A Per-ARNT-Sim-Like Sensor Domain Uniquely Regulates the Activity of the Homeodomain Leucine Zipper Transcription Factor REVOLUTA in Arabidopsis

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Class III homeodomain leucine zipper (HD-ZIP III) transcription factors regulate critical developmental programs in plants; these include leaf polarity, polarity along the shoot-root axis, and stem cell specification and proliferation. One of the defining features of HD-ZIP III proteins is the presence of a Per-ARNT-Sim-like (PAS-like) MEKHLA domain at the C terminus. PAS-like domains are known to respond to a variety of chemical and physical stimuli. Here, we provide evidence that the MEKHLA domain acts as a negative regulator of Arabidopsis thaliana HD-ZIP III REVOLUTA activity. Based on experiments in yeast and plants, we propose a model in which the MEKHLA domain inhibits dimerization through a sequence-independent steric masking mechanism. This inhibition is relieved in response to a cellular signal that requires the C terminus of the MEKHLA domain for its perception. Overexpression experiments suggest that this signal is unequally distributed and/or sensed in the plant. Our data show that the function of the REVOLUTA MEKHLA domain differs among other HD-ZIP III family members; this difference may explain the genetic differences that have been observed among family members. This finding, combined with our phylogenetic analysis, suggests that REVOLUTA is the latest type of HD-ZIP III protein to have evolved in land plants.

INTRODUCTION

Class III homeodomain leucine zipper proteins (HD-ZIP III proteins) are plant-specific transcription factors that play prominent roles in plant development. In Arabidopsis thaliana, their activity establishes the shoot pole of the shoot-root axis in embryonic development (Grigg et al., 2009; Smith and Long, 2010), establishes the adaxial (upper) domain of the leaf primordium (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003; Prigge et al., 2005; Ochando et al., 2006, 2008), promotes meristem formation (Talbert et al., 1995; McConnell and Barton, 1998; Otsuga et al., 2001; Hawker and Bowman, 2004; Prigge et al., 2005), regulates growth within the shoot apical meristem (Green et al., 2005; Williams et al., 2005; Ochando et al., 2006, 2008; Zhou et al., 2007), and patterns the vasculature (Baima et al., 2001; Zhong and Ye, 1999; Carlsbecker et al., 2010). The HD-ZIP III proteins are conserved among land plants (Sakakibara et al., 2001; Floyd et al., 2006; Prigge and Clark, 2006) and have been shown to play similar developmental roles in other flowering plants (Juarez et al., 2004; McHale and Koning, 2004).

The HD-ZIP proteins are named for the combination of homeodomain and leucine zipper domains at their N terminus (Ruberti et al., 1991; Schena and Davis, 1992). Unlike animal homeodomain proteins, HD-ZIP proteins bind DNA as dimers in vitro, where they bind a palindromic sequence (Sessa et al., 1993, 1997, 1998). The loop between helices one and two is critical in determining whether the homeodomain binds as a monomer or dimer; substitution of the loop between helices one and two with the corresponding loop from the ENGRAILED homeodomain allows HD-ZIP proteins to bind DNA as monomers (Tron et al., 2004).

Dimerization of HD-ZIP III proteins in vivo is regulated by a family of small proteins that consist almost entirely of leucine zipper sequence (Wenkel et al., 2007; Kim et al., 2008). These LITTLE ZIPPER (ZPR) proteins interact with HD-ZIP III proteins in vitro and prevent DNA binding. Overexpression of ZPR proteins causes phenotypes similar to those seen in loss-of-function HD-ZIP III mutants. These observations are the basis for a model in which ZPR proteins act to negatively regulate HD-ZIP III activity by preventing their dimerization.

The structure of HD-ZIP III proteins suggests additional mechanisms of posttranslational regulation. In addition to the homeodomain and leucine zipper domain, HD-ZIP III proteins contain, in order from N terminus to the C terminus, a steriodogenic acute regulatory protein lipid transfer domain (START), a homeodomain-START associated domain (HD-SAD), and a MEKHLA domain (Ponting and Aravind, 1999; Mukherjee and Bürglin, 2006). START domains in animals bind cholesterol, phospholipids, and carotenoids (Radauer et al., 2008). In plants, the PYR/PYL group of START domains binds ABA and appears to act as an ABA receptor (Ma et al., 2009; Park et al., 2009). It is not known what ligand, if any, binds to the HD-ZIP III START domain.

The MEKHLA domain is a member of the Per-ARNT-Sim (PAS) domain superfamily. PAS domains are signal sensors that regulate a wide range of signal transduction pathways in all
The MEKHLA Domain Is Not Required for REV Activity

Despite being subject to miRNA regulation by the same subset of miRNAs, the five Arabidopsis HD-ZIP III genes are expressed in different, overlapping patterns (Baima et al., 1995; McConnell et al., 2001; Emery et al., 2003; Prigge et al., 2005; Williams et al., 2005). Hence, the expression domain of each of the five HD-ZIP III genes is determined by their promoters as well as by localized miRNA action.

In this study, we identify and characterize the role of the MEKHLA domain in modulating REV dimerization. Our data suggest a mechanism in which the N-terminal region of the MEKHLA domain is sufficient to cause conformational changes that inhibit dimer formation. The inhibition is relieved in response to a signal that requires the C terminus of the MEKHLA domain for its perception. Overexpression analyses in planta indicate that such a signal is not equally distributed or sensed in all cell types. This biological switch lies upstream of previously known mechanisms of REV posttranslational regulation.

RESULTS

The MEKHLA domain is a conserved domain located at the C terminus of all HD-ZIP III proteins (Mukherjee and Bürglin, 2006). The MEKHLA domain consists of a PAS-like domain flanked by a conserved N-terminal domain (Mukherjee and Bürglin, 2006). The PAS-like domain is predicted to have a similar secondary structure to that of well-defined PAS domains, such as that of Sinorhizobium meliloti and Azotobacter vinelandii, whose structures are known (Figures 1A and 1B) (Möglich et al., 2009). The N-terminal flanking region has weak similarity to the WD-40 domain (see Supplemental Figure 1 online); therefore, we named it MEKHLA WD40-like (M-WD40) (note, however, that it is not at all clear that this region will have a similar structure to WD-40 domains, which consist of several strands of β-sheet rather than α-helix). The PAS domain in turn contains two regions of sequence conservation that have been named the S1 and S2 sequences (Zhulin et al., 1997).

The MEKHLA domain is predicted to adopt a similar structure to PAS domains (Mukherjee and Bürglin, 2006). PAS domains are sensors able to perceive chemical and physical stimuli (Möglich et al., 2009). After sensing a stimulus, a signal originates within the PAS domain and propagates to an effector domain through a short C- or α-helical linker. Similarly, the MEKHLA domain might regulate the activity of HD-ZIP III proteins by responding to a biological stimulus. The PAS region of the MEKHLA domain might detect such a stimulus and transmit a signal to another domain of the HD-ZIP III protein through the M-WD40 subdomain.

The MEKHLA Domain Is Not Required for REV Activity

To determine if the MEKHLA domain is required for REV function, we compared the ability of full-length REV to complement a rev-1 mutant with that of REV lacking the MEKHLA domain (REV-ΔMEKHLA). The most striking morphological phenotype of homozygous rev-1 mutants is their lack of cauline paraclades (branches born in the axils of leaves on the inflorescence stem) and their frequent failure to form pistils (Talbert et al., 1995). REV and REV-ΔMEKHLA proteins, constitutively expressed under the
Figure 1. The REV MEKHLA Domain Belongs to the PAS Domain Superfamily.
 cauliflower mosaic virus 35S promoter (35S), are nearly indistinguishable in their ability to restore cauline branch and pistil production to rev-1 plants (Figure 2A). In addition, the leaves of 35S-REV-DMEKHALA; rev-1 plants are severely curled up (Figure 2C). This curling was not observed in 35S-REV; rev-1 plants (Figure 2B). Upward leaf curling is a hallmark of ectopic HD-ZIP III expression and is commonly seen when HD-ZIP III genes are resistant to regulation by miRNAs (Juarez et al., 2004; Mallory et al., 2004; Ochando et al., 2006, 2008). Thus, REV-DMEKHALA causes upward leaf curling even though this construct is under normal miRNA control.

To further test the ability of REV-DMEKHALA to function, we tested whether deleting parts of the MEKHALA domain would affect REV ability to act as a transcriptional activator. We did this by overexpressing full-length REV, REV-DMEKHALA, or REV-DAPAS in the presence of a construct carrying the ZPR3 gene promoter (pZPR3) fused to the reporter gene uidA (uidA encodes the enzyme β-glucuronidase) (Wenkel et al., 2007). REV binds to an intron within pZPR3 and transcriptionally activates ZPR3 (M.K. Barton and S. Wenkel, unpublished data). We performed our analyses in tobacco (Nicotiana tabacum) leaf abaxial (lower) epidermal cells, where neither REV nor ZPR3 is expected to be expressed (McHale and Koning, 2004; Wenkel et al., 2007). In this experiment, REV mRNAs were made miRNA resistant through a silent mutation in the miRNA binding site to bypass the miRNA machinery active in this cell type (constructs carrying mutations that render HD-ZIP III genes miRNA resistant are designated HD-ZIP III*). All three REV* protein forms activated uidA (Figure 2D), demonstrating that the MEKHALA domain is not necessary for REV to act as a transcriptional activator. Overall, these data suggest that the REV MEKHALA domain is not required for REV activity per se but instead plays a regulatory role.

The MEKHALA Domain Inhibits REV Dimerization in Yeast

It has been speculated that a general role for PAS domains is to modulate protein hetero- or homodimerization (Möglich et al., 2009). To determine if the MEKHALA domain might play a role in regulating dimerization, we performed yeast two-hybrid (Y2H) analyses on full-length REV and REV lacking the MEKHALA domain (REV-ΔMEKHALA). Interestingly, full-length REV did not homodimerize in Y2H assays. By contrast, REV-ΔMEKHALA strongly homodimerized in Y2H experiments (Figure 3A). Thus, the MEKHALA domain prevents REV homodimerization in yeast.

Homodimerization of HD-ZIP III transcription factors is necessary for DNA binding in vitro and therefore presumably required in vivo for REV function (Sessa et al., 1997, 1998). It is generally assumed that homodimerization requires the leucine zipper domain. To test this assumption in vivo, we assayed REV proteins in which the leucine zipper had been removed for their ability to dimerize. Consistent with a role for the leucine zipper in dimerization, we found that REV proteins lacking the leucine zipper (REV-ΔLZ and REV-ΔLZ-ΔMEKHALA) are unable to homodimerize in yeast (see Supplemental Figure 2A online). Furthermore, the overexpression of REV*ΔLZ in Arabidopsis failed to generate any of the phenotypes seen in lines overexpressing full-length REV* (data not shown) and failed to activate pZPR3-reporter expression in a tobacco transient expression assay (see Supplemental Figure 2B online). REV*ΔLZ protein stability was tested by transiently expressing the protein fused to yellow fluorescent protein (YFP) in tobacco (see Supplemental Figure 2C online).

To determine which region of the MEKHALA domain inhibits REV homodimerization in yeast, we performed Y2H analyses with REV truncated in various positions. The entire MEKHALA domain was not required to prevent dimerization; the M-WD40 plus S1 subdomains were sufficient (Figure 3A). We were not able to test whether the M-WD40 region alone is sufficient to prevent dimerization because REV proteins in which both S1 and S2 subdomains are removed showed substantial autoactivation when used as bait (i.e., fused to the GAL4 DNA binding domain) (Figure 3A). Thus, in yeast, the M-WD40 domain, in the absence of S1 and S2, confers transcriptional activation capability to the REV protein and to the GAL4 DNA binding domain alone. Addition of the S1 domain is sufficient to inhibit this activation capability (Figure 3A).

The presence of the MEKHALA domain on only one member of a pair of REV proteins is sufficient to inhibit dimerization (Figure 3B), albeit less strongly than when the MEKHALA domain is present on both members of a pair. Because we could use a REV-ΔMEKHALA protein as bait in this experiment, we were able...
Figure 2. The REV MEKHLA Domain Is Not Necessary for REV Activity in Vivo.

(A) Arabidopsis rev-1 plants were transformed with 35S-REV or 35S-REV-ΔMEKHLA constructs. Three independent T2 lines (1 to 3) for each genotype were examined for the number of branches in the first three cauline leaves and for the number of pistils in the first 10 flowers. rev-1 and wild-type plants (No-0) were also analyzed as controls. Standard deviations (error bars) were calculated from more than 30 individuals. Asterisks indicate transgenic lines that are statistically different from rev-1 (Student’s t test, P < 0.05). A combined analysis of the lines 1 to 3 of 35S-REV; rev-1 and 35S-REV-ΔMEKHLA; rev-1 plants shows no statistically significant difference (Student’s t test, P > 0.05) in the number of pistils and a mild difference (Student’s t test, P = 0.026) in the number of branches. Lines 2 and 3 have no statistically significant difference (Student’s t test, P > 0.05) in the number of branches.

(B) A representative individual (6 weeks old) of 35S-REV; rev-1 transgenic plants. The white arrow points to a normally flattened leaf. Bar = 100 mm.

(C) A representative individual (6 weeks old) of 35S-REV-ΔMEKHLA; rev-1 transgenic plants. The white arrow points to an abnormally curled up leaf. Bar = 100 mm.

(D) β-Glucuronidase expression in leaf abaxial (lower) epidermal cells of tobacco transiently transformed with pZPR3-uidA and 35S (empty vector), pZPR3-uidA, and 35S-REV*, pZPR3-uidA, and 35S-REV*-ΔPAS, or pZPR3-uidA and 35S-REV*-ΔMEKHLA constructs. miRNA-resistant REV is designated REV*. REV homodimerization through the leucine zipper domain is schematically represented by two strings of Leu residues (L) touching each other.
Figure 3. The REV MEKHLA Domain Sterically Inhibits REV Homodimerization in Yeast.
to determine that the M-WD40 subdomain is not sufficient to prevent REV dimerization, at least when present on one member of a dimer (Figure 3B).

To better understand how the MEKHLA domain inhibits REV homodimerization, we conducted a reverse Y2H screening using REV-ΔMEKHLA prey against REV bait randomly mutagenized in the MEKHLA domain (REV-mMEKHLA). We then screened for mutations that would allow an interaction of REV-ΔMEKHLA and REV-mMEKHLA comparable to REV-ΔMEKHLA homodimerization. We obtained 28 positive colonies: 26 carried premature stop codons and two carried deletions promptly followed by a stop codon (Figure 1A). All mutations are located in the first 56 amino acids of the REV MEKHLA domain that include the M-WD40 subdomain (Figure 1A) and end right before the S1 region. Our screen retrieved 16 out of 22 possible premature stop codons obtainable by point mutation in the first 56 amino acids of the domain (Figure 1A). By contrast, we did not detect any of the 32 possible stop codons scattered throughout the PAS region (Figure 1A). These results confirm that part of or all the S1 region of the MEKHLA domain is necessary to inhibit REV homodimerization in yeast.

In summary, the Y2H data demonstrate that the M-WD40 plus S1 region is sufficient to prevent REV homodimerization. A simple way that this could occur would be for the MEKHLA domain to interact intramolecularly with the leucine zipper domain, thereby making it inaccessible to a partner molecule. To determine if the MEKHLA domain has the capability to engage in any intramolecular interaction, we tested the MEKHLA, PAS (S1+S2), and M-WD40 regions against several truncations and independent domains of REV by Y2H. We did not detect any interaction (Figure 3C). Furthermore, the MEKHLA domain and PAS subdomain did not inhibit REV-ΔMEKHLA homodimerization when expressed in trans in a yeast three-hybrid experiment (data not shown). Therefore, our results suggest that the MEKHLA domain does not act by binding intramolecularly to other REV domains.

An alternative possibility is that the MEKHLA domain interacts with an as yet unknown protein, thus sequestering the REV protein and inhibiting dimerization. This mechanism would predict the requirement of a specific amino acid sequence within the MEKHLA domain for such an interaction to occur. However, we find that when we replace the REV MEKHLA domain with a random peptide of similar length (REV-ΔMEKHLA-YFPa, where YFPa is the reverse complement of the YFP sequence), the random protein sequences prevent dimerization (Figure 3A). Similarly, if we replaced the S1+S2 region with a random 16-mer peptide (REV-ΔS1S2-16aa), this sequence was also able to prevent dimerization (Figure 3A). The simplest explanation for this result is that REV adopts a conformation such that the MEKHLA domain occupies space that prevents REV monomers from coming in contact with one another.

The MEKHLA Domain Modulates REV Dimerization in the Plant

To determine the role of the MEKHLA domain in regulating dimerization in the plant, we performed transient bimolecular fluorescence complementation (BiFC) assays in tobacco using the same REV truncation and fusion proteins described above. In BiFC experiments, the N-terminal portion of YFP is fused to one of the putative interaction partners and the C-terminal portion of YFP is fused to the other partner. Interaction between the two partners brings the two proteins close enough together that the YFP protein is reconstituted and can fluoresce (Hu et al., 2002). In this experiment, the mRNAs were made miRNA resistant through a silent mutation in the miRNA binding site (constructs carrying mutations that render REV miRNA resistant are designated \( \text{REV}^\ast \)). In addition, we fused all REV\(^\ast\) protein forms to YFP as a control for protein stability. In all cases, the fusion proteins were stable (Figure 4).

Similar to the Y2H data, the presence of the M-WD40 and the S1 subdomains together (REV\(^\ast\)-ΔS2) prevented dimerization in vivo (Figure 4). Also, similar to the situation in yeast, replacing the PAS domain with a random 16-amino acid sequence prevented dimerization in the BiFC assay.

In contrast with the results in the Y2H experiment, full-length REV\(^\ast\) and REV\(^\ast\)-ΔMEKHLA dimerized equally well in BiFC experiments (Figure 4). To explain this apparent contradiction, we hypothesize that the S2 domain, in the context of a cellular signal, is capable of relieving inhibition by the M-WD40-S1 region and that the cellular context within which the transgenes are expressed (abaxial tobacco leaf epidermal cells) is one in which this signal is active.

When overexpressed in plants, the REV\(^\ast\) constructs truncated for different portions of the MEKHLA domain showed various behaviors. T1 transgenic plants were assayed for the shape of their cotyledons (ectopic REV activity causes the production of inwardly curved, adaxialized cotyledons) and for the presence of leaf flaps on the abaxial (lower) leaf surfaces. Leaf flaps occur when small regions of adaxial cell fate, caused by ectopic HD-ZIP III activity, are present on the bottom of the leaf.

Compared with a full-length REV\(^\ast\) construct, which causes production of leaf flaps on leaves beginning with the third rosette leaf, REV\(^\ast\) lacking a MEKHLA domain causes the production of

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**Figure 3.** (continued).

Strong, weak, and no interactions are indicated by three plus signs, one plus sign, and a minus sign, respectively. Interaction strengths were determined by comparison to a set of standards (see Supplemental Figure 5 online). Reciprocal bait/prey couples are indicated by a number in parenthesis. B-a, bait autoactivation.

(A) Homodimerization Y2H assays. The same proteins were used as bait (fused to GAL4 DNA binding domain) and prey (fused to GAL4 activation domain).

(B) Interaction of REV-ΔMEKHLA with REV truncated for subsections of the MEKHLA domain.

(C) Interaction of isolated MEKHLA variants with REV protein domains.

[See online article for color version of this figure.]
such flaps earlier in development, beginning with the first two leaves, and on a higher proportion of leaves (Figures 4 and 5B). REV* lacking a MEKHLA domain also increases the fraction of cotyledons that are curled inward (Figure 5A). Effects on the morphologies of floral organs were also observed. REV*-ΔMEKHLA caused the production of shorter carpels with ectopic (i.e., growing out from the abaxial [outer] rather than adaxial [inner] carpel domain) ovules forming along the length of the carpel. This is in contrast with full-length REV*, which causes production of ectopic (abaxial) ovules only at the base of the carpel (Figure 5C). Sepals of plants overexpressing REV*-ΔMEKHLA were observed to be trumpet shaped, while this was not observed in plants overexpressing full-length REV* (Figure 5C). Thus, REV* lacking a MEKHLA domain acts earlier and more strongly in generating ectopic adaxial fates than a full-length REV* protein. This is consistent with a role for MEKHLA as a negative regulator.

Overexpression of a class IV HD-ZIP proteins do not carry a MEKHLA domain at their C termini. (They do, however, include the HD-ZIP, START, and HD-SAD domains.) To determine if the MEKHLA domain could prevent dimerization of the class IV HD-ZIP protein GLABRA2 (GL2), we fused the REV MEKHLA domain onto the GL2 protein (GL2-REVMEKHLA). Similar to what we observed with REV, the MEKHLA domain inhibited GL2 homodimerization in Y2H experiments but not in BiFC assays in planta (Figure 6). This inhibition was not sequence specific as, similar to the case for REV, replacing the MEKHLA domain with other sequences could prevent dimerization both in Y2H and BiFC assays. Thus, the class IV HD-ZIP proteins are similar to the MEKHLA-less HD-ZIPIII proteins in that their dimerization is sensitive to the presence of an extension at the C terminus and in that this sensitivity is not sequence specific. This is consistent with the observation that the class III and class IV HD-ZIP proteins have similar domain structures.

We also investigated whether the C. reinhardtii MEKHLA domain could function to replace the REV MEKHLA domain
and whether it could inhibit dimerization when fused to the class IV HD-ZIP protein GL2. Replacement of the REV MEKHLA domain with the \textit{C. reinhardtii} MEKHLA domain resulted in inhibition of homodimerization in yeast (Figure 6) and also failure to form homodimers in BiFC assays. Similarly, the \textit{C. reinhardtii} MEKHLA domain inhibited dimerization in both yeast and plant cells when fused to GL2. We hypothesize that the \textit{C. reinhardtii} MEKHLA domain is not able to respond to cellular signals present in \textit{Arabidopsis} to lift inhibition by the M-WD40+S1 region. We note that both the S1 and S2 domains are poorly conserved at the amino acid sequence level between the \textit{C. reinhardtii} MEKHLA domain and the REV MEKHLA domain.

\textbf{Figure 5.} Loss of the REV MEKHLA Domain Results in More Severe Overexpression Phenotypes.  
(A) Phenotypic analysis of stably transformed \textit{Arabidopsis} plants. T1 seedlings were scored for curled up cotyledons caused by ectopic REV activity. miRNA-resistant REV is designated REV*. Asterisks indicate transgenic lines that are statistically different from 35S-REV* plants (Z test, confidence > 95%).  
(B) Phenotypic analysis of stably transformed \textit{Arabidopsis} plants. First to fifth rosette leaves of T1 seedlings were scored for abaxial (lower) leaf flaps. miRNA-resistant REV is designated REV*. Asterisks indicate transgenic lines that are statistically different from 35S-REV* plants (Z test, confidence > 95%).  
(C) Row 1: Schematic representation of the REV protein forms overexpressed in the tissue samples below. Row 2: Abaxial side of the first rosette leaf of representative transgenic \textit{Arabidopsis} plants. The arrows point to leaf flaps. Bar = 2 mm. Row 3: Sepals of representative transgenic \textit{Arabidopsis} plants. Bar = 2 mm. Row 4: Pistils of representative transgenic \textit{Arabidopsis} plants. The arrows point to abaxial ovules. Bar = 2 mm.
Thus, these sequences may not be able to sense signals present in Arabidopsis that are responsible for lifting inhibition of dimerization by the MEKHLA domain.

The MEKHLA Domain of Other HD-ZIP III Proteins Behave Differently

The MEKHLA domain is conserved among all five Arabidopsis HD-ZIP III proteins (Figure 1). We performed Y2H dimerization assays with PHB and ATHB8, members of the other two HD-ZIP III phylogenetic clades (Figure 1). Both proteins behaved differently from REV as they strongly activated transcription as bait even in the absence of the MEKHLA domain (PHB-DMEKHLA and ATHB8-DMEKHLA) (Figure 7A). To avoid bait autoactivation, we tested PHB, PHB-DMEKHLA, ATHB8, and ATHB8-DMEKHLA as prey against REV and REV-DMEKHLA bait. PHB and ATHB8 interacted with REV-DMEKHLA more tightly than PHB-DMEKHLA and ATHB8-DMEKHLA did with REV (Figure 7A), suggesting that the REV MEKHLA domain has a stronger inhibitory effect than the MEKHLA domains of ATHB8 or PHB.

In addition to forming homodimers, HD-ZIP III proteins form dimers with the small ZPR proteins (Wenkel et al., 2007; Kim et al., 2008). ZPR proteins consist almost entirely of a stretch of leucine zipper that is similar to the leucine zipper found in the class III HD-ZIP proteins. We assayed HD-ZIP III prey against ZPR3 bait. PHB and ATHB8, but not REV, strongly interacted with ZPR3 (Figure 7A). By contrast, PHB-DMEKHLA, ATHB8-DMEKHLA, and REV-DMEKHLA interacted equally well with ZPRIII (Figure 7A). These results show that REV and the other HD-ZIP III proteins have diverged from each other with REV possessing a unique way to modulate homo- and heterodimerization through the MEKHLA domain.

The REV MEKHLA domain is characterized by a stretch of four amino acids (AASE) that is not present in any other Arabidopsis HD-ZIP III protein (Figure 1A). These residues are not responsible for the unique activity of the REV MEKHLA domain observed in yeast as REV deleted for this peptide (REV-ΔAASE) did not homodimerize in Y2H assays (Figure 7B). Furthermore, we replaced the REV MEKHLA domain with the PHB MEKHLA domain to create REV-PHBMEKHLA. REV-PHBMEKHLA showed mild autoactivation as bait; no additional activity was observed when tested for homodimerization, indicating that REV-PHBMEKHLA does not homodimerize in yeast. This result suggests that the difference between REV and PHB homodimerization resides outside the MEKHLA domain (Figure 7B).

To test if the REV and PHB MEKHLA domains behave differently also in planta, we overexpressed a miRNA-resistant version of PHB and PHB-DMEKHLA (35S-PHB* and 35S-PHB*-DMEKHLA, respectively) in Arabidopsis. 35S-PHB* and 35S-PHB*-DMEKHLA lines showed indistinguishable overexpression phenotypes (Figures 7C and 7D), indicating that the PHB MEKHLA domain does not exert the same inhibitory effects observed with REV.

Figure 6. The REV MEKHLA Domain Inhibits Homodimerization of the GL2 Protein.

Row 1: Schematic representation of the GL2 protein forms used in the experiments below. Row 2: Results of Y2H experiments. Strong and no interactions are indicated by three plus signs and a minus sign, respectively. Interaction strengths were determined by comparison to a set of standards (see Supplemental Figure 5 online). Row 3: BiFC assays in tobacco leaf abaxial (lower) epidermal cells transiently transformed. Nuclear expression is indicated by a white arrow. Row 4: YFP fusion assays in tobacco leaf abaxial epidermal cells transiently transformed. Bar = 100 μm.
[See online article for color version of this figure.]
One hypothesis is that REV plays unique roles in plant development that require dimerization regulation through the MEKHLA domain. In line with this model, REV is the only HD-ZIP III gene to show a loss-of-function phenotype. PHB and ATHB8 MEKHLA domains might carry out other functions. Alternatively, our experimental conditions did not allow us to detect the effect of the MEKHLA domain on PHB and ATHB8 dimerization.

REV Is the Latest Type of HD-ZIP III Protein to Have Evolved in Land Plants

To study the evolution of the MEKHLA domain we built a neighbor-joining tree based on the multiple sequence alignment of MEKHLA domains from key species in the evolution of land plants (Figure 1C; see Supplemental Figure 3 and Supplemental Data Set 1 online). To make the analysis more robust, we generated a neighbor-joining and a maximum parsimony tree with HD-ZIP III full-length sequences when available (Figure 1C; see Supplemental Figure 4 and Supplemental Data Set 2 online). The trees identify two major clades: one containing a more ancestral MEKHLA domain present in green algae, mosses, and ferns and the other containing a more recent MEKHLA domain present in angiosperms. Interestingly, gymnosperms possess both forms of MEKHLA domains. The trees could not fully resolve the evolution of angiosperm and more recent gymnosperm HD-ZIP III proteins. Nevertheless, the trees based on full-length sequences divided angiosperm HD-ZIP III proteins into two major phylogenetic clades: REV-PHB-PHV-like and ICU4/CNA-ATHB8. This phylogenetic analysis together with our functional data indicate that REV diverged considerably from the PHV/PHB and ICU4/CNA-ATHB8 clades and thus likely also from the more ancestral HD-ZIP III proteins. Therefore, we suggest that REV is the latest type of HD-ZIP III protein to have evolved in land plants.

DISCUSSION

Class III HD-ZIP proteins are master regulators of polar development in plants. They play important, conserved roles in the development of all parts of the plant: embryo, meristem, stem, leaf vasculature, root, and flower. The predicted structure of these transcription factors (the presence of a ligand binding domain and a PAS-like MEKHLA domain) indicate that there is substantial posttranslational regulation of HD-ZIP III function. In this article, we explored the function of the MEKHLA domain of HD-ZIP III REV. Our results indicate that this domain plays a negative regulatory role and support a model in which steric...
inhibition by the MEKHLA domain prevents dimerization of REV monomers. We speculate that a cellular signal recognizes the MEKHLA domain and changes its conformation to allow dimerization.

**MEKHLA as a Negative Regulatory Domain That Prevents Dimerization**

We assayed the ability of the REV protein to function with and without the MEKHLA domain in several ways; in each test, the MEKHLA-less protein was able to supply REV function. In some cases, the MEKHLA-less protein caused a more severe gain-of-function phenotype than the full-length protein. These observations all indicate that the MEKHLA domain plays a negative regulatory role. An important corollary of this is that MEKHLA negative regulation itself must be modulated to allow REV to be active at the correct time and location.

Only one point mutation has been described in the MEKHLA domain to date. This is a mutation in ICU4/CNA (Duclercq et al., 2010). A mutation at position 16 of the MEKHLA domain (this is in the beginning of the M-WD40 portion of the domain) changes a conserved Ser into a Cys in the hoc mutant. The hoc mutants were isolated in a screen for mutants that could regenerate shoots from explants in the absence of exogenous hormones (Catterou et al., 2002). The hoc mutation appears to be recessive. However, the mutation does not appear to cause a loss of protein function since mutations that disrupt the gene more severely fail to generate shoots on this medium. One interpretation of these observations is that repression by the MEKHLA domain is lifted by exogenous hormones in culture. In the hoc mutant, MEKHLA repression would be compromised and there would be no need for exogenous hormones.

Our experiments in yeast show that the presence of a MEKHLA domain on a REV protein prevents dimerization: when removed, REV dimerizes readily in yeast. We also find that the MEKHLA domain, when added to the HD-ZIP IV protein GL2, can inhibit its dimerization. The structure of class IV proteins is similar to that of class III proteins with the exception of the presence of the MEKHLA domain. Only a portion of the MEKHLA domain was needed; the M-WD40+S1 region is sufficient to carry out the inhibition. The S1 domain is critical, and the M-WD40 domain alone could not inhibit dimerization.

Interestingly, this effect was not sequence specific. The S1 domain could be replaced with random sequences, and this could inhibit REV homodimerization. In fact, the entire MEKHLA domain could be replaced with random sequences, and this prevented homodimerization in yeast. This finding would appear to rule out two possible mechanisms of MEKHLA action. In the first, the MEKHLA domain might interact intramolecularly with other regions of REV, for instance, the leucine zipper domain, and in so doing could prevent it from interacting with other proteins. This would require sequence specificity. In addition to

![Figure 8. Model for Regulation of REV Dimerization by the MEKHLA Domain.](image)
the above results, we did not find any evidence that MEKHLA interacts with other REV domains despite extensive testing.

In a second possible mechanism, the MEKHLA domain could interact with an as yet unknown partner that acts to sequester the REV protein, thus preventing it from dimerizing. Again, this would require sequence specificity, and this does not appear to be the case. Also, yeast two-hybrid screens done in our laboratory for MEKHLA-dependent interactors failed to turn up any of these (our unpublished data). HD-ZIP III proteins have been reported to interact with the APETALA2-like transcription factors DORNROSCHEN and DORNROSCHEN-like (DRNL) through the MEKHLA domain (Chandler et al., 2007). This would be evidence for a sequence-specific interaction. However, in apparent contradiction to these results, we did not detect any interaction of DRNL with full-length REV or with the REV MEKHLA domain by Y2H. We do not know why our results differ from previous results. Furthermore, DRNL did not allow REV homodimerization when coexpressed in yeast with REV prey and REV bait. Therefore, we believe DRNL is likely not responsible for lifting the MEKHLA domain inhibition and might play a role in the function of other HD-ZIP III proteins.

We are left then with a third mechanism in which the MEKHLA domain sterically inhibits dimerization, perhaps by blocking access to the leucine zipper domain. The leucine zipper domain is believed to be the site of dimerization, and our in vivo studies are consistent with this. Such a steric masking mechanism has been reported to regulate endoplasmic reticulum retention of certain plasma membrane–bound receptors (Letourneur et al., 1995).

Our results from dimerization assays in plant cells (BIFC assays) differ from those obtained in yeast cells. Similar to the yeast assays, we find that the substitution of random sequences in place of the MEKHLA domain can inhibit dimerization of REV as well as GL2. We also find that the M-WD40 plus S1 subdomains can inhibit dimerization as they do in yeast. However, full-length MEKHLA domains allow dimerization. We hypothesize that this is because the full-length MEKHLA domain is able to receive a cellular signal that alters its conformation, moving it out of the way and allowing dimerization. This would not be possible for the random sequences. The S2 subdomain appears to be required for lifting this repression by the MEKHLA domain since the REV protein carrying only the M-WD40+S1 region, without the S2 subdomain, is not constitutively active and does not cause gain-of-function phenotypes. This is consistent with their ability to prevent dimerization. Interestingly, the C. reinhardtii MEKHLA domain prevents homodimerization both in yeast and in the BIFC assay. We propose that this distantly related protein is not able to respond to a cellular signal present in tobacco cells. We note that the S1 and S2 subdomains of the C. reinhardtii MEKHLA domain are quite diverged from that of the class III HD-ZIP proteins. This may indicate that the C. reinhardtii MEKHLA domains respond to other stimuli.

Different parts of the Arabidopsis plant responded differently to the overexpression of full-length or MEKHLA-less REV. Certain cell types, such as the abaxial (lower) cells of the first two rosette leaves and the upper part of the carpel, were not responsive to REV carrying a MEKHLA domain. We speculate that the putative signal responsible for relieving the MEKHLA inhibition is absent in such cell types. Carrying this logic further, cells that show a gain-of-function phenotype in response to full-length REV would be predicted to have high levels of such signals.

Much is known about the structure of PAS domains (Möglich et al., 2009). Most if not all of this work has been done on the PAS domain alone. PAS domains have been shown to move relative to one another in response to a stimulus (Möglich et al., 2009). Less is known about how the PAS domain functions when it is part of a larger protein with other domains. It is possible that a stimulus could cause movement of the PAS domain in HD-ZIP III proteins relative to other portions of the protein. Such a mechanism could work to relieve the steric masking we propose.

**Evolution of the REV MEKHLA Domain**

Algae and bacteria have single-domain MEKHLA proteins but not HD-ZIP proteins. By contrast, HD-ZIP III proteins are the only ones to carry a MEKHLA domain in land plants. A likely scenario is that HD-ZIP III proteins acquired a MEKHLA domain from an ancestral MEKHLA domain protein similar to those present in algae. Nevertheless, the C. reinhardtii MEKHLA domain could not effectively substitute for the REV MEKHLA domain, suggesting functional divergence of the two MEKHLA domains. The C. reinhardtii MEKHLA domain inhibited REV homodimerization both in yeast and in planta, indicating that it is not recognized by the putative cellular signal responsible for inhibition relief in planta. The REV and C. reinhardtii MEKHLA domains are highly divergent in sequence and might have evolved to recognize different signals. In line with this model, our phylogenetic tree separates the MEKHLA domains of angiosperms from those of more basal plants.

The REV MEKHLA domain maintained its function when fused to GL2, a class IV HD-ZIP protein that naturally does not carry a MEKHLA domain. This result suggests that HD-ZIP proteins that do not carry a MEKHLA domain are perfectly suited to accept one, in line with the domain acquisition model. Therefore, we speculate that class III HD-ZIP proteins evolved from class IV-like HD-ZIP proteins by acquiring a MEKHLA domain.

The MEKHLA domain is conserved among all HD-ZIP III proteins, with striking sequence similarity. Nevertheless, the MEKHLA domain of REV but not that of PHB or ATHB8 inhibited heterodimerization among HD-ZIP III proteins and inhibited interaction with ZPR3 proteins. Such a difference might lie not only in the MEKHLA domain but also in the rest of the protein, as a chimeric REV protein carrying the PHB MEKHLA domain did not homodimerize in yeast. Furthermore, the overexpression of full-length PHB or PHB deleted in the MEKHLA domain resulted in indistinguishable phenotypes, in contrast with what was observed with REV. These data indicate that the function and/or regulation of the REV MEKHLA domain are substantially different from that of other HD-ZIP III proteins. In line with this model, REV is the only HD-ZIP III to show a loss-of-function phenotype. PHB and ATHB8 MEKHLA domains might carry out completely different functions or might simply recognize a more ubiquitous signal that did not allow us to highlight functional differences in the condition tested. REV is expressed in a broader domain in the developing leaf than PHB or ATHB8 are (Prigge et al., 2005). This could be the basis of a requirement for a different type of negative regulation.
Our phylogenetic trees, based on the alignment of full-length HD-ZIP III proteins, group PHB, PHV, and REV in a separate phylogenetic clade from ICU4/CNA and ATHB8. Nevertheless, our functional assays showed that PHB behaves more similarly to ATHB8 than to REV. Together, these results suggest that REV diverged from the other HD-ZIP III proteins. Note that in some cases, PHB behaves genetically more similarly to ICU4/CNA than it does to REV (Pingre et al., 2005). It is possible that functional differences in the MEKHLA domain are responsible for the observed genetic and phenotypic differences seen in combinations of loss-of-function mutants.

**METHODS**

**Bioinformatics Techniques**

Multiple sequence alignments were constructed using Muscle 3.2 (Edgar, 2004) and displayed with Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) using the BLOSSUM62 matrix and a 50% threshold for shading. Protein secondary structure was predicted using the JPRED3 (Cole et al., 2008). Phylogenetic trees were constructed using the neighbor-joining or maximum parsimony algorithm implemented in the MEGA software suite (Tamura et al., 2007). We used mean character difference, among-site rate variation, and random seed initiation; 10,000 bootstrap replicates were performed followed by identification of the consensus tree.

**Molecular Biology**

The expression level of transgenes was checked by RT-PCR analyses as described by Sambrook and Russell (2001). PCR analyses were conducted using the REV-F and REV-R4 primers (REV-F, 5'-ATGGAGATGCCTGCGTGCT-3'; REV-R4, 5'-CCTCGACGCTTCTTTCTCTGC-3'), and the amplification products were visualized with ethidium bromide. β-Glucuronidase activity was tested as described by Jefferson (1989).

**Cloning**

Coding sequences (see Supplemental Table 1 online) were PCR amplified (with a start codon and a stop codon when missing) and cloned into pENTR/D-TOPO (Invitrogen) according to the manufacturer’s instructions. PCR primers were all designed as 25-bp oligomers starting and ending at the locations indicated in Supplemental Table 1 online. The mriRNA-resistant rev and phb mutations were previously described (Mallory et al., 2004; Wenkel et al., 2007).

The REV-JMEKHLA and REV-JS1S2 coding sequences were PCR amplified with a BamHI restriction site at the 5' end and cloned into pENTR/D-TOPO (Invitrogen) according to the manufacturer’s instructions to create REV-JMEKHLA-BamHI and REV-JS1S2-16aa, respectively. The sequence coding for the Chlamydomonas reinhardtii MEKHLA domain and the YFP reverse-complement sequence were PCR amplified with a start codon and a stop codon flanked by BglII restriction sites and cloned into the BamHI restriction site of REV-JMEKHLA-BamHI to create REV-ChlamyMEKHLA and REV-YFPa, respectively. REV-ΔAASE and REV-PHBMEKHLA were obtained by overlap extension PCR. The GL2 coding sequence was PCR amplified with a BamHI restriction site at the 5' end and cloned into pENTR/D-TOPO (Invitrogen) according to the manufacturer’s instructions to create GL2-BamHI. The sequence coding for the C. reinhardtii MEKHLA domain, the REV-MEKHLA sequence, and the YFP reverse-complement sequence were PCR amplified with a start codon and a stop codon flanked by BglII restriction sites and cloned into the BamHI restriction site of GL2-BamHI to create GL2-ChlamyMEKHLA, GL2-REV-MEKHLA, and REV-YFPa, respectively.

For Y2H assays, sequences were mobilized from pENTR/D-TOPO into pDEST22 (Invitrogen) and pDEST32 (Invitrogen) according to the manufacturer’s instructions.

For overexpression assays, sequences were mobilized from pENTR/D-TOPO into pMDC32 (Curts and Grossniklaus, 2003). For BIFC assays, the sequences coding for the N terminus and C terminus of YFP were PCR amplified from pSPYNE-3SS and pSPYCE-3SS (Walter et al., 2004) using primers carrying KpnI and Ascl restriction sites and cloned into the KpnI and Ascl restriction sites of pMDC32 to create pMDC32-SPYNE and pMDC32-SPYCE, respectively. Sequences were mobilized from pENTR/D-TOPO into pMDC32-SPYNE and pMDC32-SPYCE according to the manufacturer’s instructions.

For YFP fusion analyses, sequences were mobilized from pENTR/D-TOPO into pEarleyGate-104 (Earley et al., 2008) according to the manufacturer’s instructions.

**Y2H Assays**

The Invitrogen ProQuest two-hybrid system with Gateway Technology was used in Y2H assays according to the manufacturer’s instructions. Positive interactions were detected by assaying for β-galactosidase activity at 8 and 24 h. More than six independent colonies per couple of constructs were tested. To assess the interaction strength of our protein couples, we used a collection of control strains, provided with the Invitrogen system, that contain plasmid pairs expressing fusion proteins with a spectrum of interaction strengths. †++, ++ and + correspond to interactions similar in strength to those observed with pPC97-Fos/ pPC86-Jun, pPC97-Luc/pcDNA3 and pPC97-RB/pPC86-E2F1, respectively (see Supplemental Figure 5 online).

For the reverse Y2H screening, the sequence encoding the REV MEKHLA domain was mutagenized by PCR with Mutazyme (Stratagene). The PCR products were then cotransformed with pDEST32-REV, linearized in the XbaI restriction site, into yeast cells carrying pDEST22-REV-JMEKHLA.

**Plant and Genetic Materials**

Seeds of Arabidopsis thaliana, ecotype Columbia-0 or Nossen-0, were used for all experiments. The rev-1 mutant was previously described (Talbert et al., 1995; Ratcliffe et al., 2000). Plants were grown in the greenhouse under long-day conditions or in vitro under constant fluorescent illumination.

**Transgenic Plants**

For stable transformation, Agrobacterium tumefaciens strain GV3101 was used to transform Arabidopsis plants by the floral dip method.
(Clough and Bent, 1998). Expression levels were analyzed by RT-PCR. For transient transformation, Nicotiana benthamiana abaxial epidermal cells were infiltrated with Agrobacterium strain GV3101 as previously described (Neuhaus and Boevink, 2001).

Microscopy

BiFC and YFP fusion experiments were conducted by infiltrating tobacco leaves with Agrobacterium strains carrying the appropriate fusion vectors. For each experiment, four different construct combinations were infiltrated into the same leaf. Each experiment was repeated three times. The REV constructs (either pSPYNE-3SS-REV/* or pEarleyGate-104-REV*) were infiltrated every time as reference standards for BiFC and YFP fusion experiments, respectively. YFP visualizations in tobacco epidermal cells were performed 40 to 48 h after infiltration using a Leica SP5 confocal laser scanning microscope. For each experiment, the same gain setting was used to visualize the cells infiltrated with the control constructs and the cells infiltrated with the experimental constructs.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: REV (At1g79840), pEarleyGate-104-REV* (At5g60690), PbH (At2g34710), ATHB8 (At4g32880), GL2 (At1g79840), ZPR3 (At3g52770), and C. reinhardtii gene encoding a MEKHLA domain (XM_001691785).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The REVOLUTA M-WD40 Subdomain Shows Similarities to the WD40 Domain.

Supplemental Figure 2. The Leucine Zipper Domain Is Necessary for REVOLUTA Transcriptional Activity in Vivo.

Supplemental Figure 3. Multiple Sequence Alignment of the Arabidopsis, Oryza sativa, Ginkgo biloba, Pinus taeda, Psilota nudum, Physcomitrella patens, and Chlamydomonas reinhardtii MEKHLA Domains Used to Generate the Neighbor-Joining Tree in Figure 1C.

Supplemental Figure 4. Multiple Sequence Alignment of the Arabidopsis, O. sativa, G. biloba, P. taeda, P. nudum, and P. patens HD-ZIP III Proteins Used to Generate the Neighbor-Joining and Maximum-Parsimony Trees Built to Corroborate the Tree in Figure 1C.

Supplemental Figure 5. Yeast Two-Hybrid Assays.

Supplemental Table 1. Sequences Used in This Study.

Supplemental Data Set 1. Amino Acid Sequences Used to Generate the Alignment Presented in Supplemental Figure 3 online.

Supplemental Data Set 2. Amino Acid Sequences Used to Generate the Alignment Presented in Supplemental Figure 4 online.

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