HD-ZIP III Activity Is Modulated by Competitive Inhibitors via a Feedback Loop in Arabidopsis Shoot Apical Meristem Development

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Shoot apical meristem (SAM) development is coordinately regulated by two interdependent signaling events: one maintaining stem cell identity and the other governing the initiation of lateral organs from the flanks of the SAM. The signaling networks involved in this process are interconnected and are regulated by multiple molecular mechanisms. Class III homeodomain-leucine zipper (HD-ZIP III) proteins are the most extensively studied transcription factors involved in this regulation. However, how different signals are integrated to maintain stem cell identity and to pattern lateral organ polarity remains unclear. Here, we demonstrated that a small ZIP protein, ZPR3, and its functionally redundant homolog, ZPR4, negatively regulate the HD-ZIP III activity in SAM development. ZPR3 directly interacts with PHABULOSA (PHB) and other HD-ZIP III proteins via the ZIP motifs and forms nonfunctional heterodimers. Accordingly, a double mutant, zpr3-2 zpr4-2, exhibits an altered SAM activity with abnormal stem cell maintenance. However, the mutant displays normal patterning of leaf polarity. In addition, we show that PHB positively regulates ZPR3 expression. We therefore propose that HD-ZIP III activity in regulating SAM development is modulated by, among other things, a feedback loop involving the competitive inhibitors ZPR3 and ZPR4.

INTRODUCTION

Shoot apical meristem (SAM) development is regulated by coordinate interactions of two major groups of interdependent signaling events, in which one group of signals maintaining stem cell identity is balanced with the other group of signals governing the initiation of lateral organs from the flanks of the SAM (Williams and Fletcher, 2005; Byrne, 2006). Accumulating evidence also supports that signals from the adaxial domains of lateral organs are important for SAM formation (Eshed et al., 2001). Several key regulators, including class III homeodomain-leucine zipper (HD-ZIP III) transcription factors (Emery et al., 2003; Bowman, 2004; Green et al., 2005; Kim et al., 2005; Prigge et al., 2005; Williams et al., 2005), a group of KNOX and KANADI (KAN) proteins, and miR165/166 (Juarez et al., 2004; Vaucheret et al., 2004), mediate the signaling crosstalk (Kerstetter et al., 2001). Recently, a transacting small interfering RNA has been shown to restrict the accumulation domains of miR165/166 and HD-ZIP III mRNAs (Nogueira et al., 2007), providing an additional level of signaling complexity.

The SAM exhibits a distinct cellular morphology, in which a population of slowly dividing stem cells is located in the central zone (CZ). Cells in the CZ are either used to maintain the integrity of the stem cell population or are displaced to the peripheral or rib zones (Carraro et al., 2006; Golz, 2006). A small group of cells in the peripheral zone then are switched developmentally from an indeterminate to determinate fate to initiate lateral organ formation.

A series of surgical experiments has shown that a polarizing signal arising in the meristem promotes the adaxial identity in the cells closer to the meristem and the abaxial identity of those located away from the meristem (Reinhardt et al., 2005; Byrne, 2006). Signals from the adaxial domains of leaf primordia also are essential for stem cell maintenance. Overexpression of an abaxial promoting KAN gene in the adaxial domain of cotyledons results in the loss of embryonic SAM and abaxialization of leaves (Eshed et al., 2001). By contrast, a dominant phabulosa (phb) mutant, in which an adaxial promoting PHB gene is ectopically expressed, has an enlarged SAM (McConnell and Barton, 1998).

One potential candidate for the polarizing signal is miR165/166. It is expressed predominantly in the abaxial domain of lateral organs (Juarez et al., 2004; Kidner and Martienssen, 2004). Notably, maize (Zea mays) miR166 originally is expressed basally in the developing leaf primordium and subsequently accumulates in the abaxial domain of the growing organ, possibly by migrating through the phloem (Juarez et al., 2004). However, the view of miR165/166 being a polarizing signal is not supported by the expression patterns of the HD-ZIP III genes. Each HD-ZIP III gene exhibits a distinct expression domain, and the promoter activity of each matches its mRNA distribution...
By contrast, the mRNA level also is elevated both in the abaxial and adaxial domains of lateral organs in phb-1d (McConnell et al., 2001).

A newly elucidated way of controlling transcription factors is via non-DNA binding proteins (Klemm et al., 1998). For example, the inhibitor of DNA binding protein (ID) in animals has a basic helix-loop-helix (bHLH) motif but lacks the basic DNA binding region (Benezra et al., 1990). It associates with several bHLH transcription factors, such as MyoD, E12, and E47, and represses their activities in activating transcription by forming nonfunctional heterodimers. Recently, the Arabidopsis thaliana KIDARI protein, which has a structural organization similar to ID, has been suggested to exert its role by physically interacting with a bHLH transcription factor, HFR1, involved in photomorphogenesis (Hyun and Lee, 2006).

In this work, we demonstrate that a small ZIP protein, ZPR3, is a competitive inhibitor of the HD-ZIP III transcription factors in SAM development. ZPR3 represses the activities of the HD-ZIP III members in activating transcription by forming nonfunctional heterodimers via the ZIP motifs. An activation-tagged mutant, zpr3-1d, exhibits a disrupted SAM activity and partially abaxialized leaf, as has been observed in the phb phv rev zpr3-2 zpr4-2 knockout mutant has a disorganized SAM activity, but its polar patterning of lateral organs is essentially normal. Interestingly, PHB positively regulates the ZPR3 expression. Our findings demonstrate that the HD-ZIP activities in SAM development are regulated via competitive inhibition.

RESULTS

The zpr3-1d Mutant Lacks a Functional SAM

We isolated a morphogenic mutant with notably reduced growth and narrow leaves (Figure 1A), designated zpr3-1d, from an Arabidopsis activation-tagged mutant pool generated by randomly integrating the cauliflower mosaic virus (CaMV) 3SS enhancer into the genome of ecotype Columbia (Col-0) (Weigel et al., 2000). The zpr3-1d leaves were also severely curled downward (see Supplemental Figure 1A online). Close examination of the zpr3-1d leaf surfaces by scanning electron microscopy revealed that the adaxial surface was irregular like the abaxial surface (Figure 1B). However, the polarity of the leaves was apparently unchanged in the mutant (see Supplemental Figure 1B online), indicating that the zpr3-1d leaves were partially abaxialized. Consistent with this, the expression levels of YAB3, KAN1, and KAN2, which promote the abaxial identity, were elevated by approximately twofold in the mutant (Figure 1C). By contrast, transcript levels of the HD-ZIP III genes sustaining the adaxial identity were not altered to a discernible level, except for ATHB15 (Figure 1D). The ATHB15 transcript level was elevated by ~60%. Although it was not further examined in this work, the vascular structure may be affected in the mutant (Kim et al., 2005).

zpr3-1d produced fewer secondary shoots (Figure 1E; see Supplemental Figure 2 online). Notably, some zpr3-1d lines had trumpet-shaped cotyledons and an absent or severely arrested SAM with a pin-like structure at the apex (see Supplemental Figure 3 online). The cauline leaf axils were mostly bare without lateral meristems (Figure 1E). This phenotype is quite similar to that observed in the revoluta (rev) mutant (Talbert et al., 1995), suggesting a role for ZPR3 in SAM development and lateral organ patterning.

ZPR3 Encodes a Small ZIP Protein

Three-step thermal asymmetric interlaced PCR (Liu et al., 1995) was employed to map the T-DNA insertion site in the zpr3-1d genome. The result showed that a single copy of the 3SS enhancer was inserted into the region between At3g52760 and At3g52770 (Figure 1F). In the mutant, the At3g52770 gene was drastically activated (see Supplemental Figure 4 online), while the At3g52760 gene was not. DNA gel blot hybridization confirmed that zpr3-1d contained a single copy of the T-DNA insertion (data not shown). In addition, all BASTA-resistant progeny of the zpr3-1d plants exhibited the zpr3-1d phenotype, indicating that the zpr3-1d mutation cosegregates with the T-DNA insertion.

The At3g52770 gene encodes a small protein consisting of 67 residues (see Supplemental Figure 5 online). Database searches revealed that the encoded protein is LITTLE ZIPPER3 (ZPR3), a member of a small group of leucine zipper–containing proteins consisting of ZPR1 to ZPR4 (Wenkel et al., 2007). ZPR3 possesses residue identities of 37, 38, and 75% with ZPR1, ZPR2, and ZPR4, respectively (see Supplemental Figure 5 online).

Overexpression of ZPR3 under the control of the CaMV 3SS promoter (35S:ZPR3) recapitulated the zpr3-1d phenotype (Figures 1A and 1G). Most of the transgenic plants obtained (>100 transgenic plants) exhibited reduced growth. Approximately 40% of them had downward leaf curling, and ~30% of them had no or severely arrested meristems (see Supplemental Figure 6 online), demonstrating that ZPR3 activation underlies the zpr3-1d phenotypes. By contrast, the primary root growth of 35S:ZPR3 and zpr3-1d was essentially normal, indicating that ZPR3 regulates specifically SAM development.

To confirm the correlation between ZPR3 and the zpr3-1d phenotype, an estradiol-inducible promoter was used to direct ZPR3 expression (Zuo et al., 2000). When the transgenic plants were treated with estradiol, only the newly emerging leaves were curled downward (Figure 1H), suggesting that ZPR3 functions at the early stage of leaf development.

ZPR3 Interacts with HD-ZIP III Proteins via the ZIP Motifs

The central region of ZPR3 contains a ZIP motif (see Supplemental Figure 5 online) that is most similar to those present in the HD-ZIP III proteins. However, ZPR3 lacks the region of those proteins that mediates DNA binding.

Based on the structural similarity between ZPR3 and HD-ZIP IIIIs, we predicted that they might physically interact with one another. Yeast two-hybrid assays were employed to examine the hypothesis. ATHB2, which is a HD-ZIP II member (Ruberti et al., 1991), was included as a negative control in the assays. ZPR3 indeed interacted with PHB, PHV, ATHB15, and ATHB8 (see Supplemental Figure 7 online). By contrast, it did not interact with
To confirm the results, in vitro pull-down assays were employed. A recombinant glutathione S-transferase (GST)-ZPR3 fusion and the HD-ZIP III proteins synthesized by in vitro translation in the presence of 35S-Met were used. All five HD-ZIP III members interacted with ZPR3 (Figure 2A), whereas ATHB2 did not (see Supplemental Figure 8 online). Notably, REV, which did not show any interactions with ZPR3 in yeast cells, also interacted with ZPR3. Our observations are in agreement with previous work (Wenkel et al., 2007) demonstrating that the ZPR proteins interact with multiple HD-ZIP III members.

We next examined which structural motif in the HD-ZIP III proteins was responsible for the ZPR3–HD-ZIP III interactions. We generated a series of PHB deletions and examined their interactions with ZPR3 both by yeast coexpression and in vitro pull-down assays. All the deletion constructs containing the ZIP motif efficiently interacted with ZPR3, but those without the ZIP motif did not (Figure 2B), indicating that the ZIP motif was necessary and sufficient for the interactions. The ZPR3-PHB interactions were further examined using the Nicotiana benthamiana transient expression system (Llave et al., 2002). The miR166/165
binding sequences of the PHB and REV genes were mutated to optimize protein production and transiently expressed in N. benthamiana cells (Kim et al., 2005). Total cellular extracts were then probed with the GST-ZPR3 fusion. Again, ZPR3 interacted with PHB and REV but not with ATHB2 (Figure 2C).

When a ZPR3-GFP fusion was transiently expressed in onion epidermal cells, the green fluorescent protein (GFP) signal was predominantly detected in the nucleus (Figure 2D), consistent with the ZPR3–HD-ZIP III interactions occurring there.

**ZPR3 Inhibits HD-ZIP III Dimerization**

PHV, and possibly other HD-ZIP III proteins, binds to DNA as a homodimer (Sessa et al., 1998). Our data indicated that ZPR3 binds to the ZIP motif of HD-ZIP III proteins and negatively regulates their activities. It was therefore envisioned that ZPR3 might block the homodimer formation of the HD-ZIP III proteins.

To examine the effects of ZPR3 on PHB dimerization, ZPR3 was expressed under the control of a Met-suppressible promoter, pMET25, in yeast cells (MET25:ZPR3; Figure 3A) (Olesen et al., 2000). The PHB gene was fused in frame to a gene construct encoding the activation domain (AD) of GAL4, and the AD-PHB fusion was coexpressed with the MET25:ZPR3 construct. A truncated PHB gene encoding the N-terminal half of PHB (residues 1 to 174) was fused in frame to the binding domain (BD) coding sequence of GAL4, and the BD-1C construct was cotransformed into the yeast cells.

AD-PHB efficiently interacted with BD-1C in the absence of ZPR3 induction, as examined by yeast coexpression (Figure 3A) and by β-galactosidase activity assays (Figure 3B). By contrast, ZPR3 induction substantially repressed the interaction, demonstrating that ZPR3 blocks the PHB dimerization.

We next examined whether the transcription-activation activity of PHB is influenced by ZPR3. A full-size PHB gene was fused in frame with the BD-coding sequence of GAL4, and the BD-PHB fusion was transformed into the yeast cells containing the MET25:ZPR3 construct. In the absence of ZPR3 induction, the BD-PHB protein was transcriptionally active (Figure 3B). However, ZPR3 induction drastically reduced its activity, strongly supporting that ZPR3 represses the activity of PHB in activating transcription.

Wenkel et al. (2007) have shown that ZPR3 blocks binding of REV to DNA, like the ID proteins in animals (Benezra et al., 1990; Perk et al., 2005). It is therefore likely that ZPR3 represses the activity of PHB by forming nonfunctional heterodimers.

To rule out the possibility that ZPR3 affects the PHB protein stability, we generated transgenic plants coexpressing a MYC-PHB fusion, in which six copies of the MYC epitope tag-coding sequence were fused in frame to the PHB gene, under the CaMV 35S promoter, and ZPR3 under the estradiol-inducible promoter. Protein gel blot analysis using an anti-MYC antibody demonstrated that ZPR3 blocks binding of REV to DNA, like the ID proteins in animals (Benezra et al., 1990; Perk et al., 2005). It is therefore likely that ZPR3 represses the activity of PHB by forming nonfunctional heterodimers.

**ZPR3 Inhibits PHB Activity in Planta**

To explore the effects of ZPR3 on the PHB activity in planta, zpr3-1d plants were crossed with phb-1d plants, and the phenotypes...
were compared with those of the parental mutants. Since PHB activity is inhibited by ZPR3, it was predicted that the phenotypes of phb-1d or zpr3-1d would disappear in the phb-1d zpr3-1d double mutant. For the cross, phb-1d (in Landsberg erecta background) was first backcrossed to the Col-0 ecotype twice to obtain a phb-1d-Col line. The phb-1d-Col phenotype was essentially similar to the phb-1d phenotype (see Supplemental Figure 9 online). It developed lateral organs with radial symmetry and wavy inflorescence stems (Figures 3E and 3F).

The leaves and flowers of the phb-1d-Col zpr3-1d mutant were phenotypically similar to those of wild-type plants (Figure 3G). In addition, the double mutant generated more axillary shoots than zpr3-1d, and the inflorescences and inflorescence stems were essentially normal (Figure 3F). These observations indicate that ZPR3 overexpression suppresses at least in part the phb-1d-Col phenotype and that ZPR3 negatively regulates PHB in planta.

In addition, when zpr3-1d was crossed with transgenic plants overexpressing REV (35S:REV) (see Supplemental Figure 10 online), the downward leaf curling of zpr3-1d and the upward leaf curling of the 35S:REV plants were rescued (Figure 3H; see Supplemental Figure 10 online), suggesting that ZPR3 might target multiple HD-ZIP III proteins.

**ZPR3 Is Essential for SAM Function**

To obtain more insights into the molecular mechanism of ZPR3 action, we isolated a ZPR3 mutant (zpr3-2), in which a single copy of T-DNA was inserted into the 5′ untranslated region (Figure 4A).

The zpr3-2 mutant was phenotypically indistinguishable from control plants during the vegetative growth. However, visible phenotypic changes were apparent during reproductive growth. Although its height was similar to that of control plants (Figure 4B), the internodes between siliques were often absent or very short in ~90% of the mutant plants (Figures 4B to 4D). In some plants, two or more secondary inflorescences developed at the cauline leaf axils (Figure 4C, top panel). In Arabidopsis, the vegetative SAM produces rosette leaves that are close together because of the

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**Figure 3.** Inhibition of Dimerization and Activity of PHB by ZPR3.

(A) PHB dimerization assays by yeast coexpression. ZPR3 was expressed under the control of the Met-suppressible promoter (pMET25) (MET25: ZPR3). The ZPR3 gene is not expressed on selective media without Leu, Trp, and His (-LWH) but is expressed on selective media without Leu, Trp, His, and Met (-LWHM). Cell growth on the -LW media indicates that the yeast cells contain the two plasmid DNAs. The ΔC construct of PHB includes residues 1 to 174.

(B) PHB dimerization and activity assays by β-galactosidase (β-Gal) activity measurements. For the measurements of activities in transcription-activation, a BD-PHB fusion construct containing a full-size PHB protein was used. The β-Gal activities were normalized by dividing total activity by optical cell density. Means ± SE are shown (n = 3).
lack of internode elongation. However, during the transition to reproductive growth, the internodes elongate and paraclade primordia are initiated on the cauline leaf axils. Our observations would therefore indicate that the activities of the SAM and/or axillary meristems are disrupted in zpr3-2. Transformation of zpr3-2 with a genomic DNA fragment containing the ZPR3 gene with the endogenous promoter completely rescued the zpr3-2 phenotype (see Supplemental Figure 11 online), confirming that the zpr3-2 phenotype is caused by ZPR3 inactivation.

ZPR4, consisting of 72 residues, is the most similar to ZPR3 in sequence and length among the ZPR proteins (see Supplemental Figure 5 online). It was therefore hypothesized that the absence of discernible phenotypes in the vegetative growth stage of zpr3-2 would be due to functional redundancy. To examine this, a zpr3-2 zpr4-2 double mutant was obtained by crossing zpr3-2 with zpr4-2 (see Supplemental Figure 12 online). The double mutant was verified by complementation with the ZPR3 gene with its own promoter before analysis (see Supplemental Figure 11 online).

Notably, distinct phenotypic alterations were observed in the zpr3-2 zpr4-2 mutant throughout the plant growth stages. The zpr3-2 zpr4-2 seedlings consistently had extra cotyledons, 35 out of 40 had three cotyledons while the remaining five had four cotyledons (Figure 4E, top panel). At later growth stages, the mutant produced three or four true leaves simultaneously between the cotyledons (Figure 4E, bottom panel), after which the mutant did not produce any more leaves. Interestingly, a few days later, the mutant resumed producing leaves at random positions (Figure 4F). The fully grown mutant produced >50 leaves before flowering, resulting in a bushy appearance (Figure 4G). Furthermore, phyllotaxy was also evidently altered in the mutant, similar to that in phb-1d (Figure 4H). Together, these observations indicate that leaf initiation and phyllotaxy is disturbed in the mutant, possibly because of aberrant SAM activity. Indeed, the overall shape and development of the zpr3-2 zpr4-2 SAM was quite distinct from that of control plants based on scanning electron microscopy observations. Although a SAM-like structure appeared at the apex of the mutant, its surface was irregular with several bulges (Figure 5A,
In addition, individual mutant plants exhibited an array of altered SAM-like structures, including radially symmetric organs (Figure 5C). In later developmental stages, a small bulge appeared at the center of the apex region (Figure 5A, DAS 16). Interestingly, more careful examination revealed that leaf primordia were not produced from the SAM-like structure. Instead, they originated from the adaxial side of leaf/cotyledon bases and randomly from the flat region around the SAM-like structure (Figure 5A, DAS 18 and later). Vertical sectioning of the zpr3-2 zpr4-2 apex confirmed the results (Figure 5B). The apex structure of the zpr3-2 zpr4-2 mutant explains why the mutant produces numerous leaves (Figure 4G) and why it has an altered phyllotaxy (Figure 4H). Collectively, these observations indicate that the zpr3-2 zpr4-2 SAM is non-functional, but many ectopic meristems develop around the SAM-like structure, further supporting that ZPR3 and ZPR4 are essential for proper functioning of stem cells in the SAM.

Numerous protrusions and ripples were observed around the SAM-like structure (Figure 5A, DAS 23 and 33), from which many floral primordia (as well as leaf primordia) initiated. These may be due to ectopic meristem activity and the disturbance of certain molecular mechanisms underlying the SAM development.

Our data indicated that ZPR3 negatively regulates PHB activity. It was therefore predicted that PHB activity would be enhanced in the zpr3-2 zpr4-2 mutant. If this were the case, the inactivation of PHB would reduce the phenotypes of the double mutant. To examine this hypothesis, we crossed the zpr3-2 zpr4-2 mutant with a phb mutant. Although we screened >900 F2 progeny by genomic PCR, we were unable to obtain the zpr3-2 zpr4-2 phb triple mutant. This may be because the ZPR4 and PHB genes are located close to each other on the same chromosome (only 0.57 megabases apart). Because PHB and PHV functionally overlap, at least in part, we decided to generate the zpr3-2 zpr4-2 phv triple mutant. We found that the zpr3-2 zpr4-2 phenotype was moderately recovered in the triple mutant (Figure 6). The leaf number was reduced in the zpr3-2 zpr4-2 phv mutant, comparable to that in control plants. In addition, the triple mutant flowered earlier than the zpr3-2 zpr4-2 double mutant. These results indicate that the zpr3-2 zpr4-2 phenotype is caused at least in part by the enhanced PHV activity, further supporting the
Overexpression of ZPR3 led to partial abaxialization of leaves (Figure 1B). Furthermore, the transcription of several YABBY family members regulating lateral organ polarity (Siegfried et al., 1999), particularly INO (Villanueva et al., 1999), was also altered to some degree in the zpr3-2 zpr4-2 double mutant (see Supplemental Figure 14 online). Therefore, a question was whether ZPR3 is essential for leaf polarity. We examined the surfaces of the zpr3-2 zpr4-2 leaves by scanning electron microscopy. No differences were observed in the adaxial and abaxial surfaces compared with those of the control leaves (see Supplemental Figure 14 online), showing that ZPR3 is not essential for leaf polar patterning. However, it is still possible that ZPR3 plays a redundant role with other ZPR proteins, such as ZPR1 and ZPR2, in determining leaf polarity.

**CLV3 Is Highly Expressed in zpr3-2 zpr4-2**

The WUSCHEL (WUS)–CLAVATA (CLV) feedback loop is an essential component of the molecular mechanisms governing SAM development (Williams and Fletcher, 2005). Since SAM activity was altered in the zpr3-2 zpr4-2 mutant, we examined the expression patterns of genes mediating SAM development. Transcription of CLV1 and WUS was elevated by 2 to 3 times in the mutant (Figure 7A). The CLV3 transcript level increased by more than 20 times, suggesting that CLV3 activation may contribute to the zpr3-2 zpr4-2 phenotype. This notion is also consistent with the previous observation that transgenic plants overexpressing CLV3 show a SAM phenotype similar to that of the zpr3-2 zpr4-2 mutant (Brand et al., 2000).

The expression domain of CLV3 was also expanded to the entire apical dome of the SAM-like structure in the zpr3-2 zpr4-2 mutant, as verified by in situ hybridization assays (Figure 7B). In some mutants, it was further expanded to the side of the SAM-like structure and to the whole apex area (Figure 7B), suggesting that ZPR activities are related to both the expression levels and the expression domains of CLV3.

To examine whether the elevated expression of CLV3 in the zpr3-2 zpr4-2 mutant is due to enhanced activities of the HD-ZIP III proteins, we measured the transcript levels of CLV3 and other SAM-related genes in phb-1d. CLV1 was repressed by approximately twofold, and other CLV genes and WUS were also slightly repressed (Figure 7C). The upregulation of CLV3 in the zpr3-2 zpr4-2 mutant might result from SAM malfunctioning or reflect crosstalk of multiple signals mediating the HD-ZIP III activities in SAM development.

**ZPR3 Regulates PHB via Negative Feedback**

We observed that the transcript levels of the HD-ZIP III genes were unchanged in zpr3-1d (Figure 1D). It has been reported that the ZPR3 transcript levels are elevated in phb-1d and phv-1d (Wenkel et al., 2007), indicating that PHB positively regulates ZPR3 expression. Together with the negative regulation of PHB by ZPR3 at the protein level, these observations are consistent with ZPR3 and PHB being mutually regulated via negative feedback, as has been proposed (Wenkel et al., 2007).

Meanwhile, in situ hybridization assays (see Supplemental Figure 15 online) revealed that ZPR3 expression is confined to
et al., 2007; this work), forming a negative feedback loop (Figure 8).

Along with previous work (Wenkel et al., 2007), our data strongly support that there are extensive interactions between ZPR and HD-ZIP III proteins. Interestingly, the transcription of the ZPR genes is upregulated by the activation of the HD-ZIP III genes (Wenkel et al., 2007; this work), forming a negative feedback loop (Figure 8).

An important molecular partner for HD-ZIP III function is miR165/166, which negatively regulates HD-ZIP III family members by cleaving their mRNAs (Juarez et al., 2004; Kidner and Martienssen, 2004; Kim et al., 2005; Williams et al., 2005). Since both the ZPR proteins and the miR165/166 negatively regulate the HD-ZIP III activities, it was envisioned that the ZPR proteins might be functionally related to miR165/166. As expected, the zpr3-1d mutant was phenotypically similar to the miR166-overproducing plants (Jung and Park, 2007). A similar result has also been reported with the transgenic plants overexpressing ZPR1 and ZPR3 (Wenkel et al., 2007). Accordingly, a triple mutant phb phv rev had an arrested SAM and pin-like leaves due to disrupted SAM activity (Emery et al., 2003).

Although the miR165/166 and the ZPR proteins have targets in common, it seems that they play both shared and discrete roles in SAM formation and determination of lateral polarity. Mutants overproducing miR166 exhibit radialized leaves and disrupted vascular tissues in addition to SAM enlargement (Kim et al., 2005; Williams et al., 2005). The most prominent phenotype of phb-1d and phv-1d is the severe malformation of lateral organs (leaf adaxialization and radial symmetry in severe cases), although they also exhibit an enlarged SAM. The phb phv rev triple mutants lack meristems and produce pin-shaped cotyledons. In addition, no leaves are produced. We observed that the zpr3-2 zpr4-2 leaves exhibit essentially normal leaf polarity but severely disturbed SAM activity. In addition, zpr3-1d lacks a visible SAM and has partially abaxialized leaves. We therefore propose that the regulatory role of miR165/166 is more prominent in the lateral organ patterning, whereas ZPR activity is more substantial in the SAM formation.

The structural organization of the HD-ZIP III proteins sheds lights on the molecular mechanisms by which ZPR activities are regulated. The central StAR-related lipid transfer (START) domain serves as a versatile binding interface for sterols and lipid molecules in animals (Ponting and Aravind, 1999). Although no HD-ZIP III transcription factors play diverse roles in lateral organ polarity, vascular development, and SAM development. The HD-ZIP III genes exhibit shared and distinct tissue-specific expression patterns during embryogenesis and lateral organ development. For example, PHB and PHV initiate their expression in the early stage of embryogenesis, but the expression domains are confined to the adaxial domain of the cotyledons and the central SAM (McConnell et al., 2001; Emery et al., 2003). REV is similarly expressed in the adaxial domain of lateral organs and vasculature (Otsga et al., 2001; Emery et al., 2003).

Figure 7. Altered Expression Patterns of SAM-Related Genes in zpr3-2 zpr4-2.

(A) Expression of CLVs and WUS in zpr3-2 zpr4-2 compared with the wild type. Transcript levels were measured by real-time RT-PCR. Shoot apex tissues of 2-week-old plants were used for total RNA extractions. Means ± SE are shown (n = 3).

(B) In situ hybridization showing the expression domains of CLV3 in the zpr3-2 zpr4-2 apex compared with the wild type. The apex region was sectioned and probed either with a sense (negative control) or an antisense CLV3 probe. Bars = 50 μm.

(C) Expression of CLVs and WUS in phb-1d compared with the wild type. Transcript levels were measured by real-time RT-PCR. Shoot apex tissues of 2-week-old plants were used for total RNA extractions. Means ± SE are shown (n = 3).

DISCUSSION

Regulation of HD-ZIP III Activities by Competitive Inhibitors

Dynamic protein dimerization is a critical regulatory element in transducing diverse cellular signals (Klemm et al., 1998). The dominant-negative dimerization of transcription factors represents an example, as has been illustrated with the ID proteins in animals (Benezra et al., 1990; Ruzionova and Benezra, 2003; Perk et al., 2005).

Along with previous work (Wenkel et al., 2007), our data strongly support that there are extensive interactions between ZPR and HD-ZIP III proteins. Interestingly, the transcription of the ZPR genes is upregulated by the activation of the HD-ZIP III genes (Wenkel et al., 2007; this work), forming a negative feedback loop (Figure 8).
ligands have been identified for the plant START domain yet, it is possible that certain hydrophobic molecules, such as brassinosteroids and unidentified lipids participating in signaling, may bind to the START domain and modulate the formation of heterodimers and/or homodimers. Another structural motif unique to the HD-ZIP III proteins is the MEKHLA motif located in their far C-terminal regions (Mukherjee and Bürglin, 2006). This motif is present exclusively in the class III members. Interestingly, the MEKHLA motif is structurally similar to the PAS (Per/Amt/Sim) motif that mediates diverse protein–protein interactions (Crosson and Moffat, 2002). External signals and intrinsic developmental cues may affect the conformation of the HD-ZIP III proteins, allowing ZPR3 to recognize them and form nonfunctional heterodimers.

**HD-ZIP III Regulators in SAM Function**

Our data demonstrated that ZPR3 and ZPR4 play a key role in regulating SAM development. While the SAM was apparently disrupted in the zpr3-2 zpr4-2 mutant, ectopic meristem activities were observed in several locations, suggesting that the two ZPR members are involved both in proper maintenance of SAM activity and in suppressing meristem activity in nonstem cells. First, zpr3-2 zpr4-2 leaves were produced from the adaxial sides of leaf bases and the flat regions around the SAM-like structure (Figures 5A and 5B), rather than from the central SAM. Second, some flowers were also produced from the protrusions and ripples around the SAM-like structure. Third, ectopic leaves were formed on the inflorescence stem surface in the zpr3-2 zpr4-2 mutant (Figure 5G). Interestingly, such ectopic meristem activities are also observed in the gain-of-function mutants of the HD-ZIP III genes. For example, avb1, which contains mutations in the miRNA target site of the REV gene, develops ectopic shoots from the inflorescence (Zhong and Ye, 2004). In addition, phb-1d also develops ectopic shoots on the undersides of the leaves (McConnell and Barton, 1998).

CLV3 expression is normally confined to a small region within the SAM. In the zpr3-2 zpr4-2 mutant, it was expanded to the entire apical dome of the SAM-like structure (Figure 7B), and its expression level was also greatly elevated (Figure 7A). However, it was not upregulated in phb-1d. One possible explanation is that ZPR3 and ZPR4 target all the HD-ZIP III members and therefore their activities are enhanced in the zpr3-2 zpr4-2 mutant, resulting in higher expression levels of CLV3. The activation of a single target, such as PHB, may not be sufficient for CLV3 induction. In addition, the ectopic SAM development in phb-1d is not as severe as that in the zpr3-2 zpr4-2 mutant. This is also consistent with the functional redundancy among the HD-ZIP III proteins in SAM development. The CLV3 transcript is readily detected in the meristems of the clv1-4 mutant. However, it is greatly reduced in the clv1-4 cna-1 mutant (Green et al., 2005). In addition, the cna-2 phb-13 pvh-11 and cna-1 phb mutants exhibit CLV3-deficient phenotypes, suggesting that the HD-ZIP III genes may be positive regulators of CLV3 expression (Green et al., 2005; Prigge et al., 2005).

Another possibility is that PHB upregulates ZPR3 expression, which sequentially represses PHB in the phb-1d mutant through negative feedback. It is also possible that at least some of the phb-1d phenotypes might come from altered expression domains of PHB. While PHB mRNA is detected only in the adaxial region of wild-type leaves, it is also found in the abaxial domain of phb-1d mutant leaves (McConnell et al., 2001). Although PHB activity is elevated in both phb-1d and zpr3-2 zpr4-2, the locations of increased PHB activity could be different.

At least some of the zpr3-2 zpr4-2 phenotypes can be explained by the CLV3 induction. Transgenic plants overexpressing CLV3 are phenotypically similar to the zpr3-2 zpr4-2 mutant in that leaf production is ceased after the emergence of a few leaves but reinitiated later at random positions (Brand et al., 2000). However, other phenotypes, such as the disorganized SAM morphology, the ectopic leaf formation on stems, and the carpelloid floral organs, are not observed in the CLV3-overexpressing plants, suggesting that CLV3 is not the sole mediator of ZPR function.

**Functional Diversity of the ZPR Proteins**

The zpr3-1d mutant exhibited partially abaxialized leaves and disrupted SAM activity. Wenkel et al. (2007) have also shown that ZPR3 is expressed in the adaxial region of leaves and that transgenic plants overexpressing ZPR3 have abaxialized leaves, sometimes with trumpet-shaped morphology and downward curling, in addition to meristem termination in extreme cases. Transgenic plants overexpressing ZPR1 also showed abaxialized leaves but not trumpet-shaped leaves (Wenkel et al., 2007), suggesting that ZPR1 may play a major role in generating patterns of leaf polarity. We observed that the zpr3-2 zpr4-2 double mutant had normal leaf polarity, indicating that ZPR3 and ZPR4 are not essential for the determination of leaf polarity.

The ZPR proteins are structurally similar in that they possess a ZMIP motif. However, phylogenetic analysis revealed that they can be classified into two distinct groups: one including ZPR1 and ZPR2 and the other including ZPR3 and ZPR4 (Wenkel et al., 2007; see Supplemental Figure 5 online). The two groups are also distinct in their molecular sizes and the location of the ZIP motifs within the proteins. ZPR1 and ZPR2 (93 and 105 residues, respectively) are larger than ZPR3 and ZPR4 (67 and 72 residues, respectively) and are not essential for the determination of leaf polarity.

CLV3 expression is normally confined to a small region within the SAM. In the zpr3-2 zpr4-2 mutant, it was expanded to the entire apical dome of the SAM-like structure (Figure 7B), and its expression level was also greatly elevated (Figure 7A). However, it was not upregulated in phb-1d. One possible explanation is that ZPR3 and ZPR4 target all the HD-ZIP III members and therefore their activities are enhanced in the zpr3-2 zpr4-2 mutant, resulting in higher expression levels of CLV3. The activation of a single target, such as PHB, may not be sufficient for CLV3 induction. In addition, the ectopic SAM development in phb-1d is not as severe as that in the zpr3-2 zpr4-2 mutant. This is also consistent with the functional redundancy among the HD-ZIP III proteins in SAM development. The CLV3 transcript is readily detected in the meristems of the clv1-4 mutant. However, it is greatly reduced in the clv1-4 cna-1 mutant (Green et al., 2005). In addition, the cna-2 phb-13 pvh-11 and cna-1 phb mutants exhibit CLV3-deficient phenotypes, suggesting that the HD-ZIP III genes may be positive regulators of CLV3 expression (Green et al., 2005; Prigge et al., 2005).

Another possibility is that PHB upregulates ZPR3 expression, which sequentially represses PHB in the phb-1d mutant through negative feedback. It is also possible that at least some of the phb-1d phenotypes might come from altered expression domains of PHB. While PHB mRNA is detected only in the adaxial region of wild-type leaves, it is also found in the abaxial domain of phb-1d mutant leaves (McConnell et al., 2001). Although PHB activity is elevated in both phb-1d and zpr3-2 zpr4-2, the locations of increased PHB activity could be different.

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**METHODS**

**Plant Materials and Growth Conditions**

All Arabidopsis thaliana lines used were in the Col-0 ecotype, unless specified otherwise. Plants were grown in a culture room controlled at 22°C with relative humidity of 50% under long-day conditions (16 h light/
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Isolation of Mutants

Arabidopsis ecotype Col-0 was transformed with an activation tagging vector pSKlO15 as previously described (Weigel et al., 2000). Collected F0 seedlings were sown in soil, and a Finale solution (AgriOvo), which contains 5.78% Basta, was diluted 1000 times in water and sprayed twice a week. Among the herbicide-resistant transformants, a morphogenetic mutant (zpr3-1d) showing retarded growth with altered leaf morphology was chosen for analysis.

The single insertion event of T-DNA in zpr3-1d was verified by genomic DNA gel blot hybridization using the 3SS enhancer sequence as probe, followed by analysis of segregation ratios. The sequences flanking the T-DNA insertion site were determined by three-step thermal asymmetric interlaced PCR using the LB2 and AD2 primers (Liu et al., 1995; see Supplemental Table 1 online).

The knockout mutant pph (SAIL_899_C08) was isolated from a pool of T-DNA insertion lines (Syngenta Arabidopsis Insertion Library), and the pph mutant (SALK-021684) was isolated from a pool of T-DNA insertion lines deposited in the ABRC.

The knockout mutants zpr3-2 (SALK-076134) and zpr4-1 (SALK-008069) were isolated from a mutant pool of T-DNA insertion lines (ABRC). The zpr4-2 (SM_3_15428) mutant was isolated from the John Innes Center suppressor mutator lines deposited in the Nottingham Arabidopsis Stock Center.

The zpr3-2 zpr4-2 double mutant was generated by crossing the homozygous zpr4-2 mutant with the homozygous zpr3-2 mutant. To find the homozygous double mutants, the seeds from the cross were grown on MS-agar plates. Among the ~570 seedlings examined, 40 plants had abnormal numbers of cotyledons. All the seedlings with more than two cotyledons were isolated from a mutant pool of T-DNA insertion lines (ABRC). The parental mutants were examined by two hybrid screen using pBridge vector (Clontech) and AD-PHB in the pGADT7 vector (Clontech). The expression constructs (BD–PHB–ZIP III, and AD–SMT) were transformed into E.coli DH5α competent cells and then isolated from these cells using the liquid transformation procedure.

The pBridge vector (Clontech) was used for yeast three-hybrid screen.

Quantitative real-time RT-PCR was performed in 96-well blocks with an Applied Biosystems 7500 real-time PCR system using the SYBR Green I master mix in a volume of 25 μL. The PCR primers (see Supplemental Table 1 online) were designed using the Primer Express Software installed in the system. The two-step thermal cycling profile used was 15 s at 94°C and 1 min at 68°C. A tubulin gene was included as an internal control for normalization of the variations in cDNA amounts used. Biological duplicates were performed, and the reactions were performed in triplicate for each run. The comparative ΔΔCt method was used to evaluate the relative quantities of each amplified product in the samples. The threshold cycle (Ct) was automatically determined for each reaction by the system set with default parameters. The specificity of the PCR was determined by melt curve analysis of the amplified products using the standard method installed in the system.

Scanning Electron Microscopy

Appropriate plant materials were fixed in glutaraldehyde solution (3% glutaraldehyde in 25 mM phosphate buffer, pH 7.0) at 4°C for 24 h. The samples were subsequently incubated in 1% osmium tetroxide in 25 mM phosphate buffer, pH 7.0, at 4°C for several days. They were then subjected to critical point drying and scanning electron microscopy using model JSM 5410LV (JEOL) located in the National Instrumentation Center for Environmental Management (Seoul National University). To examine the leaf surfaces, the medial regions in the nonmarginal part of the leaves were photographed.

Yeast Two-Hybrid Assay

Yeast two-hybrid assays were performed using the BD Matchmaker system (Clontech). pGADT7 was used for GAL4 AD, and pGBK7 was used for GAL4 BD. The yeast strain AH109 (teu-, trp-, ade-, his-) with chromosomally integrated reporter genes lacZ and HIS under the control of the GAL1 promoter that is activated by the GAL4 transcription factor, was used for transformation. ZPR3 and HD-ZIP III cDNAs were amplified by RT-PCR as previously described (Kim et al., 2005) using the primer sets described in Supplemental Table 1 online. The ZPR3 PCR product was digested with EcoRI and SalI and subcloned into pGBK7T. The HD-ZIP III PCR products were digested with restriction enzymes (PHB with NdeI and SacI, PHV with EcoRI and BamHI, REV with NdeI and SacI, ATHB8 with XhoI and SmaI, and ATHB15 with EcoRI and BamHI) and subcloned into pGADT7. Transformation of the AH109 cells (Clontech) was performed according to the manufacturer’s instructions. Colonies obtained were streaked on a medium without His, Ade, Leu, and Trp. To confirm the results, β-Gal assays were performed according to the system procedure.

The pBridge vector (Clontech) was used for yeast three-hybrid screening. A truncated PHB gene sequence missing the C-terminal coding sequence (PHB-JC) was amplified by RT-PCR, and the PCR products were digested with EcoRI and SalI and subcloned into the pBridge vector to generate the BD-JC construct. The ZPR3 gene was subcloned into the NotI/BgIII-digested pBridge vector so that its expression was controlled by the pMET25 promoter. The expression constructs (BD-JC and MET25ZPR3 in the pBridge vector and AD-PHB in the pGADT7 vector)

Analysis of Transcript Levels

Extraction of total RNA samples from appropriate plant materials and RT-PCR conditions have been previously described (Kim et al., 2006). Whenever possible, positive and negative control genes were included in the RT-PCR reactions to validate the assay conditions. The PCR primers for the HD-ZIP III genes have been previously described (Kim et al., 2005). Those newly synthesized in this work are listed in Supplemental Table 1 online.

For RT-PCR–based DNA gel blot hybridization, RT-PCR products were electrophoresed on 1 to 1.2% agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia). The membrane was hybridized at 65°C with gene-specific probes labeled with 32P-dCTP using the Megaprime DNA labeling system (Amersham-Pharmacia).

Phosphorylation was determined by the pMET25 promoter, the expression constructs (BD-JC and MET25ZPR3 in the pBridge vector and AD-PHB in the pGADT7 vector)
were cotransformed into the AH109 cells. The colonies were streaked on media without Leu, Trp, and His supplemented with or without Met.

Subcellular Localization Assays

The GFP-coding sequence was fused in frame to the 3’ end of the ZPR3 gene by subcloning the ZPR3 gene into the pCAMBIA1304 vector (CAMBIA). The ZPR3-GFP fusion sequence was amplified by PCR using the primer pair ZPR3-GFP (see Supplemental Table 1 online), and the PCR product was digested with XbaI and PaeI and subcloned into the pBA002 vector (Kost et al., 1998) for transient expression in onion epidermal cells. Transient expression was achieved by particle bombardment as previously described (Klein et al., 1987).

After incubation for 24 h at 23°C, the cells were subject to bright-field and fluorescence microscopy.

In Vitro Pull-Down Assays

Recombinant GST and GST-ZPR3 proteins were expressed in Escherichia coli BL21-CodonPlus (DE3)-RIL strains (Stratagene) and purified as follows. One-tenth volume of precultured cells (5 mL) was added to 500 mL of fresh Luria-Bertani medium and cultured at 37°C until the OD600 reached 0.3 to 0.6. Protein production was induced by adding isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.5 mM and shaking at 37°C for 5 h. Cells were harvested and resuspended in buffer A (25 mM HEPES, pH 7.5, 20% glycerol, 1 mM DTT, 100 mM NaCl, 0.2 mM EDTA with protease inhibitor cocktail [Sigma-Aldrich], and 1 mM PMSF). The protein extracts were incubated with buffer A. Five microliters of the 35S-labeled proteins was added and incubated for 2 h at 4°C. The beads were then washed three times with 1× PBS and one time with buffer A. Five microliters of the 35S-labeled proteins were added and incubated for 2 h at 4°C. The beads were washed for 20 min. The bound proteins were eluted with 1× SDS loading buffer by boiling for 5 min at 100°C and subjected to SDS-PAGE and autoradiography.

ZPR3-PHB Interaction Assays in Nicotiana benthamiana Cells

The miR165/166-resistant mPHB and mREV mutant genes have been previously described (Kim et al., 2005). Direct infiltration of N. benthamiana leaves was performed as previously described (Kim et al., 2006). Total proteins containing MYC-mPHB, MYC-mPHV, or MYC-ATHB2 were extracted with extraction buffer (50 mM HEPES, pH 7.3, 20% glycerol, 1 mM DTT, 100 mM NaCl, and 0.2 mM EDTA) containing a protease inhibitor cocktail (Sigma-Aldrich). The protein extracts were incubated with GST-conjugated agarose beads at 4°C for 3 h. The beads were then washed four times with fresh extraction buffer supplemented with 0.1% Triton X-100, and the bound proteins were eluted with 1× SDS-PAGE loading buffer. The eluted proteins (mPHB, mPHV, or ATHB2) were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Amer sham-Pharmacia). The HD-ZIP III proteins were detected by protein gel blot analysis using an anti-MYC antibody (Upstate).

Histological Analysis

Seedlings were embedded in paraplast (Oxford Labware). Eight-micrometer sections were prepared using the Leica RM2125RT mi-
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HD-ZIP III Activity Is Modulated by Competitive Inhibitors via a Feedback Loop in Arabidopsis Shoot Apical Meristem Development

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