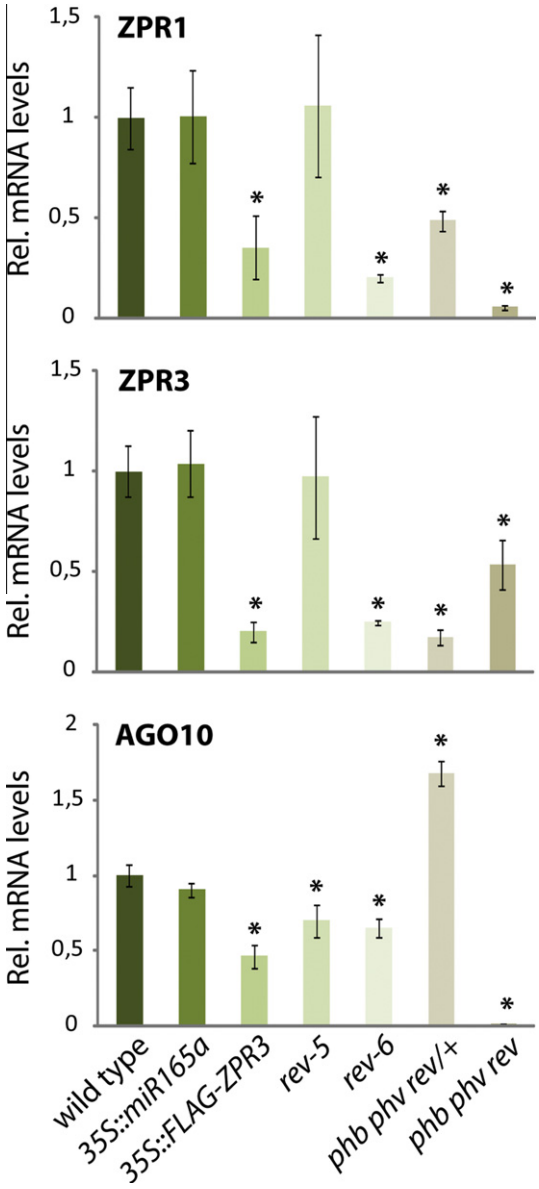

Fig. 4 – *AGO10*, ZPR1 and ZPR3 expression are altered in *hd-zipIII* mutant plants. Expression of *AGO10* and *ZPR3* was analyzed in mutants with either compromised *HD-ZIPIII* expression (*rev-5*, *rev-6*, *phb phv rev/+*, *phb phv rev* and *35S::miR165a*) or inhibited HD-ZIPIII protein activity (*35S::FLAG-ZPR3*). 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

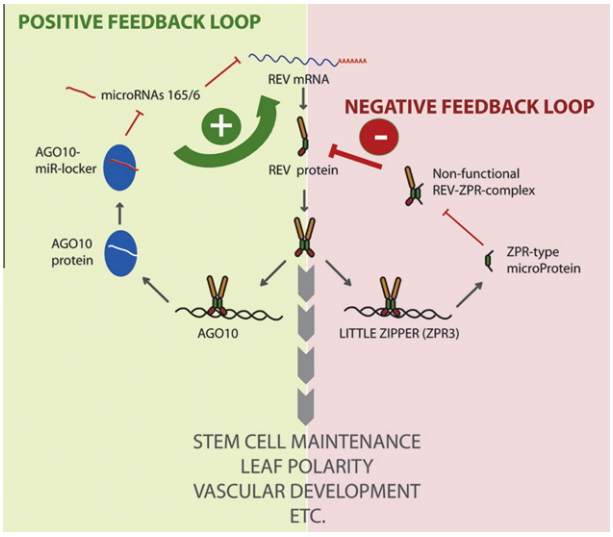

Fig. 5 – Interlocking positive and negative feedback-loops regulate stem-cell homeostasis in *Arabidopsis*. Model for the feedback loops established by AGO10 and ZPR3. Active homodimeric HD-ZIPIII proteins regulate developmental processes such as leaf polarity and stem cell maintenance. The positive 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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Homeodomain leucine-zipper proteins and their role in synchronizing growth and development with the environment

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Minireview

Abstract The *Arabidopsis* (*Arabidopsis thaliana* L.) genome encodes for four distinct classes of homeodomain leucine-zipper (HD-ZIP) transcription factors (HD-ZIPI to HD-ZIPIV), which are all organized in multi-gene families. HD-ZIP transcription factors act as sequence-specific DNA-binding proteins that are able to control the expression level of target genes. While HD-ZIPI and HD-ZIPII proteins are mainly associated with environmental responses, HD-ZIPIII and HD-ZIPIV are primarily known to act as patterning factors. Recent studies have challenged this view. It appears that several of the different HD-ZIP families interact genetically to align both morphogenesis and environmental responses, most likely by modulating phytohormone-signaling networks.

Keywords: Transcription factors; homeodomain; leucine zipper; auxin; light signaling; water stress; abscisic acid; leaf development; REVOLUTA; KANADI; microRNA

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INTRODUCTION

Plants are able to detect subtle changes in light, temperature, water and nutrient availability. In contrast to animals, plants are sessile and therefore have to cope with permanently changing environmental conditions. Adaptive growth responses feed back on intrinsic patterning programs, aligning plant growth to the environment. Among the Arabidopsis homeodomain leucine-zipper (HD-ZIP) transcription factors, several are known to be rapidly induced in response to altered environmental conditions and to integrate hormonal signals.

The Arabidopsis genome encodes for 48 HD-ZIP proteins. Based on their domain organization and biological functions, they can be subdivided into four distinct protein families (HD-ZIPI to IV). All HD-ZIPs contain an amino-terminal homeodomain (HD) (Scott et al. 1989) required for DNA-binding, followed by a small leucine zipper (ZIP)-domain enabling protein-protein interactions. HD-ZIP proteins have to dimerize via their leucine zipper domains to bind DNA with high affinity (Sessa et al. 1993). Genes encoding HD-ZIP proteins are evolutionary highly conserved and have also been identified in basal plant species (Floyd and Bowman 2006; Floyd et al. 2006; Hu et al. 2012). Another commonality among HD-ZIP proteins is the recognition of pseudo-palindromic *cis*-elements, which have been identified in DNA-binding studies. HD-ZIPI proteins interact with the CAAT(A/T)ATTG motif (Palena et al. 1999), whereas HD-ZIPII proteins preferentially bind the CAAT(C/G)ATTG motif (Sessa et al. 1993). The binding site for HD-ZIPIII proteins was found to be GTAAT(G/C)ATTAC (Sessa et al. 1998), but recent genome-wide binding site studies suggest that only the AT(G/C)AT core is essential for DNA-binding (Brandt et al. 2012). For HD-ZIPIV, the CATT(A/T)AATG element was found to be required for DNA-binding (Tron et al. 2001). Because the identified elements are very similar, these findings suggest that the different classes of HD-ZIP proteins are most likely able to associate with the same element and thus control gene expression in a competitive or antagonistic manner.

CLASS I HD-ZIP PROTEINS (HD-ZIPI)—REGULATORS OF STRESS RESPONSES

HD-ZIPI proteins are mainly associated with stress responses. This is supported by genome-wide gene expression studies, which revealed that several *HD-ZIPI* genes show transcriptional changes in response to treatments with the plant stress hormone abscisic acid (ABA) (Henriksson et al. 2005), but also function in the ABA signaling network. Expression of the *HD-ZIPI* genes *AtHB6* and *AtHB7* is for example induced by ABA application or water deficiency (Söderman et al. 1996; Himmelbach et al. 2002; Lechner et al. 2011). Furthermore, AtHB6 controls its own gene expression by recognizing a *cis*-regulatory element in its promoter and thereby reduces ABA sensitivity (Himmelbach et al. 2002), suggesting a complex interaction between HD-ZIPI transcription factors and stress. The abscisic acid-inducible *AtHB12* transcription factor is closely related to *AtHB7* (Son et al. 2010). During seed germination, it enhances ABA sensitivity, while at later stages it reduces growth of inflorescence stems by inhibition of gibberellic acid (GA) synthesis. Recently, it was shown that both ATHB7 and ATHB12 act as positive regulators of several genes encoding PP2C phosphatases and to repress multiple genes encoding

ABA-receptors (Valdes et al. 2012). These opposite activities result in the suppression of ABA-signaling and thus ATHB7 and ATHB12 down-regulate ABA-sensitivity in response to dehydration.

Besides their role in stress responses, *HD-ZIPI* genes have additional roles in controlling development. In *Medicago truncatula*, *HB1*, the homolog of Arabidopsis *ATHB7* and *ATHB12*, 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 *LATERAL ORGAN BOUNDARY1* (*LBD1*) and thereby inhibits initiation of lateral roots. A similar phenotype can be observed in response to elevated ABA levels. Beside the regulation by HB1, *LBD1* expression is also induced by auxin (Ariel et al. 2010). This auxin/ABA cross-regulation is thought to adjust root patterning to the environmental context. The recent finding that leaf patterning factors impinge on the regulation of several ABA-associated factors (discussed below) suggests that these factors have integral role in aligning development to the environmental context.

Beside the homeodomain-leucine zipper motif, HD-ZIPI transcription factors have no other known domain. The finding that they act as both positive and negative regulators of transcription suggests that they are engaged in higher order protein complexes, which mediates either transcriptional activation or repression. As outlined below, based on the structure of the leucine-zipper stretch, it seems possible that the negatively acting HD-ZIPII factors interact with HD-ZIPI proteins to mediate transcriptional repression.

CLASS II HD-ZIP PROTEINS (HD-ZIPII)—PRIME TARGETS OF LIGHT SIGNALING PATHWAYS

HD-ZIPII transcription factors are widely known to play a role in shade avoidance responses (Steindler et al. 1999; Ciarbelli et al. 2008; Sorin et al. 2009). *ATHB2*/*HAT4*, *HAT1*, *HAT2*, *HAT3* and *ATHB4* are under direct control of the phytochrome system (Ciarbelli et al. 2008) and their gene expression increases rapidly after exposure to shade (Ciarbelli et al. 2008; Sorin et al. 2009). High mRNA levels of *HD-ZIPIIs* are causal for the typical shade avoidance phenotypes: longer hypocotyls, fewer branches and smaller leaves (Schena et al. 1993; Sawa et al. 2002; Ciarbelli et al. 2008; Sorin et al. 2009). Besides shade, *HAT2* expression is also rapidly induced in response to auxin (Sawa et al. 2002) and by binding to its own promoter, HAT2 protein regulates its own mRNA expression in a direct negative manner (Ohgishi et al. 2001; Sawa et al. 2002). In addition, also HAT1, HAT4 and ATHB4 regulate expression of other *HD-ZIPIIs* in a dominant negative manner (Sorin et al. 2009), most likely to avoid excessive growth.

In contrast to *Arabidopsis* HD-ZIPI proteins that consist of a homeodomain fused to a leucine-zipper domain, most HD-ZIPII proteins contain an amino-terminal ethylene-responsive element binding factor-associated amphiphilic repression motif (EAR-domain). EAR-domain-containing proteins often act as transcriptional repressors (Kagale et al. 2010) and it was recently shown that two members of the HD-ZIPII protein family (HAT1 and HAT22) were identified as interacting proteins with the TOPLESS co-repressor protein (Causier et al. 2012). These interactions, mediated through the EAR-domain, support the idea that EAR-domain-containing HD-ZIPII proteins gain their repressive potential by interacting with TOPLESS or TOPLESS-related proteins.

CLASS III HD-ZIP PROTEINS (HD-ZIPIII)—MAJOR POLARITY DETERMINANTS

The *HD-ZIPIII* transcription factor family is the smallest gene family of all *HD-ZIPs* in Arabidopsis and consists of only five members: REVOLUTA (REV), PHABULOSA (PHB), PHAVOLUTA (PHV), ATHB8 and ATHB15/CORONA/INCURVATA4. In higher plants, HD-ZIPIIIs are involved in patterning of the embryo, shoot, root, leaves and vasculature (McConnell et al. 2001; Otsuga et al. 2001; Prigge et al. 2005; Carlsbecker et al. 2010; Smith and Long 2010; Lucas et al. 2013). Despite their close relationship within the HD-ZIPIII family, the function of the family members differs and multiple loss-of-function mutant plants display redundant but also antagonistic phenotypes in Arabidopsis (Prigge et al. 2005).

PHB determines the upper half of the embryo already at the globular stage, resulting in the subsequent formation of the upper part organ structures which are the shoot apical meristem and cotyledons (Smith and Long 2010). Later in development, *PHB*, *PHV* and *REV* are all expressed in the adaxial regions of the cotyledons and the pro-vasculature of the future hypocotyl (Williams et al. 2005; Grigg et al. 2009; Smith and Long 2010; Lucas et al. 2013). Ectopic expression of *PHB*/*PHV* in the lower part of the embryo abolishes root formation and results in lethality (Grigg et al. 2009), underpinning the importance of the spatial expression of *HD-ZIPIIIs*. During the post-embryonic growth phase, HD-ZIPIIIs are required for the maintenance of an active shoot apical meristem, polarization of newly forming leaf primordia and the initiation of lateral meristems (McConnell et al. 2001; Emery et al. 2003; Prigge et al. 2005).

Their eminent role in the regulation of developmental processes suggests that HD-ZIPIII proteins are part of growth-promoting hormone-signaling pathways. Based on overlapping patterns of expression of REV and PIN (PIN-formed) auxin transporters, a role in controlling auxin fluxes was proposed (Zhong and Ye 2001; Heisler et al. 2005). Support for this hypothesis is provided by expression studies in *rev* (*ifl1*) mutant plants in which altered gene expression for *PIN3* and *PIN4* in seedlings and stems were detected, resulting in phenotypes similar to plants with defects in polar auxin transport (Zhong and Ye 2001). A recent genome-wide ChIP-Seq study of genes directly regulated by the HD-ZIPIII protein REVOLUTA (REV) revealed that both auxin biosynthesis and signaling are directly controlled by REV (Brandt et al. 2012).

In addition to the homeodomain and leucine zipper domain, HD-ZIPIII proteins possess an additional START/HD-SAD domain (Mukherjee and Burglin 2006). The START-domain comprises a region that is complementary to the plant microRNAs miR165 and miR166 and makes *HD-ZIPIII* mRNAs highly sensitive for microRNA-regulation (Emery et al. 2003). The microRNAs are expressed in a complementary pattern of expression across the newly forming leaf

primordium, allowing *HD-ZIPIII* mRNA accumulation only in the adaxial region (Juarez et al. 2004). In this context it is important to note that the microRNA-machinery also affects *HD-ZIPIII* accumulation. AGO10, a specialized ARGONAUTE protein is able to sequester *miR165/6* thus allowing accumulation of *HD-ZIPIII* transcripts (Ji et al. 2011; Zhu et al. 2011). The finding that REV is able to directly up-regulate *AGO10* expression suggests a direct positive feed forward regulatory mechanism allowing *HD-ZIPIII* mRNA accumulation in the adaxial region (Brandt et al. 2013). High-troughput next-generation sequencing methods allowed deep insights in post-transcriptional regulation using smallRNA-Seq. Recently, Paul et al., identified a broad set of miRNAs in *Vigna mungo*, including five members of the *miR166* family to be predominantly expressed in young leaves (Paul et al. 2014).

In animals, START-domain proteins can bind lipids, steroids and steroid precursors, and some are known to be involved in shuttling these compounds between different subcellular compartments (Stocco 2001). Identification of a HD-ZIP-START-domain ligand has so far not been reported. The fact that START-domain proteins possessing homeodomain leucine zipper domains can also be identified in basal plants (Mukherjee et al. 2009) supports the idea that the START domain retains an additional function.

At the carboxy-terminal end, HD-ZIPIII proteins possess an additional MEKHLA domain. A region within this domain shows sequence similarity to the PAS (Per-Arnt-Sim)-domain which has been shown to act as intracellular sensor of light, oxygen or redox-potentials (Mukherjee and Burglin 2006). So far, no function has been assigned to the MEKHLA domain. Evidence that the MEKHLA domain has a functional relevance is provided by the identification of a point mutation in the MEKHLA-domain of ATHB15, which creates a complex shoot-regeneration phenotype (Duclercq et al. 2011). The finding that the DORNRÖSCHEN/DORNRÖSCHEN-LIKE transcription factors are able to physically interact with this domain and to genetically interact with *PHB* (Chandler et al. 2007) further supports an important function. Furthermore, it was recently shown that one of the functions of the PAS-domain might be to control the dimerization ability of HD-ZIPIII proteins (Magnani and Barton 2011).

CLASS IV HD-ZIP PROTEINS (HD-ZIPIV)—EPIDERMAL PATTERNING FACTORS

The Arabidopsis genome encodes for sixteen HD-ZIPIV proteins, commonly referred to as "GLABRA" gene family, named after its eponymous member. The large number of genes is an indicator for high functional redundancy, hampering the assignment of functions to individual genes. Indeed, the analysis of T-DNA-insertion mutants in 12 different *HD-ZIPIV* genes revealed wild type growth behavior for all examined loss-of-function mutants, except for *hdg11* (*HOMEODOMAIN GLABROUS11*), which exhibited excess branching of trichomes (Nakamura et al. 2006). Tissue-specific gene expression and promoter-GUS studies of different *HD-ZIPIV* genes revealed expression in developing shoots and reproductive organs, suggesting a general function in developmental processes (Nakamura et al. 2006). Loss-of function mutant plants of *GLABRA1, 2* or *3* (*GL1, 2* or *3*) are characterized by glabrous leaves or leaves producing trichomes with fewer branches (Marks et al. 2009; Qing and Aoyama 2012). In addition to its function in leaf epidermis patterning, GL2 has an additional role in root hair development by controlling cell fate determination of H-cells (hair cells; trichoblast) and N-cells (non-hair cells; atrichoblast) (Masucci et al. 1996). Brassinosteroid hormones are positive regulators of *GL2* gene expression, which is in agreement with *bri1* mutants (*BRASSINAZOLE INSENSITIVE1*) that are insensitive to brassinosteroids, having reduced *GL2* mRNA levels and less branched trichomes (Kuppusamy et al. 2009). This positions *GL2* downstream of the BRI1-mediated BR signaling pathway.

In addition to specifying trichome cell fate, HD-ZIPIV proteins are also involved in stomata differentiation. The HOMEODOMAIN GLABROUS2 (HDG2) protein is highly expressed in meristemoids, which are stomatal precursor cells that will undergo stomata differentiation (Peterson et al. 2013). While stomatal progression is delayed in *hdg2* mutant plants, transgenic plants over-expressing *HDG2* show multiple epidermal cell layers with ectopic stomata located in internal mesophyll tissue (Peterson et al. 2013).

It remains unknown whether HD-ZIPIV proteins integrate environmental signals to adjust patterning to altered external conditions. The analysis of publicly available microarray data using the eFP browser web-interface (Winter et al. 2007) revealed that for example *HDG2* expression increases in response to drought (Figure 1A). Whether and how this elevated expression of *HDG2* affects stomatal patterning and adaptation is not known. It is important to note that several *HD-ZIPIV* genes also contain evolutionary conserved motifs in the 3'-UTR (Ingouff et al. 2003; Javelle et al. 2011). These elements might also be involved in the regulation of translation in response to external or internal signals, thus adding an additional layer of possible regulation.

GENETIC INTERACTIONS BETWEEN DIFFERENT HD-ZIP FAMILIES: SHADE-ESCAPE CONTROL AND LEAF PATTERNING

Several recent findings point towards interactions between different HD-ZIP families. The genome-wide identification of genes directly regulated by the HD-ZIPIII transcription factor REVOLUTA (REV) revealed several members of the HD-ZIPII family (Brandt et al. 2012) underlying REV-regulation. Both *HD-ZIPII*-induction and increased levels of the plant hormone auxin seem to be essential for a full shade-avoidance response (Ciarbelli et al. 2008; Tao et al. 2008; Sorin et al. 2009). Loss-of-function *hd-zipIII* mutant plants, or plants with largely reduced HD-ZIPIII protein activity, show an impairment in their ability to respond to shade, which is manifested by a reduced hypocotyl elongation ability (Brandt et al. 2012). Mutations in *HD-ZIPII* genes also result in impaired shade avoidance responses (Sorin et al. 2009), suggesting that HD-ZIPII and HD-ZIPIII transcription factors redundantly control growth responses in the same pathway.

Beside their role in controlling shade avoidance, HD-ZIPIIs are also involved in leaf patterning. When *HAT3* and *ATHB4* are mutated, double mutant plants develop lancet-shaped

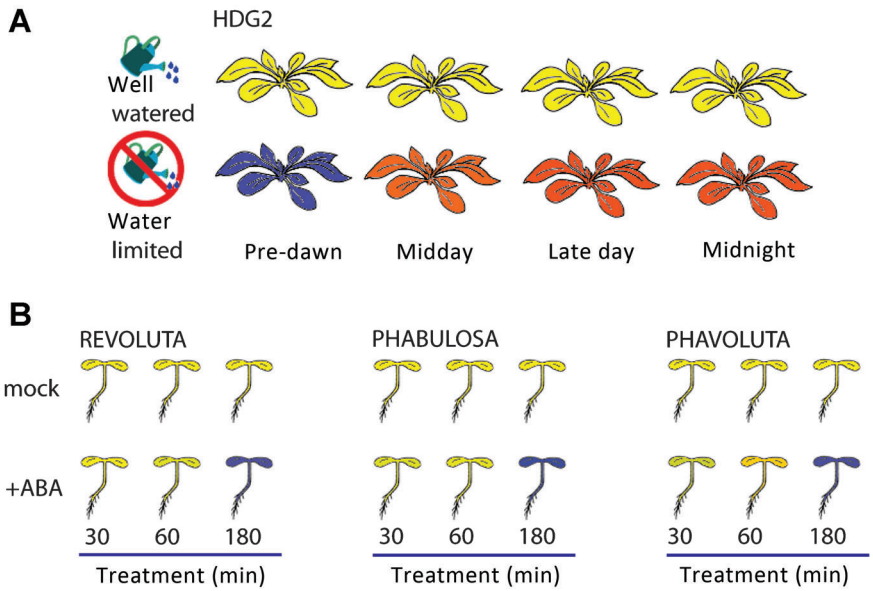

Figure 1. Response of HD-ZIP genes to environmental and hormonal cues
(A) HDG2 expression increases in response to drought. **(B)** HD-ZIPIII mRNA levels decrease in response to ABA application. Blue color indicates low level of expression, yellow color represents intermediate expression level and red color shows enhanced expression levels.

cotyledons and entirely radialized leaves with abaxial characteristics during the early growth phase (Bou-Torrent et al. 2012). When more *HD-ZIPII* genes are affected, as in the *hat3 athb4 athb2/hat4* triple mutant, growth defects increase and these plants develop radial cotyledons, which often appear as fused/single cotyledons (Turchi et al. 2013). Also in the process of establishing polarity in the early leaf primordium, both HD-ZIPII and HD-ZIPIII transcription factors appear to act in the same pathway. This finding is supported by the combination of *hd-zipII* and *hd-zipIII* mutants (as in *hat3 athb4 phb* and *hat3 athb4 rev*) that exhibit severely enhanced growth defects compared to *hat3 athb4* double mutant plants (Turchi et al. 2013).

So far, it remains unclear how HD-ZIPII/HD-ZIPIII interact in shade adaptation and leaf development. The finding that both auxin synthesis and transport are affected in *hat3 athb4* double mutant plants (Turchi et al. 2013) and that REV transcriptionally induces genes encoding auxin biosynthesis enzymes (Brandt et al. 2012), points towards signal integration at the level of hormone signaling. *HD-ZIPII* expression is strongly induced in response to both shade and auxin (Sawa et al. 2002; Ciarbelli et al. 2008; Sorin et al. 2009) and this induction appears to be independent of *HD-ZIPIII* activity. Because HD-ZIPIIs act mainly as transcriptional repressors while HD-ZIPIIIs activate transcription, it seems plausible that HD-ZIPIIs feedback regulate HD-ZIPIII activity by controlling pathways that either restrict *HD-ZIPIII* expression or HD-ZIPIII activity. Alleviating this repressive action on factors negatively regulating *HD-ZIPIII* expression or activity (as in *hat3 athb4*) would deplete *HD-ZIPIII* expression/activity and result in loss-of-function phenotypes reminiscent of *hd-zipIII* loss. The fact that *hd-zipII* multiple loss-of-function mutants resemble *hd-zipIII* mutant plants largely supports this hypothesis. Conversely, it was shown that ectopic expression of both *HD-ZIPII* and *HD-ZIPIII* genes causes adaxialization of developing leaves, which is manifested by upward curled leaf blades (Bou-Torrent et al. 2012; Turchi et al. 2013). Taken together, it seems that HD-ZIPII/III proteins have redundant functions in different pathways and their activities depend on each other, thereby reinforcing their action.

INTERACTIONS BETWEEN DIFFERENT HD-ZIP FAMILIES: LEAF DEVELOPMENT AND STRESS RESPONSES

Drought stress can have detrimental effects on plant growth and development. Our understanding how stress affects patterning processes is however scarce in comparison to our understanding of stress perception and signal transduction. One of the most prominent plant "stress hormones" is abscisic acid (ABA), which triggers a set of physiological responses, such as stomatal closure, to avoid water loss. As described above, the HD-ZIPI factors ATHB7 and ATHB12 transcriptionally down-regulate a number of genes encoding ABA-receptor proteins (Valdes et al. 2012). It was recently published that also REV is involved in positively regulating the expression of a gene encoding the ABA receptor protein PYL6 (Liu et al. 2013; Reinhart et al. 2013). Although, ATHB7/12 and REV do not seem to regulate the same genes encoding ABA receptors, it appears possible that both families oppositely regulate the expression of related genes in a cell-type specific manner.

Beside the regulation of ABA signaling by HD-ZIPIII transcription factors (here REV), expression of *HD-ZIPIII* genes can also be modulated by ABA-application. The analysis of

publicly available microarray data revealed that the expression of *REV*, *PHB* and *PHV*, three members of the *HD-ZIPIII* family, strongly decreases after three hours of ABA application (Figure 1B). According to Liu and colleagues, this decrease of *HD-ZIPIIIs* is a consequence of ectopic induction of *miR165* expression (Liu et al. 2013). Thus, ABA perception and signaling are connected by HD-ZIPIIIs and may be required to adjust leaf development to alterations in water availability.

It is tempting to speculate that ABA might also regulate HD-ZIPIII protein activity by interacting with the START domain. As described above, HD-ZIPIII/IV proteins contain a START domain for which, so far, no ligand is known to date. The ABA-receptor proteins of the PYL/PYR/RCAR family also contain a START domain (Park et al. 2009), which is, based on sequence identity, not closely related to the domain found in the HD-ZIPIII/IV proteins. It is, however, known that proteins with a low conserved amino acid sequence can fold into similar structures and perform similar tasks, wherefore a regulatory role of ABA on the activity of HD-ZIPIII proteins cannot be excluded at this stage. The fact that exogenous ABA application seems to affect leaf development and shoot apical meristem activity (Liu et al. 2013), two processes regulated by HD-ZIPIIIs, hints towards a stronger involvement.

THE LEUCINE-ZIPPER DOMAIN: A HUB FOR REGULATION?

All HD-ZIP proteins harbor a leucine-zipper domain enabling them to engage in higher order protein complexes. Leucine-zippers are coiled-coiled domains characterized by the regular arrangement of aliphatic amino acids such as leucine, methionine, valine or isoleucine residues in the "d" position of the heptad repeats (Landschulz et al. 1988; Deppmann et al. 2004). Residues in the "a", "e" and "g" positions act as specificity determinants (Deppmann et al. 2004).

It was shown that HD-ZIPIII transcription factors underlie post-translational regulation by the small leucine-zipper protein family of LITTLE ZIPPER proteins (Wenkel et al. 2007; Kim et al. 2008). HD-ZIPIII and ZPR proteins have highly similar leucine zipper domains, which can form heterodimers (Figure 2A). ZPR proteins act as microProteins, sequestering HD-ZIPIII proteins into non-functional complexes (Staudt and Wenkel 2011). If the *ZPR3* and *ZPR4* genes are mutated as in *zpr3 zpr4* double mutant plants, severe developmental defects such as enlarged meristems, altered phyllotaxis and sterile flowers are observed (Kim et al. 2008). Interestingly, the HD-ZIPIII protein REVOLUTA directly controls expression of *ZPR* genes (Wenkel et al. 2007; Brandt et al. 2012; Brandt et al. 2013). Thus, REV establishes a direct negative feedback loop adjusting HD-ZIPIII protein activity.

Based on sequence similarity, LITTLE ZIPPER proteins can be identified as potential HD-ZIPIII-interacting proteins by conducting BLAST searches with the HD-ZIPIII leucine zipper domain. By performing protein alignments and subsequent BLAST searches with the leucine-zipper domains of all other classes of Arabidopsis HD-ZIPs, we were curious whether other leucine-zipper type proteins can be identified. This analysis resulted in the identification of a bZIP transcription factor named G-BOX-BINDING FACTOR 1 (GBF1), containing a leucine-zipper domain highly similar to that of HD-ZIPII proteins (Figure 2B). GBF1 has also known roles in the adjustment of plant growth in response to light quality (Singh et al. 2012), suggesting that HD-ZIPIIs and GBF1 might act together. Predicated on the chemical nature of residues in the "a", "e" and "g" positions, it seems also likely that HD-ZIPI and HD-ZIPII proteins are able to physically interact while the interaction of either HD-ZIPI or HD-ZIPII with HD-ZIPIII proteins appears questionable, based on the published data.

INTEGRATION OF HD-ZIP TRANSCRIPTION FACTORS INTO THE NETWORK OF FACTORS CONTROLING LEAF POLARITY

Polar leaf development is regulated by the antagonistic activities of several transcriptional regulators. In this context, members of the HD-ZIPIII family specify adaxial cell fate, which is the determination of tissue that will form the upper side of the leaf. In contrast, members of the KANADI (KAN) and YABBY (YAB) transcription factor families specify abaxial cell fate and thus direct the formation of tissue making up the lower side of the leaf. While HD-ZIPIIIs mainly act as transcriptional activators, KANADI factors, which are EAR-domain-containing GARP-type proteins, predominantly repress transcription. YABBY transcription factors promote the expression of abaxial determinants but have recently been shown to also act transcriptional repressor on adaxial factors (Bonaccorso et al. 2012). It has been shown that HD-ZIPIIIs and KANs act in an antagonistic fashion exerting opposite functions (Izhaki and Bowman 2007). Using genome-wide transcriptional profiling in combination with ChIP-Seq, we have started to describe genes transcriptionally regulated by KAN1 (Merelo et al. 2013). This analysis revealed a significant overlap of genes targeted by both REV (HD-ZIPIII) and KAN1, which supports the idea that the antagonism of ad/abaxial regulation is in part mediated through opposite regulation of a set of shared target genes. In addition, we find a number of other *HD-ZIP* genes being controlled by KAN1. Two members of the *HD-ZIPIII* family (*PHB* and *ATHB8*) are potential direct negative targets, suggesting that besides oppositely controlling a common set of direct targets, mutual regulation might also contribute to polarity set-up. Beside *HD-ZIPIIIs*, *HAT2*, a member of the *HD-ZIPII* family underlies negative regulation by KAN1 (Brandt et al. 2012). Two *HD-ZIPI* genes, *ATHB22* and *ATHB12* are also potential KAN1 targets (Merelo et al. 2013). This finding is particular striking because ATHB12 is known to act in the ABA signaling pathway hypothetically antagonizing HD-ZIPIII activity (as described above). Since several ABA signaling components (such as receptors, phosphatases etc.) are also under potential KAN1 control (Merelo et al. 2013), it seems possible that ABA contributes to the patterning of leaf primordia.

Members of the different HD-ZIP families regulate a plethora of physiological pathways ranging from basal embryo patterning to adaptation responses of mature plants. The HD-ZIPIII/KANADI module, required for polarity establishment in the early leaf primordium, is also linked to adaptive responses

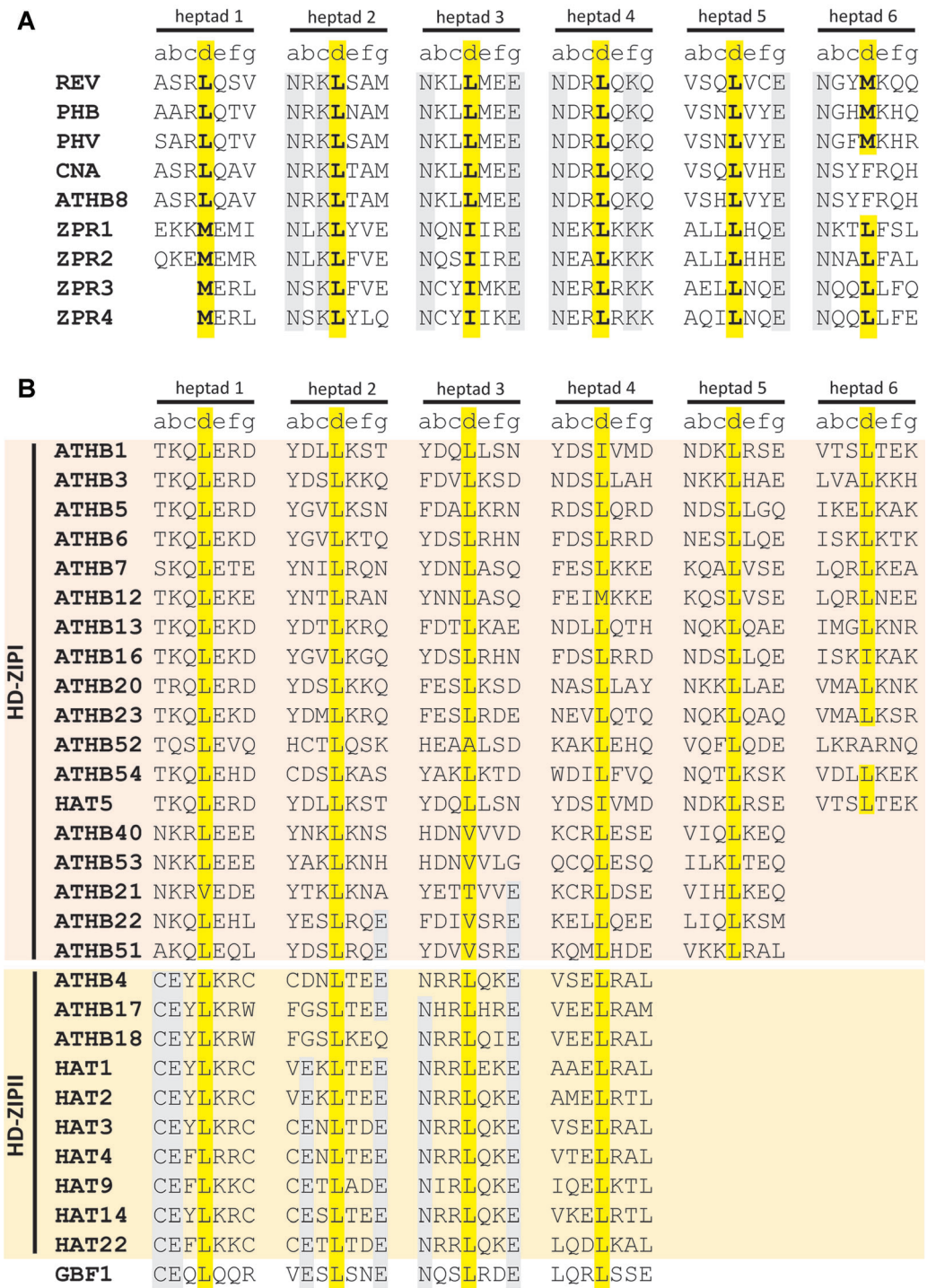

Figure 2. Alignments of Leucine-zipper domains of different classes of HD-ZIP proteins
(A) Alignment of all *Arabidopsis* HD-ZIPIII and LITTLE ZIPPER proteins. Both protein families have six heptad repeats. **(B)** Alignment of all *Arabidopsis* HD-ZIPI and HD-ZIPII families with GBF1. HD-ZIPI proteins have either five or six heptad repeats while HD-ZIPII proteins and GBF1 have only four repeats. Highly conserved residues are highlighted in grey.

such as the response to shade or the perception of drought (Figure 3). In both adaptive pathways, other members of HD-ZIP proteins (HD-ZIPII in shade and HD-ZIPI in the drought response) play integral roles in the routing and modulation of signaling cascades. Thus, the evolutionary highly conserved HD-ZIP proteins seem to be at the nexus of patterning and adaptation and may act to adjust developmental programs to the environmental context.

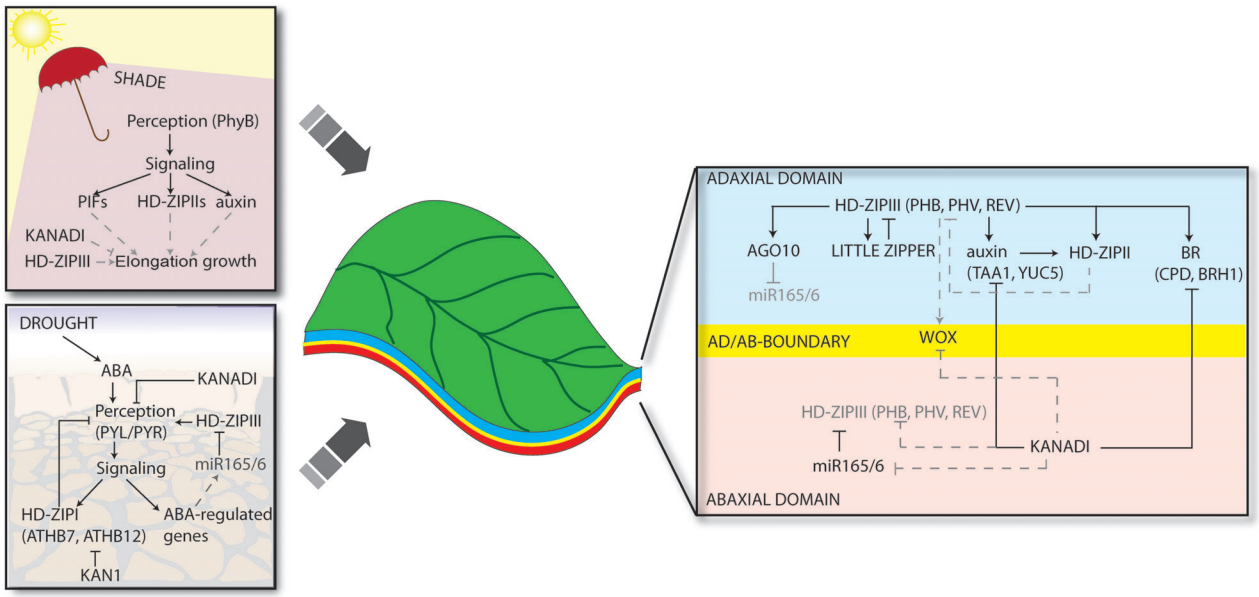

Figure 3. Model of how intrinsic and extrinsic factors control leaf development
Leaf patterning is controlled by the opposed activities of HD-ZIPIII and KANADI proteins. Both auxin- and brassinosteroid-biosynthesis seem to underlie regulation by the REV/KAN1 module. HD-ZIPII factors act in the HD-ZIPIII pathway, most likely by controlling components influencing HD-ZIPIII activity. In shade-induced elongation growth REV/KAN1 occupy opposite roles as well: while REV promotes shade-induced growth, KAN1 represses it. HD-ZIPII factors act also as growth promoting factors in shade. Drought signaling involves the phytohormone abscisic acid (ABA). ABA perception/signaling is again potentially antagonistically controlled by REV/KAN1. The HD-ZIPI proteins ATHB7/12 are involved in drought signaling and might also be regulated by KAN1. Thereby KAN1 could influence a potential HD-ZIPI/HD-ZIPIII antagonism.

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5. Discussion

5.1. The role of *REVOLUTA* in leaf senescence

Plants induce leaf senescence to reallocate nutrients and valuable substances from mature leaves to reproductive seeds. The timing of senescence is controlled by developmental age while environmental cues such as temperature, light and drought strongly influence this process. Massive changes in the transcriptome during onset and progression of senescence imply an important role for transcriptional regulators. Previous reports showed that several large groups of transcription factors are transcriptionally up-regulated in senescing leaves, such as NAC, WRKY, C2H2-type zinc finger, AP2/EREBP, and MYB proteins (Guo and Gan, 2005). In this study, we show that *HD-ZIPIII* factors have an additional formerly unknown role in the final stage of leaf development, leaf senescence. *REV* is a direct and positive regulator of *WRKY53* expression and reducing the activity of REV or other HD-ZIPIII proteins delays the onset of leaf senescence. Interestingly, *rev* mutant plants display stronger leaf senescence phenotypes compared to *wrky53* mutant plants, suggesting that *REV* also regulate other senescence-associated genes.

Here, we have identified nine *REV* targets which are differentially expressed during senescence. Among them, *HAT3* has been shown to play an important role in regulating polar leaf development (Bou-Torrent et al., 2012; Brandt et al., 2012; Turchi et al., 2013). *rev* mutant seedlings exhibit reduced levels of *HAT3* mRNA, suggesting that *HAT3* expression depends partly on the presence of REV (Brandt et al., 2012). During senescence, *HAT3* mRNA levels decrease with progression of this process. However, *HAT3* mRNA is more abundant in *rev* mutant seedlings compared to wild type (Xie et al., 2014). Overall, the transcriptome of *rev* mutant plants is dramatically altered through senescence, resulting in stage dependent misexpression of many differentially expressed senescence-associated genes.

Redox-sensitive DPI-ELISA experiments revealed a reduced DNA-binding ability of REV in response to oxidative stress, suggesting that *REV* is a redox-sensitive transcription factor. However, these results contradict the finding that *HD-ZIPIIIs* is partially required for the up-regulation of *WRKY53*, indicating a more complex regulatory cascade. Several direct *REV* target genes act as transcriptional repressors that might explain the contradiction. For instance, two *REV* targets encode EAR-domain proteins that can act as co-repressors (Causier et al., 2012). Our findings also show that the mRNA abundance of two other transcriptional repressors, *HAT3* and *ZFP8,* is altered in the senescence process. Therefore it is reasonable that the reduced REV DNA-binding activity will cause lower expression levels of these transcriptional repressors, ultimately alleviating the repressive activity of them. Thus, modulation of REV activity in response to the altered intracellular redox state will profoundly affect the REV-regulated transcriptome.

Leaf senescence can be triggered by a number of environmental factors, such as shading (Brouwer et al., 2012). Shade causes profound developmental changes in shade-sensitive plants aimed at outgrowing competitor plants. In consistence with it, our previous work showed that the *REV/KAN1* module is involved in modulating growth in response to shade (Brandt et al., 2012). Thus, *HD-ZIPIII* might play a critical role in connecting leaf patterning, leaf senescence and shade avoidance and adjusting plant growth and development to changing environmental conditions.

5.2. Identification of direct REVOLUTA targets

Based on the comparative analysis of two biologically independent ChIP-Seq experiments, we identified a number of putative REV targets which are involved in the regulation of multiple processes. Such as *STM* and *WUS* which are key regulators in meristem development. *REV* is also shown to control directly the expression of the genes that encode the auxin biosynthetic

enzymes *TAA1* and *YUCCA5* (*YUC5*). Besides, REV binds to the promoters of four members of the *HD-ZIP II* gene family, *HAT2*, *HAT3*, *ATHB2/HAT4* and *ATHB4* which are known regulators of shade signaling (Brandt et al., 2012). In conclusion, *REV* is involved in the regulation of plant development, as well as adaptive growth by regulating the expression of related target genes.

5.3. Identification of *KANADI1* target genes

a) Genes involved in organ patterning

In our study, we identified several *KAN1* targets related to the establishment of organ polarity. For instance, KAN1 binds to the promoter of *PHB* and *ATHB8* and represses their expression. It is well known that *HD-ZIPIII* activities in vascular tissue formation are antagonized by *KAN* expression, thereby affecting the canalization of auxin flow to mediate organ patterning (Ilegems et al., 2010). Here, our results suggest a new finding that there may be contexts in which *KAN1* acts directly on *PHB* and *ATHB8*. Interestingly, we also found that KAN1 binds directly to the promoters of two members of *MIR166* genes, *MIR166A* and *MIR166F* which can negatively control *HD-ZIPIII* genes. In addition, KAN1 binds to its own promoter and the promoter of *KAN2* via specifically targeting the VGAATAW motif (Merelo and Xie et al., 2013). Taken together, these results suggest that in some contexts *KAN1* may act in a negative feedback loop that limits the levels of several abaxial factors including *KAN1* itself.

Several *KAN1* targets are involved in different aspects of organ development such as *PXY/TDR*, *LNG1/2* and *SAW2*. *PXY* plays a crucial role in vasculature polarity establishment and is particularly required for the proper orientation of cell divisions in the vascular meristem (Fisher et al., 2007; Hirakawa et al., 2008). The homologous genes *LNG1* and *LNG2* regulate leaf morphology by positively promoting longitudinal polar cell elongation (Lee et al., 2006). *SAW2* is related to leaf morphology and its expression shows adaxialization in

developing lateral organs (Kumar et al. 2007). Therefore, our results suggest that *KAN1* may directly regulate genes involved in lateral organ development.

b) Genes related to Auxin

Four PIN family members (*PIN1*, *PIN3*, *PIN4* and *PIN7*) were identified as *KAN1* targets and their expression was repressed by *KAN1* as well. *PIN1* is known to mediate auxin redistribution. Previous studies showed that *kan1 kan2 kan4* embryos display ectopic expression of *PIN1* (Izhaki and Bowman, 2007). Therefore, KAN1 protein may act in patterning processes through auxin transport modulation. Additionally, several genes involved in the regulation of PIN activity and trafficking were repressed by *KAN1*, such as *PINOID* and *PLA2A* (Benjamins et al., 2001; Friml et al., 2004; Lee et al., 2010). Furthermore, we identified a set of genes involved in auxin transport and signaling. For example *YABBY5*, a transcription factor involved in specifying abaxial cell fate and auxin distribution (Sawa et al., 1999; Sarojam et al., 2010). The set of genes also includes different early auxin-responsive genes such as *GH3.3*, the *SMALL AUXIN UP RNA (SAUR)* genes (*SAUR19*, *SAUR20* and *SAUR63*), which regulate auxin polar transport and promote auxin-mediated organ elongation (Chae et al., 2012; Spartz et al., 2012). Taken together, *KAN1* may control the influence of auxin on organ development through complex interactions.

5.4. Genes subjected to dual regulation by both *KANADI1* and *REVOLUTA*

Previous studies have indicated that the *HD-ZIPIII* and *KAN* factors act oppositely in organ patterning (Eshed et al., 2001; Emery et al., 2003; Izhaki and Bowman, 2007). One hypothesis is that this interaction occurs via the mutual regulation of *KAN1* and *REV* on a set of common direct targets. Our finding that most of *KAN1* targets are down-regulated, for example *KAN1* directly represses the expression of the adaxial factor *AS2* (Wu et al., 2008),

suggests that *KAN1* acts primarily as a repressor. According to this hypothesis, HD-ZIPIII proteins should act as activators of those common targets. This is supported by the findings that *HAT2*, *TAA1* and *YUC5* genes are oppositely regulated by *REV* and *KAN1* (Brandt et al., 2012). In addition, we found 26 overlapping putative target genes after a comparison of the ChIP-Seq data for *KAN1* and *REV* respectively. Among these, we found several genes involved in auxin transcriptional response and auxin transport are repressed by *KAN1*, whereas some genes involved in auxin biosynthesis and transport are positively regulated by *HD-ZIPIII*s. Thus, our findings together with published work (Eshed et al., 2001; Emery et al., 2003; Izhaki and Bowman, 2007; Wu et al., 2008; Brandt et al., 2012) indicate that *HD-ZIPIII* and *KAN* genes function antagonistically both through mutual regulation as well as through the opposite regulation of common direct and indirect targets.

5.5. Outlook

REVOLUTA, a patterning factor, plays a critical role in early leaf development especially organ polarity processes. Taken together with our finding that *REVOLUTA* has an additional role in controlling leaf senescence via the WRKY network, there might be a complex mechanism in which *REVOLUTA* plays a central role mediating and coordinating early and late leaf development. Our finding also shows that besides *WRKY53*, a number of differentially expressed genes are regulated by *REVOLUTA* during senescence. However, much of the details remain unclear, so further investigations on these genes will unveil deep insights into the regulatory mechanism. Furthermore, induction of *WRKY53* expression in response to oxidizing conditions partially requires *REVOLUTA*, whereas hydrogen peroxide decreases DNA-binding activity of REVOLUTA. The contradiction could be partially explained by the fact that several direct *REVOLUTA* target genes act as transcriptional repressors.

Future projects should focus on studying the redox-dependent changes in the REVOLUTA protein outside the DNA-binding domain in more detail.

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