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

Ronny Brandt [a], *Yakun Xie* [a], *Thomas Musielak* [a], *Moritz Graeff* [a], *York-Dieter Stierhof* [a], *Hai Huang* [b], *Chun-Ming Liu* [c], *Stephan Wenkel* [a,*]

[a] *Center for Plant Molecular Biology (ZMBP), University of Tübingen, 72076 Tübingen, Germany*
[b] *National Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China*
[c] *Center for Signal Transduction and Metabolomics, Key Laboratory of Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*

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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

* Corresponding author. Tel.: +49 7071 29 78852.
E-mail address: stephan.wenkel@zmbp.uni-tuebingen.de (S. Wenkel).
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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

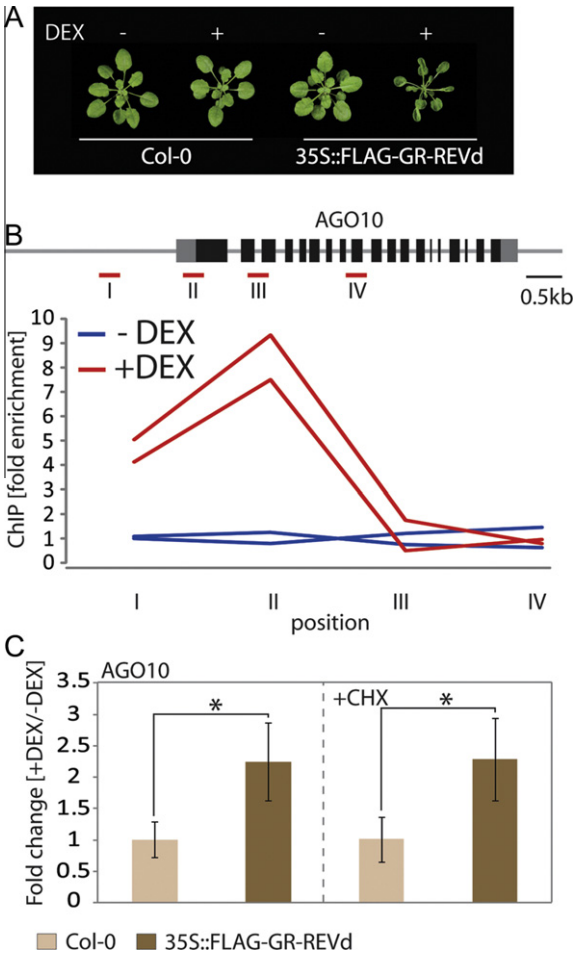

Fig. 1 – REVOLUTA directly regulates *AGO10* expression. (a) Induction of REVOLUTA causes adaxialization of leaves (Col and GR-REV +/−DEX). Plants were cultivated in long day conditions and after the production of the first true leaves sprayed daily with a 50 μM DEX solution or a mock substrate for 2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

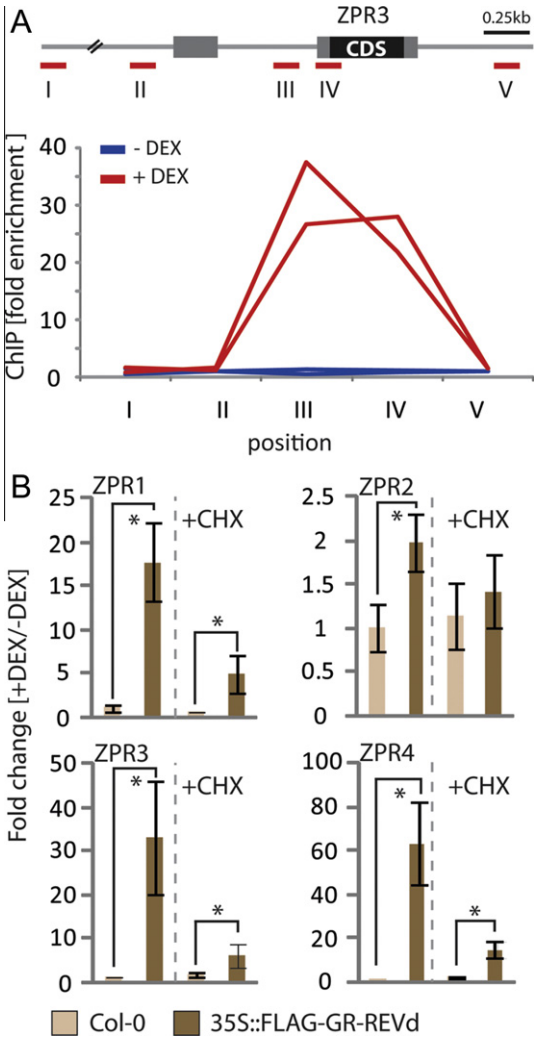

Fig. 2 – REVOLUTA directly regulates expression of *LITTLE ZIPPER* genes. (a) REV binds to the promoter of the *LITTLE ZIPPER3* gene. Chromatin-immunoprecipitation experiments with two biological replicates for *35S::FLAG-GR-REVd* without DEX (blue lines) and *35S::FLAG-GR-REVd* with DEX (red lines)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

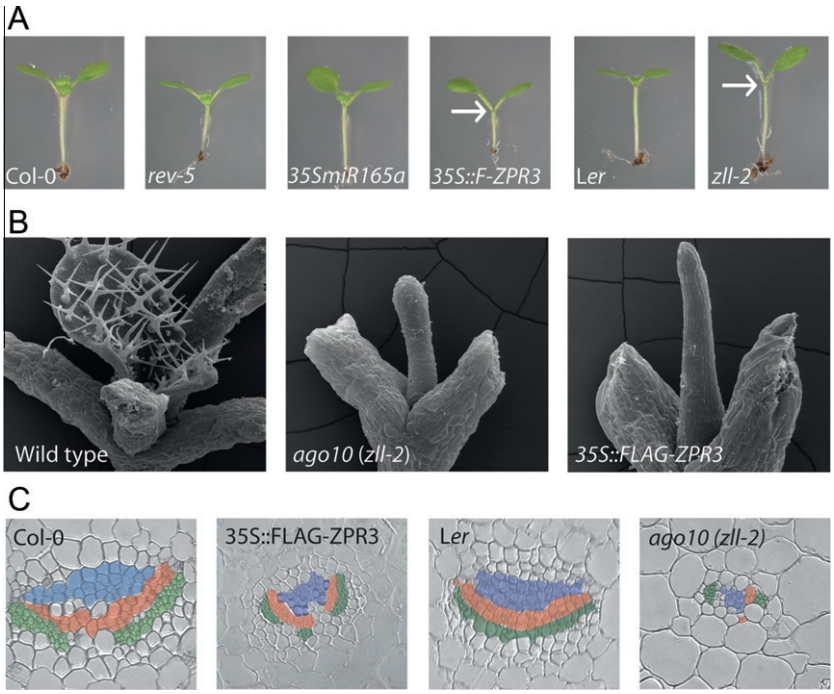

Fig. 3 – Mutations in *hd-zipIII* and *ago10* cause severe phenotypic defects. (a) Comparative growth analysis of hd-zipIII and ago10 mutant plants with corresponding wild type plants. Both *35S::FLAG-ZPR3* and *ago10* (*zll-2*) mutant plants show termination of the shoot apical meristem (arrow shows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

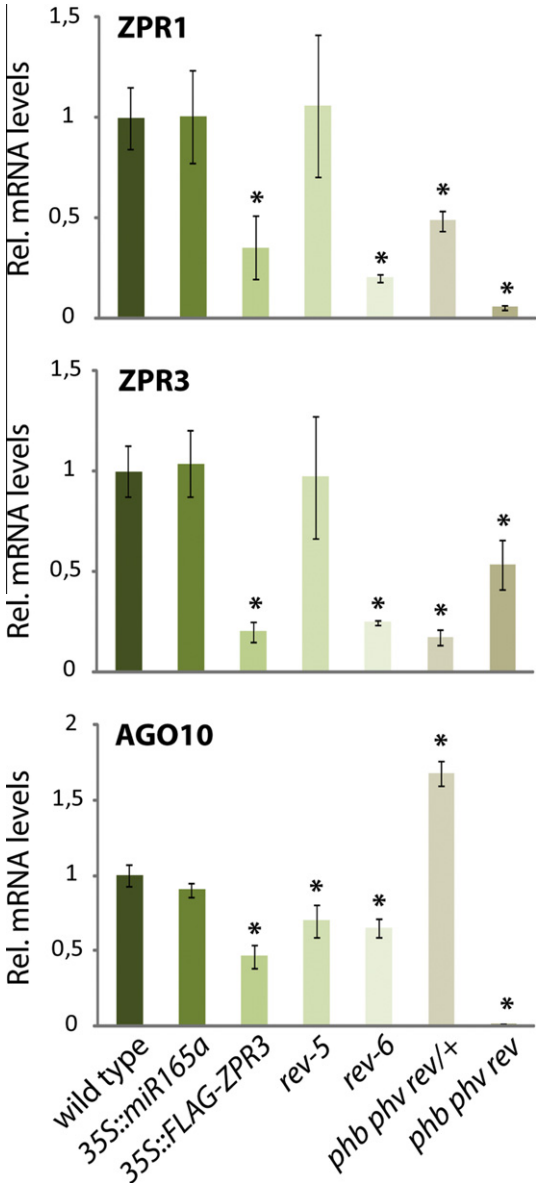

Fig. 4 – *AGO10*, ZPR1 and ZPR3 expression are altered in *hd-zipIII* mutant plants. Expression of *AGO10* and *ZPR3* was analyzed in mutants with either compromised *HD-ZIPIII* expression (*rev-5*, *rev-6*, *phb phv rev/+*, *phb phv rev* and *35S::miR165a*) or inhibited HD-ZIPIII protein activity (*35S::FLAG-ZPR3*).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

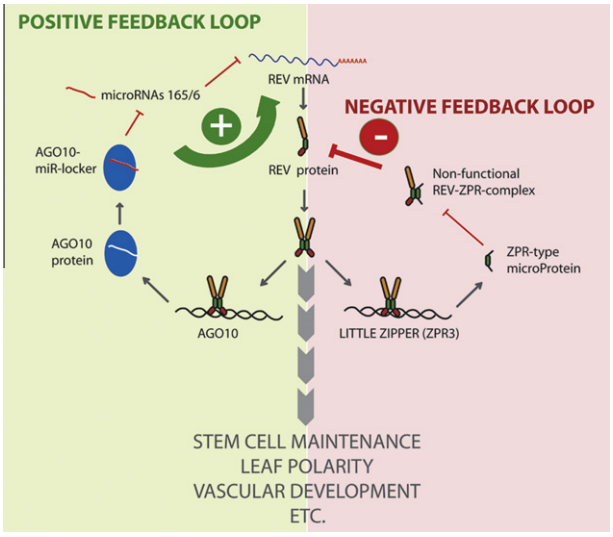

Fig. 5 – Interlocking positive and negative feedback-loops regulate stem-cell homeostasis in *Arabidopsis*. Model for the feedback loops established by AGO10 and ZPR3. Active homodimeric HD-ZIPIII proteins regulate developmental processes such as leaf polarity and stem cell maintenance. The positive 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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