UNIVERSITY OF CALIFORNIA, SAN DIEGO

Analysis of the conserved auxin signaling pathway using *P. patens*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2018

The Dissertation of Sibo Tao is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2018

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Mark Estelle, for his continuous guidance and support throughout my graduate study and research, which inspired me to become a better thinker, scientist and writer. His encouragements have led me through the difficult times and challenges during my research.

I want to thank all the other members of my committee, Professor Eric Bennett, Professor James Kadonaga, Professor Karen Oegema and Professor Martin Yanofsky for their insightful comments and suggestions to my research projects.

I would also like to thank the Estelle Lab members for their help and discussion during the past five years. Special thanks to Dr. Meirav Lavy who mentored me during my rotation in the lab and taught me every detail about moss techniques when I first joined the lab for thesis research.

Last but not least, I would like to thank my family and friends for their support and understanding during my PhD study.

Chapter 2, in part is currently being prepared for submission for publication of the material. Tao, Sibo; Estelle, Mark. The dissertation author was the primary researcher and author of this work.

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PUBLICATIONS

Tao S and Estelle M. Mutational studies of the Aux/IAA proteins in *Physcomitrella*

reveal novel insights into their function. *In press*.

Lavy M, Prigge M, **Tao S**, Shain S, Kuo A and Estelle M. (2016). Constitutive auxin response reveals complex interactions between Aux/IAA and ARF proteins in Physcomitrella. *eLife*. 5: e13325.

Tao S, Zhang Y, Wang X, Xu L, Fang X, Lu ZJ and Liu D. (2016) The THO/TREX complex active in miRNA biogenesis negatively regulates root-associated acid phosphatase activity induced by phosphate starvation. *Plant Physiology*. 171(4): 2841-2853.

ABSTRACT OF THE DISSERTATION

Analysis of the conserved auxin signaling pathway using *P. patens*

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Doctor of Philosophy in Biology

University of California, San Diego, 2018

Professor Mark Estelle, Chair

Auxin is a plant hormone that regulates a wide range of processes during plant development and response to the environment. At the molecular level, auxin signal transduction begins with perception by the TIR1/AFB-Aux/IAA co-receptor system and the degradation of Aux/IAAs by the ubiquitin proteasome pathway. Aux/IAA interacts and represses the activity of the ARF transcription factors, and its degradation leads to the activation of downstream gene transcription and physiological responses. The mechanism that determines how the diverse auxin responses are finely tuned is yet to be understood. Since there are 29 *Aux/IAA* genes in Arabidopsis, genetic studies of these genes have been hampered by extensive genetic redundancy. *P.patens* (moss), on the

other hand, has only three *Aux/IAA* genes. Here we use the *Δaux/iaa* null mutant reported previously to express and characterize PpIAA1a with different mutations *in vivo* at both the molecular and physiological levels. Our results provide unique details on how key amino acids determine Aux/IAA function both as a co-receptor and as the repressor of auxin signaling, and how they contribute to the regulation of complex auxin responses.

TIR1-Aux/IAA co-receptors showed distinct auxin-binding affinity *in vitro*. Using the transgenic moss *IAA1a* lines, we demonstrate how this difference in auxin binding affinity determines Aux/IAA protein stability, auxin-responsive transcriptional changes and the related developmental outcomes. We also reveal a novel affinity-determining residue in the TIR1-Aux/IAA interaction surface.

Based on earlier studies, repression by the Aux/IAAs is thought to require recruitment of the co-repressor TPL through an interaction with the Aux/IAA EAR motif. Our analysis indicates that this mechanism is not essential in moss. On the other hand, our analysis on *IAA1a* lines with mutations in the putative interaction centers of the C-terminal PBI domain show that Aux/IAA oligomerization is required for full repression in the natural background, while the monomer still retains partial repression. As Aux/IAAs and ARFs share homology in the interaction centers, they have a large number of combination options to oligomerize, allowing for diverse repressive strength. This, together with the difference in spatial and temporal Aux/IAA expression and in auxin-binding affinity, provides a bridge between the simple structure of auxin and the complex processes it regulates.

Chapter 1

Introduction

The plant hormone auxin has been implicated in almost every aspect of plant growth and development, from embryogenesis, primary and lateral root growth to flowering and fruit maturation, and in plant responses to the environment like abiotic and biotic stress. Because of this, many studies have been done trying to resolve the molecular mechanism that determine how auxin regulates a variety of temporal and spacial responses.

The current model for auxin signal transduction at the molecular level is shown in Figure 1.1. Recent studies have shown that auxin acts as a "molecular glue" to directly promote the interaction between the two components of its co-receptor complex, a TRANSPORT INHIBITOR RESPONSE (TIR1)/AUXIN SIGNALING F-BOX (AFB) protein and an AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressor. TIR1/AFBs are a family of F-box proteins which act as adaptors of SCF-type E3 ubiquitin ligase complexes in the ubiquitination system. In plants, one of the major types of E3 complex is the SCF complex, which consists of SKP1, Cullin-1 (CUL1), RBX1 and an F-box protein. CUL1 act as a scaffold for the assembly of the complex, with RBX1 binding to its C-terminal region to form a catalytic core. SKP1 binds to the N-terminus of CUL1, and provides an interface to dock the F-box substrate adaptors. Therefore, the auxin co-receptor complex is also an adaptor-substrate complex in the ubiquitination-proteosomal degradation system. Upon auxin perception, the F-box protein TIR1/AFBs interacts tightly with their substrate Aux/IAAs, recruiting them to the SCF complex for ubiquitination and 26S proteasome-mediated degradation. It was also shown recently that different combinations of TIR1/AFBs and Aux/IAAs form co-receptor

complexes with a wide range of auxin-binding affinities, which may function in different auxin-regulated processes (Parry et al., 2009; Hua and Vierstra, 2011; Calderón Villalobos et al., 2012).

Aux/IAAs are a group of proteins that functions as negative regulators in the auxin signaling pathway. A typical Aux/IAA protein contains four domains: domain I (DI), which contains a conserved EAR motif, DII, which contains a degron motif, and a PBI domain, which shares homology with the PB1 domain of AUXIN RESPONSE FACTOR (ARFs) downstream in the auxin signaling pathway. In earlier genetic studies, several dominant or semidominant gain-of-function mutants showing auxin-resistant phenotypes have been isolated, which turned out to have mutations in the conserved degron sequence. (Prigge et al., 2010; Wang and Estelle, 2014) Later structural studies confirmed that the degron motif in the DII domain directly interacts with TIR1 upon auxin binding, as shown in Figure 1.2. (Tan et al., 2008)

The Aux/IAA DI domain contains an EAR motif that is thought to be a transcription repression motif. (Tiwari et al., 2004) It was first identified in an Arabidopsis genetic study, that *tpl-1* is a suppressor of the auxin insensitive *iaa12*, which contains a stabilizing amino acid substitution in the degron motif, and *in vitro* protein interaction assays indicated that IAA12 interacts with the putative transcriptional co-repressor TOPLESS (TPL) through the EAR motif. (Szemenyei et al., 2008)

The Aux/IAA PB1 domain is believed to be participate in dimerization or multimerization, in interactions with other Aux/IAAs and/or ARFs. This is supported by evidence from yeast two-hybrid and transient assays in protoplasts. When auxin level is low, Aux/IAAs interact with certain ARFs and repress their activity; when auxin level is

high, Aux/IAAs are degraded by the ubiquitin-proteosome system and ARF repression is released, activating a variety of downstream gene expression. Recently, structural studies have revealed the PB1 structure of AtARF5 and AtARF7, identifying a basic lysine motif and an acidic OPCA motif as two interaction centers for their interaction with Aux/IAAs, which also have these two conserved centers. It was also shown in a DII stabilized *AtIAA16* overexpression line, that both motifs are essential for conferring the overexpression *iaa16* mutant phenotype (Korasick et al., 2014).

ARF proteins are generally comprised of an N terminal DNA binding domain, a variable middle region (MR), and a C terminal PB1 domain that shares homology with that of Aux/IAAs. In transient protoplast assays, different members of the ARF family have shown different activities, either activating or repressing reporter gene transcription (Guilfoyle and Hagen, 2007; Tiwari et al., 2003; Ulmasov et al., 1999). Structurally, these two types of ARFs mainly differ in the MR region. Recent research showed that in activating ARFs, the MR interacts with SWI/SNF chromatin remodeling ATPases that mediate changes in chromatin required for gene activation, and this interaction is blocked in the presence of Aux/IAAs (Wu et al., 2015). On the other hand, the *in vivo* activity of the putative repressing ARFs remains to be demonstrated.

One of the obstacles in further studies of auxin signal transduction mechanism is the gene redundancy. In the most widely used model organism Arabidopsis, there are 29 Aux/IAAs identified in the whole genome, making it extremely difficult to address the influence of mutation in the proteins in a defective background. *Physcomitrella patens* (moss), on the other hand, has much less gene redundancy with only three Aux/IAAs in its genome. Moreover, homologous recombination techniques are available to achieve

site-specific gene manipulation. And since moss is haploid throughout the vegetative stages, homozygous transgenic lines can be obtained directly in the transformants. With respect to auxin physiology, many developmental processes of moss are regulated by auxin, including the transition from chloroplast-rich chloronemata to the transparent filamentous caulonemata, and the formation of leafy gametophores (determined by a relative ratio of low auxin level and high cytokinin level) and brown root-like rhizoids (formed with high auxin level), as shown in Figure 1.3. Therefore, auxin sensitivity can be characterized by these related phenotypes.

These advantages make *P. patens* a very good system for studying auxin signaling. Moreover, the fact that the Aux/IAA proteins in moss and *Arabidopsis* share conserved structural motifs makes it possible to combine the knowledge and insights gained from previous *Arabidopsis* studies with the advantage of a simple genetic background in moss.

In this dissertation, we used a moss line deleted for all three native *Aux/IAA* genes to study the details of auxin signaling mechanism, by native promoter-driven expression of Luciferase-fused PpIAA1a protein with mutations in different conserved elements (Chapter 2) and by overexpression of different ARF proteins for analysis of their activities without the Aux/IAA repression (Chapter 3). In Chapter 4, we reported protein interaction study of TIR1/AFBs and Aux/IAAs carrying mutations in the PBI domain, indicating that factors outside of the Aux/IAA DII interaction surface may influence the TIR1/AFB-Aux/IAA co-receptor stability. Lastly in Chapter 5, we developed an *in vitro* ubiquitination assay with *in planta* purified SCF complex.

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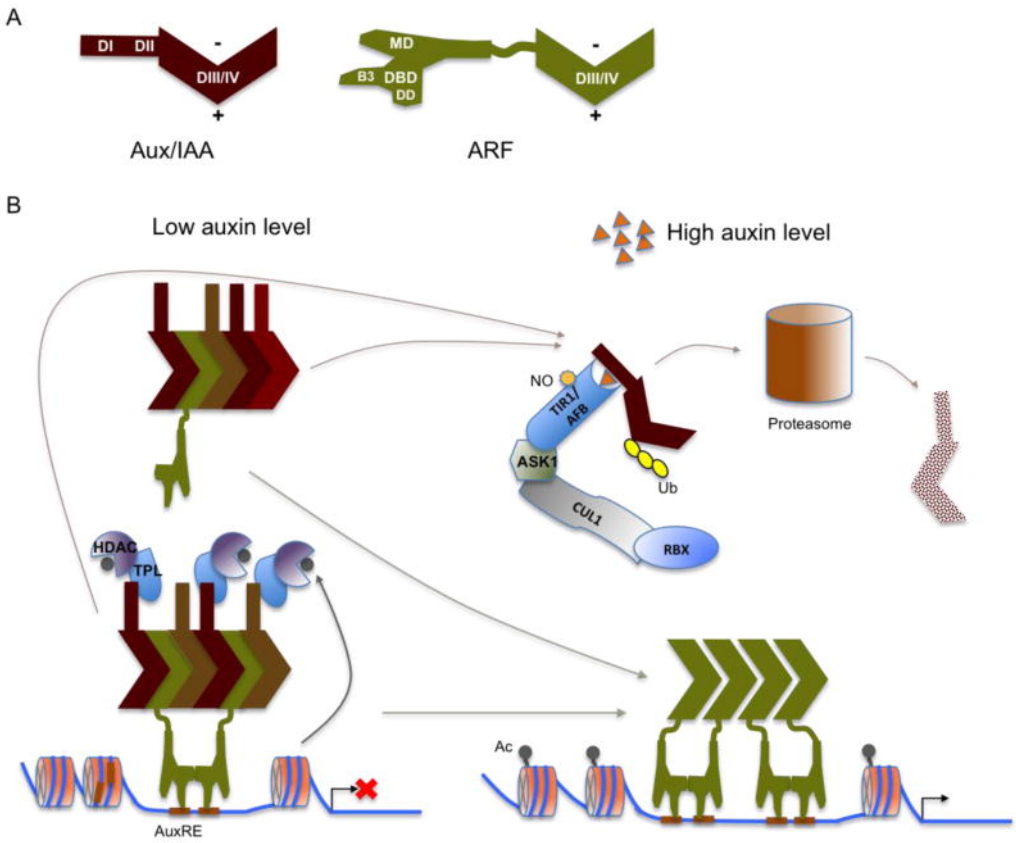

Figure 1.1 Current model for auxin signal transduction (Wang and Estelle, 2015).

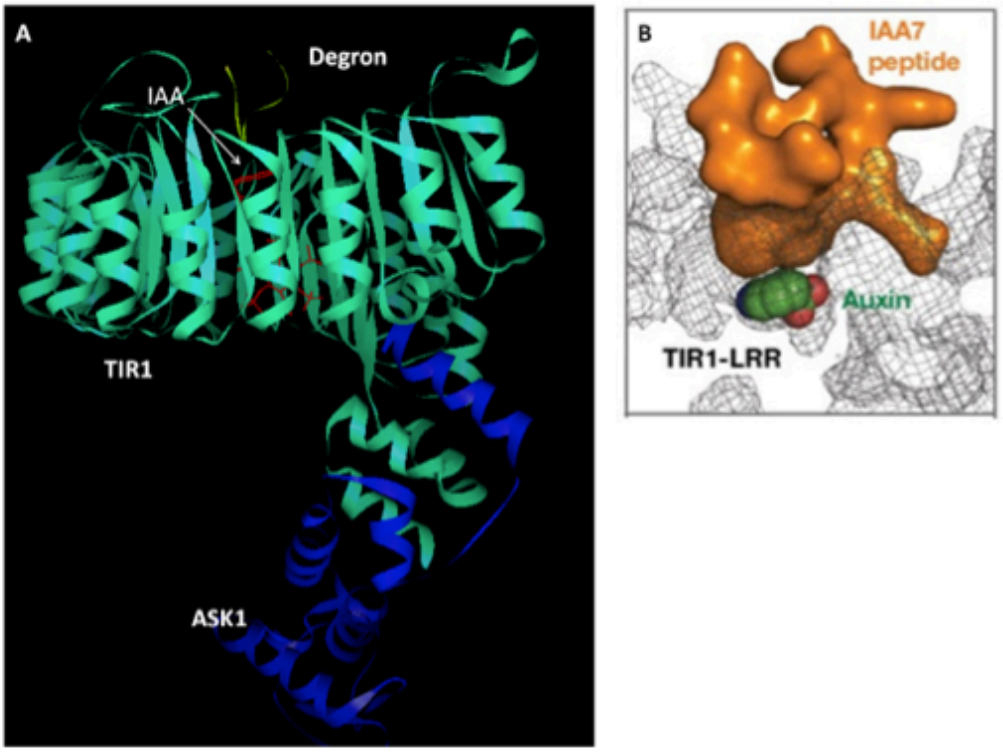

Figure 1.2 Auxin promotes the interaction between TIR1 and Aux/IAAs. (A) The structures of TIR1, ASK1, AtIAA7 DII in the presence of auxin (IAA). (B) Auxin act as a "molecular glue" to increase the affinity of TIR1-IAA7 interaction. (Tan et al., 2008)

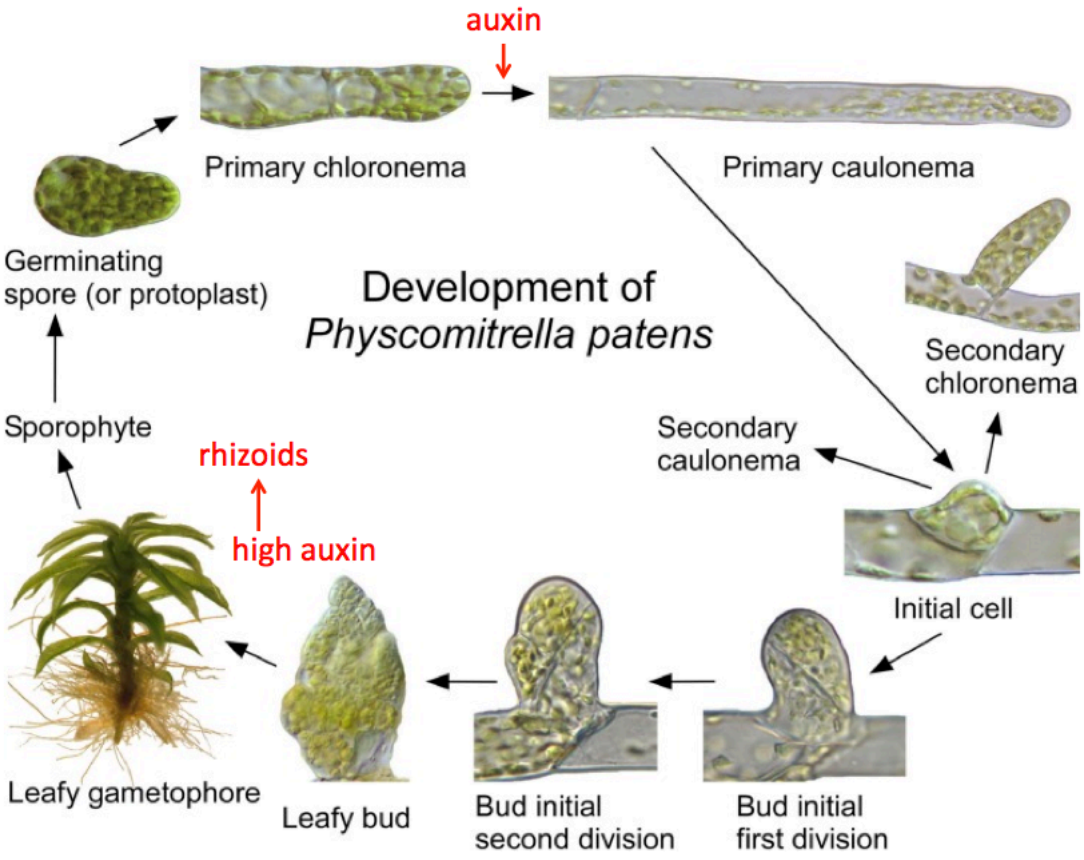

Figure 1.3 The life cycle of P. patens and the role of auxin in regulating moss development. (Adapted from Roberts et al., 2012)

Chapter 2

Novel insights into Aux/IAA functions from a moss system

Introduction

Auxin is a plant hormone that regulates a wide range of processes during plant development and response to the environment. While the chemical structure of auxin is quite simple, it triggers a complex set of downstream responses with different temporal and spatial patterns. Many studies have been devoted to understanding the mechanisms that produce this complexity at the cellular and molecular level.

Based on current knowledge, auxin acts through a transcriptional derepression mechanism. The major repressors in the pathway are the Aux/IAA proteins, which are also components of the Aux/IAA-TIR1/AFB co-receptor system for auxin perception. The TIR1/AFB proteins are F-box proteins and subunits of $SCF^{TIR1/AFB}$ ubiquitin protein ligases (E3s). In the presence of auxin, the $SCF^{TIR1/AFB}$ E3s, promote degradation of the Aux/IAAs, resulting in activation of auxin-responsive gene transcription. Several genetic studies identified stabilized forms of Aux/IAAs that escape this degradation, revealing a core degron motif in Aux/IAA Domain II. Structural studies later confirmed that this domain is the interaction surface with TIR1 in presence of auxin. There are several natural variants in this degron motif among different Aux/IAA proteins, and some of these have been shown to result in distinct co-receptor binding affinities for auxin. It is unknown whether these differences alone would lead to differences in protein stability and the level of repression.

The Aux/IAAs repress the activity of a family of transcription factors called ARFs, which share homology with Aux/IAAs in their C terminus and are thought to control auxin-responsive gene transcription. This repression may be achieved by recruiting a co-repressor called TPL through the Aux/IAA EAR motif. However, the question remains

whether Aux/IAA repression depends solely on TPL function. Thus it is possible that the Aux/IAAs act independently of TPL.

The Aux/IAA and ARF proteins interact through their homologous C-terminal domains. A recent structural study proposed that the C-terminal region constitutes a PB1 domain. The interaction between PB1 domains involves a conserved lysine residue and an acidic DxD/ExD (OPCA) motif (Fig. 2.1), which form two complimentary interaction centers. According to this hypothesis, the Aux/IAAs and ARFs could also self-interact to form oligomers. The study showed that in *Arabidopsis*, the phenotype conferred by overexpressing stabilized AtIAA16 was not maintained when either one of the interaction centers was mutated to neutral alanine, indicating that in this specific background, Aux/IAA oligomerization is required for the repression by stabilized AtIAA16. Although this result strongly suggests that repression requires oligomerization, the gain-of-function nature of the stabilized AtIAA16 protein, can be a complicating factor.

There are 29 *Aux/IAA* genes in Arabidopsis. Genetic studies of these genes have been hampered by extensive genetic redundancy among the family members. While stabilized forms of Aux/IAAs produce clear phenotypes, the gain-of-function nature of these mutations can complicate their analysis. Further, the paucity of loss-of-function phenotypes limits structure-function studies.

Unlike Arabidopsis and other higher plants, the moss *Physcomitrella patens* has only three *Aux/IAA* genes. We recently reported an *Δaux/iaa* null mutant that displayed a strong auxin-constitutive phenotype. In this study, we used the triple mutant to investigate the biological significance of conserved elements in the Aux/IAA proteins including the degron motif variations, the EAR motif and the conserved motifs in C

terminus. The results provide unique details on how key amino acids determine Aux/IAA function both as a co-receptor and as the repressor of auxin signaling, and provide new insights into how the complex auxin responses are fine-tuned.

Results

Tissue-specific expression pattern of the moss Aux/IAA genes reveals gene redundancy at the molecular level

One of the major developmental processes regulated by auxin in moss is the formation of leafy gametophores. In order to understand more about the expression pattern of each of the three Aux/IAA members in the moss genome, we dissected the gametophores from the remaining filamentous tissue and extracted total RNA from both samples. We then used qPCR to quantify and compare the expression level of the *IAA1a*, *IAA1b* and *IAA2* genes.

Our results revealed that in wild-type moss, while *IAA1a* did not show a significant difference in expression level between the filamentous tissue and the gametophores, *IAA1b* and *IAA2* were expressed at a higher level in gametophores compared to filaments. (Fig. 2.2a) We then did the same experiment using an *iaa1b iaa2* double knockout line (Fig. 2.2b). Interestingly, when the *IAA1b* and *IAA2* genes are not expressed, the *IAA1a* expression level was significantly higher in gametophores compared to filaments, in contrast to the wild type. In fact, the level of *IAA1a* transcript in *iaa1b iaa2* is roughly the same as that of *IAA1a*, *IAA1b* and *IAA2* transcripts together in the wild-type line, in both gametophores and the remaining filamentous tissue respectively. This suggests that in the absence of *IAA1b* and *IAA2*, *IAA1a* expression is up-regulated to compensate for the loss of the other two genes.

Characterization of natural variants in the degron motif in Arabidopsis and moss

Because the degron motif in Aux/IAA is the interaction surface with TIR1/AFB and auxin, it is a key element in auxin signaling. In Arabidopsis, the canonical core degron motif is VGWPPVR, which is shared by multiple members including IAA7, IAA14 and IAA28. In addition, there are several variants of this sequence including VGWPPIG (IAA12 and IAA13), VGWPPCS (IAA5, IAA6, and IAA19) and VGWPPVK (IAA29) (Fig. 2.1). The three Aux/IAA members in the moss genome share the same core degron motif VGWPPVK (degVK), which is the same as AtIAA29 (Fig. 2.1). Based on yeast two-hybrid (Y2H) data, IAA12 and IAA29 both have a much lower affinity for TIR1/AFBs than IAA7 in the presence of auxin. This may be due to the difference in the degron motif or in other regions. So far, the effects of variation in the degron motif on protein function, have not been directly addressed. Further, because of gene redundancy as well as the complexity of Aux/IAA in Arabidopsis development, it is hard to dissect the *in vivo* biological significance of these natural variants.

To address this question, we generated Luciferase-tagged *PpIAA1a* constructs with degron motifs corresponding to AtIAA7 (*IAA1a-luc*^{degVR}), AtIAA12 (*IAA1a-luc*^{degIG}), and AtIAA29/PpIAAs (*IAA1a-luc*^{degVK}). Each construct was introduced into the *iaa1b iaa2* bacground by homologous recombination. These lines allow characterization of the effects of the variation at both the physiological and molecular levels.

Treatment of moss with the auxin NAA (1-Naphthaleneacetic acid) results in reduced colony size, disruption of leafy gametophore formation and increased brown-pigmented rhizoids. When wild-type moss is treated with high NAA levels, normal cell differentiation is disrupted and the moss filaments are disorganized. The *Δaux/iaa* null

mutant displays this phenotype in the absence of auxin treatment. Interestingly, substitution of the moss degron (VGWPPVK) with the Arabidopsis canonical degron (VGWPPVR) resulted in a reduction in auxin sensitivity. *IAA1a-luc*^{degVR} shows increased leafy gametophore formation and colony size when treated with NAA compared to *IAA1a-luc*^{degVK} which carries the moss wild-type version of *IAA1a* (Fig. 2.3a). The *IAA1a-luc*^{degIG} line was even less sensitive to auxin treatment, with close-to-normal colony size and gametophore formation even under 0.5 μM NAA when the other lines showed severe auxin phenotypes.

We then used qPCR to determine the expression level of some auxin-responsive marker genes in these lines. Our results showed that that in both mock and auxin-treated conditions, the expression levels of the marker genes in the mutants was lower than in wild type (*IAA1a-luc*^{degVK}). Also, expression of the markers was lower in *IAA1a-luc*^{degIG} than in *IAA1a-luc*^{degVR} (Fig. 2.3b), consistent with their phenotypes.

We then checked whether these defects are the result of a difference in IAA1a degradation rates. The real-time amount of IAA1a protein was quantified *in vivo* by treating a fixed amount of the tissue with excess D-Luciferin and measuring the chemiluminescence at different time points after mock/auxin treatments. The normalized ratio, which is the chemiluminescence from the auxin-treated sample versus that from the mock sample, is plotted against the time of treatments (Fig. 2.3c). We found that with 0.2 μM exogenous auxin IAA (Indole-3-acetic acid), IAA1a-luc^{degIG} remained stable, while IAA1a-luc^{degVR} degrades slower than IAA1a-luc^{degVK}. When the auxin concentration is increased to 20 μM, IAA1a-luc^{degIG} level started to drop, but was still much slower than the other two variants.

In previous work by Calderón Villalobos *et al*., mutating the degron motif of AtIAA12 (degIG) to the canonical degVR resulted in higher auxin binding affinity of the TIR1-IAA12 co-receptor *in vitro*. Our results revealed the *in vivo* biological significance of this difference in multiple aspects. When auxin level is low, the lower auxin-binding affinity of degIG allows the Aux/IAA protein to be stable while Aux/IAAs with other higher affinity degrons like degVR get degraded; when auxin level is high, the Aux/IAA with degIG starts to be degraded but at a slower rate compared to high affinity degrons. This shows how auxin-binding affinity influences the receptor stability. Moreover, the qPCR of auxin-responsive genes and the moss colony phenotypes showed that this difference in Aux/IAA degradation lead to the difference in downstream gene transcription level and in long-term developmental outcomes.

Besides, although the Arabidopsis Aux/IAA member with the moss degVK, IAA29, is less characterized and shows much lower binding affinity in Y2H compared to a lot of other Arabidopsis Aux/IAAs (probably because of unknown features outside of the degron in IAA29), the degron itself (in *IAA1a-luc*^{degVK}) actually leads to increased auxin-induced degradation, higher auxin-responsive gene transcription and more severe auxin-related phenotypes compared to degVR and degIG. Therefore, the lysine residue in the last position up-regulates the degron activity.

The EAR motif is not essential for Aux/IAA function

Apart from auxin perception, another major function of the Aux/IAA is to repress downstream gene transcription. Based on earlier studies, repression is thought to require recruitment of the co-repressor TPL through an interaction with the EAR motif.

The EAR domain is conserved in the moss and Arabidopsis Aux/IAA proteins (Fig. 2.1), and previous Y2H assays show interaction between the moss EAR motif and the two moss TPL proteins. We utilized our *PpIAA1a-Luc*/*Δaux/iaa* system to study the role of the EAR motif in moss auxin signaling *in vivo*. We generated an EAR motif-deleted *PpIAA1a*$^{\Delta ear}$ line. In addition, we verified that IAAa$^{\Delta ear}$ does not interact with moss TPLs in an Y2H assay (Supplemental Fig. 2.1a).

Phenotypic characterization showed that the *IAA1a*$^{\Delta ear}$ mutant had increased caulonema formation and reduced numbers of leafy gametophores compared to wild type consistent with a reduction in *IAA1a*$^{\Delta ear}$ repression. However, the appearance of the mutant was clearly different from the Δ*aux/iaa* line indicating that IAA1a$^{\Delta ear}$ retained some function. Further, when treated with different concentrations of auxin, *IAA1a*$^{\Delta ear}$ displayed an increasingly severe auxin phenotype that correlated with auxin level (Fig. 2.4a).

We then checked the expression level of the auxin-responsive marker genes in the mutant and wild-type lines (Fig. 2.4b). In absence of external auxin, the *IAA1a*$^{\Delta ear}$ mutant had a higher level of auxin-responsive gene transcription compared to wild-type. Moreover, external auxin treatment resulted in an increased gene transcription compared to its mock condition, which correlates with the phenotypes above and suggests that IAA1a$^{\Delta ear}$ is still able to perform some repression before being directed for ubiquitination and degradation by auxin binding.

When aligning the moss and Arabidopsis Aux/IAA sequences, there is another leucine-rich region between Domain I and II in AtIAA7 (LMLNL) that is similar to the EAR motif, and the corresponding region in PpIAA1a is LKVHL. We generated another

transgenic line carrying deletion in both this region and the EAR motif, *IAA1a*$^{\Delta ear\Delta ear\text{-}like}$, and it did not show any difference from the *IAA1a*$^{\Delta ear}$ line. These results indicate that one or more EAR-independent repression mechanisms exist in the auxin signaling pathway. Since complete knockout of *IAA1a* in the *aux/iaa* null background results in fully constitutive auxin responses, these mechanisms must be mediated by some elements within the IAA1a protein.

One possible mechanism involves Aux/IAA oligomerization through the C-terminal PB1 domain. Since this domain is conserved in PpIAA1a (Fig. 2.1), we used the *PpIAA1a-Luc*/*Δaux/iaa* system to further test this hypothesis and to explore whether oligomerization contributes to an EAR-independent repression mechanism.

Mutants of putative C-terminus centers indicate repression by both single and oligo-Aux/IAAs in natural condition

We generated transgenic moss lines expressing a mutant *IAA1a* in which key amino acids in the acidic (D435 and D439) and basic (K362) centers are replaced with alanine (*IAA1a*^{K,OPCA}). An Y2H assay showed that these mutation disrupted IAA1a self-interaction and interaction with ARFs (Supplemental Fig. 2.1b). Phenotypic characterization of the transgenic line revealed that the *IAA1a*^{K,OPCA} line had an auxin constitutive phenotype similar to that of Δ*aux/iaa* null mutant, with stunted, undifferentiated and leafless filaments. It was also completely insensitive to exogenous auxin with respect to both morphology and gene expression (Fig. 2.5a and 2.5b).

Therefore, the three conserved amino acids in the IAA1a PB1 are required for its repressive function *in vivo.* This result confirms that the repressive function of the Aux/IAA protein is totally dependent on interactions involving the PB1 domain.

We then generated and characterized a mutant line carrying mutation in only one of the two interaction centers, *IAA1a* ^{K362A}. Again, Y2H assays confirmed that this single mutation disrupts the IAA1a self-interaction but retains its ability to interact with an ARF protein (Supplemental Fig. 2.1c).

When grown in plain media, the *IAA1a* ^{K362A} mutant had a higher level of auxin-related phenotypes compared to wild-type plants, represented by increased branching of caulonema filaments, reduced leafy gametophores and darker brown-pigmented rhizoids. The expression level of the auxin-responsive marker genes also indicated hypersensitivity to auxin, correlating with the phenotypes (Fig. 2.5a, b). This provides the evidence in the natural condition that Aux/IAA dimerization or oligomerization is required to achieve its full repression activity.

On the other hand, the increased auxin-response phenotype of *PpIAA1a*^{K362A} is still less severe than the Δ*aux/iaa* mutant. This differs from the results described in Korasick et al., (2014). In that case, mutation of the conserved K in the background of a stabilized gain-of-function form of AtIAA16, completely suppressed the effects of the dominant mutation. In our study, the effects of mutating the lysine were quite mild and intermediate, indicating that natural form of IAA1a monomer is still capable of repressing the downstream auxin responses.

When grown on media with exogenous auxin, the *PpIAA1a* ^{K362A} mutant shows a further increase in rhizoid formation, the complete elimination of leafy gametophores, and

induction of the marker gene expression. This further confirms that while interaction between Aux/IAA proteins is required for their full activity as auxin-signaling repressors, the single form is able to act as a partial repressor of the pathway.

Since the lysine and aspartic acid residues in the two interaction centers are highly conserved among the Aux/IAAs and ARFs in both Arabidopsis and moss genomes, the two-center interaction can result in a variety of oligomerization combinations among different Aux/IAAs and ARFs. This in turn could result in finely tuned repression, especially in Arabidopsis since it has 29 Aux/IAA members. Apart from the variety provided by auxin co-receptor combination and the fine-tuned temporal and spatial Aux/IAA expression pattern, the PB1-based interaction may contribute to the complexity of the auxin response.

Discussion

The clear auxin-related phenotypes and the reduced *Aux/IAA* gene redundancy make moss a good system for unraveling the mechanism of auxin signal transduction. The fact that the Aux/IAA proteins in moss and *Arabidopsis* share conserved structural motifs makes it possible to combine the knowledge and insights gained from previous *Arabidopsis* studies with the advantage of a simple genetic background in moss.

In our analysis of wild-type moss *Aux/IAA* expression pattern, *IAA1a* was expressed at similar levels in gametophores and filaments, while *IAA1b* and *IAA2* were expressed at a higher level in gametophores. In the *iaa1b iaa2* double knockout, on the other hand, *IAA1a* transcription was up-regulated to the same level as that of all the three *IAAs* in wild type, both in filaments and in gametophores, suggesting that *IAA1a* transcription is regulated to compensate for the loss of the other two *IAA* transcripts.

Considering the fact that loss of the *IAA1b* and *IAA2* genes does not result in an obvious phenotype, this also illustrates the redundancy in terms of the Aux/IAA protein function, which is likely achieved by the high homology in essential domains shared by the three proteins (Fig. 2.1).

The compensation of *IAA1a* expression in the double knockout suggests that this line has a wild-type level of Aux/IAA function. Based on this, we used homologous recombination to generate a series of tagged *IAA1a* lines in this knockout background (*PpIAA1a-Luc/Δaux/iaa*) carrying mutations of interest so as to study their impacts on protein behavior and developmental outcomes without the interference of other native Aux/IAAs. This is not only the first system that allows for *in vivo* study of a single intact Aux/IAA member in a null background, but also a system in which the reference line is close to the wild-type natural condition.

Although the degron motif is known to be the surface for TIR1/AFB-Aux/IAA interaction, our knowledge of how variations in the degron sequence influence protein stability is still quite limited. Apart from the gain-of-function mutants identified in forward genetic screens which lead to stabilized Aux/IAAs, it is only known that substitution of the proline or valine with alanine results in slower protein degradation and higher protein accumulation in transient assays. In our system, we compared the natural variations in the degron motif among the *Arabidopsis* and moss genomes and identified the last position in the core VGWPPVR motif as the novel point that influences the protein stability and function. Moreover, we showed that the VGWPPIG variant, which showed a lower auxin-binding affinity in the co-receptor system in Y2H assays, also shows a higher threshold for auxin-induced degradation *in vivo*. Furthermore, compared

to other degron variants investigated, it has a lower degradation rate under the same auxin concentration and leads to reduced auxin-responsive gene transcription and auxin-hyposensitive phenotypes. This is the first *in vivo* demonstration of the link between auxin-binding affinity, Aux/IAA stability and the level of downstream auxin responses. Besides, our results showed the effects of degron variations alone with other parts of the Aux/IAA protein kept the same, adding an important aspect to the knowledge on Aux/IAA stability, in parallel to previous studies on Aux/IAAs with the same degron motif but different half lives demonstrating the influence of factors outside of the degron motif.

Another major topic about Aux/IAAs is how they repress the activation of downstream genes. For a long time, recruitment of TPL and other co-repressors by the EAR motif was the only known mechanism. While moss Aux/IAAs also interact with TPLs, we found that deletion of the EAR motif does not fully abolish their repressive activity. The fact that the *PpIAA1a ear-Luc*/*Δaux/iaa* is still responsive to auxin suggests that Aux/IAAs are able to repress auxin responses in a TPL-independent way. Recently, the structures of several Aux/IAA and ARF C-terminal PBI domain were resolved, and oligomerization of Aux/IAAs through their acidic and basic centers in PBI has been proposed as another possible mechanism for repression, but the *in vivo* evidence is limited to stabilized *Aux/IAA* mutant backgrounds. Also, overexpression of stabilized AtIAA16 with mutation in a single interaction center results in the same wild-type phenotype as that with double mutations, indicating that IAA16 monomer does not have any repressing activity in this situation, which may or may not be true in natural genetic backgrounds.

Utilizing our *PpIAA1a-Luc*/*Δaux/iaa* lines, we showed the significance of Aux/IAA oligomerization in repression in a natural background. The constitutive auxin phenotype of $PpIAA1a^{K,OPCA}$ is the same as the *Δaux/iaa* null mutant, confirming that the conserved K and DxD/ExD are the two interaction centers for the PBI of Aux/IAAs and ARFs. Mutation of the conserved K results in an auxin-hypersensitive phenotype and increased auxin-responsive gene transcription, suggesting that Aux/IAA oligomerization is required to achieve the full repressive function. Furthermore, $PpIAA1a^{K362A}$ is still responsive to auxin at both phenotypic and molecular levels, indicating that Aux/IAA monomer actually retains partial repression activity in the natural genetic background.

In wild-type moss and Arabidopsis where both Aux/IAAs and ARFs have multiple variants, there are a large number of combination options for oligomerization among Aux/IAAs and ARFs, which is possible to confer a wide range of repressing strengths. This layer of diversity is in parallel with the variation in the Aux/IAA degron mentioned above, which confers difference in auxin perception and Aux/IAA stability. Taken together, these provide new insight into how the simple-structured auxin molecule triggers complex developmental outcomes.

A recent publication showed the interaction between Arabidopsis ARFs and the BRM and SYD subunits of the SWI/SNF chromatin remodeling ATPase, which mediates changes in chromatin required for gene activation. In yeast three-hybrid (Y3H) assays, this interaction was disrupted in the presence of Aux/IAAs. To investigate whether the same mechanism exists in moss, we cloned the two *BRM* and one *SYD* candidates in the moss genome that share more than 70% homology with those of Arabidopsis, but they did not interact with moss ARFs in Y2H (data not shown). Nonetheless, it is possible that

moss Aux/IAAs repress the activity of ARFs by blocking their interaction with certain unknown ARF-activating proteins, and oligomerization of Aux/IAAs may act to enhance this blockage.

Materials and Methods

Moss strains and growth conditions

Wild-type 'Gransden-2004' and mutant *P. patens* strains were grown at 25°C under continuous light at an intensity of 40-70 μmol/m2/s. When grown for phenotypic and gene expression analysis, BCD medium was used. When grown for propagation purpose, BCDAT (BCD supplemented with 5 mM Ammonium Tartrate) was used.

Molecular cloning of gene expression constructs

To make the construct for the expression of Luciferase-tagged IAA1a, the following DNA fragments were PCR-amplified with primers containing the corresponding restriction enzyme (RE) cutting sequences and cloned into the RE sites of the pBHRF2 backbone (Lavy *et al*., 2016) by enzyme digestion and ligation: the Luciferase-coding sequence was cloned into the *PstI* and *XbaI* sites; the cDNA of *IAA1a* was cloned into the *PstI* site; approximately one kilobase (kb) of the genomic sequence upstream (5') and downstream (3') of the *IAA1a* gene coding region were cloned into the *HindIII* and *SpeI* sites. This creates the *IAA1a 5' genomic:cIAA1a:Luciferase:Hyg^R (from backbone, with LoxP sites):IAA1a 3' genomic* cassette for the expression of Luciferase-tagged IAA1a in replacement of the native *IAA1a* gene. To make the constructs for expressing mutant versions of IAA1a-Luciferase, site-directed mutagenesis was done

with the construct above containing wild-type *IAA1a*. The primer sequences used for the cloning and mutagenesis were listed in Table 2.1.

Moss transformation and transgenic lines screening

Protoplast isolation and PEG-mediated transformation of the above constructs into the *iaa1b iaa2* double mutant (Lavy et al., 2016) was performed as described in (Nishiyama et al., 2000). After five days of regeneration, transformants were moved to BCDAT medium supplemented with 20mg/l hygromycin for selection. The transformants that survived the selection were screened by PCR for the presence of both left and right transgene-endogenous sequence junctions to verify the insertion of the transgene to the native locus. RT-PCR with a pair of primers flanking an *IAA1a* intron was performed to confirm the replacement of genomic IAA1a with the cIAA1a-Luc transgene. The primer sequences were listed in Table 2.1.

To remove the possible tandem repeats generated by homologous recombination, the pActin:CRE plasmid was transformed into the confirmed lines above. This removes the possible repeats as well as the hygromycin resistant marker. Therefore, the transformants that cannot survive on hygromycin-containing medium were used for further analysis.

Phenotype observation

For each line to be observed, small pieces of fresh protonemal tissue were inoculated on BCD mock or NAA-supplemented media and grown for one month into well-developed colonies. Photos were taken for each colony and typical representations were shown.

RNA isolation and qRT-PCR

For detection of tissue-specific *Aux/IAA* gene transcripts, protonemal tissue was grown on BCD plates with cellophane overlays for three weeks. Leafy gametophores were cut from the remaining filamentous tissue and the two types of tissue were collected individually for RNA isolation. For detection of auxin-responsive marker gene transcripts, protonemal tissue was grown on BCDAT plates with cellophane overlays for one week. Then the colonies were transferred into liquid BCD medium containing either 10 µM IAA or the equivalent amount of solvent (ethanol). After incubation under the moss growth condition, the colonies were collected individually for RNA isolation.

Total RNA was isolated using RNeasy plant mini kit (Qiagen). 500 ng RNA was reverse transcribed using the Superscript III First Strand cDNA Synthesis System (Life Technologies). 20 µl RT reaction was diluted to the final volumn of 200 µl. 4 µl of the diluted cDNA was used for detection by the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The primers used for detection of marker genes were described in Lavy *et al*., 2016. Normalized expression (ΔΔC(t) method) was calculated using the Bio-Rad CFX manager software using *PpEF1a* as a reference gene and plotted as relative values ± SEM. Four replicates were included in each analysis. T-test was used for statistical inference.

Luciferase-based degradation assay

Fresh protonemal tissue blended in sterile water with a homogenizer was spread and grown on BCDAT plates with cellophane overlays for four days. For each replicate of the transgenic lines of interest, two tissue samples of a fixed amount (25 mm X 25 mm area on the plate) were taken and each was blended in 50 µl of 10 mM D-Luciferin in a

well of the 96-well plate, and their chemiluminescence was measured by ImageQuant LAS 4000 Mini biomolecular imager (GE Healthcare Life Sciences) under super resolution and quantified by ImageJ. This serves as the time point 0 reference for mock/auxin treatment.

After either IAA stock solution or an equivalent volume of the solvent was added to the wells, the chemiluminescence was quantified in the same way at different time points. For data from each time point, the ratio of the signal strength of treated versus mock was calculated and normalized by the ratio of time point 0 for adjustment of difference in starting amount between the mock/treated samples. The normalized ratio represents the fold that remains after the corresponding time of auxin treatment. The degradation curve was made by plotting this normalized ratio against treatment time. Three replicates were used for each data point, and the error bar represents the SEM.

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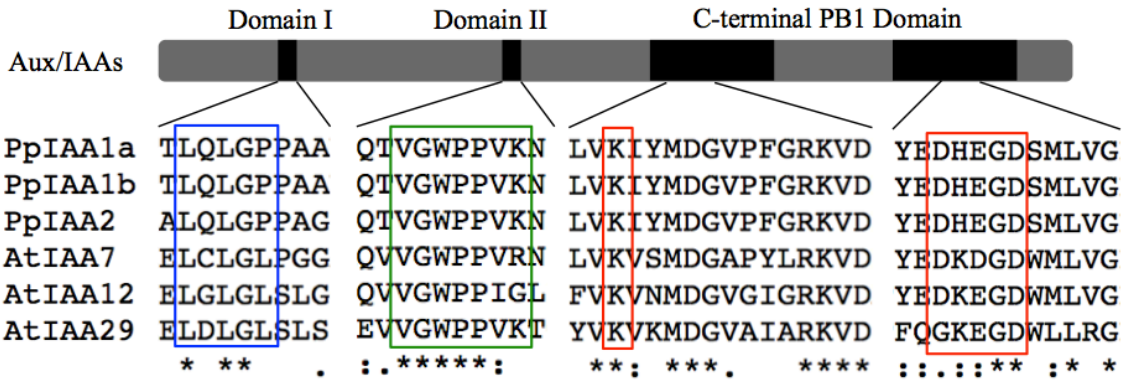


Figure 2.1. The Arabidopsis and moss Aux/IAA proteins share conserved elements in all the four functional domains. Blue box: the EAR motif in Domain I; Green box: the core degron motif in Domain II; Red box: K and OPCA motifs in Domain III/IV (also known as C-terminal interaction domain or PBI domain) which are highly conserved not only among Aux/IAAs but also among the ARF transcription factors.

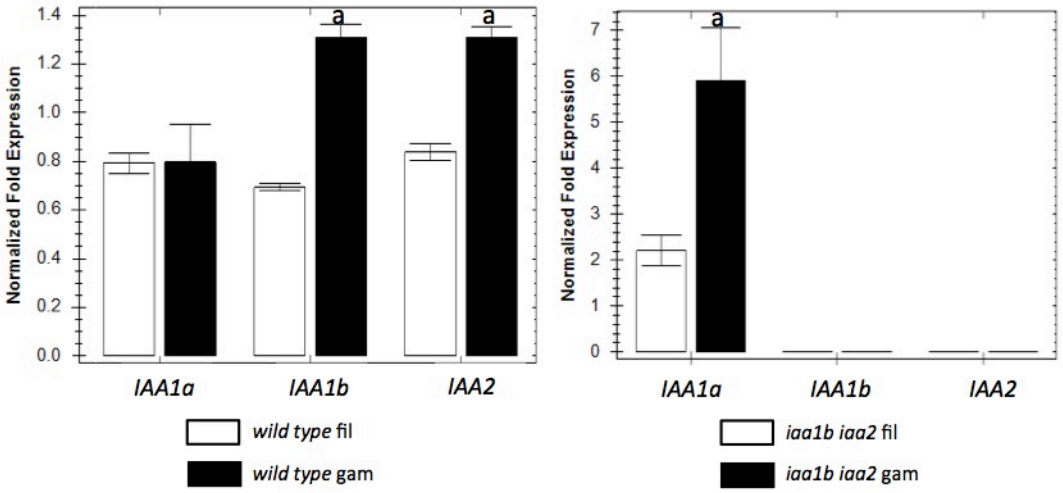


Figure 2.2. Expression pattern of the three *Aux/IAA* genes in the moss filaments and leafy gametophores checked by qPCR. Black: the gametophore (gam) sample; white: the remaining filamentous (fil) sample. Normalized expression (ΔΔC(t) method using *PpEF1a* as a reference gene) was plotted as relative values ± SEM. a: $p<0.05$ (t-test), n=4 comparing to the other tissue type for the same target gene expression.

Figure 2.3. Characterization of natural variations in the Aux/IAA degron motif. (a) The developmental phenotypes of *PpIAA1a-luc/Δaux/iaa* carrying different degron motif versions grown in BCD media with different auxin (NAA) levels, with *Δaux/iaa* null mutant as a control. The leafy gametophore formation, which indicates resistance to external auxin, is blocked in $IAA1a^{degVK}$ under 0.1 μM NAA, but still active in $IAA1a^{degVR}$ (red arrow); $IAA1a^{degIG}$ is resistant to even higher NAA concentrations compared to the other lines. (b) qPCR of auxin-responsive marker genes in the above lines treated with mock (-) or 10 μM IAA (+) for one hour. White and black: $IAA1a^{degVK}$; yellow: $IAA1a^{degVR}$; green: $IAA1a^{degIG}$; darker colors represent 10uM IAA treated samples. Normalized expression (ΔΔC(t) method using *PpEF1a* as a reference gene) was plotted as relative values ± SEM. a/b: $p<0.05$ (t-test), n=4; a: comparing to the wild-type $IAA1a^{degVK}$ in the corresponding condition; b: comparing to the $IAA1a^{degVR}$ in the corresponding condition. (c) *In vivo* degradation curve of different IAA1a versions upon 0.2 μM and 20 μM IAA treatments measured by Luciferase chemiluminescence. The normalized ratio, which is the chemiluminescence from the auxin-treated sample relative to the mock sample, was plotted against the treatment time. Error bar represents s.e.m. (n=3). (d) Comparison of normalized remaining ratio among the three variants after 550 seconds of IAA treatments. a/b: $p<0.05$ (t-test), n=3; a: comparing to the wild-type $IAA1a^{degVK}$ in the corresponding condition; b: comparing to the $IAA1a^{degVR}$ in the corresponding condition.

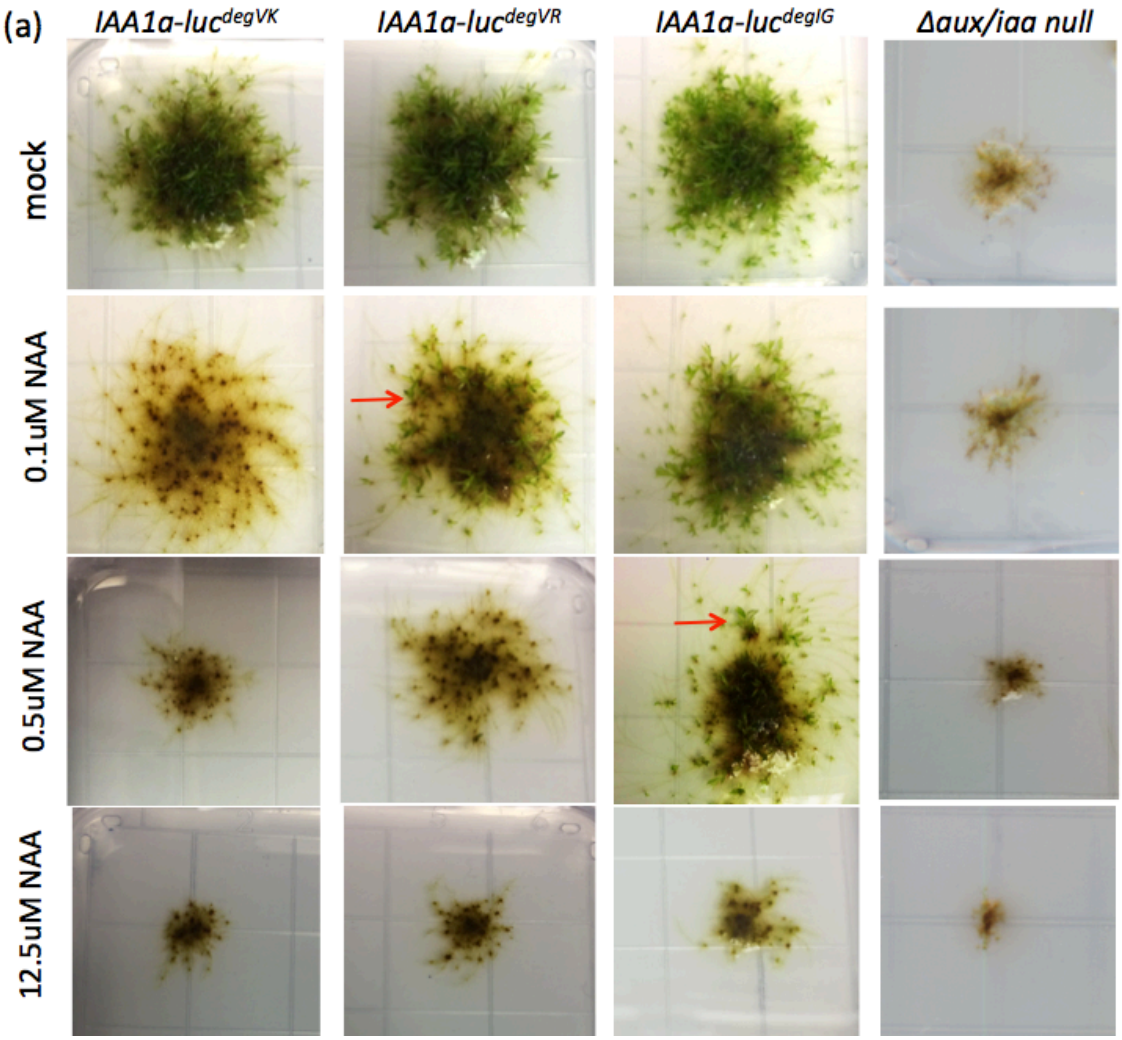


Figure 2.3. Characterization of natural variations in the Aux/IAA degron motif. (continued)

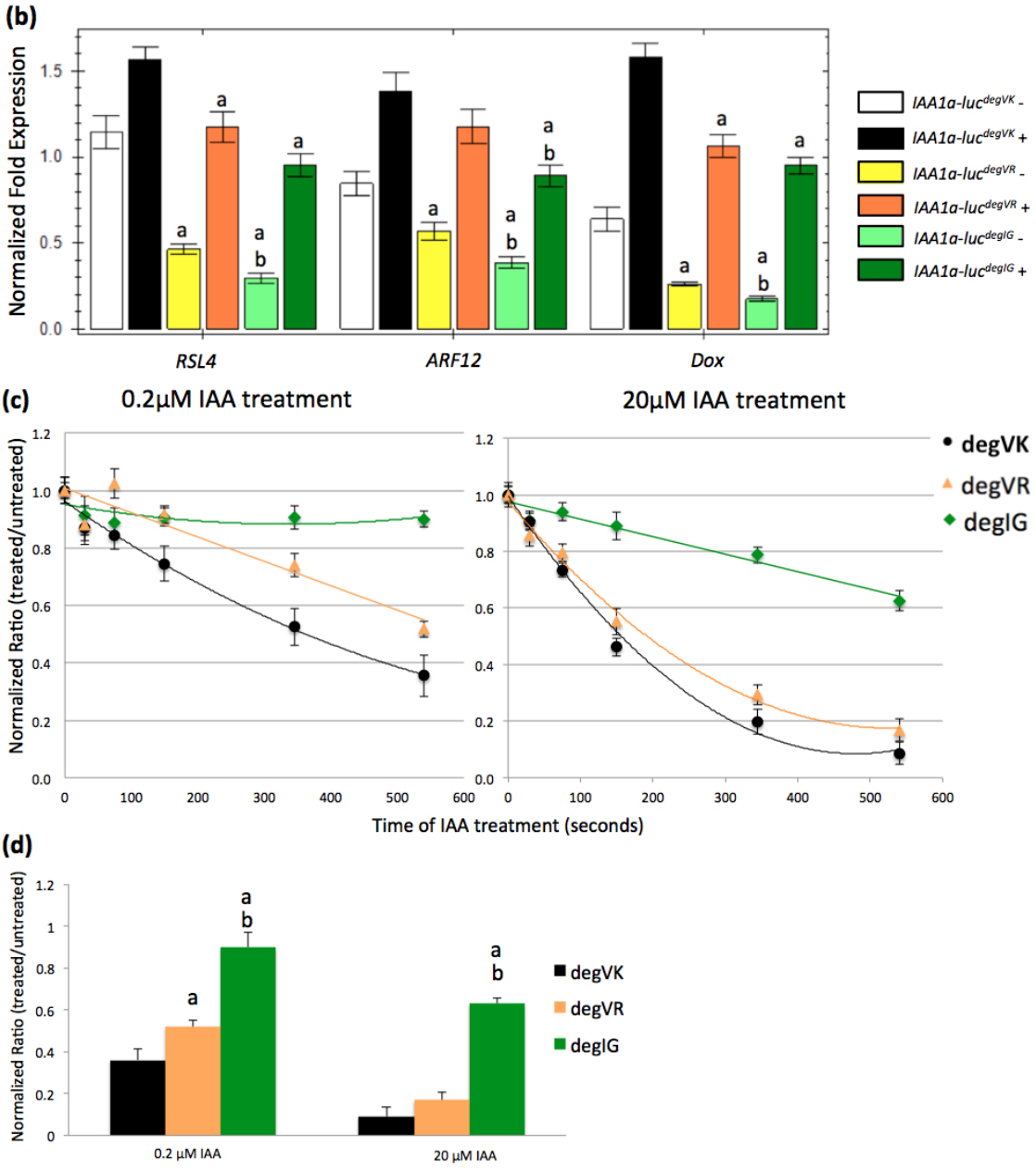


Figure 2.3. Characterization of natural variations in the Aux/IAA degron motif. (continued)

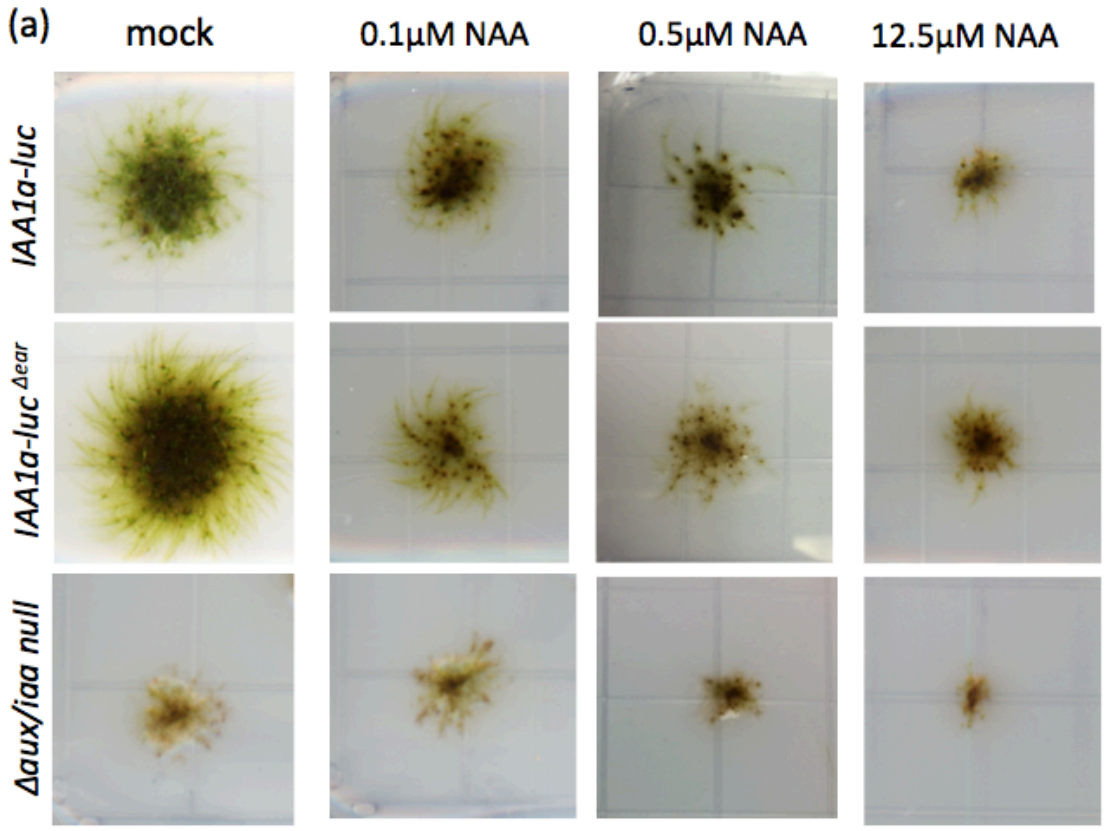


Figure 2.4. The EAR motif contributes to repression but is not essential. (a) The developmental phenotypes of *PpIAA1a-luc* $^{\Delta ear}$/*Δaux/iaa* mutant compared to wild type and the *Δaux/iaa* null mutant grown on medium supplemented with different levels of auxin. *IAA1a-luc* $^{\Delta ear}$ showed reduced leafy gametophores in plain medium compared to wild type, but was much healthier than the null mutant, and it retained responsiveness to auxin treatments. (b) qPCR of auxin-responsive marker genes in the above lines treated with mock (-) and 10 μM IAA (+) for one hour. White and black: *IAA1a-luc*; pink: *IAA1a-luc* $^{\Delta ear}$; grey: *Δaux/iaa* null mutant; darker colors represent 10 μM IAA treated samples. Normalized expression (ΔΔC(t) method using *PpEF1a* as a reference gene) was plotted as relative values ± SEM. a/b/s: $p<0.05$ (t-test), n=4; a: higher induction level than the wild-type *IAA1a-luc* in the corresponding condition; b: lower induction level than the Δ*aux/iaa null* mutant in the corresponding condition; s: different from the line itself in the other condition.

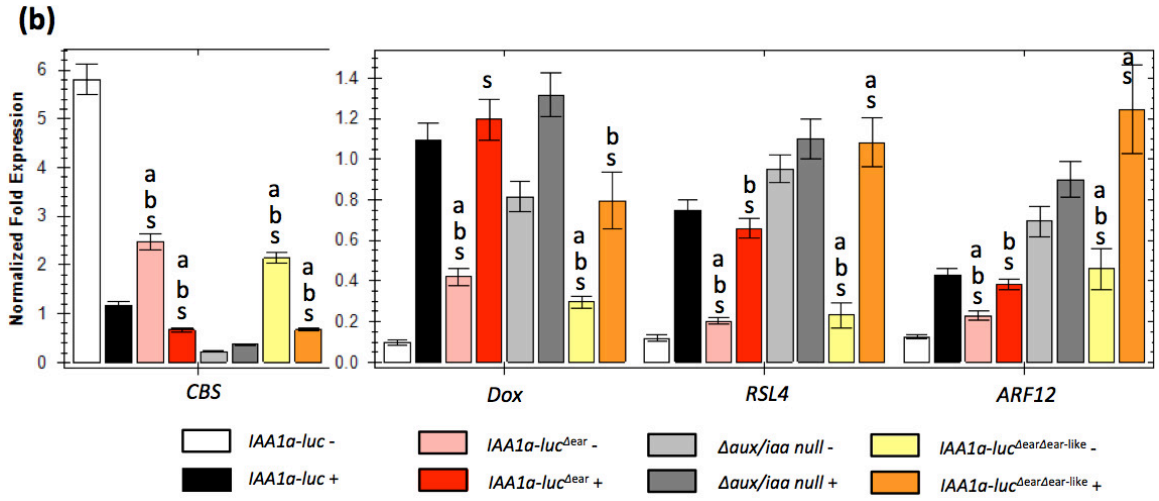


Figure 2.4. The EAR motif contributes to repression but is not essential. (continued)

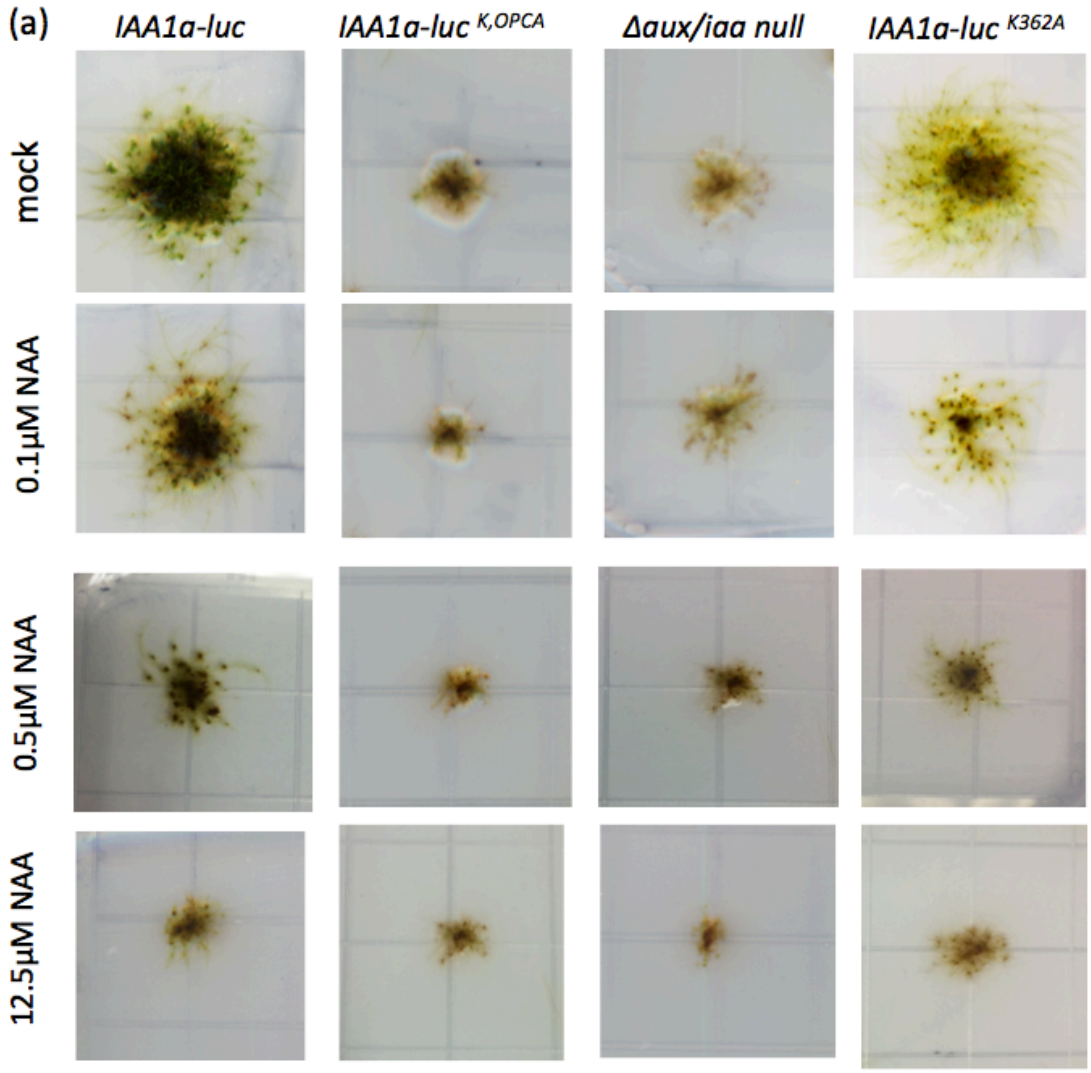


Figure 2.5. The PB1 domain has an essential role in Aux/IAA function but oligomerization is not required for repression. (a) The developmental phenotypes of *K* and *opca* single and double mutants compared to wild type and *Δaux/iaa* null mutant grown on medium with different auxin levels. (b) qPCR of auxin-responsive marker genes in the above lines treated with mock (-) and 10 μM IAA (+) for one hour. White and black: *IAA1a-luc*; blue: *IAA1a-luc* ^{K362A}; purple: *IAA1a-luc* $^{K, OPCA}$; grey: *Δaux/iaa* null mutant; darker colors represent 10μM IAA treated samples. Normalized expression (ΔΔC(t) method using *PpEF1a* as a reference gene) was plotted as relative values ± SEM. a/b/s: $p<0.05$ (t-test), n=4; a: higher induction level than the wild-type *IAA1a-luc* in the corresponding condition; b: lower induction level than the Δ*aux/iaa null* mutant in the corresponding condition; s: different from the line itself in the other condition.

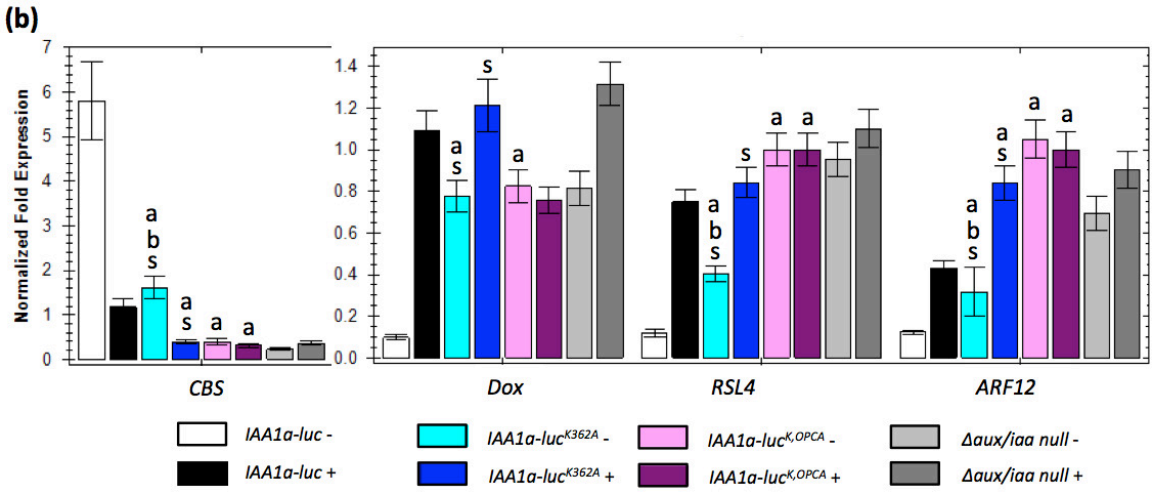
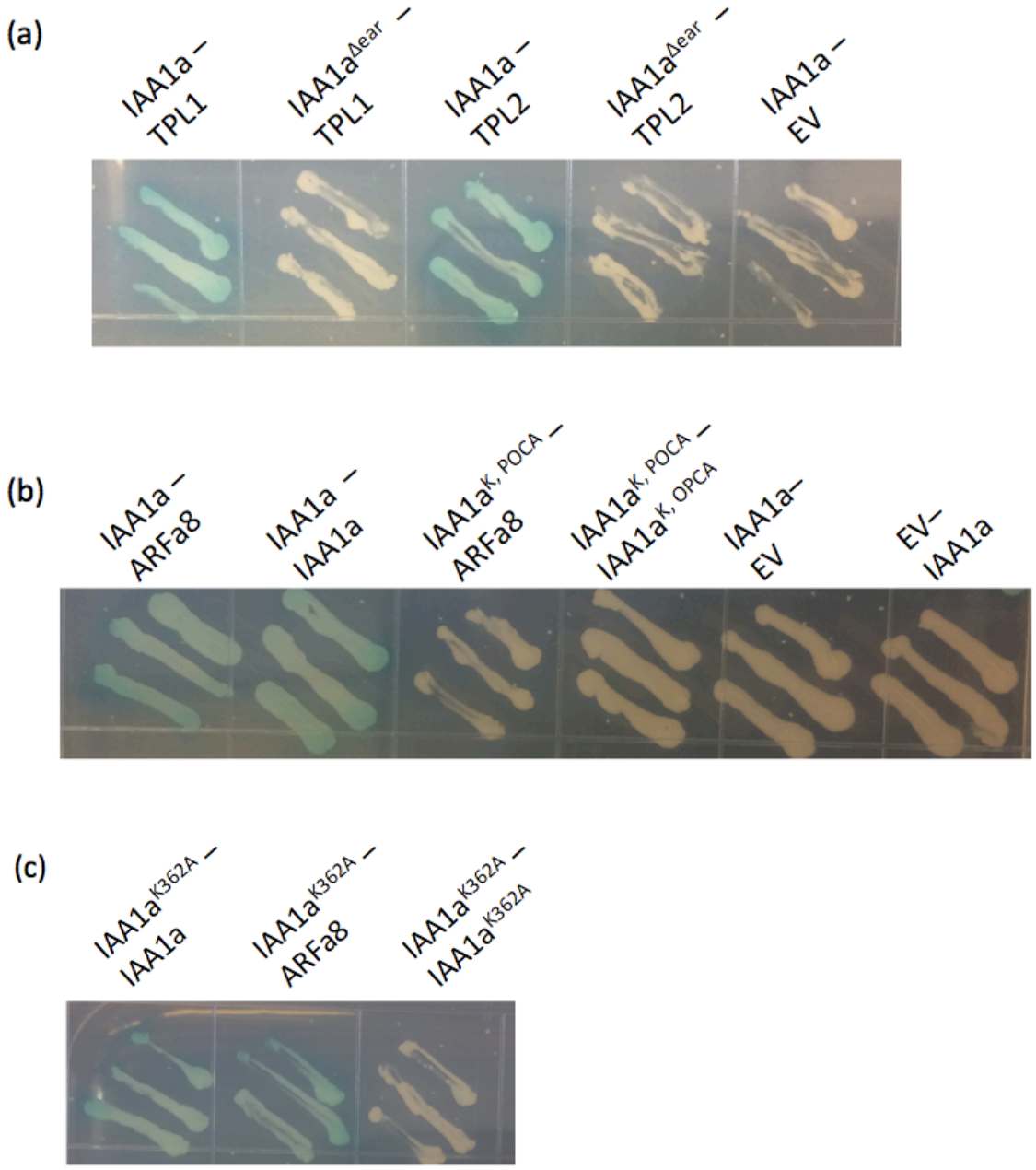
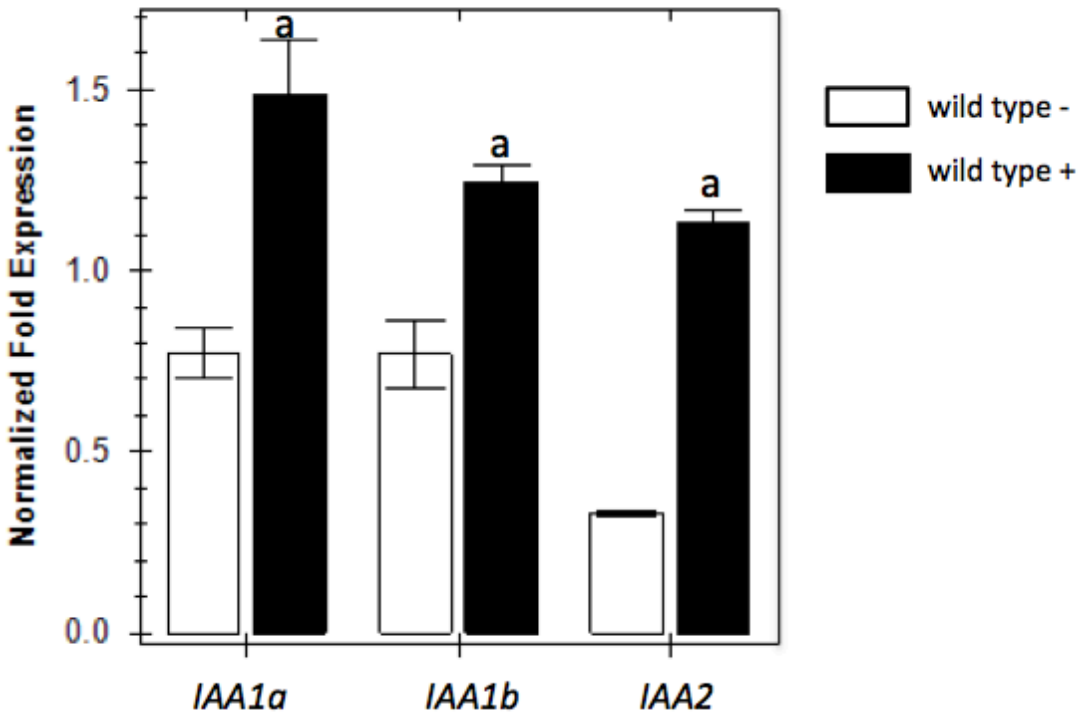


Figure 2.5. The PB1 domain has an essential role in Aux/IAA function but oligomerization is not required for repression. (continued)



Supplemental Figure 2.1. Yeast 2-hybrid analysis of the effects of IAA1a mutations on protein interaction. (a) IAA1a$^{\Delta ear}$ did not show interaction with the moss TPL1 or TPL2 proteins. (b) K and OPCA mutations disrupted the IAA1a self-interaction and its interaction with moss ARFa8. (c) IAA1a^{K362A} did not show self-interaction but still interacted with proteins that had intact PBI domain like ARFa8 and IAA1a. EV: empty vector expressing only the Y2H DNA-binding domain or activation domain.



Supplemental Figure 2.2: qPCR of the *Aux/IAA* gene expression level in the wild-type moss protonemata treated with mock (-) and 10 μM IAA (+). All the three *Aux/IAA* genes encoding the auxin signaling repressors are up-regulated upon auxin treatment, indicating a negative feedback of the signal transduction. Error bars represent SEM. a: $p<0.05$ (t-test), n=4 comparing mock and IAA treated samples for the same target gene expression.

Table 2.1 Primer sequences used in the work described in this chapter.

Primer name	Sequence	Usage
PstI-Luc-F	CGCGACCTGCAGATGGAAGACGCCAAAAACAT	Cloning of *Luciferase*
Luc-XbaI-R	CGTCTGTCTAGATTACACGGCGATCTTTCCG	
PstI-IAA1a-F	CGCGACCTGCAGATGAACGTCAGCGAAGGTTG	Cloning of *IAA1a*
IAA1a-PstI-R	ATGCCTGCAGCCCCACCGCCACTTGC	
HindIII-IAA1a5'g-F	ATGCAAGCTTGATTGAAGAGTGGCAGTGGGTG	Cloning of *IAA1a* 5' genomic DNA
IAA1a5'g-HindIII-R	CGTCTGAAGCTTTGAATAATGTCCAACTTCACTGCTAAAAG	
SpeI-IAA1a3'g-F	CACCACTAGTGGAGATTGCAATGGCGGGATGTGCG	Cloning of *IAA1a* 3' genomic DNA
IAA1a3'g-SpeI-R	CCACTAGTCGTAAAATACTTACAATCATGAAATCCTTCCC	
K325R-F	GTGGGTTGGCCACCCGTGAGGAACTTCAACAAGATGAAC	IAA1aK325R mutagenesis
K325R-R	GTTCATCTTGTTGAAGTTCCTCACGGGTGGCCAACCCAC	
VK-F	GTGGGTTGGCCACCCATCGGGAACTTCAACAAGATGAAC	V324IK325G mutagenesis
VK-R	GTTCATCTTGTTGAAGTTCCCGATGGGTGGCCAACCCAC	
K326A-F	CGAGCGGGAACCTTGTGGCGATCTACATGGATGGTGTG	IAA1aK362A mutagenesis
K362A-R	CACACCATCCATGTAGATCGCCACAAGGTTCCCGCTCG	
D435A-F	GTGTTGATATACGAGGCTCACGAGGGAGACTCG	IAA1aD435A mutagenesis
D435A-R	CGAGTCTCCCTCGTGAGCCTCGTATATCAACAC	
D439A-F	GGCTCACGAGGGAGCCTCGATGCTGGTCGG	IAA1aD439A mutagenesis
D439A-R	CCGACCAGCATCGAGGCTCCCTCGTGAGCC	
ear-F1	GAACGAAAGTGCCTCGGCCCTCCTGCGGC	IAA1aΔear mutagenesis
ear-R1	GCCGCAGGAGGGCCGAGGCACTTTCGTTC	
ear-F2	GAATGGAACAGAGCAGCTGGCACTTGCCGAAAGGC	IAA1aΔear mutagenesis
ear-R2	GCCTTTCGGCAAGTGC CAGCTGCTCTGTTCCATTC	
EF1α-F	ACGCGTTGTTGGCTTTCACTTTG	qPCR
EF1α-R	GTGGTTGCGTCCATCTTGTTGC	qPCR
IAA1a-F	ATCCGGGAGTCCGAGCTTC	qPCR
IAA1a-R	GGTTCTGCGCAGGAGGTG	qPCR
IAA1b-F	CGGTGGTCAGAATGGGTCA	qPCR
IAA1b-R	CCCACAGTCTGGTTCTGCG	qPCR
IAA2-F	TGCCTTGGGACTGGTTCATC	qPCR
IAA2-R	CACAGCACCTTGGGCTTTCA	qPCR
CBS-F	TTGGAAAGACCGCCCAGCTATC	qPCR
CBS-R	GCTCCGTTAAACTCTCAGAACCAC	qPCR
Dox-F	AGCGATCCCACCAAATTCAGCTC	qPCR
Dox-R	TGAACAACGTGGGCTCCAATCC	qPCR
RSL4-F	TCAAACGGCCGAAACATTCTACG	qPCR
RSL4-R	CAGCTCCGCTCCTTTCAGAATATG	qPCR

Chapter 2, in part is currently being prepared for submission for publication of the material. Tao, Sibo; Estelle, Mark. The dissertation author was the primary researcher and author of this work.

Chapter 3

Deletion of the Aux/IAA repressors reveals different *in vivo* functions of ARFs

Introduction

As mentioned in Chapter 1, ARFs are a family of transcription factors that bind to the promoter of auxin-responsive genes and activate their transcription. The majority of ARFs are composed of a DNA-binding domain, a middle region and a PB1 domain that is homologous to the Aux/IAA PB1 domain.

The ARFs have been characterized as activating ARFs or repressing ARFs based on their behavior in *in vitro* transcription assays and in transient protoplast assays (Guilfoyle and Hagen, 2007; Tiwari et al., 2003; Ulmasov et al., 1999). These two types of ARFs differ mainly in their middle region (MR). In activating ARFs (aARFs), the MR interacts with SWI/SNF chromatin remodeling ATPases that mediate changes in chromatin required for gene activation (Wu et al., 2015). On the other hand, the repressing activity of the putative repressing ARFs (rARFs) has not been demonstrated in plants, and the mechanism of repression is unknown.

Phylogenetic analysis of the ARF proteins among the land plants indicates that there are 8 moss ARFs (ARFa1 –ARFa8) clustering together with the Arabidopsis activating ARFs, and 4 ARFs clustering with the Arabidopsis repressing ARFs (ARFb1 – ARFb4) (Lavy et al., 2016).

Since there are multiple *ARF* genes in the Arabidopsis and moss genome, we used the overexpression approach to study the functions of the two putative ARF groups. We introduced vectors overexpressing a representative aARF (ARFa8) and a representative rARF (ARFb4) both into the WT background and into the *Δaux/iaa* knockout background mentioned in the previous chapter, and compared the phenotype and auxin-responsive gene expression in the verified transgenic lines. Comparison of the gene profiles in these

lines revealed different *in planta* functions of the aARFs and rARFs as well as complex interactions among the Aux/IAAs and ARFs in regulating auxin-induced transcriptional changes.

Results

Overexpression of putative activating and repressing ARFs in the WT background

Since the aARFs and rARFs showed distinct activities in transcriptional assays *in vitro*, we initially asked whether overexpression of these two types of ARFs have opposite effects *in vivo.* We performed mock/auxin treatment with lines overexpressing AFBb4 *(ARFb4-OE/WT),* ARFa8 (*ARFa8-OE/WT)* and a truncated form of ARFb4 lacking the PB1 domain (*ARFb4ΔPB1-OE/WT*) driven by UBI promoter, and extracted their total RNA for qPCR analysis of auxin-responsive marker genes identified from previous RNA sequencing experiments. The qPCR analysis revealed that, unlike the results in the *in vitro* assays, overexpression of both activating (ARFa8) and repressing (ARFb4) generally showed increased marker gene transcription compared to the WT in the mock and IAA-treated conditions (Figure 3.1). Except for *ARFb4ΔPB1-OE/WT*, IAA treatment resulted in up-regulation of marker gene transcription. It is also important to note that deletion of ARFb4 PB1 resulted in a significant change compared to the wild-type protein, indicating that apart from the aARFs, the rARFs are also regulated by the Aux/IAAs through interaction with the PB1 domain.

Since the *Aux/IAA* genes are themselves regulated by auxin, accumulation of active ARFs should affect *Aux/IAA* transcription. Therefore, the effects of ARF

overexpression may be influenced by a possible change in the level of Aux/IAA proteins. Thus, it is still uncertain whether the increase in auxin-regulated gene expression in the rARF overexpress is due to gene activation by the rARF, or to rARF-mediated repression of *Aux/IAA* expression.

Overexpression of the representative ARFs in the *Δaux/iaa* background

To simplify the situation, we overexpressed these representative ARFs in the *Δaux/iaa* triple mutant background, where all the ARFs are free from repression by Aux/IAAs. For ARFb4, we obtained two independent lines, *ARFb4-OE/Δaux/iaa-12* and *ARFb4-OE/Δaux/iaa-36,* for further study, and both showed less severe phenotypes compared to the *Δaux/iaa* triple mutant. For ARFa8, however, we did not recover any viable transformants that overexpressed the ARFa8 protein. It is likely that overexpression of the activating ARFs in the auxin constitutive *Δaux/iaa* triple mutant results in lethality. This indicates that rARFs and aARFs are likely to have different functions *in vivo*. In addition to the moss ARFs, AtARF1, which is an rARF in Arabidopsis, was also overexpressed in the WT and *Δaux/iaa* backgrounds to compare the outcomes.

We first did a one-hour mock/IAA treatment with the *ARFb4* and *AtARF1* lines as well as the WT and *Δaux/iaa* triple mutant, and compared their marker gene expression by qPCR, as shown in Figure 3.2. Comparing the difference in the two independent *ARFb4-OE/Δaux/iaa* lines we obtained, we found that the *ARFb4-OE/Δaux/iaa-36* had stronger ARFb4 expression level than the other line, *ARFb4-OE/Δaux/iaa-12*. The majority of the marker genes had lower expression level in *ARFb4-OE/Δaux/iaa-36* than

in *ARFb4-OE/Δaux/iaa-12* as well as the triple mutant. This means that higher *ARFb4* expression resulted in a reduction in marker gene expression level, which differs from the results in previous experiments using ARF overexpression lines in the WT background. On the other hand, the comparison between the *ARFb4-OE/Δaux/iaa* lines with the triple mutant indicates that the presence of higher level of ARFb4 in Line 36 lead to lower marker gene expression levels than those in Line 12 and triple mutant. Taken together, these results support the hypothesis that ARFb4 represses gene transcription *in vivo*. The increased marker gene expression in the *ARFb4-OE/WT* lines lines may be due to the down-regulation of Aux/IAA as repressors because of the added repression by *rARF* overexpression.

Similarly, comparison between the two independent At*ARF1-OE/Δaux/iaa* lines and the triple mutant showed that *AtARF1-OE/Δaux/iaa-1* overexpression level was higher than the other line, and the AtARF1 also had *in vivo* repressive function in this moss system.

In order to study the longer-term transcriptional change among these lines, we did an 8-hour auxin treatment with these lines. A stabilized *IAA2* mutant line was also included as a control, and the qPCR results are shown in Figure 3.3.

The results generally showed the same trend as those in the 1-hour treatment. For some genes, such as *RHD* and *818,* the expression level is further reduced by the longer treatment in the *rARF-OE/Δaux/iaa* lines.

The high auxin marker gene expression in *Δaux/iaa* and its constitutive auxin-related phenotype indicate that when both aARFs and rARFs are present without the influence of Aux/IAAs, aARFs dominates the system leading to constitutive activation of

auxin-induced gene transcription. Overexpression of the putative repressing ARFs in the triple mutant background results in repression of the constitutive phenotype, providing the evidence for the *in vivo* repressive activity of the rARFs.

In addition, marker gene expression did not change very much in *AtARF1/WT* between the mock and IAA treated conditions, in both 1-hour and 8-hour treatments. This suggests that the native moss Aux/IAAs are less efficient in repressing AtARF1 compared to the moss ARFb4.

Discussion

In the current model of auxin signal transduction, the Aux/IAA-ARF interaction is essential for the repression of auxin responses, and it is also the base for auxin-induced gene activation. While the activating ARFs fit this model directly, the role of the putative rARFs is not well understood. Our results confirmed the *in vivo* repressive activity of the rARFs for the first time, using a system in which all the Aux/IAAs are deleted.

The mechanism of repression still remains to be studied. For some repressing ARFs, the presence of an EAR domain suggests that repression could involve recruitment of the TPL co-repressor. However, many repressing ARFs, including PpARFb2, PpARFb4, and AtARF1 used in this work do not have this motif. In the recent publication Lavy *et al*., eLife, 2016, several follow-up experiments suggest that rARF can compete with aARF in binding to the same promoter of their target genes. On the other hand, considering that the work in Chapter 2 of this dissertation confirmed the role of Aux/IAA PB1 domain in repressing the activity of ARFs, and that many of the rARF C terminus share homology with Aux/IAA PB1 domain, it is possible that the rARFs

function by a similar PB1-mediated oligomerization mechanism. We have been trying to overexpress ARFb4 with mutation in one of the K and OPCA interaction centers in the *Δaux/iaa* background to see whether it confers the same phenotype and auxin-responsive gene expression level as the intact ARFb4. This would help to demonstrate whether oligomerization of rARFs is a mechanism of their repressive activity.

Previous yeast two-hybrid assays have identified very few interactions between PB1 domain of repressor ARFs and that of Aux/IAAs (Vernoux et al., 2011). Here we showed that with the presence of native Aux/IAAs, auxin treatment resulted in a much smaller change in auxin-responsive gene expression in the line expressing a truncated version of *ARFb4* line compared to the intact gene, providing evidence that rARFs are also regulated by Aux/IAAs through the C terminus *in vivo*.

Lastly, overexpression of the aARF (ARFa8) in the WT did not result in as much increase in the marker gene expression as those in the triple mutant, especially in the mock condition when there was no external auxin. This confirms the existence of an Aux/IAA negative feedback loop in regulating auxin signaling: increase in ARF activity would up-regulate the transcription of their repressors (Aux/IAAs) until they reach a dynamic balance. Therefore, the overexpression of aARFs in the WT background is limited by the native Aux/IAAs to a reasonable level for growth. This balance is disturbed in the *ARFa8-OE*/*Δaux/iaa* line by the removal of Aux/IAAs, resulting in lethality.

Materials and Methods

Moss strains and growth conditions

Wild-type 'Gransden-2004' and mutant *P. patens* strains were grown at 25°C under continuous light at an intensity of 40-70 μmol/m2/s. When grown for phenotypic and gene expression analysis, BCD medium was used. When grown for propagation purpose, BCDAT (BCD supplemented with 5 mM Ammonium Tartrate) was used.

The moss transgenic lines used in this chapter were made by Dr. Meirav Lavy and some of them were published in Lavy *et al*., *eLife*, 2016.

RNA isolation and qRT-PCR

For detection of tissue-specific *Aux/IAA* gene transcripts, protonemal tissue was grown on BCD plates with cellophane overlays for three weeks. Leafy gametophores were cut from the remaining filamentous tissue and the two types of tissue were collected individually for RNA isolation. For detection of auxin-responsive marker gene transcripts, protonemal tissue was grown on BCDAT plates with cellophane overlays for one week. Then the colonies were transferred into liquid BCD medium containing either 10 μM IAA or the equivalent amount of solvent (ethanol). After incubation under the moss growth condition, the colonies were collected individually for RNA isolation.

Total RNA was isolated using RNeasy plant mini kit (Qiagen). 500 ng RNA was reverse transcribed using the Superscript III First Strand cDNA Synthesis System (Life Technologies). 20 μl RT reaction was diluted to the final volumn of 200 μl. 4 μl of the diluted cDNA was used for detection by the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The primers used for detection of marker genes were described in Lavy *et al*., 2016. Normalized expression (ΔΔC(t) method) was calculated using the Bio-

Rad CFX manager software using *PpEF1a* as a reference gene and plotted as relative values ± SEM. Four replicates were included in each analysis. T-test was used for statistical inference.

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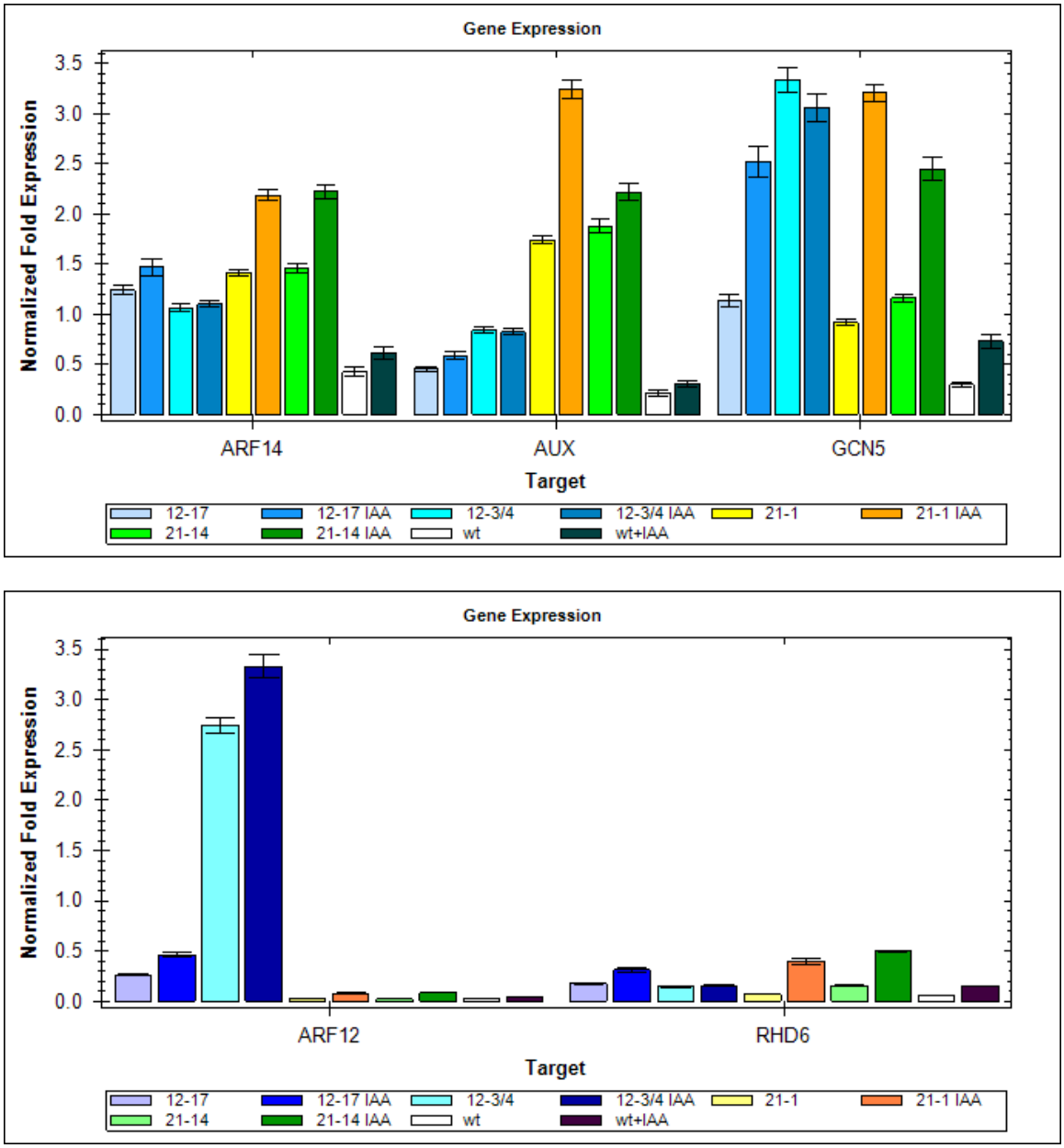

Figure 3.1 qPCR of auxin-responsive marker genes in the ARF-overexpression lines in the WT background compared to WT when treated with mock or 10 μM IAA.

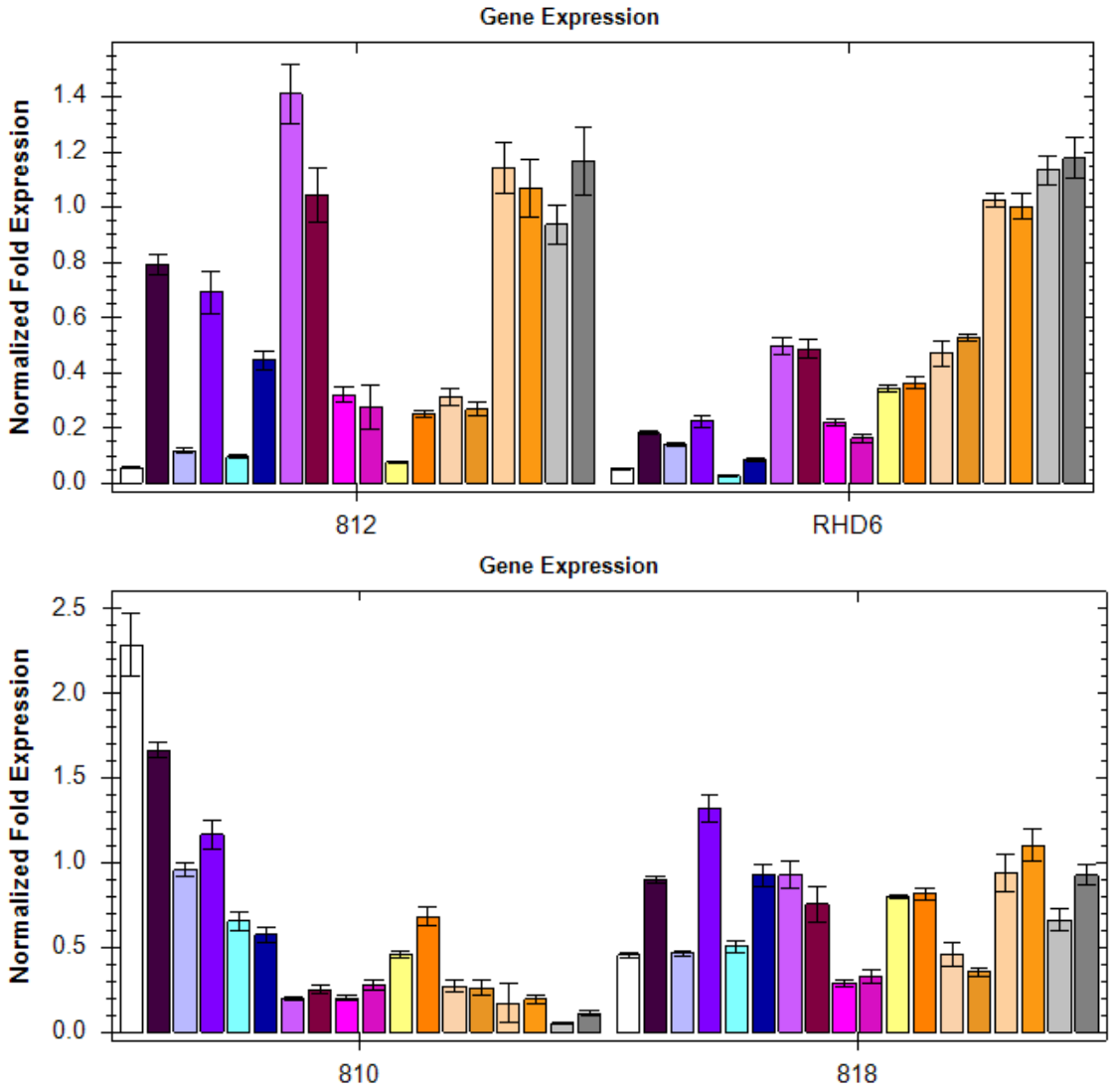

Figure 3.2 qPCR of auxin-responsive marker genes in the ARF-overexpression lines in different backgrounds after 1-hour mock/IAA treatments. 1: *WT*; 2: *ARFb4-OE/WT-17*; 3: *ARFb4-OE/WT-34*; 4: *ARFb4-OE/Δaux/iaa-12*; 5: *ARFb4-OE/Δaux/iaa-36;* 6: *AtARF1-OE/WT*; 7: *AtARF1-OE/Δaux/iaa-1*; 8: *AtARF1-OE/Δaux/iaa-80*; 9: *Δaux/iaa*. "+" indicates IAA treatment.

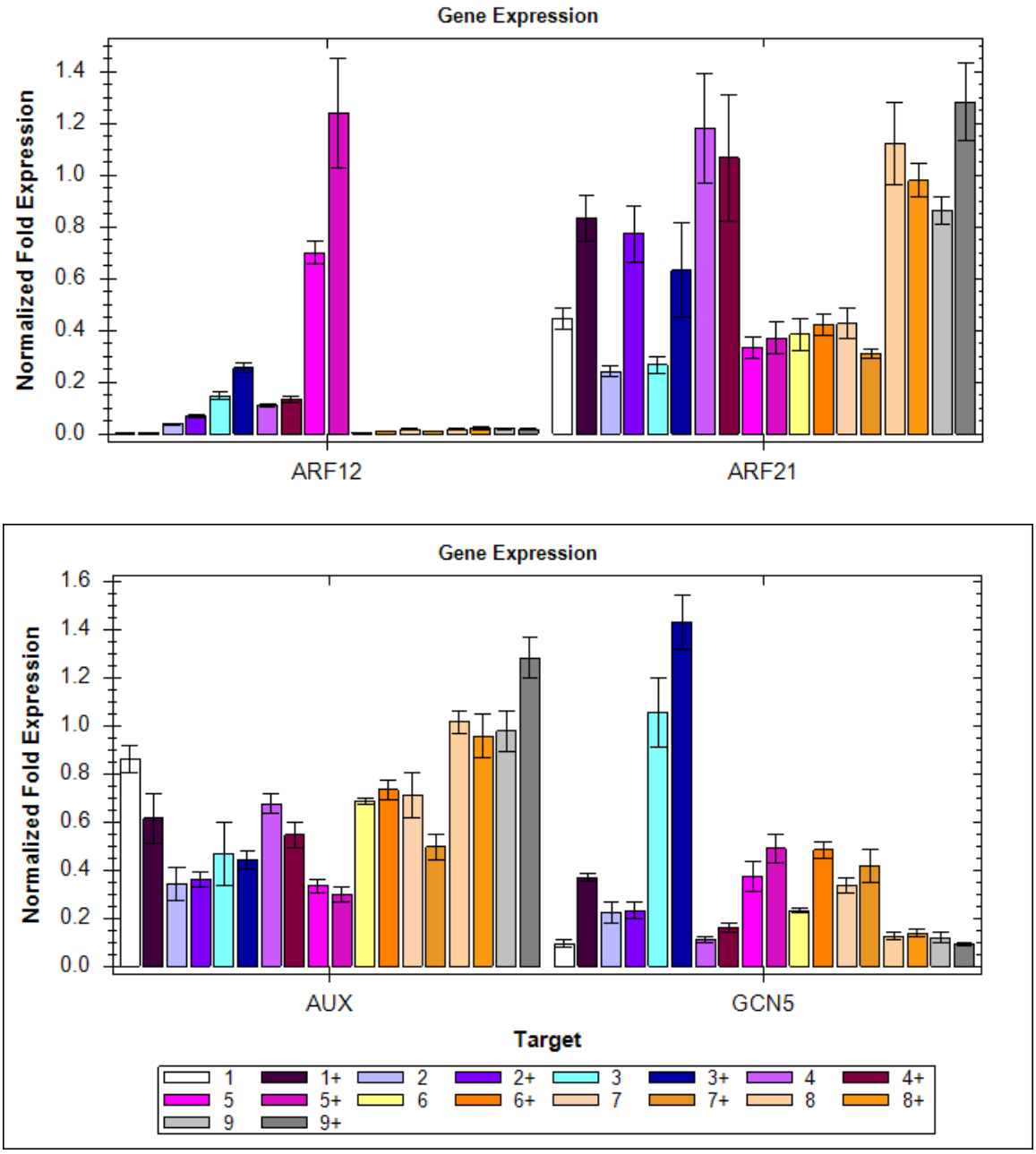

Figure 3.2 qPCR of auxin-responsive marker genes in the ARF-overexpression lines in different backgrounds after 1-hour mock/IAA treatments. (continued)

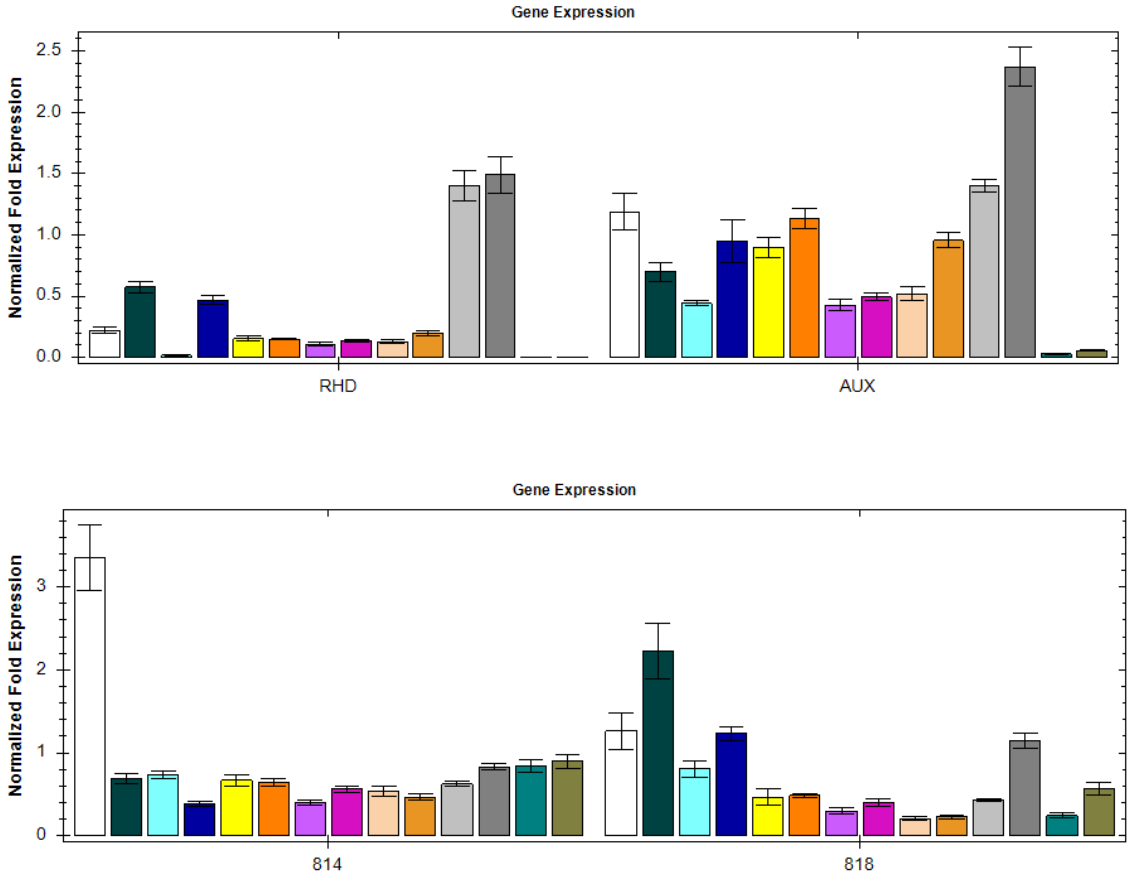

Figure 3.3 qPCR of auxin-responsive marker genes in the ARF-overexpression lines in different backgrounds after 8-hour mock/IAA treatments. 1: *WT*; 2: *ARFb4-OE/WT-34*; 3: *AtARF1-OE/WT*; 4: *ARFb4-OE/Δaux/iaa-36*; 5: *AtARF1-OE/Δaux/iaa-1;* 6: *Δaux/iaa*; 7: *mIAA2d2*. "+" indicates IAA treatment.

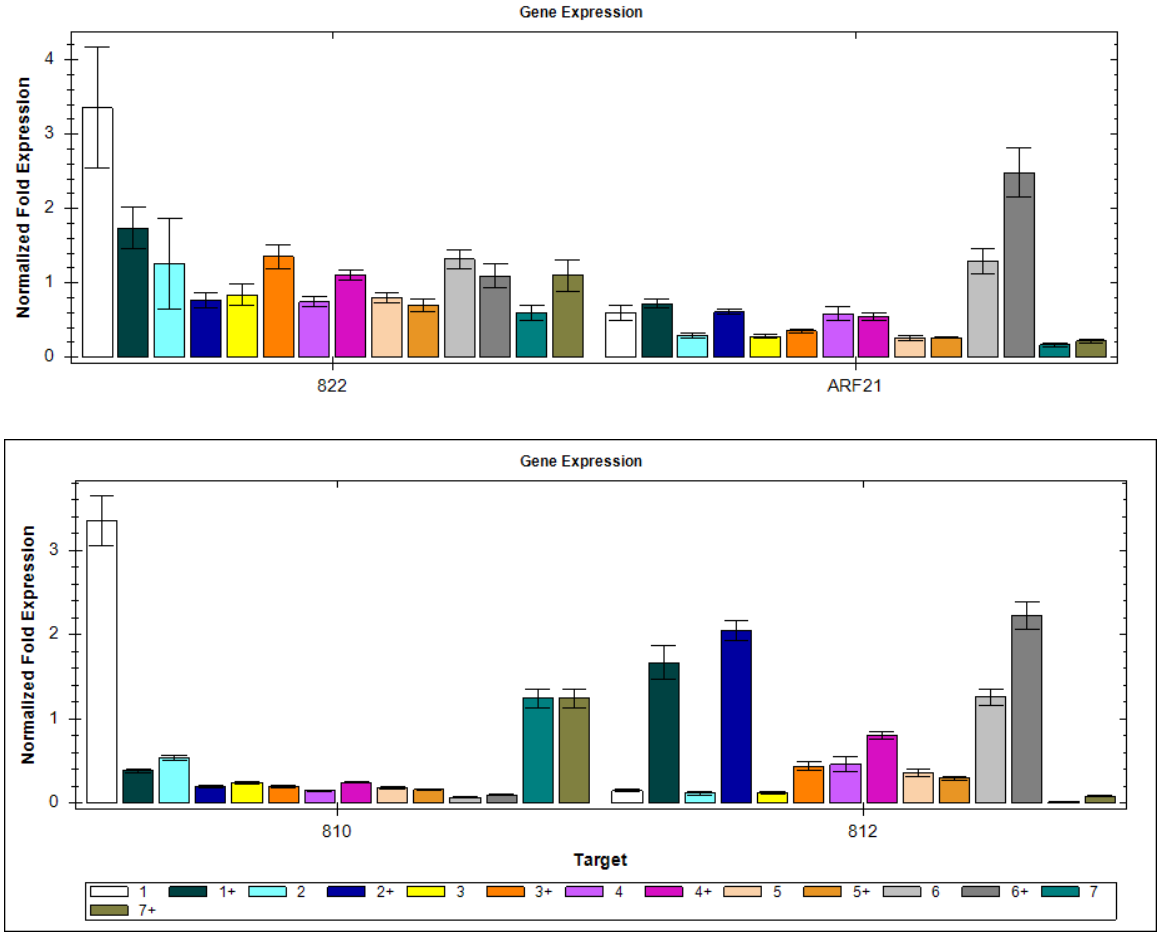

Figure 3.3 qPCR of auxin-responsive marker genes in the ARF-overexpression lines in different backgrounds after 8-hour mock/IAA treatments. (continued)

Chapter 4

Characterization of Aux/IAA DIII/IV amino acid substitutions that affects TIR1-Aux/IAA interaction

Introduction

As mentioned in Chapter 1, the degron motif in Domain II of Aux/IAAs has been shown in structural studies to be the interaction surface with TIR1 and auxin. We showed in chapter 2 that changes in the degron motif lead to a series of changes in protein behavior including auxin-binding affinity, protein stability and levels of auxin response.

On the other hand, previous yeast 2-hybrid (Y2H) studies comparing the strength of different TIR1-AtAux/IAA interaction indicate that Aux/IAAs with similar or even the same degron motif result in different interaction strength and affinity for auxin (Calderón Villalobos et al., 2012). In addition, in a study of ectopic expression of Luciferase-tagged Aux/IAA Domain I and II in wild-type Arabidopsis, a KR bipeptide between the two domains has been shown to promote the degradation of the overexpressed protein (Dreher et al., 2006). These results suggest that some factors outside of Domain II affect the auxin-binding and the stability of the co-receptor. However, other than the information mentioned above, other elements in the Aux/IAA protein that regulate degradation are largely unknown.

In a previous Y2H screen, previous lab member, Dr. Hong Yu, identified several AtIAA3, AtIAA7 and AtIAA8 mutants carrying amino acid substitutions in the PB1 domain. These mutations resulted in a decrease in TIR1-IAA interaction in yeast (Figure 4.1). Alignment of the Aux/IAA protein sequences showed that these amino acids are highly conserved among the Arabidopsis and moss Aux/IAAs (Figure 4.2). Therefore, I continued on this work to investigate whether there is a general mechanism for these amino acid residues to influence TIR1/AFB-Aux/IAA interactions in the yeast system and in planta.

Results

The reduction in TIR1-Aux/IAA interaction is a direct effect by the Aux/IAA mutations

The reduction in Y2H reporter gene expression level shown in the preliminary assay might be due to the difference in protein expression level between the WT and mutant AtIAAs. To test this, western blot analysis using antibody to the HA tag in the fused Aux/IAA protein was performed to verify the protein accumulation levels. The results showed that the mutant Arabidopsis IAA7/IAA3/IAA8 proteins were expressed at the same level as the corresponding wild-type IAA in the Y2H assay. Therefore, the reduced reporter gene activation in the assay is truly due to a decrease in TIR1/AFB-Aux/IAA interaction strength.

Since the mutations are close to the K and OPCA interaction centers that are essential for PB1-mediated Aux/IAA oligomerization, it is possible that they affect the self-interaction of the corresponding Aux/IAA. Within the yeast cell, if Aux/IAA self-interaction was enhanced, then the proteins might be less accessible to TIR1/AFB, resulting in a decrease of TIR1/AFB-Aux/IAA interaction. To test whether this is the case, constructs expressing wild-type and mutant versions of IAA7 and IAA3 fused to the DNA binding domain were made. We co-transformed these with the constructs expressing activation domain fused IAA7 or IAA3 respectively, to check their self-interaction in the system.

Y2H using IAA3 showed that in terms of the interaction strength, there was no difference among the combinations of IAA3-IAA3, IAA3 V95G –IAA3 and IAA3 V95G –IAA3 V95G. For IAA7 on the other hand, there was no self-interaction for either the

wild-type version or the mutant version in this system (Figure 4.3). The IAA7 did not show self-interaction in another Y2H assay published by Vernoux *et al*, 2011 either, suggesting that IAA7 tend to act as monomer.

Therefore, the difference shown in TIR1/AFB-Aux/IAA Y2H was not due to changes in the Aux/IAA self-interaction, but was a direct reduction of TIR1/AFB interaction.

Assays in other systems revealed contradictory results on TIR1-Aux/IAA interaction

In order to test whether these mutations influence the interaction in systems other than Y2H, we did *in vitro* pull downs following the procedures described in Parry et al., 2009. GST-fused AtIAA7 with or without the mutations were expressed and purified from *E. coli* and used for pull-down experiments with TIR1-myc expressed from the wheat germ extract *in vitro* translation system. In the result shown in Figure 4.4, for the same amount of GST-IAA7, the amount of TIR1-myc pulled down by the IAA7 mutants was more than that pulled down by the wild-type IAA7. This indicates that the interaction with the mutant IAA7 is stronger than the wild type, which is contrary to the Y2H data above.

At this point, we tried to check whether these mutations influence the processes after the TIR1-Aux/IAA binding. One available approach was to test the degradation rates of the Aux/IAA mutants in a yeast fluorescence system (Havens et al., 2012). We did this in collaboration with Britney Moss in Dr. Jennifer Nemhauser's lab. Another attempt was to use our moss system to generate a transgenic *IAA1a/*Δ*aux/iaa* line with a

mutation in the corresponding residue for comparison with the wild-type *IAA1a/*Δ*aux/iaa* line.

In the first approach, YFP-tagged AtIAAs and AtTIR1/AFB2 were integrated into the yeast ubiquitin pathway, and their degradation curve under different auxin concentrations were measured via fluorescence signal using time-lapse flow cytometry. We found that the basal expression level of the IAA3 and IAA7 mutants was much lower compared to WT IAA3 and IAA7, and the degradation curve did not show significant difference (Figure 4.5). Meanwhile, the Nemhauser lab had been doing the same degradation assay with IAA7 carrying K and OPCA mutations, and the results also showed a reduction on protein expression level compared to WT. Besides, those K and OPCA mutants did not degrade slower than WT either. Therefore, the low basal expression level may be a common issue for Aux/IAA PB1 domain mutants in this system, and judging from the available data, the mutants did not seem to affect the degradation rate of the corresponding Aux/IAAs in a significant way.

We also used the moss system that we developed in Chapter 2 to test whether the corresponding mutations in PpIAA1a result in any change in the moss developmental phenotypes. Transgenic *IAA1a V374G/*Δ*aux/iaa* was generated and characterized. The results showed that the mutant did not have any significant phenotype compared to the wild-type *IAA1a/*Δ*aux/iaa*, in both plain media and exogenous auxin treatments (Figure 4.6).

Discussion and Future Direction

The Y2H assay has been a powerful way to identify novel mutants that change the strength of protein-protein interactions. However, as an *in vitro* method, results from Y2H need to be further verified by other *in vitro* and *in vivo* methods, which may or may not be in agreement with each other. In our case, the data from Y2H and *in vitro* pull down assays showed opposite trends in terms of how the mutations in the Aux/IAA PB1 domain influence its interaction with TIR1/AFBs.

Since the TIR1/AFB-Aux/IAA interaction directs the Aux/IAA for degradation by the 26S proteasome, changes in the co-receptor interaction strength, especially with the presence of auxin, may influence the degradation rate. Based on this we tested their degradation rates in the yeast fluorescence system that worked well for testing Arabidopsis TIR1 and Aux/IAAs previously. Unfortunately our mutant Aux/IAA proteins did not seem to accumulate well in this system, although their levels were the same as wild-type Aux/IAAs in the Y2H system. And this showed that even in the same organism (yeast), different conditions might result in difference in protein accumulation levels.

In the work by Korasick *et al*., 2014 in which the ARF7 PB1 structure was resolved, they identified the lysine and OPCA interaction centers and several other conserved residues as the "interaction surface" for the domain. Although our mutations are conserved and close to the interaction centers, they do not overlap with the predicted PBI interaction surface residues. It remains to be investigated whether and how these mutations influence the TIR1/AFB-Aux/IAA interaction *in vivo*. Since the *IAA1a V374G/*Δ*aux/iaa* line did not show any significant phenotype, I did not continue on this project. However, overexpression of TIR1-myc in the *IAA1a V374G/*Δ*aux/iaa* line

followed by an *in vivo* co-IP may provide more information on how the mutation affects the TIR1-Aux/IAA interaction.

Materials and Methods

Y2H

The Y2H assays were done using the same system described in Chapter 2. Site-directed mutagenesis was done to obtain the constructs expressing mutant versions of IAA3 and IAA7 for testing self-interaction.

To check the Aux/IAA protein expression level in the Y2H assay, representative colonies of each genotype were ground in 2X SDS sample buffer. After centrifuge, 10 ul of each supernatant were used for SDS-PAGE and western blot analysis.

Plasmid constructs

The IAA7 protein coding sequence was amplified and cloned into the pDEST15 for expression of GST-IAA7 in *E. coli*. Site-directed mutagenesis was done to obtain the constructs expressing mutant versions of GST-IAA7.

Protein expression and pull-down assay

GST-Aux/IAA was expressed in *E.coli* and purified with glutathione agarose beads (Sigma) using standard methods. The TIR1-Myc proteins were synthesized by TNT coupled wheat germ extract system (Promega). 20ul TIR1-Myc were incubated with >10 ug of GST-Aux/IAA beads in 200ul of lysis buffer (50mM Tris pH 8.0, 200mM NaCl, 10% glycerol, 0.1% Tween-20, protease inhibitors) with or without IAA at room temperature for 1hr. The pull-down reaction was then transferred to a Micro Bio-Spin Chromatography Column (Bio-Rad). After washing the samples with lysis buffer,

samples were eluted using reduced glutathione (Sigma) and separated on SDS-PAGE. Products were detected by anti-c-Myc-peroxidase antibody (Roche) and chemiluminescence was visualized by ImageQuant LAS 4000 (GE Healthcare). The amount of GST-IAA7 protein was determined by the Ponceul staining of the corresponding protein molecular weight region.

Transgenic moss lines

Site-directed mutagenesis was done with the *pBHRF:IAA1a-Luc* to obtain the construct for expressing IAA1a V374G in moss. PEG-mediated transformation, transformant screening and removal of possible tandem repeats by CRE-Lox reaction were performed as described in Chapter 2.

For phenotype observation, the moss lines were grown in BCD medium with or without NAA supplement for 3 weeks and photos were taken under the dissecting microscope.

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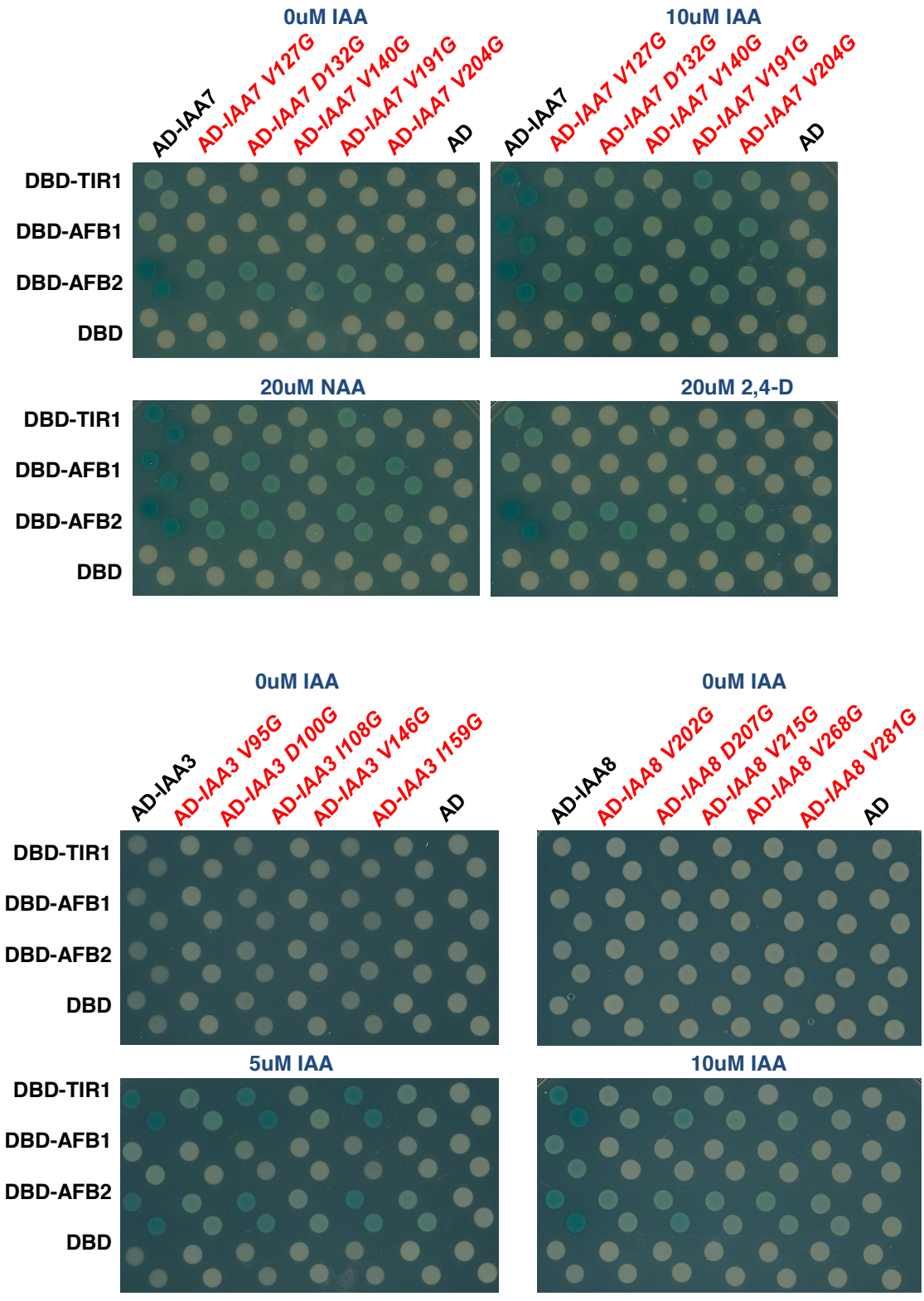

Figure 4.1 Aux/IAA PB1 domain mutations identified in a previous Y2H screen disrupt the interaction between TIR1/AFBs and Aux/IAAs. (data by Dr. Hong Yu)

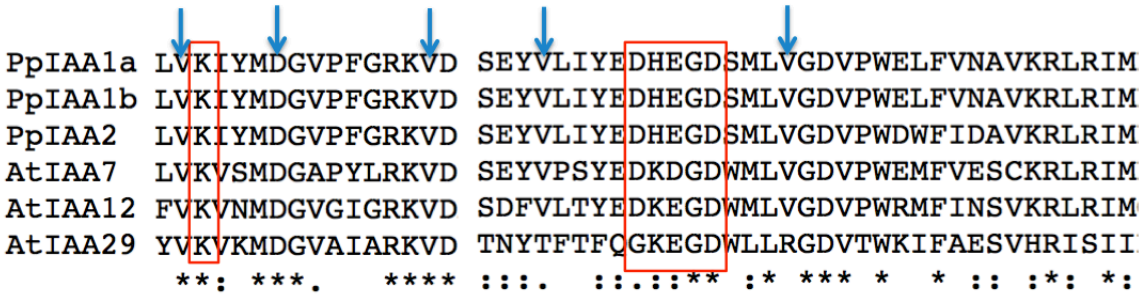

Figure 4.2 Alignment of the conserved regions of Arabidopsis and moss Aux/IAA PB1 domain amino acid sequences showed that the amino acid residues identified in the Y2H screen are highly conserved. Red box: the lysine and OPCA interaction centers. Blue arrow: mutations identified in the Y2H.

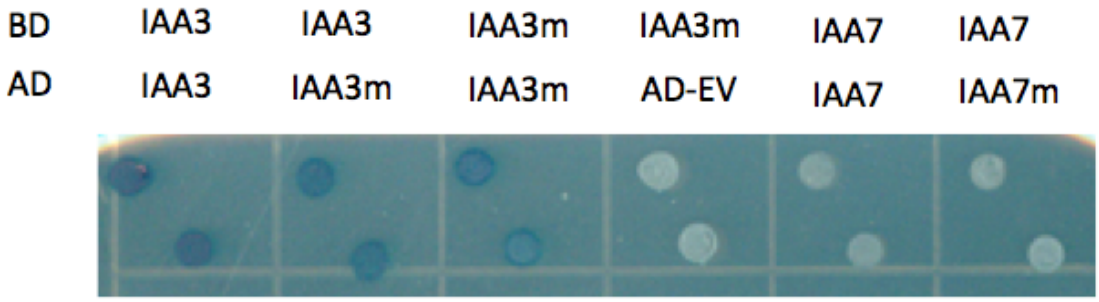

Figure 4.3 Mutations in Aux/IAA PBI domain did not change the strength of Aux/IAA self-interaction in Y2H. BD: DNA-binding domain. AD: activation domain. EV: empty vector control. IAA3m: IAA3 V95G. IAA7m: IAA7 V127G.

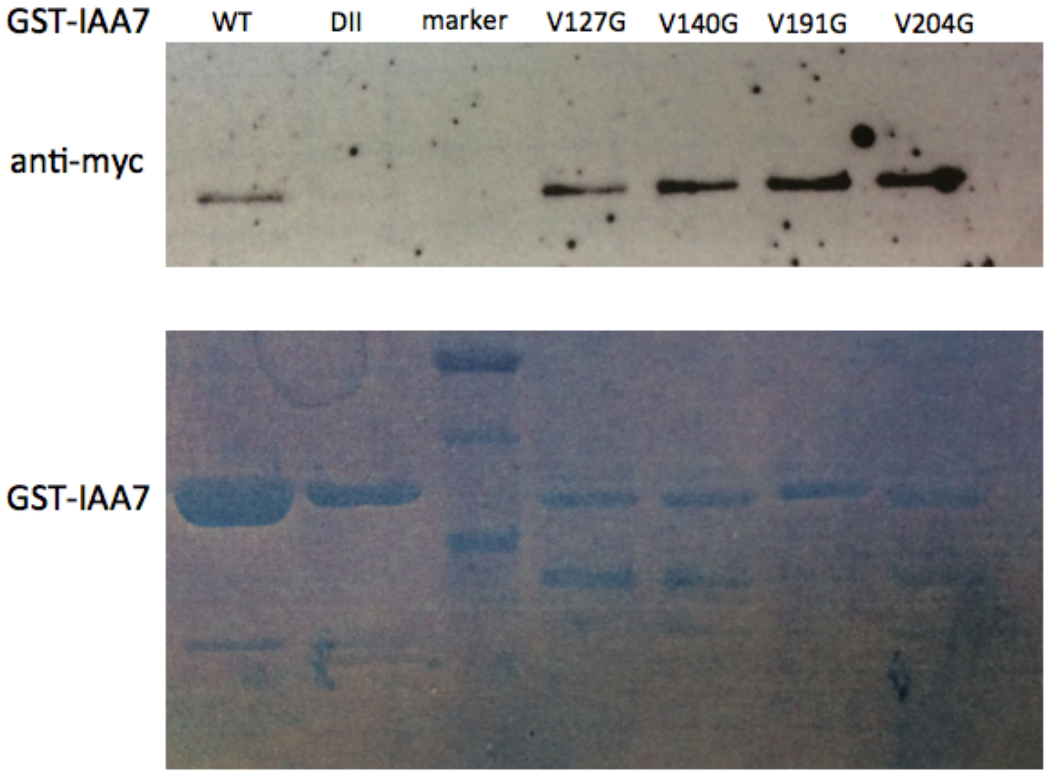

Figure 4.4 In vitro pull-downs of GST-IAA7 with TIR1-myc. Up: western blot using anti-myc antibody for detection of the TIR1-myc pulled down by different versions of GST-IAA7. DII: IAA7 with degron motif mutations resulting in disruption of TIR1 interaction. Bottom: Ponceul staining showing the amount of GST-IAA7 in each lane.

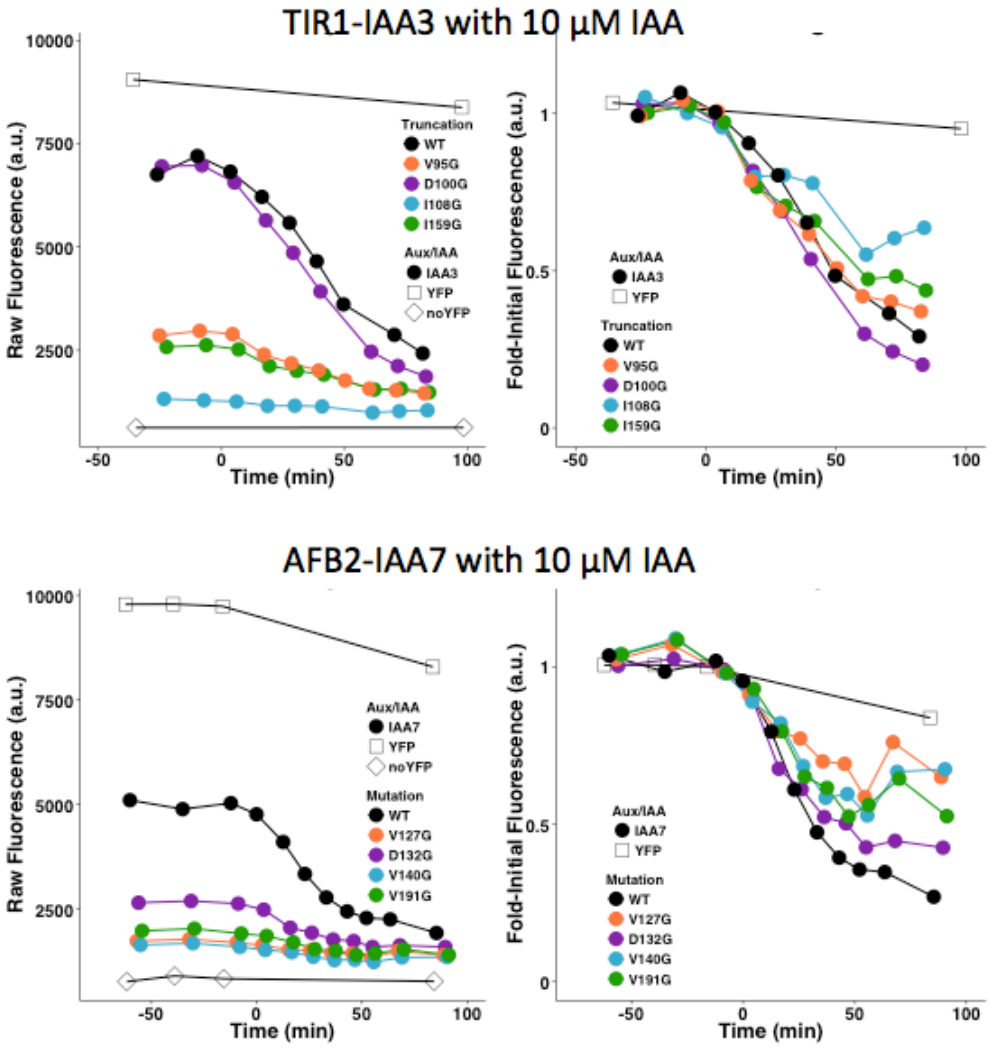

Figure 4.5 Expression levels and degradation curves of the AtIAA3 and AtIAA7 mutants in the yeast fluorescence system. The raw fluorescence signal for the mutants of IAA3 and IAA7 was much lower compared to wild type (WT) before the auxin treatment started (Time 0).

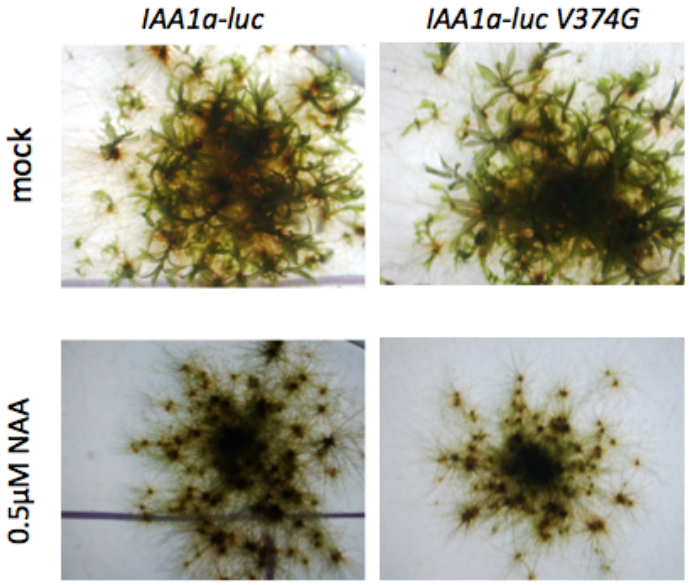

Figure 4.6 *IAA1a V374G/*Δ*aux/iaa* shows wild-type phenotype in filament growth, gametophore formation and rhizoids formation under both mock and 0.5 µM NAA treatments.