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Identification and Characterization of IAMH1 Gene in Biosynthesis of Plant Hormone Auxin

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Identification and Characterization of IAMH1 Gene in Biosynthesis of Plant Hormone Auxin

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

in

Biology

by

Yangbin Gao

Committee in charge:

Professor Yunde Zhao, Chair
Professor Mark Estelle
Professor James W. Golden
Professor Bing Ren
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Chair

University of California, San Diego

2015
DEDICATION

To Light, which makes this world being, and to Love, which make this world living. To my family, my mentors, my friends, and to myself.
One is the child of the divine law.

After one come two,

after two come three,

after three come all things.

—Laozi, Tao Te Ching
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ABSTRACT OF THE DISSERTATION

Identification and Characterization of IAMH1 Gene in Biosynthesis of Plant Hormone Auxin

by

Yangbin Gao

Doctor of Philosophy in Biology

University of California, San Diego, 2015

Professor Yunde Zhao, Chair

The plant hormone auxin plays indispensable roles in every aspect of plant life. This dissertation centered on the major natural form of auxin, Indole-3-acetic acid (IAA). In Chapter 1, we reviewed the biosynthesis and degradation of auxin. In Chapter 2 we covered an unexpected finding when crossing an auxin biosynthesis mutant, yuc1, to a floral homeotic gene mutant, agamous (ag). The severe phenotypes of ag were suppressed in the F2 population, and the suppression could be stably transmitted to later generations even without the presence of yuc1 mutation. We demonstrated that this suppression was mediated by T-DNA insertions in the two genes, and it was related to epigenetic
changes in the *ag* mutant. We also demonstrated that this phenomenon is not rare, and advised caution when interpreting results obtained from T-DNA mutants. In Chapter 3, we introduced a novel technology for improved control of guide RNA (gRNA) production when using CRISPR systems for genome editing or gene activation/suppression. We employed self-cleaving ribozymes and attached them to both ends of a gRNA sequence to enable the expression of gRNAs using RNA polymerase II promoters. We showed that the ribozyme-flanked gRNA design is functional both in vitro and in yeast, and this opens new doors for controlled gRNA expression which was not possible using RNA polymerase III promoters. In Chapter 4, we reported a surprising finding that a null mutant of an auxin-related gene *abp1* generated using our ribozyme-based CRISPR technology described in Chapter 3 did not show any developmental defects, which is contrary to previous findings that *abp1* null mutants were embryonic lethal. We continued to identify another null *abp1* mutant which also had no obvious defects under normal growth conditions, and raised questions about the previous claims that *ABP1* is an essential gene and it encodes an auxin-binding protein which may function as an auxin receptor. In Chapter 5, we described the identification and characterization of auxin-biosynthesis genes *IAMH1* and *IAMH2* involved in converting Indole-3-acetamide (IAM) into IAA. We showed that IAMH1 could hydrolyze IAM into IAA both in vitro and in vivo and this finding added another missing piece in the auxin biosynthesis pathways.
Chapter 1

Introduction to Auxin Biosynthesis and Catabolism

1.1 Background

Auxin is an essential hormone for many aspects of plant growth and development [1]. Plants have evolved a sophisticated network to control auxin levels with spatial and temporal precision in response to environmental cues and developmental signals. Indole-3-acetic acid (IAA), the main natural auxin in plants, can be produced from de novo biosynthesis. Free IAA, which is the presumed active form of auxin, can also be released from IAA conjugates including IAA esters, IAA-saccharides, and IAA-amino acids. A third probable route for producing IAA is to convert indole-3-butyric acid (IBA) to IAA using enzymes similar to those used in -oxidation of fatty acids. When auxin levels need to be lowered, plants employ several mechanisms to deactivate IAA. IAA can be quickly converted into the presumed inactive forms by reaction of the carboxyl group of IAA with amino acids, sugars, and other small molecules. The IAA conjugates may serve as a first step for the eventual complete degradation of IAA. IAA is also
inactivated by oxidation of the indole ring of IAA. For example, IAA can be converted to 2-oxindole-3-acetic acid (OxIAA). In this chapter, we discuss the progress made in the area of auxin biosynthesis and metabolism in the past few years.

### 1.2 De Novo Auxin Biosynthesis

De novo auxin biosynthesis is broadly divided into two categories: Tryptophan (Trp) dependent and Trp independent. Trp-independent auxin biosynthesis pathway was proposed two decades ago based on results from feeding plants with labeled Trp and Trp biosynthetic intermediates and from studies on Trp-deficient mutants [2, 3]. However, the molecular mechanisms and genes for the Trp-independent pathway are not known. Therefore, the Trp-independent pathway will not be discussed further in this chapter.

Trp has long been known as a precursor for the production of IAA in plants. Feeding plants with labeled Trp yields labeled IAA, indicating that plants have the enzymes to convert Trp to IAA [2, 3]. Many biosynthetic pathways have been elucidated using analytic biochemistry techniques in combination with labeled precursors and intermediates. For example, the biosynthetic routes for brassinolide and ethylene have been established long before the biosynthetic genes have been identified [4, 5]. However, the classic feeding and analytic biochemical approaches failed to identify the key components for Trp-dependent plant auxin biosynthesis pathways. There are several reasons for this apparent failure.

First, Trp is a precursor for many metabolites (Figure 1.1). Trp is a precursor for indole-3-pyruvate (IPA), tryptamine (TAM), indole-3-acetaldoxime (IAOx), indole-3-acetamide (IAM), indole-3-acetonitrile (IAN), and indole-3-acetaldehyde (IAAld) (Figure 1.1). Arabidopsis and many other plants have the capacity to produce all of the abovementioned intermediates (Figure 1.1) at a given developmental stage [6, 7]. Some
Figure 1.1: IAA Biosynthesis Pathway. Selected tryptophan metabolic intermediates. *Arabidopsis* plants produce all of the intermediates shown in the figure. The numbers in parenthesis refer to the actual concentrations in ng/g fresh weight. *IAA* indole-3-acetic acid, *IAAld* indole-3-acetaldehyde, *IAOx* indole-3-acetaldoxime, *IAM* indole-3-acetamide, *IAN* indole-3-acetonitrile, *IPA* indole-3-pyruvate, *TAM* tryptamine, and *Trp* tryptophan.

of the intermediates such as IAN exist in very high concentrations (Figure 1.1) [7]. Such a complex profile of Trp metabolism makes it difficult to identify Trp-dependent IAA synthesis intermediates. Second, some of the intermediates are intrinsically unstable in vitro and can be nonenzymatically converted to other compounds during the experimental process, therefore complicating the analysis of metabolic profiling. For example, IPA is readily converted nonenzymatically into IAA in vitro [8]. Third, most of the Trp metabolic intermediates display auxin activities during in vitro bioassays (Figure 1.2). In the presence of IAM in growth media, light-grown *Arabidopsis* seedlings have long hypocotyls and epinastic cotyledons (Figure 1.2). The IAM-induced phenotypes are identical to those observed in auxin overproduction mutants [9–11]. Therefore, the phenotypes of plants grown on IAM media are likely caused by overaccumulation of IAA in plants. *Arabidopsis* seedlings grown on IAN-containing media produce more
adventitious roots and have short primary roots [12](Figure 1.2). The IAN-induced phenotypes are very similar to those observed in plants grown on IAA-containing media, suggesting that IAN is probably converted to IAA in plants (Figure 1.2). Indeed, a genetic screen for mutants insensitive to IAN identified Arabidopsis nitrilase genes that encode enzymes for the hydrolysis IAN to IAA [12, 13]. Interestingly, treatments with IAN or IAM cause auxin overaccumulation in plants and high-auxin phenotypes. However, the IAM-induced phenotypes are dramatically different from those caused by IAN (Figure 1.2). It is speculated that both IAM and IAN need to be metabolized into IAA to show auxin activities as the observed phenotypic differences may be simply caused by different tissue specificities of the hydrolytic enzymes for IAM and IAN. Although it is very clear that Trp metabolic intermediates can be converted to IAA in plants, it is difficult to determine how important their contribution to the total IAA pool under natural conditions is. The fact that plants produce a large number of Trp metabolic intermediates (Figure 1.1) and that some of the Trp metabolites have auxin activities when added to growth media (Figure 1.2) made it very difficult to dissect Trp-dependent auxin biosynthesis pathways using classic analytic biochemistry techniques alone.

The main criterion for determining whether a Trp metabolite is important for de novo auxin biosynthesis is to use the deletion test. If the intermediate is important for auxin biosynthesis, we expect that plants show dramatic developmental defects similar to those observed in mutants defective in auxin transport or signaling if the plants lose the ability to make the intermediate. Recent results from a combination of analytic biochemical studies and Arabidopsis genetics research have established that the main auxin biosynthesis pathway in Arabidopsis is a simple two-step pathway that converts Trp to IAA (Figure 1.3). The pathway is highly conserved throughout the plant kingdom.
Figure 1.2: Auxin response of Arabidopsis to Tryptophan metabolites. Some tryptophan metabolites display auxin activities. Indole-3-acetamide (IAM) stimulates hypocotyl elongation and causes epinastic cotyledons. Indole-3-acetonitrile (IAN) inhibits primary root elongation and stimulates adventitious root initiation.

1.2.1 The TAA/YUC Pathway as the Main Auxin Biosynthesis Pathway

The YUCCA flavin-containing monooxygenases catalyze the rate-limiting step.

The YUCCA (YUC) flavin-containing monooxygenase (FMO) gene was identified as a key auxin biosynthesis gene a decade ago from an activation-tagging screen for long hypocotyl mutants in Arabidopsis [11]. The dominant yucca (later renamed as yuc1D) mutant was caused by the insertion of four copies of the CaMV 35S transcriptional enhancer downstream of the YUC gene [11]. The enhancers greatly increase the YUC expression levels, resulting in dramatic developmental defects. Physiological and molecular studies demonstrated that yuc1D is an auxin overproduction mutant [11]. Direct auxin measurements show that yuc1D contains 50% more free IAA than wild-type
Arabidopsis plants. Moreover, the auxin reporter DR5-GUS is greatly upregulated in yuc1D further supporting that yuc1D is an auxin overproduction mutant. It was suggested that YUC flavin-containing monooxygenases catalyze a rate-limiting step in auxin biosynthesis [11].

![Figure 1.3](image)

Figure 1.3: Two-step reaction from Trp to IAA. A tryptophan-dependent auxin biosynthesis pathway in plants. The TAA family of aminotransferases produces indole-3-pyruvate (IPA) from tryptophan (Trp) and the YUC flavin-containing monooxygenases catalyze the conversion of IPA into indole-3-acetic acid (IAA)

YUC was later found to be a member of a gene family with 11 genes in the Arabidopsis genome. The founding member was renamed as YUC1. Overexpression of any of the YUC family members leads to auxin overproduction phenotypes in Arabidopsis, suggesting that all of the YUC genes participate in auxin biosynthesis [14, 15]. The YUC genes have overlapping functions and inactivation of a single YUC gene does not cause any obvious developmental defects [14, 15]. The observed genetic redundancy among YUC genes may provide an explanation for why YUC genes had not been discovered previously by forward loss-of-function genetic screens. Detailed analyses of various yuc mutant combinations have demonstrated that YUC genes are essential for almost all of the major developmental processes including embryogenesis, seedling growth, vascular initiation and patterning, flower development, and plant architecture [14, 15]. For example, the yuc1 yuc4 double mutants do not make tertiary veins in rosette leaves and fail to make continuous vascular boundless in flowers. Overall yuc1 yuc4 flowers contain fewer floral organs and are completely sterile. A key piece of evidence that demonstrates the roles of YUC genes in auxin biosynthesis is the genetic rescue of yuc1
yuc4 mutants with the bacterial auxin biosynthesis gene iaaM under the control of the YUC1 promoter [14, 15].

The biochemical mechanisms of YUC-mediated auxin biosynthesis have been solved recently [16, 17]. YUC enzymes use NADPH and molecular oxygen to catalyze the oxidative decarboxylation of IPA to generate IAA (Figure 1.3). On the basis of sequence homology to the mammalian microsome FMOs, it is expected that YUCs use a flavin (FAD or FMN) as a cofactor. Expressed in and purified from E. coli, the Arabidopsis YUC6 displayed a bright yellowish colour, suggesting that YUC6 contains a flavin cofactor. HPLC and other experiments demonstrate that the cofactor in YUC6 is FAD, not FMN [17]. The YUC6-catalyzed conversion of IPA to IAA can be divided into three consecutive chemical steps: (1) reduction of FAD to FADH2 using electrons from NADPH; (2) binding of molecular oxygen to FADH2 to form the C4a-(hydro)peroxyl flavin intermediate; (3) the reaction of the C4a intermediate with IPA to produce IAA from decarboxylation of IPA (Figure 1.3 [17]. Interestingly, the reduction of YUC6 by NADPH takes place regardless of the presence of IPA. IPA also does not affect the rate of YUC6 reduction. The kinetic pattern and rate of the formation of the C4a intermediate is also not affected by IPA [17]. However, the decomposition of C4a intermediate is greatly accelerated by IPA [17]. The oxidized YUC6, reduced YUC6, and the C4a intermediate display distinct spectroscopic properties and can be monitored spectroscopically. The oxidized YUC6 shows two peaks at 376 and 448 nm in the UV-visible spectrum, while reduction of YUC6 causes the disappearance of the 448 nm peak. The YUC6 C4a-(hydro)peroxyl flavin intermediate has a maximum absorbance at 381 nm in a UV-visible spectrum [17]. The FAD cofactor in YUC6 provides a convenient handle to follow the progression of the YUC-catalyzed reactions. Besides IPA as a substrate, YUC6 can also catalyze the decarboxylation of phenyl-pyruvate (PPA) to produce phenyl-acetic acid (PAA), suggesting that YUC enzymes do not have strict
substrate specificities [17]. It is not known whether the YUC6-catalyzed conversion of PPA to PAA has any physiological significance. However, it is known that PAA displays auxin activities when added into growth media. Both YUC enzymes and mammalian FMOs share sequence homologies and form the C4a-(hydro)peroxyl flavin intermediate. Mammalian FMOs are mainly known for their ability to oxygenate soft nucleophiles such as nitrogen- or sulfur-containing molecules, whereas YUCs such as YUC6 oxygenate electrophilic substrates such as IPA and PPA [17–19]. However, mammalian FMOs recently have been shown to use electrophilic substrates as well and YUCs were previously shown to oxygenate soft nucleophiles in vitro [11, 20, 21]. The stability of the C4a intermediate is also quite different for YUCs and mammalian FMOs. The YUC6 intermediate has a half-life of about 20 s, whereas that of some FMOs from mammalian cells is more than 30 min [17–19]. It is important to use both in vitro enzymatic assays and in vivo genetic evidence to determine the physiological functions of flavin-containing monooxygenases.

In the presence of excess PPA or IPA, some uncoupled YUC6 reactions still take place and produce hydrogen peroxide. The uncouple ratio is about 4% [17]. It is not clear whether the uncoupled reaction plays any physiological role. It is conceivable that \( \text{H}_2\text{O}_2 \) produced from the uncoupled reaction may participate in deactivating YUC enzymes, providing an intrinsic mechanism for turning off auxin biosynthesis.

Genetic, physiological, and biochemical studies have unambiguously demonstrated that the YUC family of flavin-containing monooxygenases plays a key role in auxin biosynthesis. Genetic evidence suggests that the conversion of IPA to IAA is the rate-limiting and the committed step for IAA biosynthesis.
Tryptophan Aminotransferase of *Arabidopsis* (TAA) family of aminotransferases plays a key role in auxin biosynthesis

Three groups independently discovered that TAA1, the founding member of a large family of aminotransferases, is an important auxin biosynthesis enzyme [22–24]. Mutations in *TAA1*, which is also called *SAV3*, *WEI8*, and *TIR2*, alter shade-avoidance responses, cause resistance to ethylene and to the auxin transport inhibitor NPA [22–24]. Although inactivation of *TAA1* alone does not cause dramatic developmental phenotypes, simultaneously disruption of *TAA1* and its close homolog *TAR2* leads to defects in vascular pattern formation and in flower development in *Arabidopsis*. The *taa* mutants produce less free IAA compared to wild-type plants [22–24].

TAA1 and its related proteins catalyze the transfer of the amino group from Trp to pyruvate or to -ketoglutarate to produce IPA and Ala or Glu (Figure 1.3) in vitro. Therefore, it is important to keep in mind that TAA genes not only produce IPA but also affect the homeostasis of other -keto acids and other amino acids. It is not clear which -keto acid is the preferred in vivo acceptor of the amino group from Trp.

TAAs and YUCs were previously placed in two separate pathways [11, 22, 23]. But several recent genetic studies have demonstrated that YUCs and TAAs participate in the same pathway [16, 25, 26]. The *yuc* mutants and *taa* mutants share many similarities. For example, *yuc1 yuc2 yuc4 yuc6* quadruple mutants have dramatic vascular and floral defects, which are also observed in *taa1 tar2* double mutants [14, 22]. In fact, all of the characteristics of *taa* mutants can be phenocopied by inactivating certain combinations of *YUC* genes [26]. Overexpression of *YUC* genes leads to auxin overproduction phenotypes, which are dependent on the presence of functional TAA genes [26]. Furthermore, *taa* mutants are partially IPA deficient, whereas *yuc* mutants accumulate IPA, suggesting that TAA genes participate in IPA production and that YUCs use IPA as a substrate [16, 26]. Finally, recent biochemical studies on the catalytic mechanisms of YUC flavin
monooxygenases provide the final proof of the TAA/YUC two-step pathway as the main auxin biosynthesis pathway [16, 17, 27].

The TAA/YUC pathway is widely distributed throughout the plant kingdom. YUC genes from maize [28], rice [29–32], tomato [33], petunia [34], strawberry [35], and other species [36, 37] have been functionally characterized and they all participate in auxin biosynthesis. The TAA genes in maize have also been shown to participate in auxin biosynthesis [38]. The committed step for auxin biosynthesis is catalyzed by the YUC flavoproteins. Thus the YUC-catalyzed reaction has to be tightly controlled. It has been shown that YUC genes are only expressed in discrete groups of cells [14, 15]. Such tight control of YUC transcription provides a mechanism for temporal and spatial regulation of auxin production.

1.2.2 Other Trp-Dependent Auxin Biosynthesis Pathways

Trp is metabolized into several other indolic compounds (Figure 1.1), some of which show auxin activities when applied to plants (Figure 1.2). The physiological roles of the indolic compounds other than IPA in auxin biosynthesis are still ambiguous. That a compound can be metabolized into IAA both in vitro and in vivo does not mean that the compound is actually an important contributor to auxin biosynthesis in plants. Further genetic analysis of the genes responsible for generating the Trp metabolic intermediates (Figure 1.1) is needed to assess the roles of the compounds in auxin biosynthesis.

IAM pathway.

Arabidopsis and maize have detectable amount of IAM [7], which is the key intermediate in the bacterial auxin biosynthesis pathway characterized in Agrobacterium and Pseudomonas two decades ago [10, 39]. In plant pathogenic bacteria, Trp is oxidized by the iaaM Trp-2-monoxygenase to IAM that is subsequently hydrolyzed by iaaH to produce IAA. Unlike the bacterial IAM pathway, the genes and enzymes responsible
for producing IAM in plants have not been identified. It appears that plants do not have genes with high sequence homology to the bacterial iaaM gene. Therefore, IAM may be synthesized using a different mechanism. It is possible that IAM may be synthesized from IAA as a way to control free IAA levels. Conversion of IAA to IAM may be accomplished using mechanisms similar to glutamine biosynthesis.

Hydrolysis of IAM occurs in plants as feeding plants with IAM leads to elevated auxin levels and high-auxin phenotypes (Figure 1.2). It is proposed that a group of hydrolases, which are homologous to the bacterial hydrolase iaaH, plays a role in converting IAM to IAA [40, 41]. It is still inconclusive whether IAM is an important auxin biosynthesis intermediate in plants because IAM-deficient mutants have not been identified.

**TAM pathway.**

Tryptamine is presumably produced by Trp decarboxylase, but the enzymes responsible for the reaction in *Arabidopsis* have not been characterized. Sequence homology-based prediction may not lead to the correct identification of the genes. TAM was a proposed substrate for the YUC flavin monooxygenases [11, 36], which have now been shown to catalyze the conversion of IPA to IAA in vitro and in vivo. However, all of the flavin-containing monooxygenases form the C4a-(hydro)peroxyl flavin intermediates, which are the catalytically active intermediates. The C4a intermediate can do both nucleophilic and electrophilic reactions, depending on the reaction conditions. For example, mammalian FMOs have long been recognized for their roles in xenobiotic metabolism by reacting with soft nucleophiles such as nitrogen-containing compounds [19]. It has also been shown that Human FMOs can catalyze a BaeyerVilliger type reaction, in which the C4a intermediate reacts with an electrophilic carbonyl carbon [21]. To date, it has not been ruled out that TAM is an important intermediate in auxin biosynthesis; however, biosynthesis and metabolism of TAM are not well understood.
IAN pathway.

IAN is very abundant compared to other Trp metabolites (Figure 1.1). IAN stimulates adventitious root development and inhibits primary root elongation (Figure 1.2). The conversion of IAN to IAA is catalyzed by nitrilases. Inactivation of nitrilase genes leads to resistance to exogenous IAN, but the nitrilase mutants do not display obvious developmental defects observed in known auxin signaling and transport mutants [12, 13]. Arabidopsis genome contains four copies of the nitrilase gene. The developmental consequences of disrupting all four nitrilase genes have not been investigated, partially due to the fact that two of the copies are immediately adjacent to each other on the same chromosome. Therefore, it is still an open question whether IAN plays a significant role in auxin biosynthesis.

The routes for IAN production are not well understood either. It has been reported that metabolism of indolic glucosinolate yields IAN [42]. However, maize does not produce glucosinolates, but still produces IAN, suggesting that other routes can produce IAN. It has been suggested that IAN may also be produced from other indolic compounds such as IAOx [7].

IAAld pathway.

IAAld was previously proposed as an intermediate in the IPA pathway for auxin biosynthesis [1]. In plants, it is now known that IAAld is not an intermediate in the IPA pathway [16, 26] as IPA is converted to IAA by the YUC flavin-containing monooxygenases without producing IAAld [17]. In some IAA-producing bacteria, IAAld is produced from IPA by IPA decarboxylases [43]. IAAld can be further oxidized into IAA by aldehyde oxidases. In Arabidopsis, genes homologous to the bacterial IPA decarboxylases appear not to play a role in auxin biosynthesis. Inactivation of Arabidopsis aldehyde oxidases does not disturb auxin homeostasis, suggesting that it is very likely that IAAld does not contribute significantly to de novo auxin biosynthesis [16]. However, IAAld
can also be oxidized by aldehyde dehydrogenases, which have not been characterized in *Arabidopsis*.

**IAOx pathway.**

IAOx has only been detected in *Arabidopsis* and related species [16]. Monocots such as rice and maize do not have detectable levels of IAOx [16]. In *Arabidopsis*, CYP79B2 and CYP79B3 convert Trp directly to IAOx [44, 45]. Overexpression of CYP79B2 in *Arabidopsis* leads to long hypocotyl and epinastic cotyledons, a phenotype that is also observed in YUC overexpression lines, suggesting that IAOx can be a precursor for IAA biosynthesis [45]. IAOx is also a precursor for indolic glucosinolate biosynthesis [9, 46]. When the genes encoding glucosinolate biosynthesis enzymes are mutated, more IAOx is fluxed into IAA biosynthesis, causing auxin overproduction phenotypes [9, 46]. For example, the *sur1* and *sur2* mutants that are defective in glucosinolate biosynthesis overproduce auxin, which leads to the development of long hypocotyls and numerous adventitious roots.

It appears that CYP79B2 and textitB3 are the only genes responsible for producing IAOx in *Arabidopsis*. The *cyp79b2 cyp79b3* double mutants appear to completely abolish the biosynthesis of IAOx and the double mutants have no detectable amount of IAOx [7]. The double mutants have subtle growth defects when grown at high temperature, but have no obvious phenotypes under normal growth conditions [45]. Therefore, it is believed that IAOx is not an essential intermediate for auxin biosynthesis. Nor is IAOx a universal intermediate for auxin biosynthesis.

In summary, after three decades molecular genetics studies in *Arabidopsis*, the picture of de novo auxin biosynthesis has become clearer. The two-step Trp-dependent pathway catalyzed by TAAs and YUCs is the main auxin biosynthesis pathway that plays essential roles in almost all of the main developmental processes. In retrospect, *Arabidopsis* probably is not the best model for auxin biosynthesis studies for two main
reasons. First, the *Arabidopsis* glucosinolate biosynthesis pathway really complicated the analyses of IAA biosynthesis because the glucosinolate biosynthesis intermediate IAOx can be converted into IAA. The aforementioned glucosinolate mutants such as *sur1* and *sur2* had dramatic auxin overproduction phenotypes [9, 46]. Second, the genetic redundancy within *YUCs* and *TAAs* in *Arabidopsis* made it difficult for loss-of-function studies. The single *Arabidopsis yuc* or *taa* mutants do not show dramatic auxin phenotypes. Only the multiple mutants of *taa* or *yuc* display dramatic developmental defects [14, 15, 22]. In contrast, some monocots such as maize offer a relatively simpler system for analyzing auxin biosynthesis. Maize does not produce indolic glucosinolate [7]. Furthermore, inactivation of a single *YUC* gene or *TAA* gene in maize leads to dramatic developmental phenotypes, whereas inactivation of at least two *YUC* genes or *TAA* genes in *Arabidopsis* is needed to cause main developmental defects [28, 38].

### 1.3 IAA Production from Non-De Novo Pathways

Besides de novo in loco synthesis and transportation from neighboring cells, free IAA can also be made available by releasing IAA from its conjugated forms or from indole butyric acid (IBA) [47]. In fact, the majority of IAA in plants exists in the conjugated forms, which are proposed to serve as a storage pool. It is known that IAA can be conjugated via ester bonds with simple alcohols and with sugars such as glucose and myo-inositol or via amide bonds with amino acids, peptides, or proteins. Free IAA can be produced when the conjugates are hydrolyzed. Hydrolysis of conjugates provides plants with a potentially faster way to modulate free IAA levels than de novo biosynthesis. For example, in the germinating seeds of maize, large amount of IAA is released from the endosperm from its ester form to support the growth of developing seedlings [48]. Some of the enzymes responsible for hydrolyzing IAA-sugar or IAA-amino acids have been
characterized and they show different substrate specificities and are developmentally regulated [49–53].

1.3.1 Conversion of IBA to IAA

IBA has long been used in agriculture for promoting root initiation/growth from plant cuttings. *Arabidopsis* plants accumulate detectable amount of IBA. However, it is not understood how IBA is synthesized in plants. IBA is known to inhibit primary root elongation and stimulate lateral root formation. Genetic screens for *Arabidopsis* mutants resistant to exogenous IBA have identified many loci (*ibr*, IBA resistant). The majority of the *ibr* loci encode enzymes related to -oxidation of fatty acids or biogenesis of peroxisome, where -oxidation takes place. The genetic data suggest that the observed auxin activities of IBA depend on the conversion of IBA to IAA [54, 55]. However, it has not been completely ruled out that IBA itself has some biological activities [56].

The physiological roles of IBA-derived IAA are difficult to determine because the enzymes responsible for IBA to IAA conversion may also participate in other pathways such as fatty acid metabolism. Recent characterization of mutations resistant to IBA leads to the discovery that disruption of *ENOYL-COA HYDRATASE2* (*ECH2*) gene causes defects in IBA responsiveness, but appears not to affect sugar and fatty acid metabolism. Further analysis of *ech2* and other *ibr* mutants demonstrated that IBA-derived IAA plays important roles in root hair development and cotyledon cell expansion [55, 57].

1.3.2 Release of Free IAA from IAA Conjugates

IAA conjugates with ester-link to sugars and small alcohols or amide-link to amino acids and peptides have been identified in plants. The various conjugates may serve as a storage form of IAA and can release free IAA when needed. The most studied case
of releasing free IAA from conjugates is the hydrolysis of IAA-amino acid conjugates. Among the 20 potential amino acid conjugates, 19 (except IAA-Arg) were tested for their ability to release free IAA in a bioassay based on root elongation in *Arabidopsis* [52]. It was shown that IAA-Ala, -Leu, -Phe, -Asn, -Gln, -Glu, -Gly, -Met, -Ser, -Thr, and -Tyr inhibited root elongation by more than 50% at 40 µM, suggesting that these amino acid conjugates can be hydrolyzed to release free IAA. In contrast, IAA-Asp, -Cys, -His, -Ile, -Lys, -Pro, -Trp, and -Val appeared not a source for free IAA [52]. Genetic screens for *Arabidopsis* mutants resistant to IAA-Leu and IAA-Ala identified a family of hydrolases including *IAALeu Resistant 1 (ILR1)*, *IAAAla resistant (IAR3)*, and the *ILR1-like protein (ILL2)* responsible for releasing free IAA from the IAA-amino acid conjugates [50–53]. The *ilr1 iar3 ill2* triple mutants are resistant to several IAA-amino acid conjugates and have shorter hypocotyl and fewer lateral roots than wild-type plants, suggesting that releasing free IAA from conjugates plays important roles in IAA homeostasis and plant development [53].

### 1.4 Deactivation of IAA

The active form of IAA is believed to be free IAA. The carboxyl group in IAA is essential for its auxin activities. IAA is inactivated by complete oxidation, a process that is still not well understood. IAA can also be taken out of action by forming various conjugates with alcohols, sugars, and amino acids [47].

#### 1.4.1 Synthesis of IAA Conjugates

Great progresses have been made in recent years towards understanding the enzymes responsible for synthesizing IAA esters and amide conjugates. In maize, synthesis of IAA-ester with sugar starts with the formation of IAA-glucose that is
preceded by activation of glucose by the formation of glucose-UDP that is then joined with IAA. IAA-glucose is further converted to other IAA-sugar ester conjugates that are mostly believed to be a storage form of IAA [58–60]. The formation of methyl IAA by the IAMT1 methyl transferase has been implicated in regulating leaf development in Arabidopsis [61].

In Arabidopsis, 20 amidosynthases encoded by the large Gretchen Hagen 3 (GH3) family of genes conjugate IAA as well as some other plant hormones such as jasmonic acid and salicylic acid with amino acids to form amide conjugates [62–64]. GH3 genes are among the early-induced genes by auxin treatments [62]. Originally discovered as being able to adenylate IAA in vitro, GH3 amidosynthases are later shown to be responsible for synthesizing IAA-amino acid conjugates. The adenylyl-IAA serves as the activated intermediate and readily reacts with some amino acids [64]. Some of the IAA-amino acid conjugates can be hydrolyzed to release free IAA, while some of the conjugates appear non-hydrolyzable in vivo [52]. The latter group of IAA-conjugates may serve as a way to inactivate IAA. For example, once IAA-Asp is formed, it would not be hydrolyzed and the conjugated IAA is consequently permanently deactivated. IAA-Asp is also known as a target for oxidative degradation. GH3 proteins have also been shown to play roles in response to environmental stimuli such as light and wounding processes, possibly through the regulation the formation of IAA, jasmonic acid, and/or salicylic acid conjugates [47]. Interestingly, some of the IAA conjugates possesses antagonist effects against IAA. Externally applied IAA-Trp effectively antagonizes the inhibitory effects of IAA treatment in Arabidopsis roots [65, 66]. IAA-peptide and IAA-protein conjugates have also been discovered [67], indicating that IAA may serve as a small molecular tag, but their functions are still unclear.
1.4.2 IAA Degradation via Oxidation

IAA starts the oxidative degradation either with decarboxylation on the side chain or with oxidation of the indole ring. Very little is known regarding oxidative degradation of IAA. It has been reported that peroxidase may be involved in the oxidative decarboxylation of IAA [68]. Oxidative intermediates including OxIAA have been discovered in plants [69–72]. In *Arabidopsis*, other IAA metabolites such as N-(6-hydroxyindol-3-ylacetyl)-phenylalanine (6-OH-IAA-Phe), N-(6-hydroxyindol-3-ylacetyl)-valine (6-OH-IAA-Val), and 1-O-(2-oxoindol-3-ylacetyl)-beta-d-glucopyranose (OxIAA-Glc) have been observed with OxIAA-Glc being the main oxidative product. Recently, it was reported that in *Arabidopsis* roots, OxIAA is the major catabolic product of IAA [73]. Because OxIAA has little auxinic effects, irreversible oxidation of IAA into OxIAA effectively removes the IAA from the auxin pool. Another recent discovery in rice shed light on the genes underlying the conversion of IAA to OxIAA [27]. Rice plants with a mutation in the *Dioxygenase for Auxin Oxidation* (*DAO*) gene have elevated free IAA levels in anthers and ovaries and are defective in anther dehiscence, pollen fertility, and seed development [27]. The *dao* mutants also do not have detectable level of oxIAA, and the purified DAO protein expressed in *E. coli* could convert IAA to oxIAA in vitro [27]. The new findings mark the beginning of understanding the molecular and genetic mechanisms underlying IAA oxidation and the roles of oxidative degradation of IAA in auxin homeostasis.

1.5 Acknowledgments

Chapter 1 in full, is a reprint of the material as it appears in Part 1.1 in Auxin and Its Role in Plant Development, 2014. Gao, Yangbin; Zhao, Yunde. Springer, 2014. The dissertation/thesis author was a primary investigator and author of this paper.
Chapter 2

Epigenetic Suppression of T-DNA Insertion Mutants in *Arabidopsis*

2.1 Abstract

T-DNA insertion mutants have been widely used to define gene functions in *Arabidopsis* and in other plants. Here, we report an unexpected phenomenon of epigenetic suppression of T-DNA insertion mutants in *Arabidopsis*. When the two T-DNA insertion mutants, *yuc1-1* and *ag-TD*, were crossed together, the defects in all of the *ag-TD* plants in the F2 population were partially suppressed regardless of the presence of *yuc1-1*. Conversion of *ag-TD* to the suppressed *ag-TD* (named as *ag-TD*\(^*\) did not follow the laws of Mendelian genetics. The *ag-TD*\(^*\) could be stably transmitted for many generations without reverting to *ag-TD*, and *ag-TD*\(^*\) had the capacity to convert *ag-TD* to *ag-TD*\(^*\). We show that epigenetic suppression of T-DNA mutants is not a rare event, but certain structural features in the T-DNA mutants are needed in order for the suppression to take place. The suppressed T-DNA mutants we observed were all intronic T-DNA mutants and the T-DNA fragments in both the trigger T-DNA as well as in the suppressed T-DNA
shared stretches of identical sequences. We demonstrate that the suppression of intronic T-DNA mutants is mediated by trans-interactions between two T-DNA insertions. This work shows that caution is needed when intronic T-DNA mutants are used.

### 2.2 Introduction

Agrobacterium-mediated plant transformation is achieved when the T-DNA (Transfer DNA) fragment from the modified Ti plasmids is integrated into chromosomes in plant cells. T-DNA transformation can be used as a tool for insertional mutagenesis and also serves as an efficient vehicle for delivering target genes into plant cells. T-DNA fragments randomly insert into a plant genome during transformation and, when a T-DNA insertion is inserted in an exon or an intron, it often leads to the inactivation of the gene. As part of the different functional genomic initiatives in *Arabidopsis*, a number of T-DNA insertional mutagenesis have been conducted and currently we have access to large libraries of sequence-indexed T-DNA insertion lines in *Arabidopsis* [74–76]. The T-DNA insertion mutants are tremendous resources for the determination of gene function and the elucidation of metabolic/signaling pathways. T-DNA mutants in *Arabidopsis* have become the first choice for many scientists because (1) the mutants are easily accessible through the *Arabidopsis* stock centers and (2) the mutants are often null alleles. T-DNA insertion mutants have been extensively used in reverse genetics and in studies of genetic interactions in *Arabidopsis*.

Studies on genetic interactions between two non-allelic mutants often provide insights into the functions of the two genes and the relative positions of the genes in a genetic pathway [77, 78]. Phenotypes of a mutant can be suppressed or enhanced by mutations in other genes. Synergistic genetic enhancement between two mutants often suggests that the two genes have overlapping functions or participate in parallel
pathways [77]. If the two mutants are not null alleles and the two genes have no sequence homology, synergistic enhancement can also suggest that the two genes function in the same pathway [79]. Genetic suppression of the phenotypes of one mutant by a mutation in another gene could be achieved through several mechanisms [78]. A mutant could be rescued if the general machinery of transcription and/or translation is altered. For example, a mutation that converts a sense codon to the stop codon UAG in a gene can be suppressed if the anti-codon in Trp-tRNA is mutated from CCA to CUA. Additionally, genetic suppression could also take place if the suppressor removes toxic proteins or metabolic intermediates. A mutant can also be suppressed if protein interactions or gene dosages are altered. Genetic screens for enhancers and suppressors for mutants have led to the discoveries of the regulatory mechanisms of major signaling and metabolic pathways.

In general, the phenotypes of a mutation are suppressed when an extragenic suppressor is present. Removal of the suppressor leads to the restoration of the original mutant phenotypes. In this paper, we report an unexpected phenomenon that phenotypes of a T-DNA insertion mutant are partially suppressed by another T-DNA insertion at another locus. Remarkably, the suppressed phenotypes could be stably transmitted for generations even in the absence of the suppressor T-DNA insertion. We crossed an auxin biosynthesis mutant yuc1-1 to a floral mutant ag-TD in order to generate the yuc1-1 ag-TD double mutants for analyzing the roles of auxin in flower development. Both yuc1-1 and ag-TD are T-DNA insertion mutants (Figure 2.1A) and both are loss-of-function, recessive mutants. The YUC1 gene encodes a flavin-containing monooxygenase involved in auxin biosynthesis [11, 14, 15]. The yuc1-1 mutant has no obvious developmental defects because of the existence of other homologous YUC genes in Arabidopsis [14]. AGAMOUS (AG) is an essential gene for reproductive organ formation in Arabidopsis [80]. The ag-TD mutant displays the characteristic ag loss-of-function phenotypes including
the transformation of stamens into petals, loss of floral meristem determinacy, and a lack of carpels and stamens [80]. Surprisingly, none of the *ag-TD* plants in the F2 population displayed the typical *ag-TD* phenotypes, regardless of the presences of *yuc1-1*. We demonstrate that suppression of *ag-TD* is mediated by trans-interaction between the T-DNA insertions in *yuc1-1* and *ag-TD*. Although gene silencing mediated by trans-interaction between two T-DNA insertions has been well documented [81], it has never been reported previously that such a trans-interaction among T-DNA insertions can lead to the restoration of gene functions inactivated by the same T-DNA insertions. We show that suppression of intronic T-DNA insertional mutants is frequently induced by other T-DNA insertions, suggesting that caution is needed when intronic T-DNA mutants are used in *Arabidopsis*.

### 2.3 Results

#### 2.3.1 Suppression of an *agamous* T-DNA Insertion Mutant by *yuc1-1*

To investigate the mechanisms of local auxin biosynthesis in specifying flower development, we combined the auxin biosynthesis mutant *yucca1* (*yuc1*) [14] with a known floral homeotic mutant *agamous* (*ag*) [80]. We chose the recessive T-DNA insertion mutants *ag-TD* and *yuc1-1* because both mutants are in the Columbia background. In both *ag-TD* and *yuc1-1*, the T-DNA is inserted in an intronic region (Figure 2.1A). The *yuc1-1* does not show obvious developmental defects [14], but *ag-TD* fails to produce any stamens and carpels (Figure 2.1). We crossed *ag-TD*/*- to *yuc1-1* and genotyped the F1 plants to select the *ag-TD*/*+ yuc1-1*/*- plants (Figure 2.1B), which did not have obvious defects as expected. We let the F1 *ag-TD*/*+ yuc1-1*/*+ plants self-pollinate and collected the F2 seeds (Figure 2.1B). We analyzed the F2 population in order to identify the *ag-TD yuc1-1* double mutants. Unexpectedly, none of the *ag-TD* plants
Figure 2.1: Suppression of ag-TD by Crossing ag-TD+/− to yuc1-1. (A) A diagrammatic presentation of the two T-DNA insertion mutants: yuc1-1 and ag-TD. (B) The crossing scheme. The ag-TD+/+ yuc1-1+/− F1 plants were discarded. (C) Suppression of the floral defects in ag-TD in the F2 population. From left to right: WT, yuc1-1, ag-TD, and ag-TD*. (D) Phenotypic difference between ag-TD and ag-TD* inflorescence. (E) Production of petal-like stamens in ag-TD*. (F) Normal floral organs in ag-TD*.

displayed the typical ag phenotypes, indicating that ag-TD phenotypes were partially suppressed (Figure 2.1C). We named the plants with ag-TD genotype but without ag flower phenotypes as ag-TD* (Figure 2.1C). Note that ag-TD* is still homozygous for the T-DNA insertion as shown in Figure 2.1A, but the AG function is no longer inactivated by the T-DNA insertion in ag-TD*. The ag-TD* plants were fertile and produced viable seeds (Figure 2.1D). The suppression of ag-TD was only partial, because some ag-TD* flowers still contained petal-like stamens (Figure 2.1E) and indeterminate flowers (Figure 2.1D). However, the majority of ag-TD* flowers had flower with four sepals, four petals, and one gynoeceum consisting of two fused carpels (Figure 2.1F).

2.3.2 The yuc1-1 Is Not Required in the F2 Population for ag-TD* Phenotypes

We genotyped the F2 plants from the cross between ag-TD and yuc1-1 for the presence of ag-TD and yuc1-1. Among the 176 F2 individual plants, 56 were ag-TD,
indicating that the T-DNA insertion at the AG locus segregated normally. Among the ag-TD plants, 43 did not contain T-DNA insertion at YUC1, 13 were yuc1-1+/−, and zero were yuc1-1. Because both AG and YUC1 are on chromosome IV and they are about 15 cM apart, it was expected that very few ag-TD yuc1-1 would be observed in the F2 population. Floral defects in all of the ag-TD plants in the F2 population were partially suppressed. Overall, 80% of the ag-TD YUC1 plants were suppressed well enough to be fertile. We noticed that all of the ag-TD−/− yuc1-1+/− plants were able to set seeds, suggesting that the presence of the yuc1-1 mutation enhanced the suppression. However, the continued presence of the yuc1-1 mutation was not required to suppress ag-TD.

2.3.3 The ag-TD* Is Genetically Stable

Figure 2.2: Inheritability of ag-TD*. The ag-TD* has been transmitted for five generations. Note that the fifth generation of ag-TD* produced more seeds than the earlier generations of ag-TD*.

To test whether ag-TD* phenotypes could be stably transmitted, we let the ag-TD* plants self-fertilize and studied the progeny for five generations. All of the progeny of ag-TD* was fertile in every generation and set a good number of seeds. We also noticed that the later generation of ag-TD* produced more seeds than the earlier generation of
We concluded that, once *ag-TD* was converted to *ag-TD*\(*\), the *ag-TD*\(*\) does not spontaneously revert to *ag-TD* over generations (Figure 2.2).

We crossed *ag-TD*\(*\) to wild-type (WT) Columbia (Col) and let the F1 plants self-pollinate to generate an F2 population for analysis of the genotypes and phenotypes. Among the 98 plants analyzed, 23 were homozygous for the T-DNA insertion at the *AG* locus and all of the *ag-TD* plants displayed the *ag-TD*\(*\) phenotypes, indicating that *ag-TD*\(*\) is very stable.

### 2.3.4 The *ag-TD*\(*\) Is Able to Convert *ag-TD* to *ag-TD*\(*\)

We tested whether *ag-TD*\(*\) could induce similar changes in *ag-TD*. We crossed *ag-TD*\(*\) to *ag-TD*\(+/−\) plants and half of the resulting F1 plants were homozygous with the T-DNA insertion as expected. The F1 plants that presumably had the *ag-TD*\(**/ag-TD* genotype were fertile and set a good number of seeds. We further analyzed the F2 plants generated from *ag-TD*\(**/ag-TD* selfing. Among the 68 F2 plants analyzed, 66 plants behaved like *ag-TD*\(*\). Two plants had weak *ag-TD* phenotypes and did not set seeds. Our data suggest that *ag-TD*\(*\) has the capacity to convert *ag-TD* into *ag-TD*\(*\).

### 2.3.5 The *ag-TD*\(*\) Cannot Suppress Non-T-DNA *ag* Alleles

We have shown that *ag-TD*\(*\) allele induced the conversion of *ag-TD* into *ag-TD*\(*\). We investigated whether *ag-TD*\(*\) could also restore the *AG* functions in other non-T-DNA *ag* mutant alleles. We used the strong *ag-3* mutant and the weak *ag-4* mutant alleles for the experiments (Figure 2.2A). Both *ag-3* and *ag-4* carried point mutations at splice junction sites (Figure 2.2A) [82,83]. We crossed *ag-TD*\(*\) to *ag-3*\(+/−\), and the resulting F1 *ag-TD*\(**/ag-3* plants still displayed the typical *ag* mutant phenotypes and were sterile, suggesting that *ag-TD*\(*\) could not rescue *ag-3*. When we crossed *ag-TD*\(*\) to the weak
Figure 2.3: Suppression of ag Involves Special Alleles of ag and yuc. (A) The two non-T-DNA alleles of ag used in this study. (B) Allele of yuc1-3, yuc1-3 has a T-DNA insertion in the third exon. (C) The ag-TD was not suppressed by yuc1-3.

ag-4+/− plants, the ag-TD*/ag-4 plants were partially fertile. Normally, the ag-4 plants produce some stamens and carpel-like structures, but are sterile in our growth conditions. The ag-TD*/ag-4 plants could set seeds and their phenotypes were intermediate when compared to ag-TD* and ag-4 plants. We further analyzed the F2 population produced from selfing the ag-TD*/ag-4 plants. All of the homozygous ag-TD plants from the F2 population displayed the same phenotypes as those of ag-TD*. The ag-TD*/ag-4 plants in the F2 population were fertile, but all of the ag-4 plants were sterile. Our data indicate that ag-TD* could not rescue non-T-DNA ag mutants.

2.3.6 Conversion of ag-TD to ag-TD* Depends on a Specific yuc1 T-DNA Allele

The ag-TD mutant was rescued when it was crossed to yuc1-1 (Figure 2.1). We tested whether other T-DNA insertion mutants in yuc1 could also convert ag-TD to ag-TD*. We crossed ag-TD to yuc1-3 (Figure 2.3B). The yuc1-3 contained a T-DNA insertion at the third exon in the YUC1 gene (Figure 2.3B). Although both yuc1-1 and
yuc1-3 were T-DNA insertion lines, they were generated using two different plasmids. The yuc1-1 was generated using the plasmid pROK2, which renders kanamycin resistance in Arabidopsis. The yuc1-3 was produced using a different plasmid that contains the SPM transposase gene and the BAR gene.

We genotyped the F2 population generated from selfing ag-TD+/− yuc1-3+/− to identify ag-TD plants. All of the ag-TD plants in the F2 population displayed the typical ag-TD phenotypes regardless of the existence of yuc1-3 mutation and none of the ag-TD plants set any seeds (Figure 2.3C). We also isolated ag-TD yuc1-3 plants from the progeny of a single ag-TD+/− yuc1-3 plant and the double mutants behaved like ag-TD. These results suggest that inactivation of YUC1 is not sufficient to trigger the suppression of ag-TD and that the suppressor and the suppressed T-DNA mutants need to be generated from similar plasmids.

2.3.7 Production of Full-Length AG cDNA Using mRNAs from ag-TD and ag-TD*

We investigated whether the ag-TD to ag-TD* conversion is caused by an increased expression of AG in ag-TD*. We designed PCR primers to amplify the portion of AG cDNA starting from the start codon to the stop codon. To our surprise, ag-TD produced the full-length AG cDNA, suggesting that ag-TD is a partial loss-of-function mutant. We sequenced the AG cDNA from WT plants, ag-TD, and ag-TD*, and discovered that there were no structural differences among the cDNAs from the analyzed genotypes. It is difficult to compare the expression levels of AG in WT and in ag-TD using RTPCR or Northern blot because the floral structures are quite different for the two genotypes. We used RNA in situ hybridization to detect the expression levels of AG in WT, ag-TD, and ag-TD*. The AG expression in ag-TD was weaker than that in WT, but ag-TD* clearly had more AG expression than ag-TD, suggesting that the conversion of
ag-TD to ag-TD* correlates with an increased AG mRNA level in ag-TD* (Figure 2.4A).

2.3.8 Kanamycin Resistance Gene Is Silenced in ag-TD*

![Figure 2.4](image)

**Figure 2.4**: Suppression of ag-TD Is Probably Mediated by Trans-Interaction between Two T-DNA Insertions. (A) In situ analysis of AG expression in WT, ag-TD, and ag-TD*. (B) Conversion of ag-TD to ag-TD* correlates with the loss of kanamycin resistance. (C) Conversion of ag-TD to ag-TD* can be achieved by the introduction of a T-DNA fragment that expresses the NPT II gene. About 75% of the T1 plants with ag-TD genotype did not show the typical ag phenotypes.

We hypothesized that perhaps the partial restoration of AG function in ag-TD* might be caused by structural changes in DNA/chromatin in or near the T-DNA insertion. Such DNA/chromatin structural modifications might also alter the expression of the Neomycin phosphotransferase II (NPT II) gene, which renders plants resistant to kanamycin, within the T-DNA fragment. The NPT II gene in the T-DNA insertion made ag-TD plants resistant to kanamycin (Figure 2.4B) and, accordingly, about 25% of the progeny from ag-TD^{+/-} plants were kanamycin-sensitive, suggesting that ag-TD
contains a single T-DNA insertion. In contrast, all of the ag-TD* plants were kanamycin-sensitive (Figure 2.4B), although the NPT II gene still existed in ag-TD*. These data suggest that transcripts from the T-DNA fragment are also affected by the epigenetic modifications that suppressed ag-TD.

2.3.9 Suppression of ag-TD by Trans-Interactions between T-DNA Loci

The observation that kanamycin resistance was lost in ag-TD* suggested that trans-interactions between the T-DNA fragment in yuc1-1 locus and the T-DNA in ag-TD may be responsible for the suppression of ag-TD. To test this hypothesis, we transformed ag-TD+/− plants with a construct that expressed both the NPT II and the BAR gene (Figure 2.4C). Transformants were selected on basta-containing media. Among the 26 T1 plants with ag-TD genotype, 76% were partially suppressed and 44% were fertile (Figure 2.4C), demonstrating that introduction of another T-DNA insertion that expresses NPT II gene is sufficient to suppress ag-TD. The suppression of ag-TD is likely mediated by trans-interactions among T-DNA insertions.

2.3.10 Suppression of T-DNA Mutants by Other T-DNA Insertions Is Not Rare

We have demonstrated that ag-TD is suppressed by yuc1-1 and also by transforming a T-DNA fragment into ag-TD. We investigated whether other T-DNA insertion mutants can also be suppressed by similar T-DNA interactions. We crossed yuc1-1 to cob-TD, which also contains a T-DNA insertion in the large intron (Figure 2.5A). The COB gene encodes a glycosylphosphatidylinositol (GPI) anchored protein and plays an important role in cellulose microfibril orientation in Arabidopsis [84, 85]. Inactivation
of COB by the T-DNA insertion led to very short roots and other defects (Figure 2.5B). However, all of the cob-TD plants in the F2 plants from the cross between yuc1-1 and cob-TD had longer roots than the original cob-TD lines, indicating that yuc1-1 also converted cob-TD to cob-TD*, which was partially suppressed (Figure 2.5). The presence of yuc1-1 made the suppression of cob-TD better (Figure 2.5B). This result is consistent with the observation that the ag phenotypes in ag-TD yuc1-1+/− were better suppressed than those in ag-TD alone. Interestingly, the conversion of cob-TD to cob-TD* also led to the loss of kanamycin resistance (Figure 2.5D).

2.4 Discussion

In this paper, we presented the analyses of an unexpected epigenetic phenomenon in Arabidopsis. We showed that some Arabidopsis T-DNA mutants were stably suppressed by T-DNA insertions in other non-homologous loci. We proposed that a T-DNA insertion in one locus could trigger undefined epigenetic modifications at a different T-DNA insertion site. The epigenetic modifications in the T-DNA mutants were heritable.
in the absence of the T-DNA suppressor. Because T-DNA mutants have been widely used in reverse genetics and in analyzing genetic interactions in *Arabidopsis*, this work suggests that we should be cautious about intronic T-DNA mutants.

2.4.1 Suppression Reported in this Work Violates Rules of Mendelian Genetics

When *ag-TD* was crossed to *yuc1-1*, all of the *ag-TD* plants in the F2 population were partially suppressed no matter whether *yuc1-1* was present or not, although the presence of *yuc1-1* rendered better suppression (Figure 2.1). The *ag-TD* not only could be stably transmitted for many generations in the absence of *yuc1-1* (Figure 2.2), but also had the ability to trigger new epigenetic suppressions in *ag-TD*. There are many similarities between the epigenetic suppression of T-DNA mutants and paramutation, a well-studied epigenetic phenomenon in Maize [86–89]. In paramutation, one allele (*B’*) causes heritable changes in another allele (*B-I*) of the same locus. Both the conversions of *B-I* to *B’* and *ag-TD* to *ag-TD* were triggered by a cross. Once *B-I* is converted to *B’*, the new *B’* from *B-I* can be stably transmitted. We have shown that *ag-TD* could also be stable for many generations (Figure 2.2). The newly converted *B’* can convert *B-I* to *B’* and we showed that *ag-TD* can convert *ag-TD* to *ag-TD*.

The end results of paramutation appear to be unidirectional because it is always that *B-I* is converted to *B’*. In the cases of T-DNA suppression that we have analyzed, the suppression appeared to be one-directional as well. When *ag-TD* was crossed with *ag-TD*, *ag-TD* was always converted to *ag-TD*. 
2.4.2 Epigenetic Suppression of T-DNA Mutants Is Triggered by Trans-Interaction between T-DNA Insertions

Several lines of evidence support the hypothesis that the conversion of $ag$-TD to $ag$-TD* is probably caused by trans-interaction between T-DNA insertions. First, $ag$-TD mutant displays strong kanamycin resistance whereas $ag$-TD* is kanamycin-sensitive (Figure 2.4). It has been demonstrated that trans-inactivation between homologous genes causes the loss of antibiotic resistance in T-DNA insertion mutants [81]. The T-DNA insertions in both $yuc1$-1 and $ag$-TD are from the same plasmid; therefore, the $NPT$ II transcripts from the T-DNA insertion at the $yuc1$-1 locus have the capacity to induce the silencing of the $NPT$ II gene in the T-DNA fragment at the $ag$-TD locus. Second, the conversion of $ag$-TD to $ag$-TD* could also be achieved by transforming $ag$-TD$^+/−$ plants with a construct that expresses $NPT$ II from the 35S promoter (Figure 2.4C).

We propose that transcripts such as the $NPT$ II mRNA from the T-DNA insertions in $yuc1$-1 and $ag$-TD interact in trans to cause the silencing of $NPT$ II. It has been well documented that trans T-DNA interactions can lead to the silencing of homologous genes [81]. What is very unusual is that the silencing of the genes located in the T-DNA fragments such as $NPT$ II is correlated with the restoration of the gene function inactivated by the T-DNA insertion.

It is often hypothesized that intronic T-DNA insertions disrupt gene function because transcripts cannot be properly spliced. However, some genes have very large introns that are spliced out properly from the primary transcripts, suggesting that other factors may also contribute to the inactivation of gene function by intronic T-DNA insertions. When a T-DNA fragment is inserted into an intron of a gene, the primary transcript from the gene contains the entire intron plus the T-DNA fragment if the transcription is not prematurely terminated within the T-DNA region. Therefore, it is
conceivable that transcripts from the T-DNA fragment such as the *NPT II* transcript may be able to form a partial duplex with the primary transcript when the *NPT II* gene is transcribed from the opposite direction. Such a duplex may affect proper processing of the primary transcript or even lead to degradation of the transcript. When those T-DNA-generated reverse transcripts are silenced by transcripts from another homologous T-DNA insertion (a process very similar to co-suppression), the duplex between the *NPT II* transcript and the primary transcript would be resolved. Consequently, the intronic T-DNA mutants are partially suppressed and the *NPT II* gene is silenced. We recognize that the *NPT II* transcript from the T-DNA insertion is similar to long intronic non-coding RNA, which causes epigenetic changes and affects gene expression levels [90].

Our findings indicated that intronic T-DNA insertion mutants can be easily suppressed by *trans*-interaction with another T-DNA. Therefore, the use of intronic T-DNA insertion mutants sometimes may lead to incorrect interpretations. We would like to point out that *trans*-interaction with another T-DNA insertion may not be the only trigger that is capable of causing the suppression of the phenotypes of an intronic T-DNA mutant. Environmental factors may also be able to cause the suppression of T-DNA mutants. For example, the intronic T-DNA insertion mutant *opr3* has long been recognized as a null allele and it produced no detectable Jasmonic acids (JAs) following wounding and looper infestation [91]. However, recently it was shown that the same *opr3* mutant became activated upon fungal infection and accumulated substantial levels of JAs. It was suggested that splicing of the T-DNA-containing intron might be responsible for the reactivation of *OPR3* [91]. In light of our findings, it is also possible that epigenetic modifications induced by fungal infection may play a role. Our study indicates that we should be careful about the use of intronic T-DNA mutants because some intronic T-DNA insertion mutants may undergo epigenetic changes that complicate interpretations of genetic interactions in *Arabidopsis*. 
2.5 Methods

The T-DNA insertion mutants cob-TD and yuc1-3 were obtained from the ABRC at Ohio. The ag-TD was from Dr Yanofsky. The yuc1-1 was previously described [14,26]. For genotyping T-DNA mutants, we used PCR-based methods as previously described [76]. The gene-specific primers for genotyping ag-TD were 5’- ACGGCGTACCAAT-CGGAGCTAGGAGGA -3’ and 5’- TCTAGCTAGTTTCACCTTATTCACTCTC -3’. Primers for genotyping yuc1-1 and yuc1-3 were 5’- GGTCATGTGGTTGCCAAGGGA -3’ and 5’- CCTGAAGCCAAGTAGCAGCCTTT -3’. Gene-specific primers for cob-TD were 5’- TCCACTCCTCCTCAAGCAAGGC -3’ and 5’- CCATTTCATTGTAATGTCGCCCTC -3’. The T-DNA specific primer for genotyping ag-TD, cob-TD, and yuc1-1 was JMLB1 (5’- GGCAATCAGCTGCCCGTCTCACTGGTG -3’). T-DNA primer for yuc1-3 was Spm32 (5’- TACGAATAAGAGCGTCCATTTTAGAGTGA -3’). RNA in situ hybridization was performed as described previously [14].

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Chapter 3

Self-processing of ribozyme-flanked RNAs into guide RNAs in vitro and in vivo for CRISPR-mediated genome editing

3.1 Abstract

CRISPR/Cas9 uses a guide RNA (gRNA) molecule to execute sequence-specific DNA cleavage and it has been widely used for genome editing in many organisms. Modifications at either end of the gRNAs often render Cas9/gRNA inactive. So far, production of gRNA in vivo has only been achieved by using the U6 and U3 snRNA promoters. However, the U6 and U3 promoters have major limitations such as a lack of cell specificity and unsuitability for in vitro transcription. Here, we present a versatile method for efficiently producing gRNAs both in vitro and in vivo. We design an artificial gene named RGR that, once transcribed, generates an RNA molecule with ribozyme
sequences at both ends of the designed gRNA. We show that the primary transcripts of \( RGR \) undergo self-catalyzed cleavage to generate the desired gRNA, which can efficiently guide sequence-specific cleavage of DNA targets both in vitro and in yeast. \( RGR \) can be transcribed from any promoters and thus allows for cell- and tissue-specific genome editing if appropriate promoters are chosen. Detecting mutations generated by CRISPR is often achieved by enzyme digestions, which are not very compatible with high-throughput analysis. Our system allows for the use of universal primers to produce any gRNAs in vitro, which can then be used with Cas9 protein to detect mutations caused by the gRNAs/CRISPR. In conclusion, we provide a versatile method for generating targeted mutations in specific cells and tissues, and for efficiently detecting the mutations generated.

### 3.2 Introduction

The CRISPR/Cas9 system (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated endonuclease Cas9) has been shown to mediate efficient genome editing in human cells [92,93], mice [94], rat [95], zebrafish [96,97], \( Caenorhabditis \) \( elegans \) [98], \( Drosophila \) [99], yeast [92, 100], \( Arabidopsis \) [101], and crop plants [101–103]. CRISPR/Cas9 relies on RNA-guided DNA cleavage to generate double-stranded breaks [104]. CRISPR provides a very simple approach for targeted gene disruption and targeted gene insertion. To disrupt a gene by CRISPR, only two components are needed: (1) the Cas9 protein that contains the nuclease domains, and (2) the guide RNA (gRNA) that provides sequence specificity to the target DNA. The first 20-nucleotide sequence at the 5’-end of the gRNA is complementary to the target sequence and it provides the specificity for the CRISPR/ Cas9 system [104]. The 3’ portion of the gRNA forms certain secondary structures and is required for Cas9 activities. The
gRNA brings the Cas9 nuclease to the specific target and subsequently Cas9 generates double-stranded breaks in the target DNA at the protospacer-adjacent motif (PAM) site. Non-homologous end-joining repair of the double-stranded breaks often leads to deletions or small insertions that disrupt the target DNA.

There are two major challenges in using CRISPR for targeted mutagenesis: (1) production of the gRNAs, and (2) analysis of the CRISPR-generated mutations. The first 20-nucleotide sequence of the gRNA is used to guide targeted DNA cleavage. Additional bases at the 5’-end of gRNA or other modifications may abolish the gRNA’s ability to guide DNA cleavage by Cas9 [104]. RNAs transcribed by RNA polymerase II (pol II), which is the polymerase responsible for the production of the majority of mRNAs, cannot be used as gRNAs because they undergo extensive processing and modification at both ends. Additionally, most mRNAs are actively transported from the nucleus into the cytoplasm after transcription, while the Cas9/gRNA only has access to the genomic DNA inside the nucleus. Most of the well-characterized promoters are transcribed by pol II and have not been used to produce gRNA for CRISPR. Therefore, promoters such as U3 and U6, which are transcribed by the RNA polymerase III (pol III) were previously chosen to produce gRNA in various organisms. There are many limitations to U6- or U3-based gRNA production. First, U6 snRNA and U3 snRNA are housekeeping genes and they are ubiquitously expressed. Therefore, they cannot be used to generate gRNAs with cell or tissue specificity. Second, the U6 and U3 promoters in many organisms have not been characterized, making it difficult to choose the correct U6/U3 promoters for CRISPR. Third, the U6 and U3 promoters are not suitable for routine in vitro production of gRNAs because RNA pol III is not commercially available. Furthermore, the U6 and U3 promoters limit the CRISPR target sequences to G(N)_{20}GG and A(N)_{20}GG, respectively. Improved methods for producing gRNAs in vivo are needed in order to conduct targeted mutagenesis with spatial and temporal control in a wide
range of organisms.

The second challenge in using CRISPR for genome editing is detecting and analyzing the mutations generated. Mutations are often detected by enzyme digestion of a PCR product that contains the target region, followed by DNA sequencing. Restriction digestion can only work when a restriction site is altered and many useful mutations may not be detected by restriction digestion. CEL I-based enzyme digestion can also be used to detect mutations [105]. However, CRISPR often generates many different mutations in a tissue or an organism, making a CEL I-based method less effective. We believe that the best way to detect mutations caused by CRISPR is to use the specific gRNA and Cas9 protein to digest the PCR products that contain the target sequence [104]. However, such a method requires an easy and efficient way to produce gRNAs in vitro.

In this paper, we present a method that successfully overcomes the aforementioned challenges in using CRISPR for genome editing. We take advantage of the nuclease activity of ribozymes [106, 107] to design an artificial gene \( RGR \) (Ribozyme-gRNA-Ribozyme). We hypothesize that the primary transcripts of \( RGR \) undergo self-catalyzed cleavage to precisely release the designed gRNA. We show that gRNA is specifically released from the primary transcripts of \( RGR \) by self-processing in vitro. The produced gRNA efficiently guides Cas9-mediated cleavage of target DNA in vitro. Furthermore, we introduce the \( RGR \) gene into yeast under the control of the \( alcohol dehydrogenase 1 \) (ADH1) promoter, which is transcribed by pol II, and we observe the targeted DNA cleavage in yeast. Our results demonstrate that production of gRNAs is no longer limited to a specialty promoter such as the U6 promoter, thereby enabling us to conduct genome editing with spatial and temporal precision if proper promoters are chosen. In addition, we demonstrate that the target sequences are no longer limited to \( G(N)_{20}GG \) or \( A(N)_{20}GG \) because our method does not require the specific G or A for transcription initiation for gRNA production as is the case for U6 and U3 promoters. We also show that the
efficient production of gRNAs by in vitro transcription from a commonly used promoter such as SP6 makes it very easy to use gRNA and Cas9 to detect mutations caused by CRISPR/Cas9.

### 3.3 Results

#### 3.3.1 Design an RNA molecule with self-processing capacity for g-RNA production

We took advantage of the nuclease activities of ribozymes that catalyze the cleavage at a specific site within an RNA molecule. We designed an RNA molecule (pre-gRNA) that was predicted to undergo self-catalyzed processing (Figure 3.1A). The RNA molecule we designed contained a Hammerhead (HH) type ribozyme [108] at the 5’-end, a gRNA that targets a green fluorescent protein (GFP) gene in the middle, and a hepatitis delta virus (HDV) ribozyme [109] at the 3’-end (Figure 3.1A). After the self-cleavage at the predicated sites, the mature gRNA was released (Figure 3.1A). The gRNA was predicted to guide Cas9 to cut DNA at the targeted sites (Figure 3.1B). By altering only the first six nucleotides of the HH ribozyme, our design can be employed to generate gRNAs that target any DNA sequence with a PAM site (NGG). Previous CRISPR targets were limited to either G(N)20GG or A(N)20GG.

#### 3.3.2 Production of a gRNA by in vitro transcription and self-processing

The designed pre-gRNA molecule can be generated by in vitro transcription of the corresponding DNA sequence, which we named the Ribozyme-gRNA-Ribozyme (RGR) gene (Figure 3.2A). We placed the RGR gene under the control of the SP6 promoter and
Figure 3.1: The schematic design of a self-processing RNA molecule for gRNA production. (A) The modular structure of the pre-gRNA, which contains a Hammerhead ribozyme at the 5’-end, the sequence-specific gRNA portion in the middle (shaded yellow), and a HDV ribozyme at the 3’-end. The predicted secondary structures of both ribozymes are shown. The hairpin (stem) regions in the Hammerhead ribozyme are labeled H1, H2, and H3. P1, P2, P3, and P4 refer to the hairpin regions in the HDV ribozyme. The pre-gRNA undergoes self-catalyzed processing to release the mature gRNA. The 5’-end (in red) of the mature gRNA is complementary to the target sequences and the 3’-end (in green) is universal for all gRNAs in this work. (B) Schematic representation of gRNA and Cas9-mediated cleavage of target DNA. Note that the target sequence contains the NGG PAM site, which is not in the gRNA.

conducted in vitro transcription using the commercially available SP6 RNA polymerase. As shown in Figure 3.2B, the primary transcripts of the RGR were self-processed into
several RNA bands. The smallest RNA band was the predicted gRNA (Figure 3.2B). We introduced mutations in the HH ribozyme and in the HDV ribozyme individually to disrupt their self-processing ability. We also mutated the two ribozymes simultaneously. We then tested the self-processing ability of the mutated pre-gRNAs (Figure 3.2B, Lanes 2, 3, and 4, respectively). Disruption of the two ribozymes simultaneously led to a complete failure to self-process the transcripts (Figure 3.2B, Lane 4). Inactivation of the HH ribozyme (5’-end) blocked the separation of the 5’-end ribozyme from the rest of the RNA molecule, but did not affect the processing of the 3’-end HDV ribozyme (Figure 3.2B, Lane 2). On the other hand, mutations in the HDV ribozyme only disrupted the removal of the 3’-end portion of the pre-gRNA molecule (Figure 3.2B, Lane 3). We noticed that the processing ability of the HDV ribozyme was not as strong as that of the HH ribozyme because partial cleavage directed by the HDV ribozyme was observed (Figure 3.2B, Lanes 1 and 2).

3.3.3 Guide RNA molecules produced in vitro guided specific cleavage of the target DNA

We next investigated whether the gRNA molecules produced from in vitro transcription and self-processing have the ability to guide Cas9 to perform sequence-specific cleavage of the target DNA in vitro. When the gRNA, Cas9, and the PCR fragment containing the target sequence were mixed and incubated for 60 min, we observed efficient and complete cleavage of the target DNA (Figure 3.2C, Lane 1). The cleavage appeared to be specific because the sizes of the resulting DNA fragments were the same as predicted. We discovered that unprocessed pre-gRNA molecules generated from the in vitro transcription of the mutated RGR gene failed to guide the cleavage of the targeted sequences (Figure 3.2C, Lane 4). Removal of the HDV ribozyme alone was also insufficient to support Cas9 digestion (Figure 3.2C, Lane 2). However, gRNA with the
Figure 3.2: gRNA production and gRNA-mediated specific cleavage of a DNA target in vitro. (A) DNA sequence of the artificial gene \textit{RGR} that encodes the pre-gRNA. The first six nucleotides (in red) of the Hammerhead (HH) ribozyme must be complementary to the first six nucleotides of the target sequence (in red). The entire mature gRNA sequence is in bold and is underlined. The HDV ribozyme is in green. The two arrows mark the cleavage sites for the ribozyme-catalyzed reactions. (B) Analysis of the self-processing capacity of transcripts generated by in vitro transcription. The primary transcripts are 416 bp long (extra bases are added to both ends of the pre-gRNA during in vitro transcription). The predicted size of mature gRNA is 100 bp, the length from the transcription initiation site to the Hammerhead cleavage site is 131 bp, and the length from the HDV cleavage site to the end is 185 bp. Lane 1: gRNA is released from the pre-gRNA with both functional ribozymes. Note that the cleavage of the HDV ribozyme is incomplete. Lane 2: the Hammerhead ribozyme is mutated, which does not prevent the processing of the 3’-end of HDV ribozyme. However, the processing of HDV ribozyme is incomplete. Lane 3: the HDV ribozyme is mutated and only the Hammerhead ribozyme is released. The self-processing of the 5’-end of the pre-gRNA is complete, but no mature gRNA is released. Lane 4: both Hammerhead and HDV ribozymes are mutated and no self-processing is observed. (C) The gRNA-mediated cleavage of target DNA in vitro. The PCR fragment of the \textit{GFP} gene is used as a substrate for gRNA/Cas9 digestion. gRNA released from wild-type pre-gRNA leads to a complete digestion of the target DNA (Lane 1). However, RNAs from pre-gRNAs with mutations in the Hammerhead ribozyme fail to guide the target cleavage (Lanes 2 and 4). Interestingly, the gRNA with the 3’-end HDV ribozyme mutated is still partially active (Lane 3).

HDV ribozyme at the 3’-end still retained sufficient activity to guide Cas9 to cut target DNA (Figure 3.2C, Lane 3). The gRNAs used in the assays were not purified, suggesting
that the free ribozymes and other components from the in vitro transcription did not interfere with the Cas9/gRNA-mediated cleavage. These results bode well for using this method in vivo, where many other RNAs and proteins exist.

3.3.4 Guide RNAs produced from the ADH1 promoter guide specific DNA cleavage in yeast

We next tested whether our method for producing a self-processing RNA molecule to generate a gRNA could succeed in vivo. We placed the RGR gene under the control of the ADH1 promoter, which is transcribed by pol II (Figure 3.3A). The transcripts of the RGR gene contained the gRNA portion that was designed to target the GFP gene (Figure 3.3A). We introduced the plasmid along with another Cas9-expressing plasmid to a yeast strain that harbors a GFP gene in its chromosomes and that is brightly fluorescent (Figure 3.3B). We first analyzed whether our constructs disrupted the fluorescence displayed in the yeast cells. The yeast cells that harbored the plasmids failed to produce any fluorescence, indicating that the GFP gene had likely been disrupted in the cells (Figure 3.3B). Interestingly, partially processed pre-gRNA with the HDV remaining at the 3’-end displayed significant activity in vitro (Figure 3.2C, Lane 3). However, such an RNA molecule did not function in yeast as we did not observe any silencing of the GFP signal when the HDV was mutated in the pre-gRNA (data not shown).

We extracted the genomic DNA from the yeast cells and amplified the GFP gene by PCR. The PCR fragments were resistant to Cas9/gRNA digestion (Figure 3.3C). By sequencing, we found that deletion mutations were generated in the GFP gene as was expected for CRISPR-mediated mutations (Figure 3.3D).
Figure 3.3: ADH1 promoter-driven expression of the pre-gRNA is sufficient to guide Cas9-mediated disruption of a target gene in yeast. (A) Schematic representation of the constructs that express Cas9 and the pre-gRNA. (B) Yeast cells that harbor the GFP gene display bright green fluorescence (left). The DIC image of the control is also shown. Expression of the Cas9 and the gRNA silences the fluorescence of GFP (right two panels). (C) PCR fragments of the GFP gene amplified from the genomic DNA of different yeast clones expressing Cas9 and RGR. The GFP fragments are resistant to Cas9/gRNA in vitro cleavage, indicating that the target sites in the GFP gene have likely been mutated. Lanes 1-5: PCR fragments amplified from different yeast colonies. Lane 6: wild-type GFP fragment as a positive control. Lane 7: no gRNA was added. (D) DNA sequencing confirms that the GFP gene is mutated by expressing Cas9 and the pre-gRNA in yeast. The target sequence and the 1 bp deletion are indicated (red). The PAM site (blue) is also marked.

3.4 Discussion

We demonstrate that gRNAs can be efficiently produced in vitro and in vivo from essentially any promoters when the primary transcripts are flanked by self-cleaving ribozymes. The produced gRNAs can guide Cas9-mediated specific cleavage of DNA targets both in vitro and in vivo. This work opens the door to conducting more sophisticated CRISPR-mediated genome editing in many organisms. Because gRNAs can now be produced using tissue-specific promoters, hormone-responsive promoters, environ-
mental signal-regulated promoters, and other well-characterized promoters; this work lays the foundation for studying the roles of specific genes in various developmental and pathological processes. For example, temporal control of gRNA production is necessary when sequential disruption of different genes is preferred, which cannot be achieved by modulating the Cas9 expression alone. Additionally, when multiplex gene targeting is desired, different RGR genes can be expressed as a single transcript under one single promoter. Furthermore, gRNA and Cas9 together enable us to cut specific DNA in vitro, thus greatly enhancing our ability to manipulate DNA in vitro.

There are two main structural differences between the gRNAs produced in this work and the gRNAs generated using U6 and U3 promoters. First, gRNAs transcribed from U6 and U3 promoters have a triphosphate group at the 5’-end whereas gRNAs generated from self-processing of the pre-gRNAs have a hydroxyl group at the 5’-end. Second, the 3’-end of the gRNAs reported in this work is 2’,3’-cyclic phosphate while transcripts from U6 or U3 often end with hydroxyl groups at both 2’ and 3’ positions. Although both types of gRNAs possess the ability to guide the cleavage of target DNA in vivo, the structural features in the ends of our gRNAs may have advantages. For example, our gRNAs may be more stable because some nucleases require the 5’-terminal phosphate group for specific cleavage [110].

We have shown that we can effectively use Cas9 as a restriction enzyme in vitro to cut specific DNA sequences that complement the 20 nucleotides of the 5’-end of the gRNA. Because we have shown that the specific gRNAs can be easily produced from in vitro transcription (Figure 3.2), we can now generate specific Cas9 sites in DNA molecules to facilitate routine molecular cloning. This application is especially useful when no other restriction enzyme sites are available in the region. Another application we have successfully demonstrated in this work is using Cas9/gRNA to detect the mutations generated by CRISPR (Figure 3.3C).
Our system has the potential for automation and high-throughput production of gRNAs, thus laying the foundation for systematically knocking out every single gene in an organism using CRISPR technology. Because the RGR genes we designed mainly differ in the 20-nucleotide sequence encoding for the specific portion of the gRNA, every RGR gene can potentially be amplified by PCR with a pair of universal primers. If the SP6 or T7 promoter sequences are included in the primers, we can easily transcribe all of the RGR genes and produce the corresponding gRNAs using commercially available RNA polymerases (Figure 3.2). Amplification and in vitro transcription can be programmed for automation if thousands of different gRNAs are needed.

Only one construct is needed for generating a specific gRNA in vitro and in vivo. The construct can be transformed into a cell/organism to generate targeted modifications in the genome. The same construct also serves as the DNA template for amplifying the RGR gene using universal primers. The PCR fragments can be used for in vitro transcription to produce gRNAs, which along with Cas9 can be used for detecting mutations generated by the same gRNA.

3.5 Materials and Methods

3.5.1 Design ribozyme-flanked gRNAs

The gRNA sequence except for the target sequence was adapted from [93]. The sequence in the GFP gene targeted by the Cas9/gRNA in this study was 5’- CGTGCT-GAAGTCAAGTTTGAAGG -3’, with the first 20 bp as the beginning of the gRNA. At the 5’-end of the gRNA was a HH ribozyme, and at the 3’-end of the gRNA was a HDV ribozyme. The design of both ribozymes was based on the work of Avis et al. (2012) [111]. The mutated HH ribozyme (mHH) had a 13 bp deletion at its 5’-end, which affected the H1 and H2 stem-loop region as well as the conserved CUGANGA
domain of the HH ribozyme (Figure 3.1). The mutated HDV ribozyme (mHDV) had a 15 bp deletion at its 3’-end, which affected the P2 and P4 region (Figure 3.1). Four different ribozyme-flanked gRNAs (pre-gRNA) were generated by overlapping PCR reactions: HH-gRNA-HDV (referred as Full), mHH-gRNA-HDV (referred as mHH), HH-gRNA-mHDV (referred as mHDV), and mHH-gRNA-mHDV (referred as mm).

3.5.2 Cloning, expression, and purification of Cas9

The human-codon-optimized Cas9c gene template was a generous gift from Luhan Yang (G. Church Laboratory, Harvard University). The Cas9 gene was cloned into pET28a plasmid in order to express the N-terminally His-tagged Cas9 protein. The pET28a-Cas9 plasmid was transformed into BL21(DE3) (Invitrogen, Carlsbad, CA, United States). One single colony harboring pET28a-Cas9 was inoculated into 5 mL of Luria-Bertani (LB) media and grown at 250 rpm, 37 °C for 7 h. All of the 5 mL culture was transferred into 50 mL of LB and then grown overnight at 250 rpm, 17 °C. Of the overnight culture, 50 mL was transferred into pre-chilled 1000 mL of Terrific Broth (TB) media, and the resulting culture was grown at 250 rpm, 17 °C for 24 h. When the OD600 reached 1, the cells were chilled on ice for 30 min. Isopropylthio-β-galactoside was then added to the final concentration of 1 mmol/L, and MgCl₂ was added to the final concentration of 10 mmol/L. The cells were then grown at 250 rpm, 17 °C for 48 h before harvesting.

Cells were collected by centrifugation at 5000 rpm for 10 min. Cells were then frozen at -80 °C for 30 min, followed by thawing on ice for 15 min. Cells were resuspended in 18 mL lysis buffer (50 mmol/L HEPES, pH 7.5, 300 mmol/L NaCl, 10 mmol/L imidazole). Lysozyme was added to the final concentration of 1 mg/mL. Cells were incubated on ice for 30 min and DTT was added to the final concentration of 2 mmol/L. Cells were then lysed by sonication on ice for 80 s.
His-tagged Cas9 protein was purified from the cell lysate using Ni-NTA Agarose from Qiagen, Hilden, Germany following the manufacturer’s instructions. The wash buffer contained 50 mmol/L HEPES, pH 7.5, 300 mmol/L NaCl, 20 mmol/L imidazole and the elution buffer contained 50 mmol/L HEPES, pH 7.5, 300 mmol/L NaCl, 250 mmol/L imidazole. The buffer was then exchanged to Cas9 storage buffer (20 mmol/L HEPES, pH 7.5, 150 mmol/L KCl, and 1 mmol/L TCEP) using the PD-10 Desalting Columns (GE Healthcare Life Sciences, Waltham, MA, United States). The purified protein was kept at 4 °C.

3.5.3 Yeast strains and constructs

The yeast strain LPY16936 expressing GFP as a C-terminal fusion protein of the GDH1 gene was used for the Cas9/gRNA in vivo assay. The yeast strain LPY142 was used as a negative control for GFP fluorescence imaging. Both yeast strains were gifts from Bessie Xue Su (L. Pillus Laboratory, UCSD).

To express Cas9 in yeast cells, the Cas9 gene with SV40 NLS signal at its C-terminal was cloned into the HindIII sites in the pACT2 vector (Leu selection marker) between the ADH1 promoter and ADH1 terminator. The sequence between the HindIII sites, including the region for the GAL4 activation domain, was removed. To express ribozyme-flanked gRNAs, the DNA fragment corresponding to the designed pre-gRNA molecules was cloned into pRS316 (Ura selection marker) between the BamHI and EcoRI sites by overlapping PCR. The pACT2-Cas9 and pRS316-pre-gRNA constructs were sequentially transformed into LPY16936.
3.5.4 In vitro transcription for gRNA production

The templates for in vitro transcription were amplified by PCR from pRS316-pre-gRNA constructs using common primers 5’- GTCACTATTTAGGTGACACTATA-GAAGCGCCTCGTCATTGTTCTCGTTCC -3’ and 5’- ACGTATCTACCAACGATT-TGACC -3’. In vitro transcription was carried out at 40 °C for 3 h in a total volume of 50 µL with 700 ng purified DNA template, 2 µL of SP6 RNA polymerase (19U/µL, Promega, Madison, WI, United States), 0.5 mmol/L rNTPs, 1X Transcription Optimized Buffer (Promega), 10 mmol/L DTT and 1 µL of RNasin Ribonuclease Inhibitor (Promega). 1 µL of 500 mmol/L EDTA was added to each tube to terminate the reactions. The RNA transcripts were not further purified. Of the in vitro transcription products, 4 µL were analyzed by electrophoresis in 12% denaturing urea polyacrylamide gels. The RNA bands were stained with ethidium bromide and visualized using a UV transilluminator.

3.5.5 In vitro cleavage assay using Cas9 protein and gRNA

For each in vitro cleavage assay, approximately 100 ng of purified PCR products were digested with 0.2 µL of the purified Cas9 and 0.8 µL of the gRNA from the in vitro transcription reaction in 1X cleavage buffer (20 mmol/L HEPES pH 7.5, 150 mmol/L KCl, 1 mmol/L TCEP, and 10 mmol/L MgCl₂) in a total volume of 20 µL, at 37 °C for 60 min. The reaction was stopped by adding 2 µL of 10% SDS, and was then chilled on ice for 2 min, and centrifuged at 13,000 rpm for 2 min. The supernatant was analyzed by 1%-1.5% agarose gel electrophoresis. The DNA bands were stained with ethidium bromide and visualized using a UV transilluminator.
3.5.6  Green Fluorescent Protein fluorescent imaging of yeast

To observe the collective GFP fluorescence of yeast cells in different constructs, each yeast strain harboring the corresponding plasmids (if any) were grown in SD-Ura-Leu media overnight. The OD600 of each strain was measured (around 1.0) and concentrated to the OD600 of 20 in 50% glycerol. The 0.15 µL concentrated culture of each strain was carefully spotted onto ProbeOn Precleaned slides (Fisher Biotech, Waltham, MA, United States), covered and photographed under a DIC or fluorescent microscope (10X objective lens).

3.6  Acknowledgments

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Chapter 3 in full, is a reprint of the material as it appears in Journal of Integrative Plant Biology, 2014, Gao, Yangbin; Zhao, Yunde. The dissertation/thesis author was a primary investigator and author of this paper.
Chapter 4

Auxin Binding Protein 1 (ABP1) is not required for either auxin signaling or Arabidopsis development

4.1 Abstract

Auxin binding protein 1 (ABP1) has been studied for decades. It has been suggested that ABP1 functions as an auxin receptor and has an essential role in many developmental processes. Here we present our unexpected findings that ABP1 is neither required for auxin signaling nor necessary for plant development under normal growth conditions. We used our ribozyme-based CRISPR technology to generate an Arabidopsis abp1 mutant that contains a 5-bp deletion in the first exon of ABP1, which resulted in a frameshift and introduction of early stop codons. We also identified a T-DNA insertion abp1 allele that harbors a T-DNA insertion located 27 bp downstream of the ATG start codon in the first exon. We show that the two new abp1 mutants are null alleles. Surprisingly, our new abp1 mutant plants do not display any obvious
developmental defects. In fact, the mutant plants are indistinguishable from wild-type plants at every developmental stage analyzed. Furthermore, the \textit{abp1} plants are not resistant to exogenous auxin. At the molecular level, we find that the induction of known auxin-regulated genes is similar in both wild-type and \textit{abp1} plants in response to auxin treatments. We conclude that \textit{ABP1} is not a key component in auxin signaling or \textit{Arabidopsis} development.

4.2 Introduction

The auxin binding protein 1 (ABP1) was first isolated from maize plants based on its ability to bind auxin [112]. The crystal structure of ABP1 demonstrated clearly that ABP1 has an auxin-binding pocket and, indeed, binds auxin [113]. However, the elucidation of the physiological functions of ABP1 has been challenging because the first reported \textit{abp1} T-DNA insertion mutant in \textit{Arabidopsis} was not viable [114]. Nevertheless, \textit{ABP1} has been recognized as an essential gene for plant development and as a key component in auxin signaling [115–120]. Because viable \textit{abp1} null mutants in \textit{Arabidopsis} were previously unavailable, alternative approaches have been used to disrupt ABP1 function in \textit{Arabidopsis} to determine the physiological roles of the protein. Cellular immunization approaches were used to generate \textit{ABP1} knockdown plants [121, 122]. Inducible overexpression of the single chain fragment variable regions (scFv12) of the anti-ABP1 monoclonal antibody mAb12 both in cell lines and in \textit{Arabidopsis} plants presumably neutralizes the endogenous ABP1 activities [121, 122]. Two such antibody lines, SS12S and SS12K, have been widely used in many ABP1-related studies [115, 117, 120–122]. The results obtained from the characterization of the antibody lines suggest that ABP1 regulates cell division, cell expansion, meristem activities, and root development [115, 117, 121, 123, 124]. Transgenic plants that overexpress \textit{ABP1}
antisense RNA were also used to elucidate the physiological functions of \textit{ABP1} [115, 121]. Moreover, missense point mutation alleles of \textit{abp1} have also been generated through the \textit{Arabidopsis} TILLING project. One such TILLING mutant, named \textit{abp1-5}, harbors a mutation (His94 \text{>} Tyr) in the auxin-binding pocket and has been widely used in many \textit{ABP1}-related studies [115, 119, 120]. Previous studies based on the antisense lines, antibody lines, and \textit{Arabidopsis} mutant alleles have led to the conclusion that \textit{ABP1} is essential for embryogenesis, root development, and many other developmental processes. However, the interpretation of results generated by using the \textit{ABP1} antisense and antibody lines are not straightforward and off-target effects have not been completely ruled out. We believe that characterization of \textit{abp1} null plants is urgently needed to unambiguously define the roles of \textit{ABP1} in auxin signaling and in plant development.

In the past several years, studies of the presumed \textit{ABP1}-mediated auxin signal transduction pathway were carried out in several laboratories. It has been hypothesized that \textit{ABP1} is an auxin receptor mediating fast, nongenomic effects of auxin [115, 117, 123, 124], whereas the TIR1 family of F-box protein/auxin receptors are responsible for auxin-mediated gene regulation [125, 126]. One of the proposed functions of \textit{ABP1} is to regulate subcellular distribution of PIN auxin efflux carriers [117, 120, 124]. Furthermore, a recent report suggests that a cell surface complex consisting of \textit{ABP1} and transmembrane receptor-like kinases functions as an auxin receptor at the plasma membrane by activating the Rho-like guanosine triphosphatases (GTPases) (ROPs) in an auxin-dependent manner [119]. ROPs have been reported to play a role in regulating cytoskeleton organization and PIN protein endocytosis [116, 117]. However, it is important to unequivocally determine the biological processes that require \textit{ABP1} before extensive efforts are directed toward elucidating any \textit{ABP1}-mediated signaling pathways.

In this paper, we generate and characterize new \textit{abp1} null mutants in \textit{Arabidopsis}. We are interested in elucidating the molecular mechanisms by which auxin regulates
flower development because our previously identified auxin biosynthetic mutants display dramatic floral defects [14, 15, 127]. Because ABP1 was reported as an essential gene and ABP1 binds auxin [113, 114], we decided to determine whether ABP1 plays a role in flower development. We used our recently developed ribozyme-based CRISPR gene editing technology [128] to specifically inactivate ABP1 during flower development. Unexpectedly, we recovered a viable abp1 mutant (abp1-c1, c stands for alleles generated by using CRISPR) that contains a 5-bp deletion in the first exon of ABP1. We also isolated a T-DNA abp1 allele (abp1-TD1) that harbors a T-DNA insertion in the first exon of ABP1. We show that both abp1-c1 and abp1-TD1 are null mutants. Surprisingly, the mutants were indistinguishable from wild-type (WT) plants at all of the developmental stages we analyzed. Our data clearly demonstrate that ABP1 is not an essential gene and that ABP1 does not play a major role in auxin signaling and Arabidopsis development under normal growth conditions.

4.3 Results and Discussion

4.3.1 Generation of Loss-of-Function abp1 Mutants in Arabidopsis Using CRISPR Technology

In an attempt to determine the roles of ABP1 in Arabidopsis flower development, we used the latest CRISPR technology [128] to specifically knockout the ABP1 gene during Arabidopsis flower development. We designed a ribozyme-guide RNA-ribozyme (RGR) unit that specifically targets a stretch of DNA in the first exon of ABP1 gene (Figure 4.1). The RGR unit was placed under the control of the strong constitutive CaMV 35S promoter. Primary transcripts of RGR undergo self-processing to release the mature functional guide RNA (gRNA) as we demonstrated [128]. We controlled the
expression of the Cas9 nuclease by using the APETALA 1 (API) promoter (Figure 4.1A).
We expected that the gRNA would bring the Cas9 protein to the ABP1 target site where it will generate double-stranded breaks. Deletions and insertions will be produced during nonhomologous end joining repair of the double-stranded break. We hypothesized that the gene editing will take place only during flower development as the expression of the Cas9 nuclease is under the control of a floral meristematic promoter.

**Figure 4.1:** Generation of a null allele of *abp1* mutant using the ribozyme-based CRISPR gene editing technology. (A) A schematic description of the CRISPR construct that contains a Cas9 expression cassette and a CaMV 35S promoter controlled gRNA production unit. (B) A 5-bp deletion was detected in genomic DNA of *abp1-c1* mutants. The intron sequences are in lowercase and in red. (C) The *abp1-c1* cDNA also contained the same 5-bp deletion. (D) There was no detectable ABP1 protein in *abp1-c1* as shown in this Western blot image.

We were disappointed that no obvious floral defects were observed in the T1
transgenic plants that contained the expression cassettes for *Cas9* and the *RGR*. We then grew T2 plants to identify homozygous *Cas9/RGR* insertion plants, which may have higher efficiency of editing *ABP1* because of potentially higher expression of *RGR* and *Cas9* in the homozygous lines. Unexpectedly, we recovered T2 plants that are homozygous *abp1* deletion mutant plants (named *abp1-c1*). The *abp1-c1* contains a 5-bp deletion in the first exon (Figure 4.1B). The deletion presumably leads to a frameshift and would generate premature stop codons. Therefore, *abp1-c1* is likely a null mutant. Because our *abp1-c1* results appear to contradict a previous report that a T-DNA insertion *abp1* mutant was embryo lethal [114], we hypothesized that perhaps the Cas9 protein or the CRISPR construct or an off-target site mutation partially rescued the presumed embryo lethal phenotypes of *abp1-c1*. We then backcrossed the *abp1-c1* to WT plants to segregate out the CRISPR construct and potential off-target background mutations. We genotyped the F2 population generated from the backcross and identified *Cas9* free, *abp1-c1* homozygous plants. It was clear that *abp1-c1* plants were not embryo lethal. The mutation in *abp1-c1* was stable and transmitted to next generations in a Mendelian fashion (Figure 4.2).

**Figure 4.2:** The CRISPR allele of *abp1* is stably transmitted to next generations according to Mendel genetics. Ninety-six progenies from a single *abp1-c1* plant were genotyped by using methods described in the text. The gel picture shows the patterns of WT, heterozygous, and homozygous *abp1-c1* samples. The actual number of plants for each genotype is shown in parentheses.
Figure 4.3: The *abp1-c1* and WT plants display no significant differences at various developmental stages. (A) Seven-day-old seedlings on regular MS plates. (B and C) Hypocotyl length and root length of 7-d-old WT and *abp1-c1* seedlings. Shown are average SD (n = 50). (D and E) Root cell shape of 7-d-old WT and *abp1-c1*. (F, G, and I) Phenotype of WT and *abp1-c1* at juvenile stage (F), floral transition stage (G), and mature plant stage (I). (H) Flowers and floral organs of WT and *abp1-c1*.

### 4.3.2 The *abp1-c1* Mutant Is a Null Allele

The 5-bp deletion in the first exon is predicted to cause a frameshift and to introduce several early stop codons. Because our results were not consistent with what was previously reported regarding an *abp1* null mutant, we investigated whether the 5-bp deletion in *ABP1* might generate cryptic splicing junctions that might still lead to the production of functional *ABP1* mRNA and ABP1 protein. We extracted mRNA from *abp1-c1* and WT plants, and amplified *ABP1* cDNAs by RT-PCR. The *ABP1* cDNA from WT plants was the same as reported [114]. The *ABP1* cDNAs from *abp1-c1* all contained the 5-bp deletion (Figure 4.1C). The mutant *abp1-c1* cDNA contained several premature stop codons and was unlikely to produce a functional ABP1 protein. To further
demonstrate that our \textit{abp1-c1} is a null allele, we performed a Western blot by using anti-ABP1 polyclonal antibody [119]. The results in Figure 4.1D show that the antibody detected ABP1 and several nonspecific bands. Although both the WT and \textit{abp1-c1} lanes had the same nonspecific bands, the ABP1 band in \textit{abp1-c1} sample was clearly missing, demonstrating that the \textit{abp1-c1} is a null mutant.

\textbf{Figure 4.4:} Pavement cell development in \textit{abp1-c1} and WT. Confocal images of cotyledon pavement cells of WT (A and C) and \textit{abp1-c1} (B and D) with auxin (C and D) and without auxin (A and B) treatments. Five-day-old light-grown seedlings were transferred to MS plates with or without 25 nM NAA for 2 d. Samples were treated with 5 \(\mu\)g/mL FM1-43 (Life Technologies; F-35355) for 30 min before confocal imaging. (E) Quantification of pavement cell lobes. One hundred fifty cells for each treatment and each genotype were quantified. Images were gridded to 25 of 20,000 \(\mu m^2\) squares by using ImageJ before counting. Error bars are SD.
4.3.3 The abp1-c1 Plants Are Indistinguishable from WT Plants

In previous studies, ABP1 knockdown was associated with a number of developmental defects including changes in root and hypocotyl elongation, leaf expansion, and maintenance of the root meristem [115, 121, 122, 129–132]. To determine whether abp1-c1 plants exhibited any of these defects, we compared them to WT plants grown under the same growth conditions. As shown in Figure 4.3A, light grown abp1-c1 seedlings looked similar to WT seedlings. Both WT and abp1-c1 plants had similar hypocotyl lengths (Figure 4.3B). Hypocotyl elongation is sensitive to changes in auxin concentration or auxin response [11, 133]. The length of primary roots of abp1-c1 seedlings was also like that of WT plants (Figure 4.3A and C), and the cellular organization of primary roots of the mutant, including the meristem, appeared similar to that of WT plants (Figure 4.3D and E). We did not observe any alterations of cell size or changes in spatial arrangement of the different cell types (Figure 4.3D and E). The microscopic structure of abp1-c1 roots is not different from that of WT plants. At young adult stages, abp1-c1 plants developed normally and appeared as healthy as WT plants (Figure 4.3F). WT plants and abp1-c1 plants had similar flowering time (Figure 4.3G). Flowers of abp1-c1 had the same numbers of floral organs as WT flowers (Figure 4.3H). Lastly, mature abp1-c1 plants and WT plants had similar architecture and abp1-c1 plants were as fertile as WT plants (Figure 4.3I). Dark-grown seedlings of the ABP1 antibody lines were partially de-etiolated with short hypocotyls and lacked an apical hook [122]. However, the abp1-5 weak allele was indistinguishable from WT when grown in total darkness [134]. Because dark-grown conditions vary little from laboratory to laboratory, we tested whether abp1-c1 displayed any phenotypes in the dark. Dark-grown abp1-c1 appeared similar to WT seedlings in terms of hypocotyl length and the formation of an apical hook (Figure 4.5).

One of the key phenotypic readouts of abp1 knockdown or weak alleles in previous studies is a reduction of pavement cell interdigitation [119, 120].
Figure 4.5: The \textit{abp1-c1} seedlings grown in the dark were similar to WT grown under the same conditions. (A) Four-day-old seedlings grown in total darkness: WT (Left) and \textit{abp1-c1} (Right). Note that both WT and the mutant had an apical hook. (B) Quantification of hypocotyl length of dark-grown seedlings.

of interdigation in \textit{abp1} knockdown lines or \textit{abp1-5} cannot be rescued by exogenous auxin [119,120]. We analyzed pavement cell interdigation in both WT and \textit{abp1-c1} with and without auxin treatments (Figure 4.4). In the absence of exogenous auxin, \textit{abp1-c1} and WT showed the same levels of pavement cell interdigation (Figure 4.4). Auxin treatments slightly increased interdigation of pavement cells in both WT and \textit{abp1-c1} (Figure 4.4). We did not observe any differences between \textit{abp1-c1} and WT plants in terms of pavement cell interdigation.

Overall, the \textit{abp1-c1} plants were indistinguishable from WT plants at the various developmental stages we analyzed, demonstrating that \textit{ABP1} probably does not play a major role in \textit{Arabidopsis} development under normal growth conditions.
4.3.4 The *abp1-c1* Plants Are Not Auxin Resistant

Several studies have reported changes in auxin response in *ABP1* knockdown lines [129, 130]. We used a classic root elongation assay [135] to determine whether *abp1-c1* had altered sensitivity to exogenous auxin. We tested both the natural auxin indole-3-acetic acid (IAA) and the synthetic auxin 1-naphthaleneacetic acid (NAA), because ABP1 has been reported to have a higher affinity for NAA than IAA [136]. In the presence of increasing concentrations of auxin in the growth media, primary roots of WT plants became progressively shorter (Figure 4.6). Both auxins also inhibited the elongation of primary roots of *abp1-c1* (Figure 4.6). The dose-response curves to IAA treatments for WT and *abp1-c1* were almost superimposable, indicating that there was not a significant difference between WT and *abp1-c1* plants in response to auxin treatments (Figure 4.6A). Similar results were also observed when NAA was used in the treatments (Figure 4.6B).

![Figure 4.6](image)

**Figure 4.6:** Effects of auxin treatments on *abp1-c1* root elongation. Quantification of root elongation of WT and *abp1-c1* with various concentrations of IAA (A) or NAA (B) for 2 d. Shown are average SD (n = 50).
4.3.5 The abp1-c1 and WT Plants Respond to Auxin Similarly at the Molecular Level

Although ABP1 was suggested to mainly function in nongenomic pathways, several studies have reported that reduction in ABP1 function affects auxin-regulated gene expression [121, 129, 130]. Furthermore, it was recently reported that ABP1 regulates the degradation of AUX/IAA proteins [118]. Therefore, we analyzed the expression levels of a set of well-characterized auxin inducible genes in both abp1-c1 and WT plants with and without auxin treatments to determine whether disruption of ABP1 affects auxin signaling. The tested auxin responsive genes were induced by auxin in WT plants (Figure 4.7). The same set of auxin-inducible genes was also induced in abp1-c1 plants (Figure 4.7). The overall expression levels of the genes in abp1-c1 and WT were similar, indicating that disruption of ABP1 did not affect auxin-mediated gene expression.
4.3.6 A New T-DNA abp1 Null Mutant Was Not Embryo Lethal and Displayed No Obvious Developmental Defects

We have provided clear evidence that abp1-c1 is a null mutant and that abp1-c1 plants do not display any obvious defects at the various developmental stages we analyzed. Further, the abp1-c1 plants did not show altered auxin responses. Because of the lack of any visible and molecular phenotypes in abp1-c1, it is difficult to completely rule out the possibility that a tightly-linked unknown abp1 suppressor may have completely
masked the effects of abp1 mutation. We believe that analysis of additional alleles of abp1 that were generated by using non-CRISPR methods will help us to further confirm our findings. We obtained a T-DNA insertion mutant from the Arabidopsis stock center (Figure 4.8). The mutant (abp1-TD1) had a T-DNA insertion at 27 bp downstream of the ATG start codon in the first exon (Figure 4.8A). Interestingly, the T-DNA insertion site was close to the previously reported embryonic lethal T-DNA insertion mutant, which had an insertion at 51 bp from the ATG [114]. The abp1-TD1 plants were viable and displayed no obvious differences from WT plants (Figure 4.8B). At the mature stage, abp1-TD1 and WT were similar in size and both were fertile (Figure 4.8C). We investigated whether abp1-TD1 still produced ABP1 mRNA by RT-PCR analysis. We first used a pair of primers (A5P + A3P, please see Table 4.1 for primers used in this study) (Figure 4.8A) that can amplify the entire ORF from ATG start codon to the TAA stop codon. It was clear that the primers efficiently amplified the ABP1 cDNA from WT samples, whereas no ABP1 cDNA was amplified in the abp1-TD1 sample (Figure 4.8D). We then used another pair of primers (A2E and A3P) (Figure 4.8A) to determine whether abp1-TD1 can produce partial ABP1 mRNA, which might still produce functional ABP1 protein. As shown in Figure 4.8D, abp1-TD1 did not produce such partial mRNA. Moreover, our Western blot analysis (Figure 4.8E) indicated that abp1-TD1 is a null allele. The finding that abp1-TD1 was viable, normal, and fertile further supports the conclusions that ABP1 is not essential.

In summary, the new abp1 mutants presented in this paper offer the genetic materials needed to unambiguously define the physiological roles of ABP1. The mutants are viable, stable, and more importantly, they are nulls. Moreover, the mutants are generated by using different methods and the abp1-c1 and abp1-TD harbor different types of mutations. Our results clearly demonstrate that plants do not need ABP1 for auxin signaling and for their growth and development under normal growth conditions.
At this point, the reasons for the differences between the phenotype of our mutants and previously described ABP1 knockdown lines are not clear. However, both cellular immunization and antisense approaches can be susceptible to off-target effects. For example, a recent study in zebrafish showed that 80% knockdown mutants induced by Morpholinos (antisense) were not recapitulated by true null mutants [137].

4.4 Materials and Methods

4.4.1 Plant Materials

The abp1-TD1 (SK21825) was obtained from the Arabidopsis stock center. All plants were grown under long-day conditions (16-h light and 8-h darkness) at 22 °C if not otherwise specified. For hypocotyl and root length measurements, seedlings were grown on Murashige and Skoog (MS) media containing 1% sucrose under long-day conditions on vertical plates for 7 d. The plates were scanned, and NIH Image J software was used to quantify hypocotyl and root lengths.

4.4.2 Generation of abp1-c1 using CRISPR technology

Our ribozyme-based CRISPR technology was described [128]. WT Arabidopsis plants, Columbia-0 ecotype, were transformed with the CRISPR construct by floral dipping. The abp1-c1 plants were identified at the T2 stage.

4.4.3 Genotyping abp1 Mutants

The T-DNA insertion mutant was genotyped by using a PCR-based method described [76, 138]. Genotyping primers for abp1-TD1 were as follows: ABP1-U409F, ABP1-586R, and the T-DNA specific primer pSKTAIL-L3 (please see Table 4.1). For
genotyping *abp1-c1*, we amplified an *ABP1* fragment by PCR using the following two primers: ABP1-U409F and ABP1-586R. The resulting PCR product was digested with the restriction enzyme *B*<sub>s</sub>*l*<sub>I</sub>, which cuts WT PCR product once and does not cut the mutant band (Figure 4.2).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
</tr>
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<tr>
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</tr>
<tr>
<td>ABP1-3P (A3P)</td>
<td>TAAAGCTCTCCTTTTGTGATTCT</td>
</tr>
<tr>
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</tr>
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<td>pSKTAIL-L3</td>
<td>ATACGACGGATCGTAATTTGTCG</td>
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<tr>
<td>ABP1-U409F</td>
<td>CCTATACACACACAAAGAGTCACACTC</td>
</tr>
<tr>
<td>ABP1-586R</td>
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<td>PP2A (At1g69960)-R</td>
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</tr>
<tr>
<td>GAP2 (At1g13440)-R</td>
<td>AAACCTTGTGCTCATAAGC</td>
</tr>
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</table>

**4.4.4 Western Blot**

Plant extracts were loaded onto SDS/PAGE gels. The gel was run until bromophenol blue was approximately 1 cm above the bottom of the gel, and the proteins were transferred to a PVDF membrane. The membrane was blocked in 5% (wt/vol) nonfat
milk overnight at 4 °C and with anti-ABP1 antibody at room temperature for 3 h. The membranes were washed in TBST (20 mM Tris, 150 mM NaCl, pH = 8.0 plus 0.05% Tween 20) three times, incubated with goat anti-rabbit secondary antibody for 3 h, and washed in TBST three times. Results were visualized by ECL Plus Western Blotting Detection System (Amersham; RPN2232).

### 4.4.5 Analysis of Auxin Responses

Five-day-old seedlings grown on MS plates were transferred to MS plates containing various concentrations of IAA or NAA, or mock. The root tips of seedlings were marked. After grown vertically for 2 d, plates were scanned. The root elongation that occurred during the 2-d period, and hypocotyl length was measured by using NIH ImageJ.

To analyze auxin-induced gene expression, 7-d-old seedlings were treated with or without 1 µM NAA for 2 h. Five biological replicates were prepared for both WT and abp1-c1 mutant, with or without the treatment. Total RNAs were extracted by using the RNeasy Plant Mini Kit (Qiagen; 74904) according to the instructions from the manufacturer. RNA samples were treated with DNase and purified before performing quantitative RT-PCR. PCR primers are listed in Table 4.1.

### 4.5 Acknowledgments

We thank Dr. Alan Jones for kindly providing us the ABP1 antibody. This work was supported by NIH Grants R01GM068631 (to Y. Zhao) and R01GM43644 (to M.E.), National Science Foundation (Plant Genome Grant DBI-0820729 (to Y. Zhao), the Gordon and Betty Moore Foundation (M.E.), and the Howard Hughes Medical Institute (M.E.).
Chapter 4 in full, is a reprint of the material as it appears in Proceedings of the National Academy of Sciences, 2015, Gao, Yangbin; Zhang, Yi; Zhang, Da; Dai, Xinhua; Estelle, Mark; Zhao, Yunde. The dissertation/thesis author was a primary investigator and author of this paper.
Chapter 5

Identification and Characterization of IAMH1 Gene In Biosynthesis of Plant Hormone Auxin

5.1 Abstract

Plant hormone auxin is a small-molecule growth regulator that is involved in almost every aspect of plant life. In Arabidopsis plants, the major active form of auxin, indole-3-acetic acid (IAA), could be synthesized through many different pathways via different intermediates. The two-step auxin biosynthesis by YUC and TAA family proteins has recently been established as the main auxin biosynthesis pathway using indole-3-pyruvic acid (IPA) as the intermediate. IAA could also be synthesized from other intermediates found in Arabidopsis, such as tryptamine (TAM), indole-3-acetaldoxime (IAOx) and indole-3-actamide (IAM). Although genes capable of converting IAM into IAA in Arabidopsis have been discovered, their knockouts do not confer IAM resistance, indicating the presence of other genes responsible for the auxin-overproduction phenotype.
in IAM-treated plants. In this paper, we report the identification of the IAMH1 gene, which is involved in converting IAM into IAA in Arabidopsis. We show that iamh1 point mutation lines are resistant to IAM treatment. We also demonstrated that IAMH1 could convert IAM into IAA both in vitro and in vivo. The expression pattern of IAMH1 gene is also examined, and its expression is found in various tissues and stages of Arabidopsis plants. We also identified another close homolog of IAMH1, the IAMH2 gene. Interestingly, IAMH2 gene appears as a tandem repeat on the chromosome with IAMH1 gene. To better understanding the function of IAMH genes, we generated iamh2 mutants in the iamh1 background using ribozyme-based CRISPR genome-editing technology and we identified two lines in iamh1 background with different iamh2 mutations. The possible interactions between auxin-overproduction mutant sur1 and iamh1 mutant are also examined.

### 5.2 Introduction

Auxin plays essential roles in many aspects of plant growth and development [1]. Auxin concentrations in plant cells need to be tightly controlled so that plants can grow properly in response to developmental and environmental signals. Plants have evolved a complex network to effectively modulate auxin concentrations. Auxin biosynthesis, degradation, and transport all contribute to establishing proper auxin concentrations in cells [11, 14, 15, 27, 139, 140]. Recent studies have shown that spatially and temporally regulated auxin biosynthesis is involved in determining almost all of the major developmental processes including embryogenesis, seedling growth, vascular pattern formation, and flower development [14, 15, 141]. Understanding the molecular mechanisms of auxin biosynthesis provides the necessary tools for effectively modulating auxin levels in plants, thus allowing us to improve agriculturally important traits such as branching and root
Auxin is generally believed to be synthesized through both tryptophan (Trp)-dependent and Trp-independent pathways [1]. Very little is known about the Trp-independent pathway. Only recently a report demonstrates that the cytosolic indole synthase (INS) is a key enzyme in the elusive Trp-independent pathway and that mutants defective in INS functions display phenotypes during early embryogenesis [142]. Trp-dependent pathways have not been fully elucidated either. It has been proposed that Trp may be converted to IAA, the main natural auxin in plants, through several routes [1]. Trp can be metabolized into tryptamine (TAM) and indole-3-pyruvate (IPA) by PLP-dependent decarboxylases and aminotransferases, respectively. It is also known that Trp can be converted into indole-3-acetaldoxime (IAOx) by CYP79B2 and CYP79B3 P450 monooxygenases [45]. Moreover, plants also produce indole-3-acetamide (IAM) and indole-3-acetonitrile (IAN) from Trp [7]. All of the aforementioned Trp metabolites including TAM, IPA, IAOx and IAM have been proposed as intermediates for auxin biosynthesis in plants. However, so far only IPA has been firmly established as an important auxin biosynthetic intermediate in plants [16, 25, 26]. Disruption of either IPA biosynthesis or metabolism in Arabidopsis, maize, and rice leads to dramatic developmental defects [14, 15, 22, 23, 26, 28, 38, 143]. It has been shown that Trp is converted into IAA using IPA as the intermediate in two steps in the so-called TAA/YUC pathway [144]. Trp is first metabolized into IPA by the TAA family of aminotransferases and subsequently the YUC family of monooxygenases catalyzes the conversion of IPA into IAA [144]. The TAA/YUC pathway is evolutionary conserved among plant species and it is required for all of the major developmental processes in Arabidopsis. Therefore, the TAA/YUC pathway has been recognized as a major auxin biosynthesis pathway.

The roles of the other Trp metabolites in auxin biosynthesis and plant development have not been fully resolved. IAOx has long been recognized as a potential
auxin biosynthesis precursor. Over accumulation of IAOx in *Arabidopsis* by either overexpressing the biosynthetic enzyme CYP79B2 or by inactivating IAOx metabolizing enzymes such as SUR1 and SUR2 leads to auxin overproduction [9, 45, 145]. Although IAOx can be metabolized into IAA in *Arabidopsis*, the exact mechanisms by which IAOx is converted into IAA are not understood at present. It is generally accepted that IAOx probably is not a major intermediate for auxin biosynthesis for two reasons. First, complete elimination of IAOx production in *Arabidopsis* by knocking out both CYP79B2 and B3 does not lead to dramatic developmental defects. Second, IAOx is only produced in limited number of plant species that produce indolic glucosinolates [7].

The biosynthetic route for IAOx is well understood, but the reactions from IAOx to IAA have not been elucidated. In contrast, enzymes responsible for converting other Trp metabolites such as IAN into IAA are known [13], but the biosynthetic route for IAN and IAM are not understood. IAN can be converted into IAA in plants when added to plant growth media. It was shown more than a decade ago that IAN is converted into IAA by a family of nitrilases [13]. Mutations in *nitrilase 1* (*nit1*) in *Arabidopsis* render the mutant resistant to exogenous IAN [13]. Under normal growth conditions, *nit1* mutants do not display obvious developmental defects probably because of the compensatory effects provided by NIT1 homologs in *Arabidopsis*. It is still an outstanding question whether nitrilases and IAN play an important in auxin biosynthesis and plant development.

IAM was the first definitively identified intermediate used in Trp-dependent auxin biosynthesis pathways in bacteria. Plant pathogens such as agrobacterium and pseudomonas synthesize auxin from Trp when they infect plants [39, 146, 147]. The bacteria-produced auxin alters the growth and developmental patterns of the infected plant cells so that the pathogens can use the plant cells to produce carbon- and nitrogen-rich compounds for their growth. The pathogens convert Trp into IAM using the bacterial iaaM Trp-2-monooxygenase and subsequently the pathogen-encoded hydrolase iaaH converts
IAM to IAA [39, 146, 147]. *Arabidopsis* and other plants produce IAM in the absence of a bacteria infection, suggesting that plants may use IAM as an auxin biosynthetic intermediate as well. Furthermore, IAM was proposed as an intermediate in a route that converts IAOx into IAA [7]. It is well known that *Arabidopsis* and other plants have the capacity to convert IAM into IAA. Overexpression of *iaaM* in *Arabidopsis*, petunia, and tobacco led to auxin overproduction phenotypes [10, 148, 149]. It is hypothesized that plant hydrolases can convert IAM produced by the *iaaM* transgene to generate IAA. Bioinformatic analyses have identified a small family of IAM hydrolases named as amidases that share significant homology to the bacterial iaaH proteins [40, 150, 151]. *Arabidopsis* AMIDASE I (*AMI1*) has been shown to have the capacity to hydrolyze IAM into IAA in vitro and in *Arabidopsis* [151]. However, the amidase mutants do not display much reduced sensitivity to exogenous IAM [151], suggesting that plants probably also use other unidentified hydrolases to convert IAM to IAA. Identification of additional enzymes that are responsible for converting IAM to IAA will help us to unambiguously determine whether IAM is a key auxin biosynthetic intermediate in plants and whether IAM-derived auxin plays an important role in plant growth and development. Understanding of how IAM is converted into IAA in plants will also clarify whether IAM is an important intermediate in metabolizing IAOx into IAA.

In this paper, we present the identification of two homologous genes that encode hydrolases responsible for converting IAM to IAA in *Arabidopsis*. We conducted a genetic screen for mutants that displayed reduced sensitivity to exogenous IAM. We mapped one of the strong IAM-resistant mutants to chromosome IV and discovered that the mutant contained a G to A conversion that generated a premature stop codon in the gene *At4g37550*, which encodes a predicted hydrolase. We named *At4g37550* IAMH1 (*IAM HYDROLASE 1*). We demonstrated that IAMH1 had the capacity to convert IAM into IAA both in vitro and in *Arabidopsis*. Furthermore, we show that IAMH1 was
localized in the cytosol and IAMH1 is almost ubiquitously expressed in the shoot and root tip. Interestingly, *Arabidopsis* genome contains two copies of the IAMH genes and the two genes are arranged as tandem repeats on the chromosome IV. We found that disruption of either IAMH gene lead to a decreased sensitivity to IAM. We successfully used the latest CRISPR genome editing technology to generate mutations in the IAMH2 gene in the *iamh1-1* mutant background. *Arabidopsis* plants lacked any IAMH activities were resistant to IAM, but did not display any obvious growth and developmental defects, suggesting that the IAM-derived auxin is not required for *Arabidopsis* development under normal growth conditions. The work identified the main enzymes for hydrolyzing IAM to IAA in *Arabidopsis* and clarified the roles of IAM in auxin biosynthesis and plant development.

### 5.3 Results and Discussion

#### 5.3.1 IAM promotes plant growth and activates the auxin reporter DR5-GUS

IAM is the key intermediate used by some plant pathogenic bacteria to synthesize auxin (Figure 5.1A) [39, 146, 147]. The two-step pathway catalyzed by the bacterial *iaaM* and *iaaH* effectively converts Trp into IAA (Figure 5.1A). The *iaaM* gene has been widely used to genetically modulate auxin levels in plants [10, 14, 149]. *Arabidopsis* seedlings grown on IAM-containing media in the light had much elongated hypocotyls and developed epinastic cotyledons (Figure 5.1B). IAM also slightly inhibited the elongation of primary roots. IAM-treated plants resembled closely to the well-characterized *Arabidopsis* auxin overproduction mutants such as *YUC* overexpression lines and *sur1* mutants [9, 11], suggesting that IAM either activates an auxin signaling pathway directly
Figure 5.1: Indole-3-acetamide (IAM) is a potential auxin biosynthetic intermediate in plants and IAM treatments affect plant growth and activate auxin reporter DR5-GUS. (A) A proposed Trp-dependent auxin biosynthetic pathway using IAM as the intermediate. (B) Five-day old Arabidopsis seedlings grown on MS media and media containing 20 µM IAA or IAM. Note that IAA inhibits primary root elongation and IAM stimulates hypocotyl growth. (C) Activation of DR5-GUS expression by IAA and IAM. Interestingly, IAM mainly activates DR5-GUS expression in aerial tissue whereas IAA increases DR5-GUS signal in the root.

or IAM is converted into IAA, the active natural auxin. Interestingly, seedlings grown on IAA plates did not display long hypocotyls and epinastic cotyledon (Figure 5.1B). Rather IAA mainly inhibited primary root elongation and stimulated lateral root initiation and elongation (Figure 5.1B). We investigated whether IAM activated the expression of the auxin reporter DR5-GUS. As shown in Figure 5.1C, seedlings grown on IAM-containing media had much elevated expression levels of DR5-GUS in the cotyledons and true leaves compared to seedlings grown on regulate media. Activation of DR5-GUS expression in aerial part by IAM is consistent with the observation that IAM mainly stimulated hypocotyl elongation and changed the shape of cotyledons (Figure 5.1B). In contrast, IAA activated DR5-GUS expression in the roots (Figure 5.1C). Our results indicated that IAM and IAA caused different developmental phenotypes in Arabidopsis seedlings
(Figure 5.1B & C). The observed differences were probably caused by differences in uptake and transport of the two compounds. It is very clear that IAM treatment could activate the auxin reporter and cause phenotypes related to elevated auxin levels.

5.3.2 Genetic screens for mutants resistant to IAM

Arabidopsis seedlings grown on 20 μM IAM phenocopied the YUC overexpression plants, which produce elevated levels of auxin due to the overexpression of the YUC flavin monooxygenase, a rate-limiting enzyme in auxin biosynthesis [11, 14]. Because auxin overproduction mutants display phenotypes different from those caused by IAA treatments and because previous genetic screens for auxin resistant mutants were mainly conducted using IAA or synthetic auxin 2,4-D, we hypothesized that a genetic screen for mutants that can suppress YUC overexpression lines would uncover novel auxin genes. We hypothesized that such a genetic screen might be able to identify genes that are important for auxin biosynthesis, conjugation, degradation, transport or auxin signaling. Unfortunately, the YUC overexpression lines were not stable and the strong lines were completely sterile. Therefore, genetic screens for suppressors/enhancers of YUC overexpression lines were not feasible. Because of the strong phenotypic similarities between IAM-treated plants and YUC overexpression lines, we believe that genetic screens for IAM resistant mutants would mimic the screens for suppressors of YUC overexpression lines.

We mutagenized Arabidopsis seeds using EMS and conducted the genetic screen using 7- to 9-day old seedlings grown on 20 μM IAM under light. The putative mutants should have short hypocotyls and normal cotyledons. We screened M2 seeds from 1000 individual M1 plants and identified more than 100 putative IAM-resistant mutants, which were transplanted to soil. Among the putative mutants, many were dwarf with dark-green leaves, which are very similar to the brassinolide (BR) biosynthesis and signaling mutants.
Figure 5.2: Isolation and cloning of an IAM resistant mutant (iamh1). Isolation of an IAM-resistant mutant, which does not have elongated hypocotyl and does not display epinastic cotyledons when grown on 20 µM IAM-containing media. (B) The expression of DR5-GUS auxin reporter is not induced by IAM treatments in the iamh1 mutant. (C) The iamh1 mutation was identified by map-based positional cloning. The IAMH1 gene is At4g37550. The iamh1-1 mutant harbors a G to A mutation in At4g37550 that results in a premature stop codon. (D) The iamh1 phenotypes are rescued by wild type IAMH1 cDNA or IAMH1 cDNA fused with GFP driven by the IAMH1 promoter.

After discarded the obvious BR-related mutants and conducted second round screens with M3 seeds, we had isolated 24 confirmed IAM-resistant mutants. One of the mutants, #483, was almost insensitive to IAM. Light grown mutant #483 had a short hypocotyl and
flat cotyledons (Figure 5.2A) when grown on 20 \( \mu \text{M} \) IAM. We backcrossed the mutant to Wild-Type (WT) Columbia (Col) and out-crossed it to Wild-Type (WT) Landsberg (Ler). About 25% seedlings from either F2 populations displayed the IAM resistant phenotype, suggesting that the phenotype was caused by a single locus.

We mapped the mutation in the #483 mutant to the bottom of chromosome IV and narrowed the mapping interval down to about 330 Kb region. Among the ORFs in the mapping interval, At4g37550 encodes a putative Acetamidase/Formamidase, which potentially has the hydrolase activities that can break an amide bond. We hypothesized that a mutation in At4g37550 probably would abolish the conversion of IAM into IAA, thus causing the IAM-insensitive phenotypes. We sequenced the genomic DNA of At4g37550 from the mutant #483 and identified a G to A conversion in the first exon of At4g37550 (Figure 5.2A). The mutation converted a Trp codon to a stop codon (Fig. 2A), suggesting that the mutant is likely a null allele.

To confirm that the identified mutation in the At4g37550 gene caused the observed IAM insensitive phenotype of mutant #483, we obtained a T-DNA insertion mutant of At4g37550 from the ABRC stock center. The T-DNA mutant was also resistant to IAM treatment, demonstrating that #483 mutant phenotypes were caused by disruption of At4g37550. We renamed #483 mutant iamh1-1 (IAM HYDROLASE 1) and At4g37550 gene IAMH1. The T-DNA allele was named iamh1-2. To further demonstrate that we had identified the causal mutation in iamh1-1, we transformed iamh1-1 plants with a construct that harbored a cassette that expresses At4g37550 cDNA under the control of At4g374550 promoter. As shown in Figure 5.2C, the IAMH1 transgene fully restored the IAM sensitivity of the iamh1-1 mutant. We also expressed an IAMH1-GFP fusion under the control of the IAMH1 promoter in the iamh1-1 background. The GFP fusion could also fully rescue the iamh1-1 phenotypes. Interestingly, the complementation transgenic lines appeared to have longer hypocotyls than wild type plants grown under
the same conditions (Figure 5.2C). The differences probably were caused by a slight overexpression of the transgenes. Such an observation actually further supports the hypothesis that IAMH1 is involved in converting IAM to IAA in *Arabidopsis*.

*IAMH1*-like genes have been identified in all of the plant genomes that have been sequenced. IAMH1 appears to be a plant specific protein. The only animal genome that contains a close homolog of IAMH1 is the Tibetan antelope genome. However, it has not been ruled out whether the antelope gene is contaminated from a plant source. IAMH1 is highly conserved throughout the plant kingdom. For example, the maize IAMH1 homolog shares 89% amino acid sequence identity with the *Arabidopsis* IAMH1.

### 5.3.3 The IAMH1 is broadly expressed and is not localized in the nucleus

We expressed the *GUS* gene driven by the *IAMH1* promoter in *Arabidopsis*. At seedling stage, the *GUS* expression was broadly distributed in cotyledons, true leaves, and root tips (Figure 5.3). At reproductive stage, *GUS* expression was observed in young flowers, gynoecia, and in inflorescences (Figure 5.3A). Expression of IAMH1-GFP fusion driven by *IAMH1* promoter showed that *IAMH1* was clearly not expressed in the nucleus (Figure 5.3B)

### 5.3.4 IAMH1 has the capacity to hydrolyze IAM into IAA and ammonia

Our genetic data suggest that IAMH1 functions as a hydrolase that converts IAM to IAA in *Arabidopsis*. We expressed IAMH1 as a His-tagged fusion protein in *E. coli* and purified it to homogeneity (Figure 5.4A). IAM and IAA can be easily separated on a TLC plate (Figure 5.4B). In the presences of recombinant IAMH1, IAM was converted
Figure 5.3: Expression pattern of IAMH1 and sub-cellular localization of IAMH1 protein. (A) The GUS expression patterns of IAMH1 prom:GUS Transgenic lines. Note that the reporter has a broad expression pattern. (B) Expression of the IAMH1-EGFP fusion in Arabidopsis roots driven by the IAMH1 promoter. IAMH1 appears to be located in the cytosol.

into IAA (Figure 5.4). In contrast, heat-inactivated IAMH1 protein failed to hydrolyze IAM to IAA in vitro (Figure 5.4B). We also used a colorimetric assay and successfully detected the other product ammonia. Further quantitative analysis showed that IAMH1 was a rather slow enzyme with kcat of 1.5 min−1 and Km for IAM as 437 µM. The in vitro data suggest that the assay conditions probably were not optimal and that additional in vivo factors might affect the catalysis. Nevertheless, our in vitro data was consistent
Figure 5.4: IAMH1 can hydrolyze IAM into IAA and ammonia. (A) IAMH1 with a His-tag was expressed in *E. coli* and purified to homogeneity. Lane 1: Prestained protein marker. Lane 2: *E. coli* cell lysate before IPTG induction. Lane 3: *E. coli* cell lysate after IPTG induction. Lane 4: Purified IAMH1 protein using Ni-NTA agarose. (B) IAM and IAA are separated on a TLC plate. Ammonia produced in the reaction was detected colorimetrically.

with a role for IAMH1 in converting IAM into IAA in *Arabidopsis*.

5.3.5 *Arabidopsis* genome contains two copies of IAMH genes

Both *iamh1-1* and *iamh1-2* did not show obvious developmental defects under normal growth conditions, despite that both mutant alleles were resistant to IAM. Blastp analysis using IAMH1 protein as query identified At4g37560 as a close homolog of IAMH1 in the *Arabidopsis* genome (Figure 5.5). We named At4g37560 *IAMH2*. *IAMH2* and *IAMH1* share 90% amino acid sequence identity. Because of the high sequence homology, we hypothesized that *IAMH2* might also play an important role in converting IAM into IAA in *Arabidopsis*. Functional redundancy between *IAMH1* and *IAMH2* may explain our observation that *iamh1-1* and *iamh1-2* did not show obvious developmental defects.

We obtained a T-DNA insertion mutant from the ABRC stock center to test
Figure 5.5: IAMH2 gene is also involved in converting IAM into IAA. (A) IAMH1 has a close homolog, IAMH2. The two genes are tandem repeats located on Chromosome IV. (B) A T-DNA insertion in IAMH2 also caused resistance to exogenous IAM. (C) Generation of iamh2 alleles by CRISPR. TGG in red is the PAM site for CRISPR/Cas9. The iamh2-2 allele harbors one T insertion and iamh2-3 contains a 20 bp deletion.

whether iamh2 was also resistant to IAM. As shown in Figure 5.5B, iamh2-1 had short hypocotyls and normal cotyledons when grown on 20 µM IAM whereas wild type plants developed long hypocotyls and epinastic cotyledons, demonstrating that disruption of IAMH2 also led to IAM resistance. These data suggest that IAMH2 likely has overlapping functions with IAMH1.
5.3.6 Construction of *iamh1* *iamh2* double mutants

In order to assess the roles of the *IAMH* genes in auxin biosynthesis and *Arabidopsis* development, we need to inactivate both *IAMH* genes simultaneously. The two *IAMH* genes are located at Chromosome IV as tandem repeats (Figure 5.5A). It is virtually impossible to generate *iamh1* *iamh2* double mutants by crossing two single mutants together because of the extremely tight linkage between the two genes. We employed our recently developed ribozyme-based CRISPR technology [128, 152] to generate *iamh2* mutations in the *iamh1-1* background. We obtained two *iamh2* alleles (Figure 5.5C): *iamh2-2* and *iamh2-3*. The *iamh2-2* contained a single bp insertion after the nucleotide 330 from the ATG start codon in the cDNA (A in the ATG start codon counts as the first nucleotide), which generated an immediate stop codon (Figure 5.5C). Therefore, *iamh2-2* is likely a null allele. The *iamh2-3* allele harbored a 20 bp deletion from nucleotide 320 to 339 in the cDNA. Such a large deletion in *iamh2-3* was also likely to completely abolish *IAMH1* function. We backcrossed both *iamh2* alleles to wild type Col plants to segregate out the CRISPR/Cas9. Both *iamh1-1 iamh2-2* and *iamh1-1 iamh2-2* double mutants were viable and fertile. In fact, we did not observe any obvious developmental defects in the *iamh* double mutants under normal plant growth conditions. Our data suggest that auxin-derived from IAM probably is not required for *Arabidopsis* growth and development under laboratory growth conditions.

5.3.7 Auxin overproduction phenotypes of *sur1* is not suppressed by *iamh1*

*SUR1* is a key enzyme for indolic glucosinolate biosynthesis. Disruption of *SUR1* leads to the accumulation of IAOx, which is metabolized into IAA through an undefined pathway [9, 145]. One of the proposed intermediate in converting IAOx into IAA is
Figure 5.6: Disruption of the IAMH1 gene could not suppress the auxin overproduction phenotypes of sur1. Note that the iamh1 sur1 double mutants still have long hypocotyl and epinastic cotyledons.

IAM [7]. We introduced the iamh1-1 mutation into sur1-2 to test whether the auxin overproduction phenotypes of sur1 could be suppressed by compromising the IAMH functions. As shown in Figure 5.6, the iamh1-1 sur1-2 double mutants still developed long hypocotyls and epinastic cotyledons, suggesting that IAM probably is not the main intermediate for metabolizing IAOx into IAA.

In this paper, we uncovered two IAMH genes that encode hydrolases capable of converting IAM into IAA both in vitro and in Arabidopsis. Our data demonstrated that the two IAMH genes are the main players in metabolizing exogenous IAM into IAA. Inactivation of the IAMH genes renders Arabidopsis plants insensitive to IAM treatments. Our preliminary data indicate that IAMH genes do not play an essential role in Arabidopsis development. Further detailed characterization of the iamh1 iamh2 double mutants is still needed to definitively determine their functions in auxin biosynthesis and
plant development. We still need to biochemically determine whether concentrations of IAA, IAM, IAN, and IAOx in the iamh1 iamh2 double mutants are affected. The genetic interactions between the iamh mutants with known auxin biosynthetic mutants such as yuc, taa, and cyp79b2 cyp79b3 will further clarify the auxin biosynthetic landscape.

5.4 Materials and Methods

5.4.1 Plant Materials and Growth Conditions

The iamh1-2 and iamh2-1 T-DNA mutants was obtained from the Arabidopsis stock center. Plants were grown under long-day conditions (16-h light and 8-h darkness) at 22 °C. Seeds were surfaced sterilized by 70% ethanol and air-dried on filter papers in the hood before placed on plates containing Murashige and Skoog (MS) media (supplemented with IAA or IAM when indicated). The plates with seeds were then incubated at 4 °C for 2 days before placed in the growth chamber. Seedlings were grown on the plates in the growth chamber until 7-9 days old, and then transfered to grow in soil if needed.

5.4.2 IAM-resistant mutant screening

WT Col plants were mutagenized using Ethyl methanesulfonate (EMS). Seeds from each mutagenized plants were harvested individually and grown on MS plates containing 20 μM IAM. WT plants would show obviously elongated hypocotyl. IAM-resistant seedlings with reduced hypocotyl length were selected and further analyzed. The iamh1-1 mutant was backcrossed 2 times to WT Col to remove background mutations.
5.4.3 Plant Transformation

Arabidopsis plants with different genetic background were transformed using corresponding T-DNA constructs in agrobacteria GV3101 via floral dipping method described previously [153].

5.4.4 Constructs and Transgenic Plants

IAMH1<sub>pro</sub>:GUS construct was made using pBI101.3 with the 2.8 Kb promoter region before the ATG start codon cloned before the GUS gene. WT Col plants were transformed and T1 plants were selected on MS plates containing kanamycin.

IAMH1<sub>pro</sub>:IAMH1 construct was made using pART27 with the whole 5.5 Kb genomic region, which includes the 2.8 Kb promoter region before the start codon, the 2.0 Kb region from the start codon to the stop codon and the 657 bp region after the stop codon. iamh1-1 plants were transformed and T1 plants were selected on MS plates containing 20 µM IAM. Plants showing restored IAM sensitivity were selected as complemented lines.

IAMH1<sub>pro</sub>:IAMH1-EGFP construct was made using pART27 with the 4.9 Kb genomic region (which includes the 2.8 Kb promoter region before the ATG start codon, the 2.0 Kb region from the start codon to just before the stop codon), the EGFP gene coding region, and an OCS terminator. iamh1-1 plants were transformed and T1 plants were selected on MS plates containing 20 µM IAM. Plants showing restored IAM sensitivity were selected as complemented lines.

CRISPR construct targeting IAMH2 gene was generated using our ribozyme-based guide RNA CRISPR system described previously [128, 152]. The CRISPR target site chosen for IAMH2 gene was TGCAACTTGGGTCCTCTTCCAGG, which is in the second exon and the 314 to 336 bp region counting from the ATG start codon in
the cDNA. *iamh1-1* plants were transformed and T1 plants were selected on MS plates containing hygromycin. Mutant plants were identified using restriction enzyme Bs/I (recognition site CCNNNNNNNGG) to cut PCR fragments containing the CRISPR target site amplified from the genomic DNA.

### 5.4.5 *iamh1-1* genotyping

Plants with *iamh1-1* mutation were genotyped using primers 5’- GATGACGCC-AAGCGTGTAAGC -3’ and 5’- CTGGGAATTCAGAGGTAAGCAC -3’ to amplify the genomic DNA and then digested with NcoI. PCR products from WT plants would be cut into two fragments (0.6 Kb + 0.9 Kb) while the PCR products from the *iamh1-1* mutants would appear as a single band (1.5 Kb).

### 5.4.6 Beta-glucuronidase (GUS) staining

GUS staining of *IAMH1* :GUS plants and *DR5-GUS* plants were performed according to the previously described protocol [154].

### 5.4.7 IAMH1 protein expression, purification and SDS-PAGE analysis

*IAMH1* cDNA was cloned into pET28a vector, and transformed into *E. coli* strain BL21 (DE3). The IAMH1 protein is expressed at 15 °C induced by 1mM IPTG. The protein is purified via the His-tag fused on the N-terminal using Ni-NTA Agarose from Qiagen, Hilden, Germany following the manufacturer’s instructions. The *E. coli* cell lysate before and after the IPTG induction as well as the purified protein were loaded onto 12% SDS-PAGE gel and stained with coomassie blue to visualize the bands.
5.4.8 IAMH1 in vitro activity assay

IAMH1 protein was tested in a buffer solution containing 50 mM NaCl, 1 mM DTT, 20 mM Tris-Cl (pH = 7.5) and 0.1 mg/mL BSA. Boiled or active IAMH1 protein was tested at the final concentration of 50 µg/mL in a total volume of 100 µL. Reaction was carried out at room temperature for 24 h. 50 µL of the reaction mix was transferred into a new tube, and acidified by adding 3 µL of 1 M HCl. After mixing, 250 µL of ethyl acetate was added and vortexed for 30 s to extract the indole derivatives. The mix was then centrifuge at 12,000 rpm for 1 min. 200 µL of the top layer was transferred to a new tube, and concentrated by spin vacuuming at 60 °C for 7 min. The concentrated samples were spotted onto a TLC plate by repeatedly spotting 1.5 µL with drying in between. Control samples of IAM or IAA were dissolved in 100% ethanol and spotted onto the TLC plate. TLC was performed in 100% ethyl acetate for 10-12 min (before the solvent front reach the top) and briefly dried in air. Then plate was visualize under UV light and the indole derivatives appeared as dark spots on the plate.

To test the ammonia produced and calculate the IAMH1 enzymatic kinetics, a colorimetric assay based on the Berthelot indophenol reaction was used [155]. For each 50 µL sample after reaction, 10 µL of 10% Phenol (ethanol solution), 2 µL of 0.5% sodium nitroprusside (SNP), 7 µL of (20% Na3Citrate + 1% NaOH) and 2 µL of NaClO solution (12% chlorine) were sequentially added and mixed after each addition. The color was developed at room temperature for 30 min and OD640 was measured. Standard curves were produced using NH4Cl following the same protocol.

5.4.9 Confocal imaging of root tips

Root tips of IAMH1pro::IAMH1-EGFP plants were stained using Propidium Iodide and visualized using a confocal microscope. The cell contour appeared as red florescence
signal and IAMH1-EGFP fusion proteins appeared as green fluorescence signal.

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Chapter 5, in part is currently being prepared for submission for publication of the material. Gao, Yangbin; Zhang, Da; Dai, Xinhua; Guo, Xiuhua; Zhao, Yunde. The dissertation/thesis author was a primary investigator and author of this material.
Appendix A

Final notes

Just as “All roads lead to Rome”, the plants use many different intermediates via different pathways to synthesize the important plant hormone auxin. With the main auxin biosynthesis route being identified as the YUC/TAA pathway, the importance of the other pathways remains to be fully elucidated. The identification of the IAMH genes put one more piece in the puzzle, and more await to be discovered in the years to come.
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