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Characterization of UNUSUAL LATERAL ORGANS:
A miRNA Regulated F-Box Protein.

A Thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

Peter Thomas Smith

Committee in charge:

Professor Jeff Long, Chair
Professor Mark Estelle, Co-Chair
Professor Robert Schmidt

2009

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Chair

University of California, San Diego

2009

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ABSTRACT OF THE THESIS

Characterization of UNUSUAL LATERAL ORGANS:
A miRNA Regulated F-Box Protein.

by

Peter Thomas Smith

Master of Science in Biology

University of California, San Diego, 2009

Professor Jeff Long, Chair
Professor Mark Estelle, Co-Chair

The high quality annotation of the *Arabidopsis thaliana* genome allows biologists to predict the target transcripts of known microRNAs (miRNAs). The transcript of At1g27340, e.g. *UNUSUAL LATERAL ORGANS* (*ULO*), is the predicted target of the miRNA394 family. The ULO protein is predicted to contain

an F-BOX domain and a Kelch protein-protein interaction domain by amino acid sequence similarity. Given these predicted domains, it is possible *ULO* may be recognizing proteins and mediating their decay. miRNA resistant versions of *ULO* result in polarity defects in all lateral organs derived from the shoot apical meristem. These defects are similar to what is seen in loss-of-function alleles of the *CLASS III HD-ZIP* genes, indicating *ULO* may negatively regulate this gene family. Genetic interactions between *HD-ZIP III* mutants and miRNA resistant alleles of *ULO* support this finding. Using molecular biology tools discussed here, we have attempted to characterize the expression and function of *ULO* and its corresponding microRNA family 394.

Introduction

Arabidopsis thaliana has proven to be an indispensable resource for biologists in studying developmental processes in plants. The utility of this small angiosperm has been proven throughout the years allowing for the discovery of new genes within diverse developmental pathways. The benefits of using *Arabidopsis* include its minimal diploid genome, allowing for relatively simple genetics when compared to more complex polyploidy plants, as well as highly annotated and reliable sequencing information. The other benefits of short seed-to-seed generation time (approximately 6-8 weeks) as well as the small amount of space needed to cultivate *Arabidopsis* make it ideal for study in laboratories. Maintaining the evolutionary mechanisms essential for proper development of most angiosperms, *Arabidopsis* has been used to make scientific discoveries that can lead to better understanding of development of other flowering plants such as agricultural plants (e.g. corn, rice, wheat, etc.).

Utilizing the benefits of *Arabidopsis* within the past few decades researchers have been able to fully sequence this small genome with astounding coverage and fidelity. Along with genome sequencing has been transcriptome and proteomics work which has allowed for accurate prediction of coding regions within the *Arabidopsis* genome. Utilizing the advanced sequencing technology and molecular biology techniques proven over the past few decades, researchers working on the “*Arabidopsis* Small RNA Project” (aka ASRP) at Oregon State University have even been able to identify microRNAs and other small RNAs

within the *Arabidopsis* genome [1]. With this sequencing information, as well as the current understanding of how RNAi occurs, the targets of these small RNAs can be predicted.

One such microRNA (miRNA) identified by the ASRP is designated miRNA 394. This microRNA family, of which there are only two members shown in Figure 1, was predicted to bind the transcript of At1g27340, a predicted F-Box containing gene referred to from here on as *UNUSUAL LATERAL ORGANS (ULO)*[1]. As seen in Figure 1, the predicted structure of the *ULO* gene includes a single intron toward the beginning of the coding sequence and small 5' and 3' untranslated flanking regions. The entire coding sequence of *ULO* without introns is only about 1.7 kilobases, a relatively short gene, with the miRNA binding region coded within the second exon. This gene's miRNA 394 binding region appears to have been well conserved throughout plant evolution as seen in Figure 2. It is only when analyzed via amino acid similarity that the possible function of *ULO* becomes apparent.

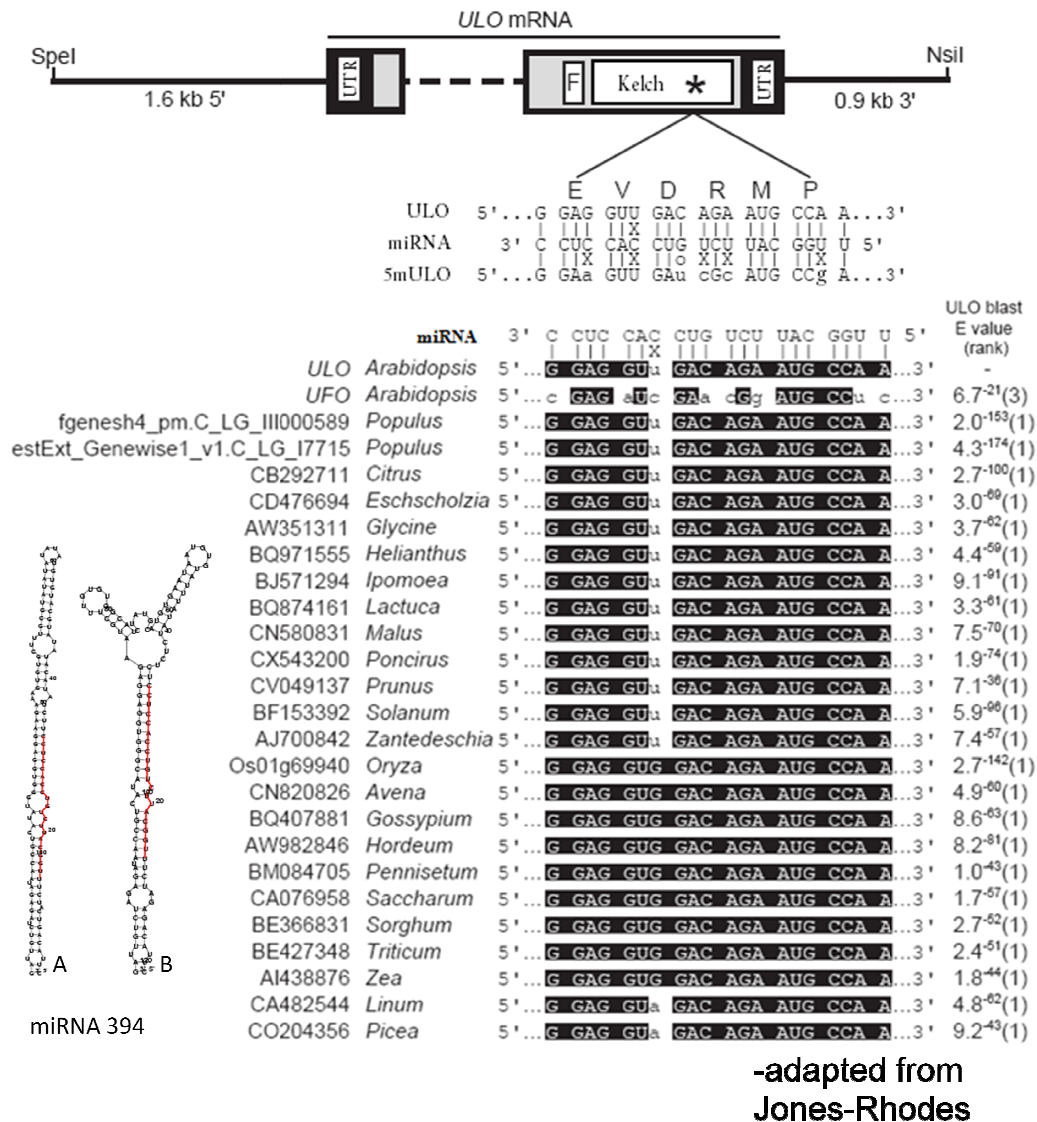


Figure 1: ULO structure and Phylogeny

ULO has 1 intron as well as a predicted F-Box domain and Kelch (7 beta-propeller protein-protein interaction) domain. *ULO* orthologs and the miRNA binding site have been well conserved throughout plant evolution. *ULO* being a single gene family member and regulated by its own family of miRNAs; *ULO* appears to have an important role in plant development. *ULO* knockout mutants show no phenotypes on their own; nor do miRNA overexpressor lines. However, mutating the miRNA binding site with 5 silent mutations (5mULO) gives distinct phenotypes. The predicted stem-loop structures of the two miRNA 394 family members are shown at left.

F-Box Proteins and the SCF Complex

The highly conserved F-Box domain at the beginning of the second exon indicates the possible function of ULO as a substrate recognition domain within an SCF complex. F-Box proteins are thought to act as the substrate recognition domains of SCF complexes. These SCF complexes, so-called because of the complexes that form via Skp- Cul1- F-Box interactions, have been hypothesized and shown to act as E3 ligases leading to protein ubiquitination and destruction[2]. The proposed model in Figure 2 shows the F-Box protein acting as the substrate recognition domain of the SCF complex, which is composed of several connected scaffolding proteins that allow the E2 domain to attach ubiquitin to a substrate thus polyubiquitinating said substrate[3]. The polyubiquitinated substrate is then recognized and degraded by the 26s proteasome pathway[4]. Studies have also shown that F-Box proteins can act as dimers or heterodimers for substrate binding[5], and it has also been shown that phosphorylation of the substrate is often required to allow binding by F-Box proteins[6]. Also, some studies have shown that the F-Box protein may be ubiquitinated itself after assembly of the complex, allowing for turnover of the complex for use with other F-Box proteins and recognition of different substrates[7].

The fact that F-Box proteins are the key component in giving specificity to the SCF complex makes them an intriguing subject. F-Box proteins have been shown in plants to have the essential and novel role of direct hormone signaling

as shown by TIR1 (TRANSPORT INHIBITOR RESPONSE 1), which requires auxin to bind its substrate AUX/IAA[8,9]. Within the *Arabidopsis* genome 694 F-Box proteins have been predicted while relatively few of these have been characterized[10]. Those F-Box proteins that have been characterized appear to be important factors in regulating development, such as UFO (UNUSUAL FLOWER ORGANS) which has the most homology to ULO even though there is only ~30% amino acid similarity between the two. At the end of the coding region of *ULO* is another clue as to its function - amino acid similarity to a Kelch protein-protein interaction domain. These domains have been studied and crystallized in other organisms showing them to possess 7-beta-propeller domains which are used to interact with other proteins [11]. The presence of a protein-protein interaction domain and an F-Box domain suggest ULO may be acting in an SCF complex signaling for the degradation of a substrate protein.

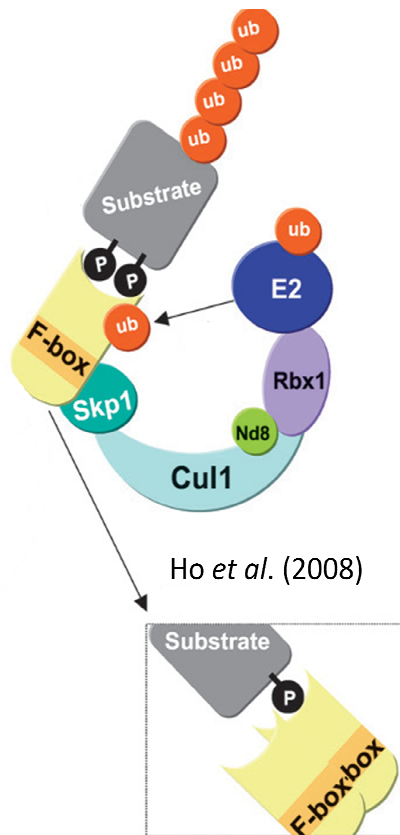


Figure 2: The SCF Complex

F-Box proteins act as the substrate recognition domain of the SCF complex. There are 694 predicted F-Box proteins in *Arabidopsis thaliana*. These proteins have been shown to act as dimers and heterodimers in other organisms for substrate recognition. Post-translational modification of the substrate has been shown to be required for recognition by F-Box proteins in several systems. Often this modification is by phosphorylation and subsequent conformational changes allowing binding by the complementary F-Box protein. Along with polyubiquitination of the substrate allowing for recognition and degradation by the 26s proteasome pathway, it has been shown that ubiquitination of the F-Box itself allows for turnover of the F-Box within the SCF complex.

miRNA Resistant ULO (a.k.a 5mULO)

The knowledge of *ULO*'s regulation by a single family of miRNAs and its possible role in protein turnover indicate that it could be important for *Arabidopsis* development because of the multiple levels of regulation it undergoes and affects. Transgenic lines carrying miRNA resistant versions of *ULO* show severe developmental phenotypes in *Arabidopsis* plants, suggesting an important function in developmental pathways even though knockout mutants of *ULO* showed no phenotypes on their own. Transgenic lines were created with the native *ULO* promoter 1.6kb 5' and the 0.9kb sequence 3' to the coding sequence as well as *ULO* itself. However, there were five silent mutations made in the miRNA binding site within *ULO*'s predicted Kelch coding domain to disrupt binding of miRNA 394 to the *ULO* transcript as seen in Figure 1. While there were many varying phenotypes among these new *5mULO* (named so because of the 5 mutations) mutants, the most prominent were leaf curling defects and the apparent stochastic initiation of lateral organs. The most severe transgenics showed strongly abaxialized phenotypes reminiscent of other HD-ZIP loss of function mutants, resulting in terminated meristems and wholly abaxialized (tissue resembling the bottom of the leaf) cotyledons or pin-like structures which are seen in Figure 3.

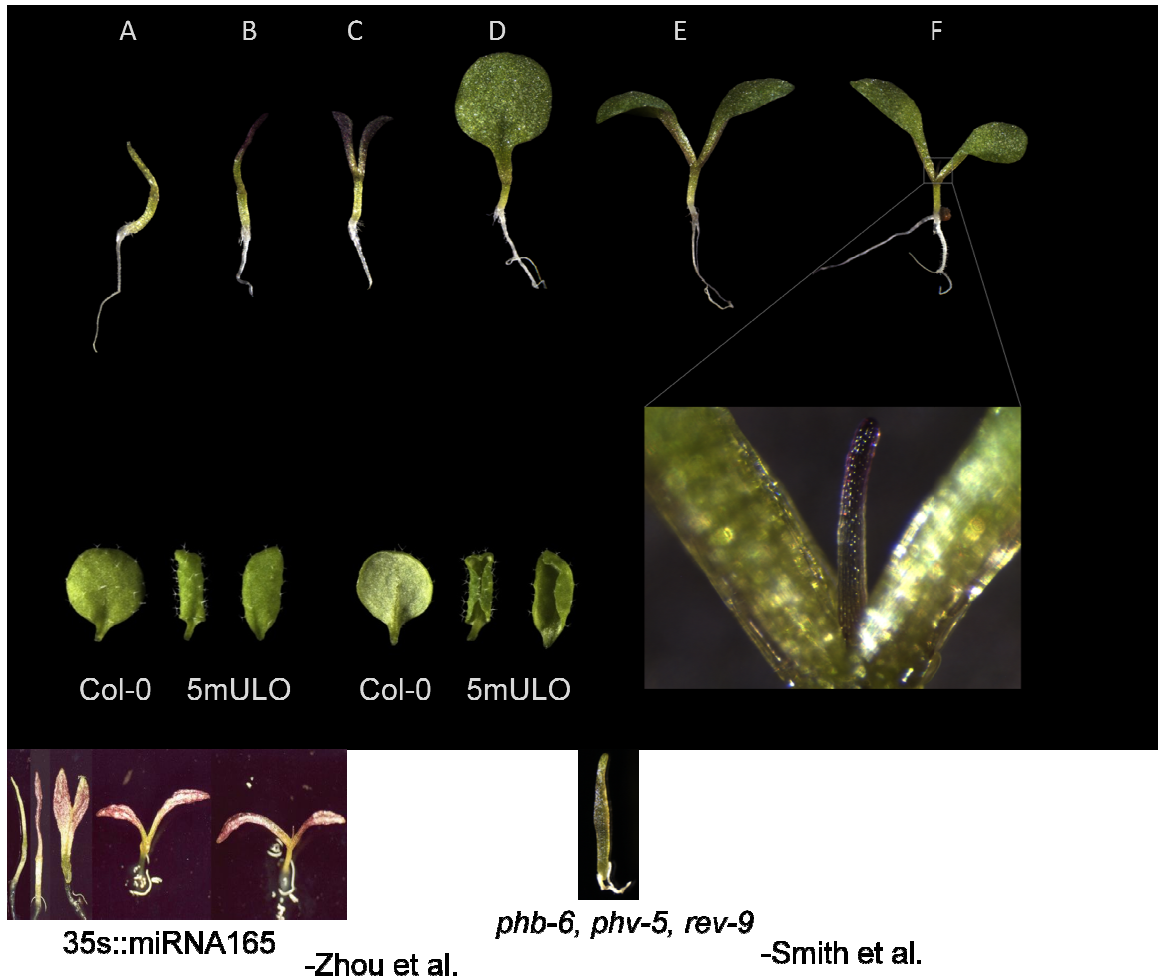


Figure 3: miRNA Resistant ULO Phenocopies Loss-Of-Function HD-ZIP III Mutants

Phenotypes of the 5mULO mutant plants show strong abaxialized phenotypes. These phenotypes include abaxialized pins (A); single abaxialized radialized cotyledons (B); double abaxialized radialized cotyledons (C); single cotyledons with a terminated meristem (D); two cotyledons with a terminated meristem (E); and terminated meristems with pin-like lateral organ initiation which terminates prematurely (F). Also in older flowering plants there are distinct leaf curling defects as well as lateral organ initiation patterning defects. Many mutants are partially sterile with severe floral initiation defects. Axillary meristem development also seems to be deficient. These phenotypes are consistent with strong loss-of-function class III HD-ZIP mutants as shown by over-expression of miRNA 165, a miRNA which binds and regulates the HD-ZIPs, and in the triple loss-of-function *phb-6, phv-5, rev-9* mutant plants.

Class III HD-ZIPs and Polarity

Many different pathways have been implicated in the formation of lateral organs and the maintenance of the shoot apical meristem during development as seen in Figure 4. The developing cotyledons and leaves of *Arabidopsis* have distinctly morphological differences which allow the plant to maximize photosynthesis and metabolism. From trichome arrangement and cell size to stomatal density and vasculature organization, these morphological differences have evolved to maximize the survival of the plant. The 'top' of the leaf arises from the central region of the stem cell population in the meristem and is referred to as the adaxial (or dorsal) side of the leaf which will ultimately face the sun and carry out photosynthesis. The 'bottom' of the leaf arises from the peripheral stem cell domain of the meristem and is known as the abaxial (or ventral) side of the leaf, where gas exchange away from the effects of the sun occurs through stomatal openings. Auxin flow and antagonism between adaxial and abaxial determining factors ultimately lead to the patterning seen in the leaves of the developed plant. *CLASS III HD-ZIP* genes have been shown to be essential genes for meristem maintenance, adaxial lateral organ identity, and apical polarity during embryogenesis [12]. The Class III HD-ZIP genes have two main domains which give them their name: the homeodomain (HD) binds DNA, and the leucine zipper domain which allows for homo and heterodimer formation. Within the transcripts of the Class III HD-ZIPs is a miRNA binding site complementary to miRNA 165/166. Mutations in this miRNA binding site disrupting binding of miRNA 165/166 have been found in the adaxialized gain of

function *HD-ZIP III* mutants that have been isolated over the years. These gain-of-function *HD-ZIP III* mutants display severe phenotypes including adaxialized leaves and overproliferation of the meristem[12]. However, of these genes only triple loss of function mutants of *Class III HD-ZIPs* *revoluta* (*rev*), *phabulosa* (*phb*), and *phavoluta* (*phv*) resulted in severe abaxialized phenotypes which were not seen in single loss of function mutants. *REV* mutants such as *rev-9* produce the only single loss-of-function *HD-ZIP III* phenotypes, and these phenotypes are limited in effect to lateral organ initiation defects[13]. However, the triple loss of function *phb-6^{-/-}*, *phv-5^{-/-}*, *rev-9^{-/-}* mutants give abaxialized pin-like phenotypes during embryogenesis, indicating failure to properly form the apical meristem during development[14]. Also, similar pin-like and abaxialized phenotypes were seen when the miRNAs which target the HD-ZIPs (miRNAs 165/166) were over-expressed[15]. The presence of these abaxialized phenotypes in *5mULO* mutants indicated that *ULO* mutants could be working in a related pathway to the HD-ZIPs during development. It is also worth noting that like *ULO*, abaxial determinant genes such as the *KANADIs* and the *YABBYs* also have no significant single loss-of-function phenotypes. The large amount of redundancy within these pathways suggests how essential leaf polarity patterning is to plant development and survival.

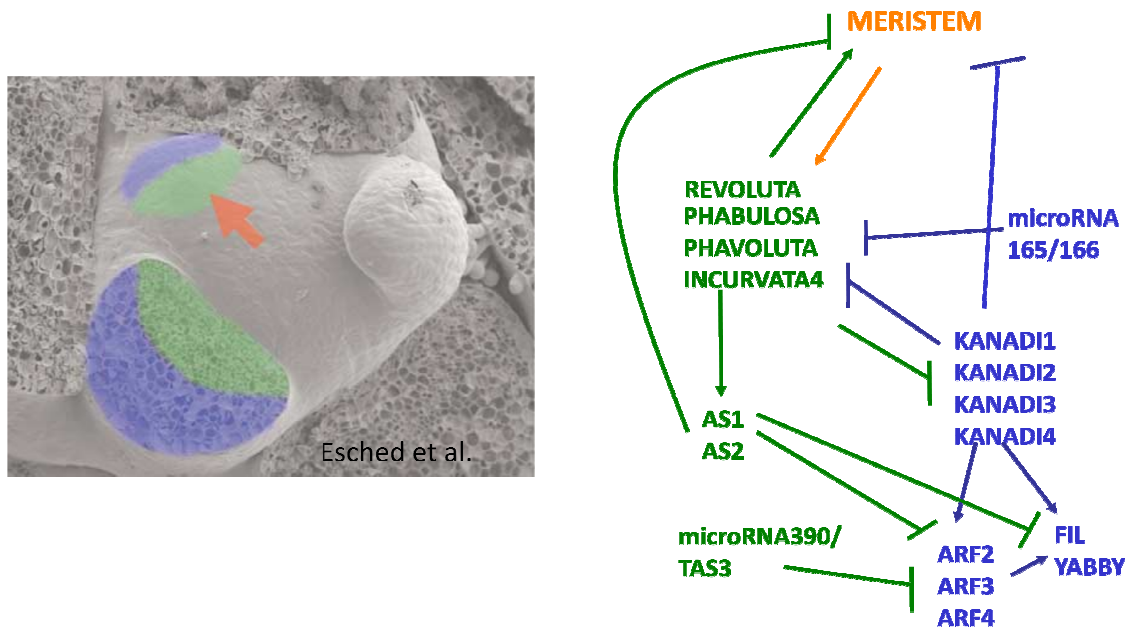


Figure 4: Lateral Organ Development

Image on left is a false colored electron micrograph illustrating domains of developing leaf primordia on the flanks of the meristem (at center). The adaxial, or 'top', of the leaf is first patterned at this early stage (false colored green), as is the abaxial, or 'bottom', of the leaf shown in blue. There are many levels of regulation during lateral organ development. Signals to and from the meristem by the *HD-ZIP III* genes allows for maintenance of the meristem, axillary meristem development and adaxial (proximal) lateral organ patterning. miRNA families 165/166 have been shown to negatively regulate the *HD-ZIP III* transcripts. There are also many proposed antagonisms between abaxial (distal) patterning genes and the *HD-ZIP III* genes as seen in the diagram above. (adapted from Smith et al.)

Analysis of ULO Expression

To determine what function ULO has during development, one of the first tasks we undertook was to try to identify its expression pattern. This proved difficult, which is logical when one considers the many levels of regulation ULO is predicted to undergo (e.g. transcriptional, miRNA, and possible proteosomal degradation). Clear post-translational expression of ULO was only seen when using tobacco injections to transiently transform leaves with a miRNA resistant version of ULO fused to three tandem fluorescent proteins as seen in Figure 5. With these tobacco injections a clear nuclear localization of ULO protein was observed, indicating ULO may function to negatively regulate transcriptional regulatory proteins in the nucleus such as the HD-ZIP IIIs (which contain DNA binding domains). Using *ULO* RNA probes with *in situ* hybridization in the embryo does not reveal a clear pattern of *ULO* transcripts at any point observed during embryogenesis. However, the *ULO* transcript levels measured by *in situ* hybridization are elevated in *5mULO* miRNA 394 resistant mutant embryos. The higher levels of *ULO* transcript reveal that these *5mULO* mutants have successfully inhibited the activity of miRNA 394.

Because of the difficulty of seeing stable *ULO* transcript patterns in the embryo, it was determined that further study of miRNA 394 activity may reveal where *ULO* is expressed during development. The miRNA sensor diagrammed in Figure 5 provides a readout for the activity of the native miRNA, which the sensor is designed to bind to. This miRNA activity can be visualized by the loss

of GFP fluorescence. The activity of miRNA 394 is apparent throughout the adaxial region of a sectioned leaf where the sensor transcript is not being translated into fluorescent protein. If *ULO* is indeed specifying abaxial fate as indicated by the *5mULO* gain of function mutants, then clearing of *ULO* transcripts via activity of miRNA 394 in the adaxial region of the leaf is to be expected. Due to unclear miRNA 394 activity patterns in the embryo when visualizing the erGFP directly, we attempted to look at the actual transcript levels of the miRNA 394 sensor using *in situ* hybridizations with an anti-GFP probe. The *in situs* of the miRNA 394 sensor revealed an early pattern of adaxial miRNA activity in the embryo. miRNA 394 appeared to have the most consistent activity in the developing meristematic and adaxial cotyledon regions of embryos as early as the heart stage. This early activity of miRNA 394 in the developing embryo suggests *ULO* may be functioning in and be important for regulating *Arabidopsis* adaxial/abaxial identity during early embryogenesis.

Figure 5: ULO Expression and miRNA 394 Activity

Using *ULO* as a probe has shown inconclusive results when trying to identify an expression pattern in wild type *Arabidopsis* embryos as seen in the top images. One conclusion that can be made is that the transcript levels of *ULO* are greatly enhanced in the *5mULO* mutants. By injecting tobacco with agrobacterium containing a desired construct we are able to see the effect of a transient transformation via microscopy as seen in the middle set of pictures. Using a series of fluorescent tags along with the miRNA resistant version of *5mULO* we were able to see the sub-cellular nuclear localization of the protein. The activity of the regulatory miRNA 394 for *ULO* can be measured using a miRNA sensor which contains the miRNA 394 binding site between an ER-localization signal and a fluorescent tag. In the picture on the bottom left a constitutive 35s promoter drives miRNA 394 sensor expression in the leaves. The lack of fluorescence in the adaxial region of the leaf indicates miRNA 394 is active in these regions, sequestering or cleaving *ULO* transcripts. By looking at the transcript of this construct by RNA *in-situ* hybridization we can see with better resolution the activity of the miRNA in the developing plant embryo. On the bottom right an embryonic promoter (UBI3p) driving a miRNA 394 sensor shows distinct patterns of miRNA activity in the embryo. The miRNA appears to be active in the adaxial side of the developing cotyledons as well as in the developing meristem.

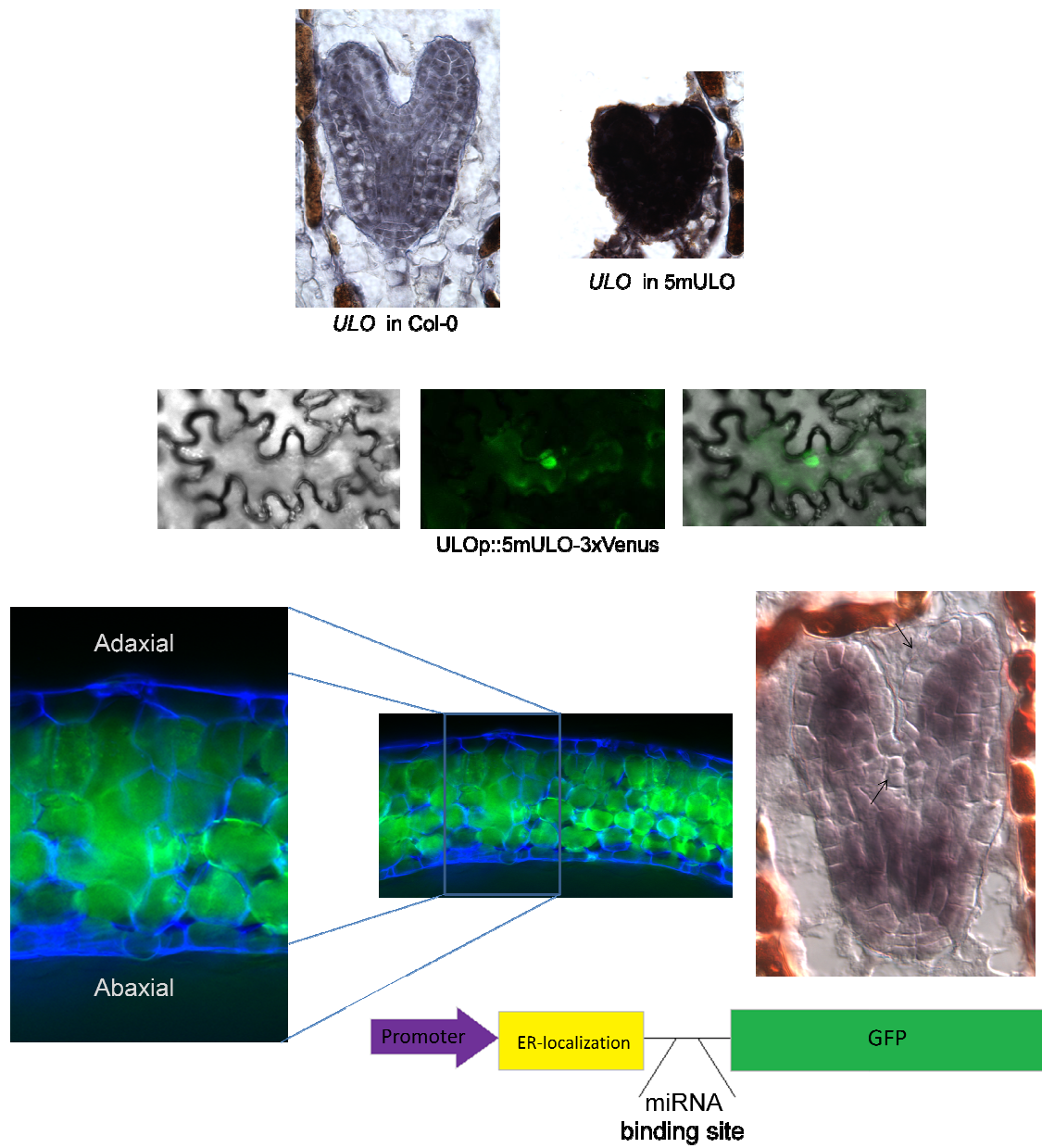


Figure 5: ULO Expression and miRNA 394 Activity

Class III HD-ZIPs and ULO Interaction

Given the apparent similarities between gain-of-function *5mULO* phenotypes and loss of function *HD-ZIP III* phenotypes, experiments were conducted to elucidate any possible interaction between them. *FILAMENTOUS FLOWER1 (FIL)* is a well documented abaxial cotyledon marker during embryogenesis [16]. Examination of *5mULO* mutant embryos reveals a spread of *FIL* RNA expression and thus abaxial identity throughout the apical region of the developing embryo, as is also seen in triple loss-of-function HD-ZIP mutants in Figure 6. Given this evidence genetic interactions could further show interaction of ULO and the HD-ZIP IIIs within the same pathway. Such genetic interactions do appear to occur as *5mULO* mutants crossed to *revoluta* loss-of-function mutants produce dramatically enhanced phenotypes as seen in Figure 7. The large increase in instances of meristem arrest in *5mULO* x *rev-1* and *rev-9* mutants indicates an enhanced loss of HD-ZIP III function in developing *Arabidopsis* plants.

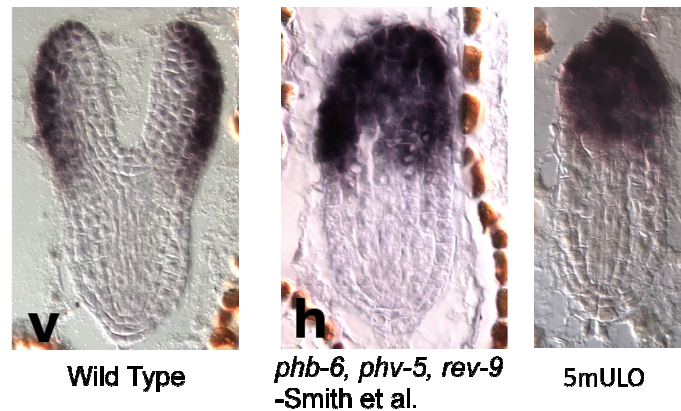


Figure 6: *FIL* Expression Patterns

The abaxial identity marker *FIL* (*FILAMENTOUS FLOWER*) can be used in RNA *in-situ* hybridization to see where abaxial patterning is occurring. When severe phenotypes such as pin formation occur in 5mULO embryos, there is an evident spread of *FIL* into the entire apical domain of the embryo. The expression pattern of *FIL* in these 5mULO embryos matches the expression pattern found in triple loss-of-function HD-ZIP III mutant embryos; indicating that the spread of abaxial identity within these two mutants is working in a similar way.

The enhanced *HD-ZIP III* loss of function phenotypes when crossed to gain of function *5mULO* indicates a possible direct interaction between the two genes. It is possible that *ULO* is directly negatively regulating *HD-ZIP III* expression or function given such enhanced phenotypes as shown in the model in Figure 8. To test whether *ULO* is regulating transcription of the *HD-ZIP III*s, or the transcription or activity of miRNA 165/166, we performed RNA *in situ* with *HD-ZIP III* probes on *5mULO* mutants. If *ULO* were negatively regulating the HD-ZIPs in a pre-translational fashion, there would presumably be less *HD-ZIP III* transcripts within developing embryos as compared to wild-type embryos. However, there is no apparent difference in *HD-ZIP III* transcript patterns in

5mULO embryos as is seen in Figure 7. This consistent transcriptional pattern was also observed for the HD-ZIP IIIs *REV*, *PHV*, and *PHB* respectively.

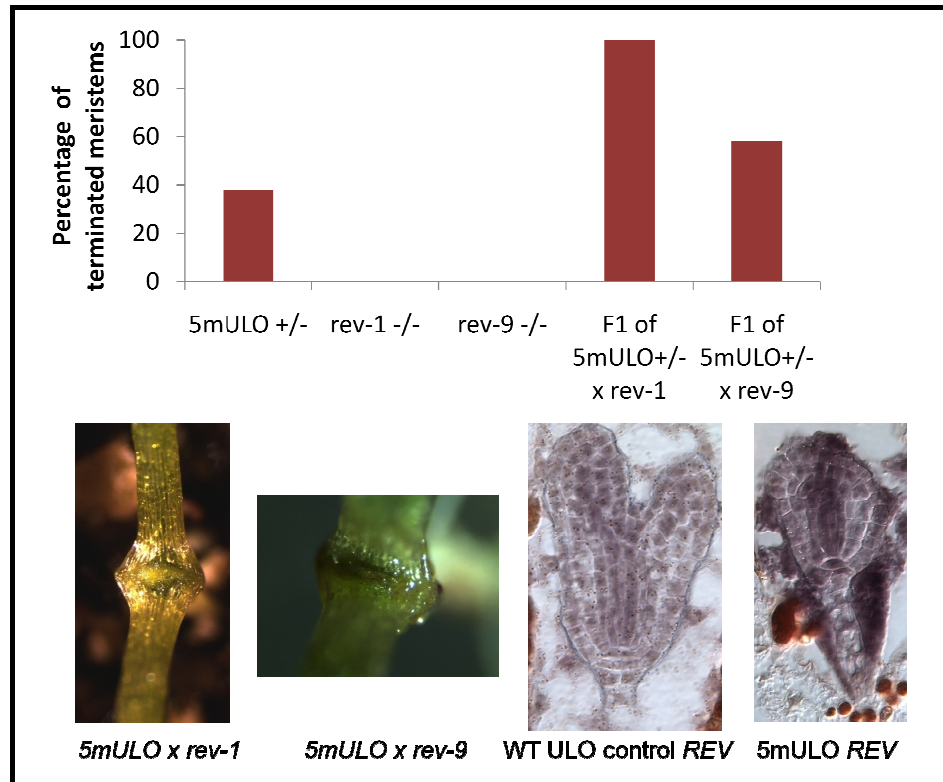


Figure 7: ULO and the Class III HD-ZIPs Genetically Interact

Testing genetic interactions between ULO and the HD-ZIPs showed a severe enhancement of phenotypes. *rev-1* and *rev-9* have two of the strongest phenotypes on their own of any of the HD-ZIP III loss-of-function mutants. However, even the strongest mutant, *rev-1* rarely has any defects aside from axillary meristem initiation. When crossed to the gain-of-function ULO mutant, the HD-ZIP III mutants severely enhance the phenotypes of the 5mULO mutants. Preliminary data shows that genetic interactions between ULO and other HD-ZIPs also occurs. Also, according to the preliminary data there appears to be some suppression of phenotypes when gain-of-function HD-ZIP III mutants (with mutated miRNA binding sites) are crossed to 5mULO. Because of the undefined level of regulation that ULO has on the HD-ZIP III's we looked at whether the RNA levels of the HD-ZIPs were changed in the gain-of-function ULO mutants. There did not appear to be any change in the HD-ZIP RNA expression in these 5mULO mutants, suggesting that ULO is not acting to regulate the HD-ZIPs at the transcriptional or miRNA level as seen in the two bottom right hand images.

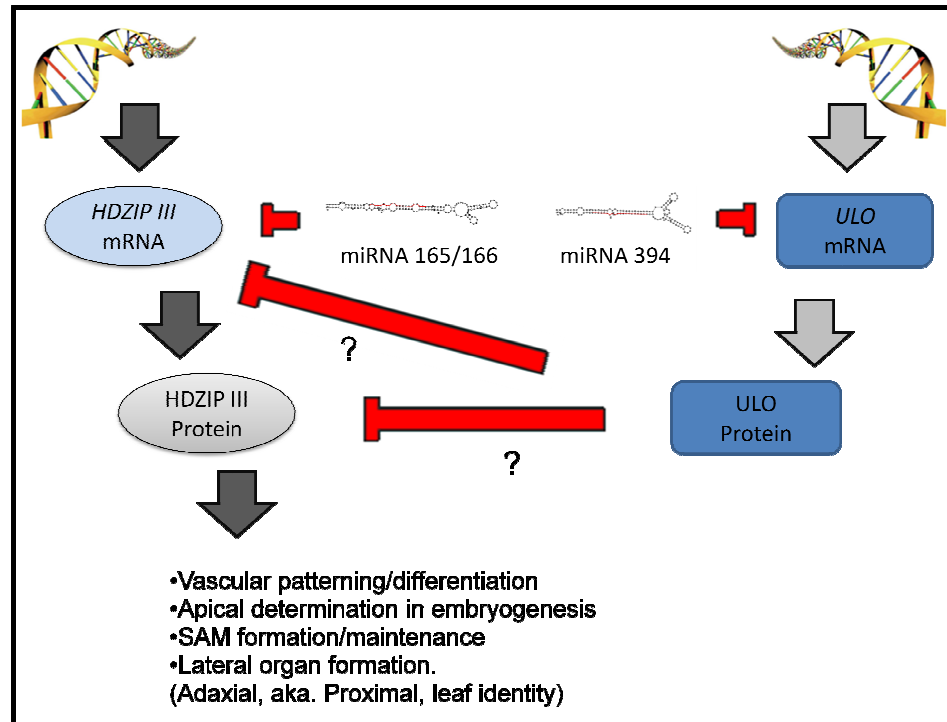


Figure 8: Proposed ULO Model

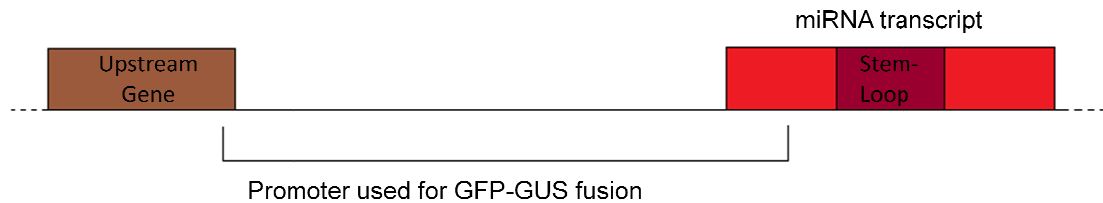
Given ULO's genetic interactions with the HD-ZIPs and the nature of F-Box proteins to negatively regulate substrates via protein degradation, we are questioning if and how ULO could be acting to negatively regulate the HD-ZIPs. Due to the numerous levels of regulation already known about the HD-ZIPs, ULO could be negatively regulating a positive regulator of HD-ZIP transcription; negatively regulating a negative regulator of miRNA families 165 or 166; or ULO could be directly interacting with and degrading the HD-ZIP proteins themselves. Given the class III HD-ZIP's function in so many developmental processes, ULO's effect on the HD-ZIPs could be important on many developmental levels. Since pre-translational regulation seems unlikely due to stable HD-ZIP transcript levels in 5mULO mutants, the most likely level of regulation is post-translational.

miRNA 394 Expression and Contrasting Expression of ULO and HD-ZIPs

The ability to attach GUS (beta-glucuronidase) reporter enzymes to the ends of cloned fragments allows molecular biologist to see expression of genes even at low levels when substrates such as 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) are allowed to react with the enzyme. Following a long incubation with substrate, preliminary results of homozygous stable transgenic lines with miRNA 394 A and B promoters fused to GUS reveal distinct patterns of expression. This expression as seen in Figure 9 reveals that miRNA 394 is expressed in the developing vasculature and meristem of 10 day old seedlings. The presence of miRNA 394 expression in the vasculature prompted analysis of microarray data comparing *ULO* and *HD-ZIP III* expression in the vasculature. Using the Genevestigator utility to view compiled microarray data, contrasting levels of expression of *REV* and *ULO* in the vasculature may reveal another place that *ULO* regulation of the HD-ZIP IIIs could be occurring [18]. HD-ZIP IIIs have been implicated in xylem differentiation in the past [19], and the high levels of *REV* in the xylem support this claim. However, the low expression levels of *ULO* in the xylem contrast with these high *REV* levels, and in the cork the inverse is true. *ULO* as a backup mechanism for negative regulation of HD-ZIP IIIs in surrounding tissues seems more and more likely given these findings. Contrasting levels of *REV* expression and *ULO* expression throughout the plant can also be seen when compared through the Genevestigator microarray data

[18]. Further study of the distinct patterns of differing ULO expression and *HD-ZIP III* expression could better prove this hypothesis.

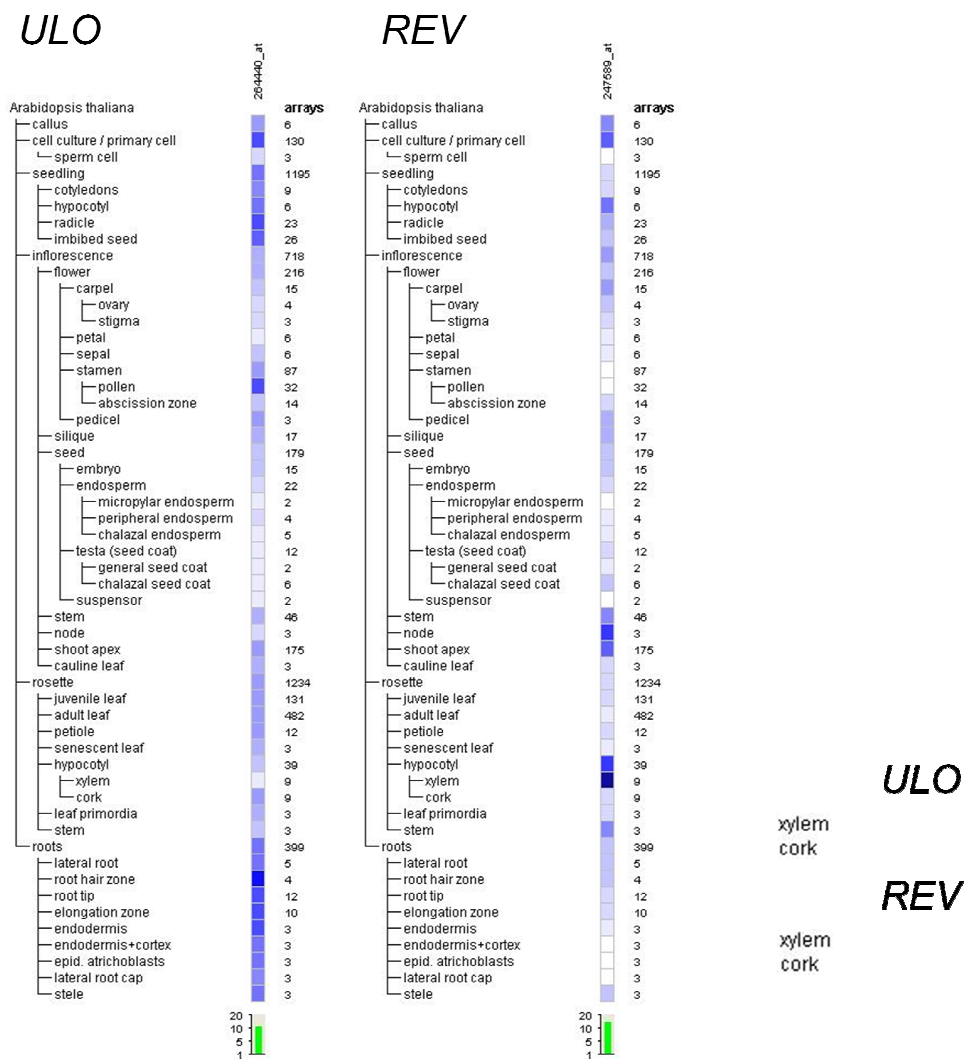
Looking at the miRNA 394 activity sensors when compared to the regular expression levels of *REV* and the *HD-ZIP III*s as shown earlier, a pattern similar to the microarray data emerges. miRNA 394 appears to only be active in tissues that would normally have *HD-ZIP III* expression, e.g. meristematic tissue and adaxial cotyledon and leaf tissues. It would appear that miRNA 394 is keeping ULO from being expressed in tissues where *HD-ZIP III* expression is found. Better resolution of the vascular tissues in the miRNA 394 promoter GUS fusion lines may reveal how tightly negatively regulated ULO is in vascular tissues like the xylem where *HD-ZIP* expression is known to exist.



miRNA 394 A promoter :: GFP-GUS

Figure 9: miRNA and ULO Expression Patterns

Because of the presumed low expression levels of ULO, GUS staining seems to give the best results. Seen above, miRNA promoter constructs can be made by designing the promoter region to include everything up to the next 5' gene and going into the miRNA transcript sequence, stopping before the actual predicted stem-loop structure. Using this technique to make promoter-GFP-GUS fusion constructs, the miRNA promoter activity can be seen *in planta*. The promoter activity of one of the miRNA family members can be seen above. The expression in the meristem and vasculature as well as the apparent expression in the adaxial side of the cotyledons matches the activity of the miRNA via the miRNA sensors. Looking at the vasculature in more detail may prove helpful in explaining ULO's expression and regulation by the miRNAs because of the HD-ZIP III's role in xylem differentiation and development.



Hruz et al. 2008

Figure 10: Comparing ULO and REV Expression Using Genevestigator.

Looking at the differing expression patterns of *ULO* and *REV* via microarray data reveals distinctly differing expression patterns. Tissues in which one is expressed the other seems to be missing. This is especially clear in the vasculature as shown at left. Higher resolution expression patterns of *ULO* and miRNA 394 will elucidate the role *ULO* may be playing in regulation of the HD-ZIP IIIs.

Conclusions

The many levels of regulation in embryonic and leaf polarity developmental pathways indicate an essential nature of these processes for *Arabidopsis* and plants in general. Since plants are unable to move, the proper formation of organs and maintenance of the stem cell populations in the meristems are essential to the survival of the plant. Given the diverse ways in which the HD-ZIP IIIs act in developing plants (from apical determination in the embryo to the patterning and differentiation of vasculature, meristems, and lateral organs), many forms of regulation must exist within a plant in order to develop correctly and survive. Thus, this new proposed level of regulation of the HD-ZIP IIIs by ULO is not surprising when viewed in the context of the already extremely complex regulatory pathways which ensure proper expression of these developmentally essential genes. The lack of phenotypes in *ulo* knockout mutants suggests ULO's role as being redundant to other negative regulatory pathways of the HD-ZIP IIIs, but other abaxial determinants such as *YABBY* and *KANADI* loss-of-function single mutant phenotypes as well. Only when definitive evidence of direct ULO and HD-ZIP III protein-protein interaction is ascertained will we know if ULO is directly regulating the HD-ZIPs, but the complimentary expression patterns of the *HD-ZIP IIIs* and miRNA 394 activity suggests that ULO may be a backup system for keeping the highly influential HD-ZIP IIIs from being expressed in inappropriate tissues during development. Accurate expression patterns of ULO itself would also be extremely helpful in proving our hypothesis,

and experiments are being carried out in the lab using recombineering technology to see where ULO accumulates at different stages of development.

In order to test if and how ULO interacts with the HD-ZIP IIIs a series of experiments can be performed. Yeast two hybrid protein-protein interaction experiments were conducted using the Dualhunter split-ubiquitin system [17]. Using this yeast two hybrid assay, ULO and REV were tested for direct protein-protein interaction, which yielded negative results with all controls working. The lack of interaction in the yeast assay could be explained by possible phosphorylation states required by the HD-ZIP proteins for interaction with ULO that cannot be achieved in yeast. Preliminary *in planta* results co-injecting REV and ULO fluorescently tagged constructs into tobacco gave mixed results and needs further study to conclusively show the presence or absence of interaction. Another problem with using conventional protein-protein interaction assays is that ULO is predicted to be an F-Box protein possibly working in an SCF complex as mentioned previously, so if the HD-ZIP IIIs are the target of ULO they will be degraded upon interaction. If one or both of the interactors are degraded during an assay there is no way to report this interaction by conventional means. However, there are proteasome inhibitors which have been shown to inhibit the degradation of F-Box protein substrates by inhibiting activity of the 26s proteasome pathway. With the proteasome inactive, polyubiquitination of SCF substrates have been observed by other researchers and such experiments were conducted on *REVp::REV-GFP* stable transgenic seedlings with proteasome

inhibitors mg115 and mg132. These proteasome inhibitor assays initially showed some increased size laddering of the REV protein indicating possible polyubiquitination. However, these experiments were not reliably reproducible. Use of these proteasome inhibitors in interaction assays such as co-immunoprecipitations may show interactions between ULO and the HD-ZIP proteins *in planta*. Another possible use of these techniques may be to treat tagged ULO transformed plants with proteasome inhibitor and then perform mass spectrometry on pulled-down ULO to determine possible interactors. This technique may also elucidate some of the machinery that ULO requires in acting in the SCF complex as there are many different ASK (*Arabidopsis* SKP1-like) proteins in *Arabidopsis* as well as the other components of the hypothesized complex.

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