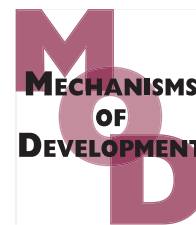


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Control of stem cell homeostasis via interlocking microRNA and microProtein feedback loops

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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.

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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 cytokinin 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-ZIPIII 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-ZIPIII. Because AGO10 is able to capture *microRNA165/6* and thereby protect HD-ZIPIII 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-ZIPIII emphasizing the biological importance of these feedback loops.

2. Results

2.1. An inducible system to study REVOLUTA DNA-binding

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

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

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

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