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Elucidating Molecular Mechanisms of Auxin Metabolism in *Arabidopsis thaliana* Using the Bacterial *iaaB* Gene

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The Thesis of Hanchuanzhi Yu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Elucidating Molecular Mechanisms of Auxin Metabolism in *Arabidopsis thaliana* Using the Bacterial *iaaB* Gene

by

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The phytohormone auxin regulates nearly every aspect of plant growth and development. How plants control auxin biosynthesis, conjugation, degradation and auxin transport has been a major research topic in auxin biology. Great progress has been made in auxin biosynthesis and conjugation as well as auxin transport in the past decades. The recent discovery of *DAO* genes also contributes to the knowledge of auxin catabolism in plants. The bacteria *Aromatoleum aromaticum* is able to degrade auxin in anaerobic conditions. The *iaaB* gene encodes an indole

acetate-CoA ligase that converts IAA to IAA-CoA in the auxin degradation pathway in *A. aromaticum*. IAA-CoA can be potentially converted back to IAA providing a source of auxin, or form IAA conjugates or IBA. We overexpressed the *iaaB* gene in *Arabidopsis thaliana* using the *CaMV* 35S promoter to investigate the potential roles of IAA-CoA in auxin metabolism in *Arabidopsis*. Distinct auxin-related phenotypes of the *iaaB*-overexpression *Arabidopsis* plants were observed. The overall size of the *iaaB*-overexpression plants was much smaller than that of Col-0 (wild-type), suggesting that the overall auxin homeostasis is altered. Moreover, we found that the auxin reporter was activated at the basal end of hypocotyls and resulted in longer hypocotyl. We also observed that the DR5-GUS expression was reduced at root tips, which correlates with reduced gravitropism and fewer lateral roots. We conclude that overexpression of the *iaaB* gene alters the auxin homeostasis and affects the growth and development of *Arabidopsis.* We believe that different cells/tissues may have different sensitivities to *iaaB* overexpression. Future studies are needed to demonstrate the exact role of the *iaaB* gene and IAA-CoA in *Arabidopsis*.

Introduction

Plant hormones, also known as phytohormones, are a group of growth-promoting molecules, which include auxin, cytokinin, the gibberellins (GAs), abscisic acid (ABA), ethylene, brassinosteroids (BRs), and jasmonic acid (JA) (Gray, 2004). These hormones regulate different aspects of plant development, such as seed germination, vascular formation, and root development (Davies, 1987; Li et al., 2016). Auxin, the first identified and isolated plant hormone, has been studied for almost a hundred years. Overwhelming evidence clearly demonstrated that auxin is essential for embryogenesis, leaf development, root initiation and elongation, flower and vascular tissue formation (Cheng, Dai, & Zhao, 2006, 2007).

Indole-3-acetic acid (IAA) is the most common form of naturally occurring auxin. IAA controls plant growth and development by regulating gene expression (Strader & Zhao, 2016). The IAA biosynthesis pathways have been studied extensively (Yunde. Zhao, 2018). IAA can be synthesized through both tryptophan-dependent and tryptophan-independent pathways. In the tryptophan-dependent pathway, tryptophan is converted to various metabolites that are then converted to IAA. Based on the intermediates, the tryptophan-dependent pathways can be divided into four pathways – IAOx (indole-3-acetaldoxime) pathway, IAM (indole-3-acetamide) pathway, TAM (tryptamine) pathway, and IPA (indole-3-pyruvate) pathway (Rosquete, Barbez, & Kleine-Vehn, 2012). The IPA pathway is the main auxin synthesis pathway in *Arabidopsis* and maize, and it is a simple two-step pathway (Y. Zhao, 2010). The first step is to remove the amino group from tryptophan by the Tryptophan Aminotransferase of *Arabidopsis* (TAA) to generate indol-3-pyruvate (IPA). The second step is to convert IPA to indole-3-acetic acid (IAA) catalyzed by YUCCA flavin monooxygenases (YUC) (Gao & Zhao, 2014). The tryptophan-independent pathway has been proposed to synthesize IAA from indole-3-glycerol phosphate

(IGP) and/or indole (Normanly J, 1993). The cytosol localized indole synthase (Jackson RG) has been identified as an essential enzyme in the tryptophan-independent pathway, and it is reported to be essential for early embryogenesis in *Arabidopsis* (Wang et al., 2015). However, its exact function and molecular mechanism remain controversial (Nonhebel, 2015). Moreover, IAA can be synthesized through the β-oxidation of IBA (indole-3-butryic acid) or the hydrolysis of IAA-conjugates (Woodward & Bartel, 2005).

High concentration of auxin is toxic and even causes lethality to plants. Auxin interacts with other phytohormones to optimally regulate plant growth and development. High level of auxin can disrupt the balance among the phytohormones (Woodward & Bartel, 2005). For example, high concentrations of auxin can inhibit cytokinin production, causing deficient shoot development (Woodward & Bartel, 2005). Conversely, auxin can induce overproduction of ethylene. Ethylene can inhibit auxin transport and consequently inhibit growth (Woodward & Bartel, 2005). Therefore, excessive free auxin has to be removed to maintain auxin homeostasis. Local free auxin levels can be regulated by biosynthesis, transportation, conjugation and degradation.

Auxin can be transported to other cells or tissues to control the local auxin levels. Two transport system is known so far: the non-directional transport from auxin synthetic sites to recipient sites through phloem, and the directional cell-to-cell polar auxin transport (Adamowski & Friml, 2015). Auxin can enter cells by diffusion because of its protonated state when it is in the slightly acidic pH of the apoplast, but once auxin is in cytosol that has a pH about 7, a membrane transporter is required to facilitate auxin to transport out of the cell (Adamowski & Friml, 2015). The PIN proteins, a family of plasma-membrane proteins that localized only one side of the cell, are found to play crucial role in auxin efflux, and its localization is dynamic and

changeable depending on the development stage and the environmental cues (Teale, Paponov, & Palme, 2006). The PIN1 auxin transporter, the first identified PIN protein, is found to be localized at the basal side of the cell indicating that auxin is transported from shoot to root (GaLweiler, 1998). AUX1, an auxin influx carrier, is shown to regulate root gravitropism (Bennett, 1996).

IAA can also be converted to an inactive form by conjugation to amino acids or sugar. Often times, the conjugation protects IAA from degradation. Some IAA conjugates, such as IAA-Ala, IAA-Leu, and IAA-Phe can be converted back to free IAA for later use, but IAA-Asp and IAA-Glu are thought to be precursors for IAA oxidative degradation while IAA-Trp is reported to inhibit auxin activities (Ludwig-Muller, 2011; Staswick, 2009). The Gretchen Hagen 3 (GH3) family of IAA-amido synthetases is found to catalyze the conjugation of IAA to amino acids (Hagen, 1991). Different *GH3* genes have different functions in specific species. In *Arabidopsis*, GH3.6 can conjugate IAA to Ala, Asp, Phe, and Trp (Staswick et al., 2005). In grape, GH3.6 can catalyze conjugation of IAA to Gln, Met, Tyr, Val, Phe, Gly, Asn, Ile, Trp, Asp, Glu, Ala, and His (Bottcher et al., 2012). The *GH3* genes can be rapidly induced by applying exogenous IAA and conjugate free IAA to amino acids, preventing excessive IAA accumulation (Zheng et al., 2016). IAA can also conjugate to glucose to form IAA-Glc. In maize, *IAGLU* genes encode IAA-Glc synthase to catalyze the conjugation (Ludwig-Müller et al., 2005). In Arabidopsis, the multigene family of Group 1 glucosyltransferases (UGTs) can transfer glucose from UDP-glucose to IAA (Jackson RG, 2002).

Another way to reduce auxin levels is enzymatic oxidation that generates two main metabolites: 2-oxoindoleo-3-acetic acid (oxIAA) and oxIAA-glucose (oxIAA-Glc) (Enders & Strader, 2015; Ljung, 2013; Rosquete et al., 2012). The *Dioxygenase for Auxin Oxidation* (*DAO*)

gene in rice has been identified to encode a 2-oxoglutarate-dependent-Fe(II) (2OG-Fe(II) oxidase and catalyzes the oxidation of IAA to oxIAA. *DAO* gene is crucial for reproductive development of rice (Z. Zhao et al., 2013). *DAO1* and *DAO2* genes are reported to oxidize auxin in *Arabidopsis thaliana*. DAO1 is the main auxin catabolic enzyme in *Arabidopsis*. It regulates the development of lateral roots, leaves and floral organs (Zhang et al., 2016). UGT74D1 is found to convert oxIAA to oxIAA-Glc via glucosylation in *Arabidopsis* (Zhang & Peer, 2017). IAA can also be catabolized through oxidative decarboxylation of the IAA side chain with the simulation of ethylene (Davies; Normanly, 2010).

Bacteria have close relationships with plants. They are able to affect plant development through their interaction with plants (Duca, 2014). Most of the bacteria in the soil around the root area can produce auxin to affect plant auxin level (Přikryl, 1985). The auxin produced by bacteria can function as a signal to trigger the defense response of plants against bacteria (Bari, 2009; Spaepen & Vanderleyden, 2011). Bacteria also benefit from plants. When plants die and decompose in the soil, auxin is released to the soil that provides a good food source for bacteria. (Schuhle, Nies, & Heider, 2016). Bacteria genes have been used to elucidate the mechanisms of auxin biosynthesis in plants. The auxin biosynthesis pathways in bacteria and plants have similar genes and enzymes (Spaepen & Vanderleyden, 2011). One example of bacterial genes functioning in plants is the *Agrobacterium tumefeaciens auxin synthesis genes*, which have *iaaM* gene that can be expressed in *Arabidopsis* and induce auxin overproduction (Klee, 1987). Some bacteria can also synthesize auxin conjugate. For example, *iaaL* gene from *Pseudomonas savcastanoi* encodes an IAA-lysine synthetase and conjugate IAA to lysine. Overexpression of *iaaL* in tobacco and *Arabidopsis* lowered the free auxin levels, and *iaaL*-overexpression plants

showed abnormal phenotypes (Jensen, 1998; Kim et al., 2011; Romano C, 1991). Genes of these bacteria may be used to study the biosynthesis and metabolism of auxin in plants.

A recent study has shown that Betaproteobacterium *Aromatoleum aromaticum* is able to degrade IAA in anaerobic conditions (Ebenau-Jehle et al., 2012). *A*. *aromaticum* has an *iaa* operon that includes the genes encoding for the enzymes for IAA degradation. The proposed auxin degradation pathway requires several genes, including *iaaL* and *iaaB* genes. The *iaaL* gene encodes for a CoA-transferase that converts 3'-(2-aminophenyl)succinyl-CoA or 2'-(2-aminophenyl)succinyl-CoA in the anaerobic auxin degradation pathway (Schuhle et al., 2016). Note that the *iaaL* gene here is different from the *iaaL* gene from *Pseudomonas savcastanoi.* The *iaaB* gene encodes an indoleacetate-CoA ligase that can convert indole-3-acetic acid (IAA) to indoleacetyl-CoA (IAA-CoA) in an ATP-dependent process right after IAA goes into the cytoplasm. The CoA-ligase is very specific, as it can only convert IAA and some structurally-related molecules to CoA-thioester (Schuhle et al., 2016). IAA-CoA might be converted back to IAA through thioester hydrolysis, which could contribute to the IAA synthesis. IAA-CoA is a high energy compound and has the potential for synthesis of IAA conjugates with amino acids or sugar. Moreover, IAA-CoA may serve as a precursor for the biosynthesis of indole-butyric acid, which is a natural auxin and whose biosynthesis is still a mystery (Gao & Zhao, 2014; Woodward & Bartel, 2005). We are interested in using the bacterial *iaaB gene* to investigate whether IAA-CoA plays important roles in auxin homeostasis.

Arabidopsis thaliana is a good model to study auxin biology because its small genome has been sequenced and it has a rapid life cycle (Weigel, 2002). We want to use *iaaB* gene as a tool to elucidate the molecular mechanism of auxin metabolism in *Arabidopsis thaliana*. Specifically, we want to investigate the role of IAA-CoA in the auxin metabolic pathways. The

iaaB gene participates in the auxin degradation pathway in *A*. *aromaticum*, and it may affect the degradation of IAA in *Arabidopsis*, but the IAA-CoA generated by *iaaB* could also provide a source for IAA and IBA synthesis. Our main question is whether *iaaB* gene overexpression can affect auxin levels in *Arabidopsis*. Our hypothesis is that the *iaaB*-overexpression plants will have low level of auxin level because the CoA-ligase encoded by *iaaB* gene will convert IAA to IAA-CoA. Our alternative hypothesis is that the auxin level increases in *iaaB*-overexpression plants because IAA-CoA is hydrolyzed back to IAA or converted to IBA. To test our hypotheses, we used *Agrobacteria*-mediated transformation to overexpress *iaaB* gene in *Arabidopsis* under the control of *CaMV 35S* promoter and compared the phenotypes of *iaaB*-overexpression lines with wild-type ones.

Materials and Methods

Growth of Arabidopsis

The *Arabidopsis thaliana* ecotype Col-0 was used in the experiments. Seeds were sterilized in 800 µL 75% ethanol for 10 minutes followed by 1 mL 100% ethanol wash for 10 minutes, and then sown on ½ Murashige and Skoog (MS) plates. Seeds were stratified for 2 days at 4 ℃. Plates were then transferred to a growth chamber and plants were grown under long-day conditions (16 h light/8 h dark) at 22℃ for 5 days. Seedlings were then transplanted to soil and grew in the growth room under long-day conditions (16 h light/8 h dark) at 22℃.

Construction of vector

The pCHF3 plasmid was used to overexpress the *iaaB* gene. We have two versions of the construct—pCHF3 plasmid with mCherry at C terminal and at N terminal. The pCHF3 plasmid with mCherry allows mCherry fused to the N terminal or C terminal of *iaaB* (Fig. 3A) The pCHF3 plasmid was digested by kpnI restriction enzyme. For the pCHF3 plasmid without mCherry, the *iaaB* gene was amplified by PCR using primers with kpnI restriction sites at the 5' and 3' ends. For pCHF3 plasmid with mCherry, the *iaaB* gene was fused with mCherry fluorescent protein gene. Linearized pCHF3 plasmid (0.3 µL) and *iaaB* gene (0.7 µL) were assembled together using 3 µL 1.33 Gibson assembly mixture. Heat-shock method was used to transform the plasmid to E. coli. The ligated product was added to a tube with 50 µL E. coli DH-5α competent cells, and placed on ice for 20 min. The tube was transferred to a 42℃ water bath for 1.5 min, and then placed on ice for another 3 min. The product was added to 200 µL LB media and placed in a 37℃ incubator for 30 min. The bacteria were spread onto LB plates containing 50 µg/mL spectinomycin and incubated at 37℃ overnight (~15 hours).

Positive colonies were identified by PCR using the same primers for amplification of *iaaB* gene. Each positive colony was added to a test tube with 3 mL LB and 3 µL 50 mg/mL spectinomycin, and then inoculated on a 37℃ shaker overnight. The overnight culture was transferred to a microfuge tube and centrifuged, and the supernatant was removed. The pellet was resuspended using 200 µL P1 buffer (100 µg/mL RNase A, 50 mM Tris-Cl pH 8.0, 10 mM EDTA), followed by 200 µL P2 buffer (200 mM NaOH, 1% SDS). The tube was inverted gently and placed on ice for 2-5 min. 200 µL P3 buffer (3 M KAC) was added, and the tube was inverted gently and placed on ice for 10 min. After 10 min centrifuge, 550 µL supernatant was transferred to a new microfuge tube, and 550 µL isopropanol was added. The mixture was placed on ice for 10 min and centrifuged for 10 min. The supernatant was discarded, and 500 µL 75% ethanol was added and centrifuged for 2 min. The liquid was discarded, and the plasmid was dried. 30 µL DI water was added to dissolve the plasmid. 2 µL plasmid was used to run 1% agarose gel to check if the size of plasmid was correct. Sequencing was performed to confirm the sequence of the desired product.

Agrobacterium-mediated transformation using floral dipping

The pCHF3 plasmid with *iaaB* gene was transformed to Agrobacteria strain GV3101 by electroporation. The product was added to 200 µL LB media and incubated at 28℃ for 1 hour. The LB product was spread onto LB plate containing 50 µg/mL spectinomycin and incubated at 28℃ for 2-3 days. Each colony was added to a test tube with 3 mL LB and 3 µL 50 mg/mL spectinomycin, and then inoculated on a 28℃ shaker overnight. 100 µL Agrobacterium solution was transferred to a flask with 100 mL LB containing 50 µg/mL and placed on a 28℃ shaker overnight. The overnight culture was centrifuged, and the precipitate was resuspended in100 mL

3% sucrose MS solution with 30 µL Silwet L-77. Floral dipping method was used to transform the Col-0 *Arabidopsis*.

Selection of iaaB-overexpression (OX) Seeds

To select *iaaB-OX* seeds with mCherry fluorescent protein, T1 seeds were screened under fluorescent microscope with a mCherry filter (Fig. 3B). Seeds with red fluorescence were grown on ½ MS plate for a week and transplanted to soil. T2 seeds obtained from T1 plants were screened under fluorescent microscope with mCherry filter. Seeds with a 3:1 ratio of red to black seeds had desired single insertion. The T2 red seeds from these seeds were selected for later use (Fig. C).

Dark Treatment

The Col-0 and *iaaB*-OX seeds were sterilized and then sown on ½ MS plate. Seeds were stratified for 2 days at 4 ℃. After stratification, seeds were placed in white light for 2 hours, and then incubate in the dark for 3 days. The hypocotyl length was measured from the apical meristem to the beginning of the roots using ImageJ. A student's t-test was performed to determine if the hypocotyl length of *iaaB*-OX plants is significantly different from the one of Col-0.

Root Growth Analysis

The Col-0 and *iaaB*-OX seeds were sterilized and placed in a line on square ½ MS plates and stratified for 2 days at 4℃. The plates were placed vertically in the growth chamber. After 1 week, the root length of the seedlings was measured using ImageJ. A student's t-test was performed to determine if the root length of *iaaB*-OX plants is significantly different from the one of Col-0. The number of lateral roots were counted. A student's t-test was performed to determine if the number of lateral roots of *iaaB*-OX plants is significantly different from the one of Col-0.

GUS Staining

The *iaaB*-OX T2 plants were crossed with DR5::GUS plants. The F1 seeds were screened under fluorescence microscope with mCherry filter, and the red seeds were selected for GUS staining. F1 red seeds were incubated on ½ MS media in the growth chamber for 1 week with DR5::GUS seeds as control. The seedlings were placed in cold 90% acetone in glass scintillation vials on ice and then transferred to vacuum for 10 min at room temperature. The seedlings were removed from vacuum and placed in room temperature for 20-30 min. The acetone was removed and GUS staining buffer (0.2% Triton X, 50 mM PO_4 Buffer, 2 mM Potassium Ferrocyanide, 2 mM Potassium Ferricyanide, 2 mM X-Gluc) without X-Gluc was added. The seedlings were placed in vacuum for 10 min. The GUS staining buffer without X-Gluc was removed, and the staining buffer with X-Gluc was added to the vials on ice. The seedlings were infiltrated under vacuum for 15-20 min. The seedlings were placed in 37°C incubator overnight. The staining buffer was removed, and the seedlings were incubated successively in 20%, 35% and 50% ethanol at room temperature for 30 minutes each. Then the seedlings were incubated in FAA

(50% ethanol, 3.7% formaldehyde, 5% acetic acid) for 30 min at room temperature. FAA was removed, and 70% ethanol was added to store the seedlings.

Exogenous IAA treatment

The Col-0 and *iaaB*-OX seeds were sterilized and placed in a line on square ½ MS plates with 5 nM IAA and without IAA. Seeds were then stratified for 2 days at 4℃. The plates were placed vertically in the growth chamber. After 10 days, the root length of the seedlings was measured using ImageJ, and the number of lateral roots were counted.

Results

Constitutive overexpression of iaaB gene in Arabidopsis thaliana

In order to test our hypothesis, we overexpressed the *iaaB* gene in *Arabidopsis thaliana* using the *CaMV 35S* promoter. The *iaaB*-overexpression plants displayed distinct auxin-related phenotypes. The inflorescence length of *iaaB*-OX plants was much shorter than that of Col-0 (WT) (Fig. 4A). The rosette diameter of *iaaB*-OX plants was smaller than that of Col-0 (Fig. 4B). The leaves of the *iaaB*-OX lines were smaller and rolling down (Fig. 4C). The cotyledons of *iaaB*-OX seedlings were smaller than those of Col-0, and the hypocotyl of *iaaB*-OX seedlings was longer (Fig. 5A). *iaaB*-OX seedlings had fewer root hair than Col-0 (Fig. 5B).

The hypocotyls of iaaB-OX lines were short in dark

We analyzed three-day old dark-grown seedlings under microscope. The *iaaB*-OX plants showed short hypocotyl and lacked an apical hook (Fig. 6A). We then measured the hypocotyl length of *iaaB*-OX and Col-0 plants. The average hypocotyl length of 76 seedlings from *iaaB*-*iaaB*-OX was 0.634 ± 0.130 cm. The average hypocotyl length of 52 seedlings from Col-0 was 1.064 ± 0.163 cm. The student's t test result showed that the average hypocotyl length of *iaaB*-OX was significantly shorter than that of Col-0 (p-value < 0.05) (Fig. 6B).

iaaB-OX lines had root defects

After 1 week of growing on vertical MS plate in white light, the *iaaB*-OX plants had shorter primary roots and fewer lateral roots than Col-0, and they had reduced gravitropic responses (Fig. 7A). We measured the primary root length of 1-week old *iaaB*-OX and Col-0 plants. The average root length of 29 seedlings from *iaaB*-OX was 1.811 ± 0.437 cm. The

average root length of 29 seedlings from Col-0 was 2.243 ± 0.330 cm. The student's t test results showed that the average primary root length of *iaaB*-OX was significantly shorter than that of Col-0 (p-value < 0.05) (Fig. 7B).

We also counted the number of lateral roots of 10-day old *iaaB*-OX and Col-0 plants. The average number of lateral roots of 61 seedlings from *iaaB*-OX was 0.049 ± 0.384. The average number of lateral roots of 73 seedlings from Col-0 was 4.329 ± 3.245. The student's t test result showed that the average number of lateral roots of *iaaB*-OX was significantly smaller than that of Col-0 (p-value < 0.05) (Fig. 7C).

DR5-GUS expression pattern in iaaB-OX Arabidopsis

We crossed *iaaB*-OX lines (father plants) with DR5::GUS auxin reporter lines of Col-0 (mother plants) to generate *iaaB*-OX lines with DR5::GUS auxin reporter so that we can visualize the auxin response patterns in *iaaB*-OX seedlings. The DR5::GUS results showed that *iaaB*-OX seedlings had less DR5-GUS expression at the root tip and more DR5-GUS expression at the basal end of hypocotyl (Fig. 8).

Exogenous IAA application partially rescued the root defect in iaaB-OX lines

We noticed that the agravitropic root phenotype of *iaaB*-OX lines was partially rescued by exogenous IAA application after growing on vertical MS plate with 5 nM exogenous IAA in white light for one week (Fig. 9A and 9B). We measured the primary root length of 10-day old *iaaB*-OX and Col-0 plants. The average root length of 15 seedlings from *iaaB*-OX on ½ MS plate was 2.932 ± 0.689 cm. The average root length of 14 seedlings from *iaaB*-OX on ½ MS plate with 5 nM IAA was 3.858 ± 0.471 cm. The average root length of 13 seedlings from Col-0

on ½ MS plate was 3.410 ± 0.555 cm. The average root length of 13 seedlings from Col-0 on ½ MS plate with 5 nM IAA was 3.712 ± 0.596 cm. The student's t test result showed that the average primary root length of *iaaB*-OX was not significantly shorter than that of Col-0 (p-value > 0.05) (Fig. 9C). The defect in root length was rescued by application of exogenous IAA.

We also counted the number of lateral roots of 10-day old *iaaB*-OX and Col-0 plants. The average number of lateral roots of 15 seedlings from *iaaB*-OX on ½ MS plate and 14 seedlings from *iaaB*-OX on ½ MS plate with 5 nM IAA were both 0. The average number of lateral roots of 13 seedlings from Col-0 on ½ MS plate was 2.929 ± 1.385. The average number of lateral roots of 10 seedlings from Col-0 on ½ MS plate with 5 nM IAA was 3.600 ± 1.4302. The student's t test result showed that the average number of lateral roots of *iaaB*-OX on both MS plate with and without 5 nM IAA was significantly lower than that of Col-0 (p-value < 0.05) (Fig. 9D). Application of 5 nM exogenous IAA cannot recover the defect in lateral roots.

Discussion

Previous studies have suggested that IBA can be potentially converted to IAA-CoA and sequentially to IAA. IAA is also proposed to serve as a precursor for IBA synthesis, but the molecular mechanism of IBA biosynthesis in plants has remained unresolved (Woodward & Bartel, 2005). The indoleacetate-CoA ligase encoded by *iaaB* gene converts IAA to IAA-CoA in *Aromatoleum aromaticum*, and there is no evidence showing if plants have the *iaaB* gene. The protein BLAST result shows that *Arabidopsis* has similar CoA ligase, but the function is unclear.

We overexpressed *iaaB* gene in *Arabidopsis*, and *iaaB*-overexpression plants had distinct auxin-related phenotypes. Some of the phenotypes indicated that the auxin level in the *iaaB*-OX plants was higher than Col-0, including small and down-curled leaves, short primary roots, long hypocotyl when grown in white light and short hypocotyl when grown in darkness, and lack of apical hook when grown in darkness. These phenotypes were similar to those of yucca mutants (Y. Zhao, et al. , 2001) and *iaaM*-overexpression *Arabidopsis* (Romano CP, 1995) that had elevated level of free auxin. There could be several potential explanations to the elevation of auxin level in *iaaB*-OX lines. IAA-CoA has the potential to be hydrolyzed back to IAA through enzymatic reaction or spontaneous conversion, because IAA-CoA is an unstable and high-energy compound. IAA-CoA could also be converted to IAA-amino acid conjugates or IAA-ester conjugates to function as an auxin storage. IBA, another form of auxin, could also be a possible metabolite of IAA-CoA to induce the phenotypes of high-auxin level.

While the above phenotypes implied increased auxin level in the *iaaB*-OX plants, there were some phenotypes that could not be explained by auxin overproduction. The *iaaB*-OX lines had shorter petioles while yucca mutants have elongated petioles (Y. Zhao, et al. , 2001). Although yucca mutants had shorter roots as *iaaB*-OX lines, but they had more plentiful and

longer root hairs (Y. Zhao, et al. , 2001), which was in contrary to the extremely reduced amount of root hair in *iaaB*-OX lines. The yucca mutants were also reported to have increased apical dominance (Y. Zhao, et al. , 2001) while *iaaB*-OX lines had reduced apical dominance. The *iaaM*-overexpression *Arabidopsis* also showed increased apical dominance that was not shown in *iaaB*-overexpression plants (Romano CP, 1995). Phenotypes of low auxin level have been documented. The *iaaL* gene from *Pseudomonas savcastanoi* that encodes an IAA-lysine synthetase had been overexpressed in tobacco and *Arabidopsis*. *iaaL*-OX plants had phenotypes, such as smaller overall plant size and wrinkled leaves (Jensen, 1998; Romano C, 1991). The yucQ mutants that have low auxin level exhibited defect in primary root length and gravitropic response (Chen et al., 2014). Those phenotypes of *iaaB*-overexpression plants might support the hypothesis that overexpression of the *iaaB* gene reduced the auxin level in *Arabidopsis*, at least in the root tissue. We expected that the level of free IAA in *iaaB*-overexpression lines would be lower than that in Col-0, because the *iaaB* gene encodes a CoA ligase that catalyzes the conversion of free IAA to IAA-CoA, thus reducing the level of free IAA (Schuhle et al., 2016).

The different phenotypes of *iaaB*-OX plants led us to propose a cell or tissue specific mechanism of the sensitivity to *iaaB* overexpression in *Arabidopsis*. In order to visualize the auxin distribution pattern in the *iaaB*-OX plants, we used the auxin-responsive promoter DR5 fused with GUS to show. The GUS staining result showed that *iaaB*-OX lines had more DR5-GUS expression at the end of hypocotyl indicating that more auxin might be expressed in hypocotyl, which correlated to the phenotype of elongated hypocotyl. Extremely reduced DR5-GUS expression at the root tip suggested low auxin level at the root tip, which was consistent to the root defect phenotypes of *iaaB*-OX lines. The *iaaB* gene was presumably expressed through

the whole body of *Arabidopsis*, but the sensitivity to *iaaB* overexpression is dependent on the specific cell or tissue.

In order to determine if the root defects occurred because of auxin deficiency, we applied 5 nM exogenous IAA to *iaaB*-OX lines. The exogenous IAA application rescued the defect in primary root length and partially rescued agravitropism but not the defect in lateral roots initiation, root hair initiation and elongation. One explanation could be that the reason for the defects in lateral root initiation is not auxin deficiency and applying exogenous IAA would not facilitate the lateral root growth. IBA has crucial role in lateral root initiation, and it can serve as a precursor for the synthesis of IAA-CoA. IBA concentration in the roots of *iaaB*-OX plants might be reduced because it is possible that IBA is converted to IAA-CoA. Reduced level of IBA may cause the inhibition of lateral root growth. Another explanation could be that the concentration of exogenous IAA was not high enough to compensate the IAA depleted by CoA-ligase, and different concentrations of IAA could be used to test this explanation.

To understand the exact role of *iaaB* overexpression and IAA-CoA in *Arabidopsis*, several experiments need to be conducted. First, the overall and tissue-specific IAA level as well as its metabolites including IAA-CoA, IAA conjugates and IBA need to be measured to fully determine the auxin expression. In addition, a RT-PCR of *iaaB* gene would allow us to know the gene expression level in *iaaB*-OX plant compared to Col-0. To investigate if each different phenotype is caused by auxin reduction or elevation, the *iaaB*-OX lines could be crossed to auxin-overproduction and auxin-deficient lines. To confirm if the phenotype is due to auxin reduction, we could cross the *iaaB*-OX lines to auxin-overproduction lines including *iaaM*-OX and gain-of-function *YUC* overexpression mutants to see if the phenotype could be rescued. If F2 plants do not show a particular phenotype in *iaaB*-OX lines, it suggests that the phenotype is due

to auxin deficiency. On the other hand, to determine if the main cause of the phenotypes is the elevated auxin level, one possible way is to cross the *iaaB*-OX lines to auxin-deficient lines, such as *iaaL*-OX plants and loss-of-function *yuc* mutants. If some of the phenotypes in F2 plants are rescued, this indicates that the phenotypes are caused by auxin overproduction.

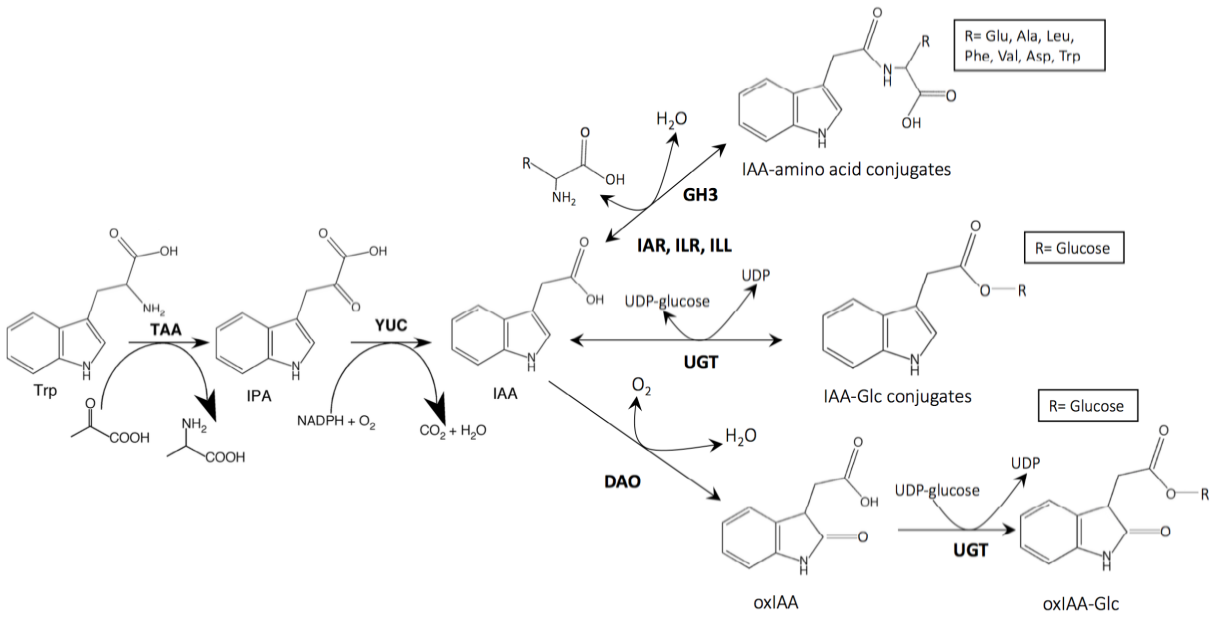

Figure 1. The primary auxin biosynthesis and metabolic pathway. IAA is synthesized via a simple two-step pathway. First, the amino group on Tryptophan is removed by TAA (Tryptophan Aminotransferase of *Arabidopsis*) to generate IPA (indol-3-pyruvate). Second, IPA is converted to IAA by YUC (YUCCA flavin monooxygenases). IAA can conjugate to amino acids by GH3 or to glucose by UGT. IAA-amino acid conjugates can be hydrolyzed to IAA by IAR, ILR or ILL. IAA-glucose conjugates can be converted back to IAA by UGT. IAA can also be degraded through oxidation by DAO to synthesize oxIAA and further conjugated to glucose to form oxIAA-Glc by UGT.

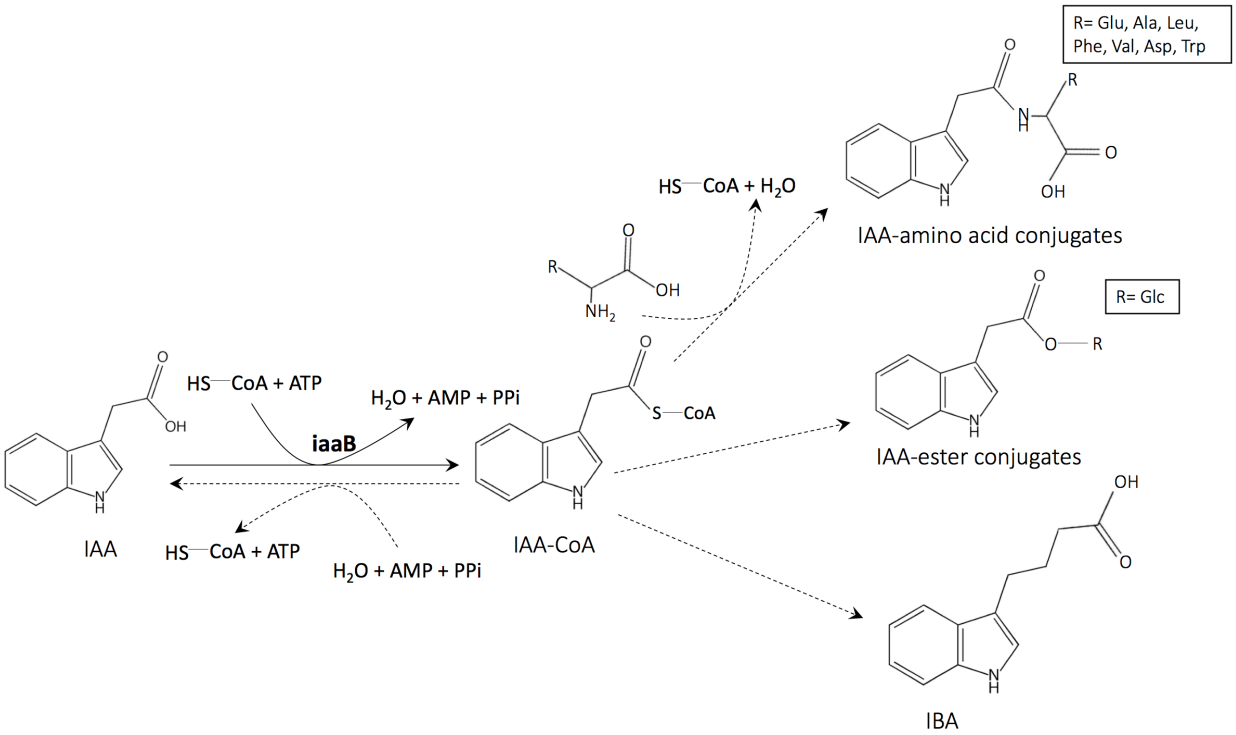

Figure 2. Hypothetical roles of IAA-CoA in Arabidopsis. IAA is converted to IAA-CoA by the Co-A ligase encoded by *iaaB* gene. IAA-CoA can potentially be converted back to IAA by hydrolysis, or converted to IAA-amino acid conjugates, IAA-ester conjugates, or IBA (indole-3-butryic acid). Solid lines indicate established reactions. Dash lines indicate hypothetical reactions.

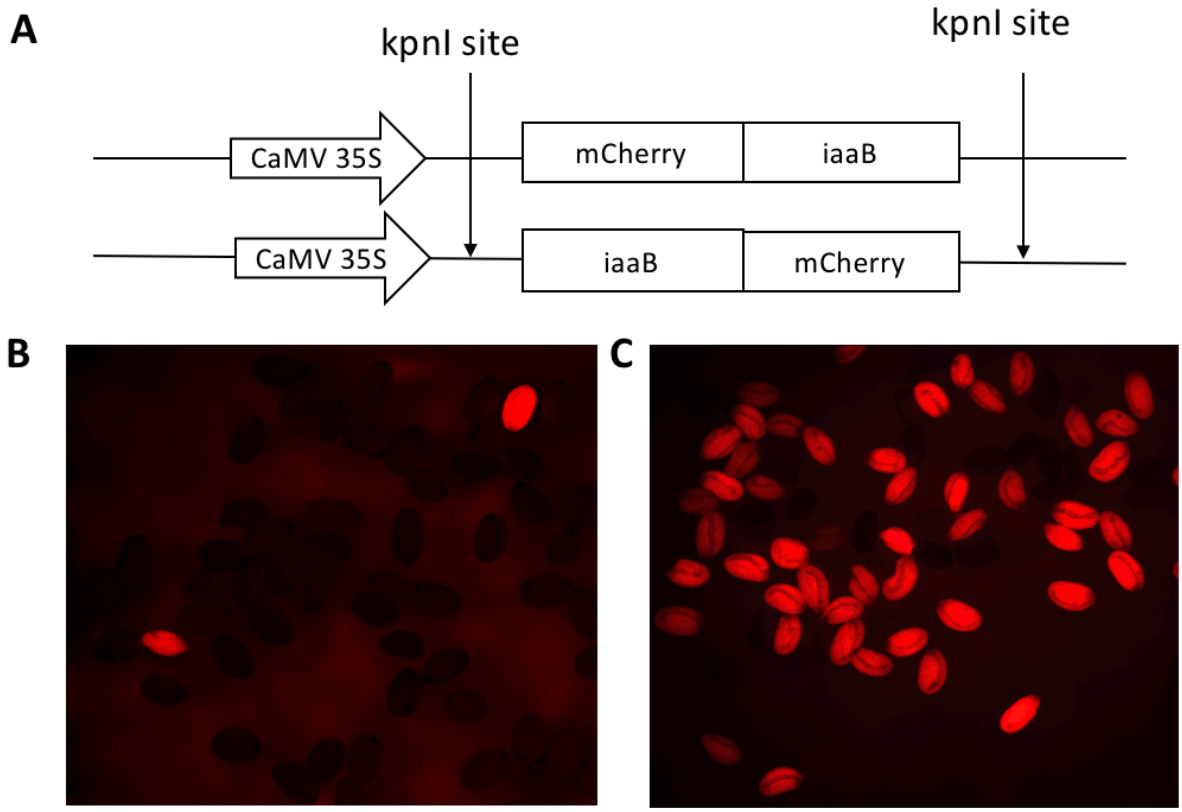

Figure 3. Vector constructs and seeds selection. A) Two versions of constructs: pCHF3 plasmid with iaaB fused with mCherry at N terminal or at C terminal. B) T1 red seeds were selected. C) T2 seeds with a 3:1 ratio of red to black seeds were selected.

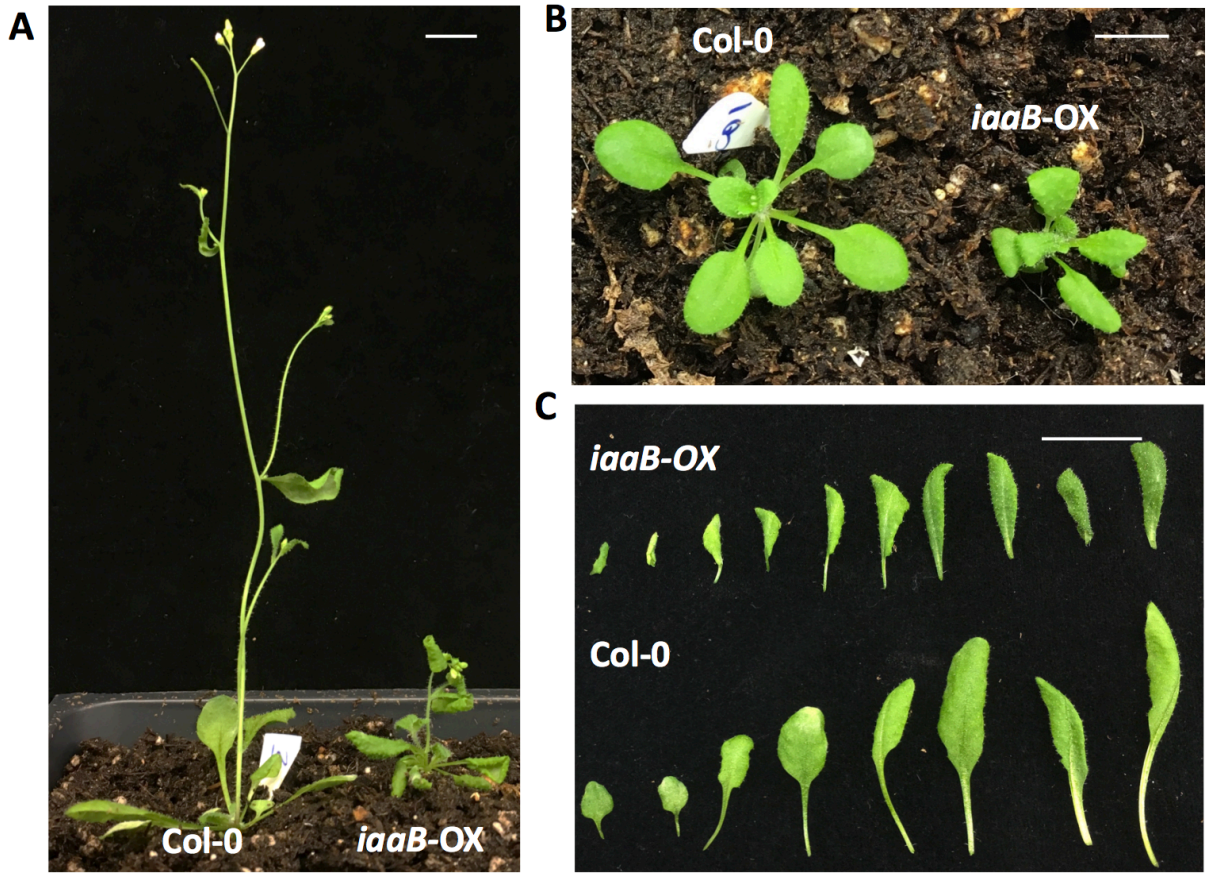

Figure 4. Overexpression of the *iaaB* gene affects the growth and development of *Arabidopsis.* A) 3-week old Col-0 (wild-type) and *iaaB*-OX (*iaaB*-overexpression) plants. *iaaB*-OX plants have shorter inflorescence length. B) 2-week old Col-0 and *iaaB*-OX plants. *iaaB*-OX plants have smaller rosette diameter. C) Rosette leaves of 3-week old Col-0 and *iaaB*-OX plants. The scale is 1 cm.

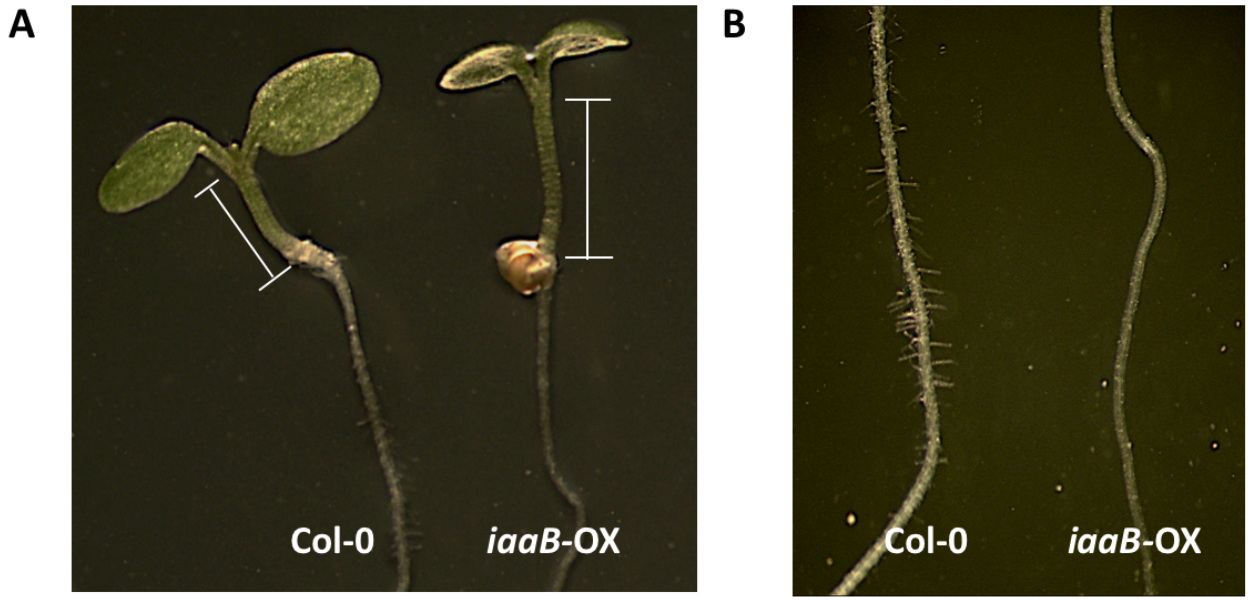

Figure 5. Overexpression of the *iaaB* gene affects the hypocotyl elongation and root hair initiation. A) Hypocotyls of 5-day old Col-0 (wild-type) and *iaaB*-OX (*iaaB*-overexpression) seedlings. B) Root hair of Col-0 and *iaaB*-OX seedlings.

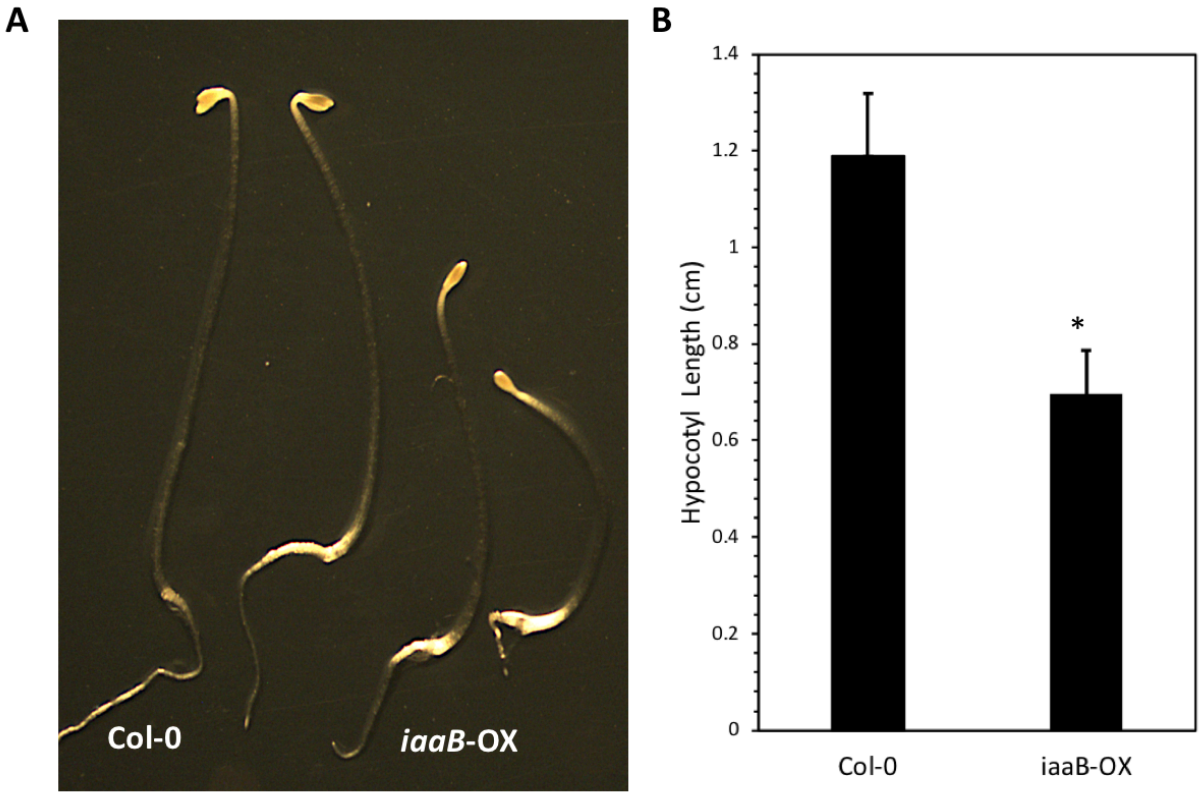

Figure 6. Overexpression of the *iaaB* gene inhibits hypocotyl elongation in darkness. A) 3-day old Col-0 (wild-type) and *iaaB*-OX (*iaaB*-overexpression) lines with dark treatment. B) The hypocotyl of seedlings *iaaB*-OX and Col-0 plants were measured after 3 days of dark treatment. *iaaB*-OX line had shorter hypocotyls. N = 52 for Col-0, N = 76 for *iaaB*-OX. *significantly different from Col-0, p-value < 0.05.

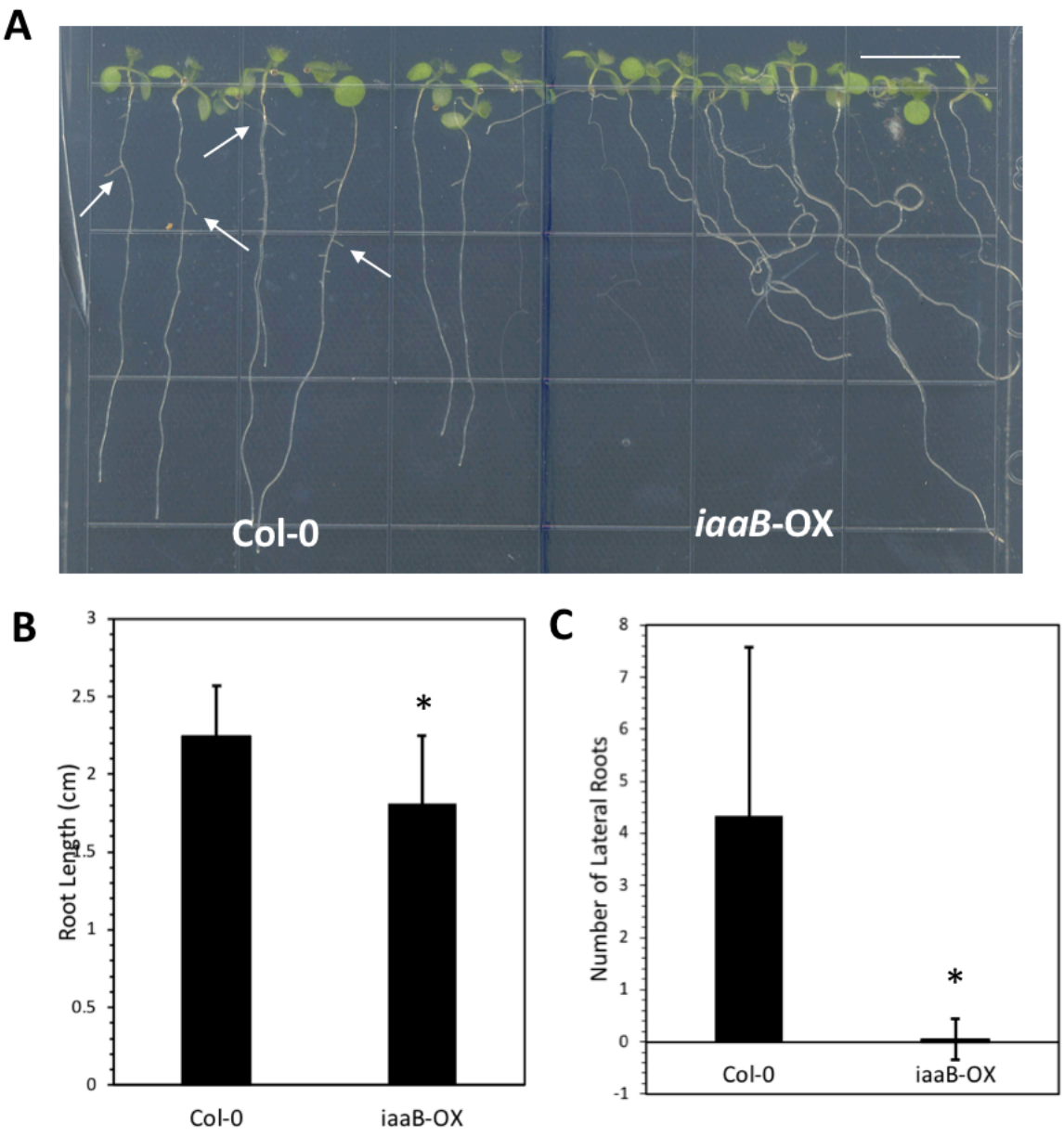

Figure 7. Overexpression of the *iaaB* gene leads to abnormal growth and development of roots. A) 10-day old Col-0 (wild-type) and *iaaB*-OX (iaaB-overexpression) plants. *iaaB*-OX plants have reduced gravitropism and less lateral roots. B) The roots of 1-week old seedlings from *iaaB*-OX and Col-0 plants were measured. *N = 29 for iaaB*-OX and Col-0. *significantly different from Col-0, p-value < 0.05. C) The number of lateral roots of 10-day old seedlings from *iaaB*-OX and Col-0 plants was counted. N = 73 for Col-0, N = 61 for *iaaB*-OX2. *significantly different from Col-0, p-value < 0.05.

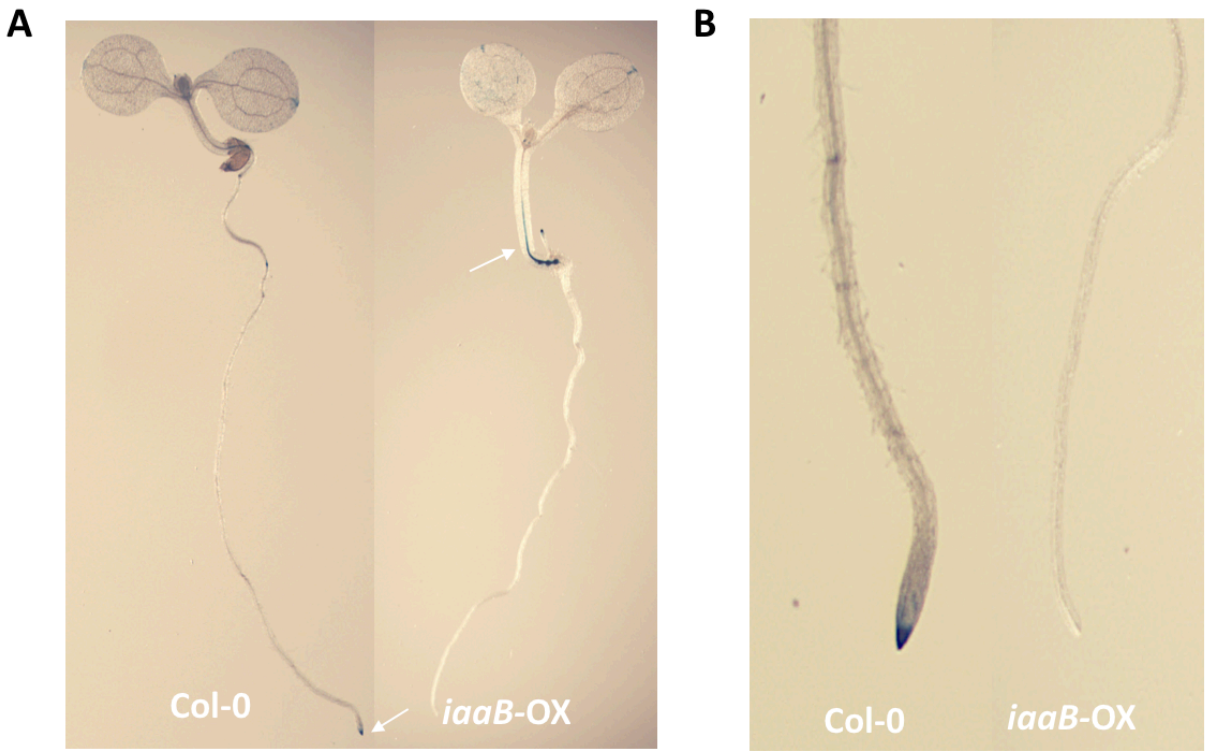

Figure 8. The *iaaB* gene overexpression alters the expression of the auxin reporter DR5:GUS. A) GUS staining of the DR5::GUS reporter lines of Col-0 (wild-type) and *iaaB*-OX (*iaaB*-overexpression) lines. *iaaB*-OX plants have less DR5-GUS expression at primary root tips, and more DR5-GUS expression at the end of hypocotyl. B) Root from the DR5::GUS reporter lines of Col-0 and *iaaB*-OX plants.

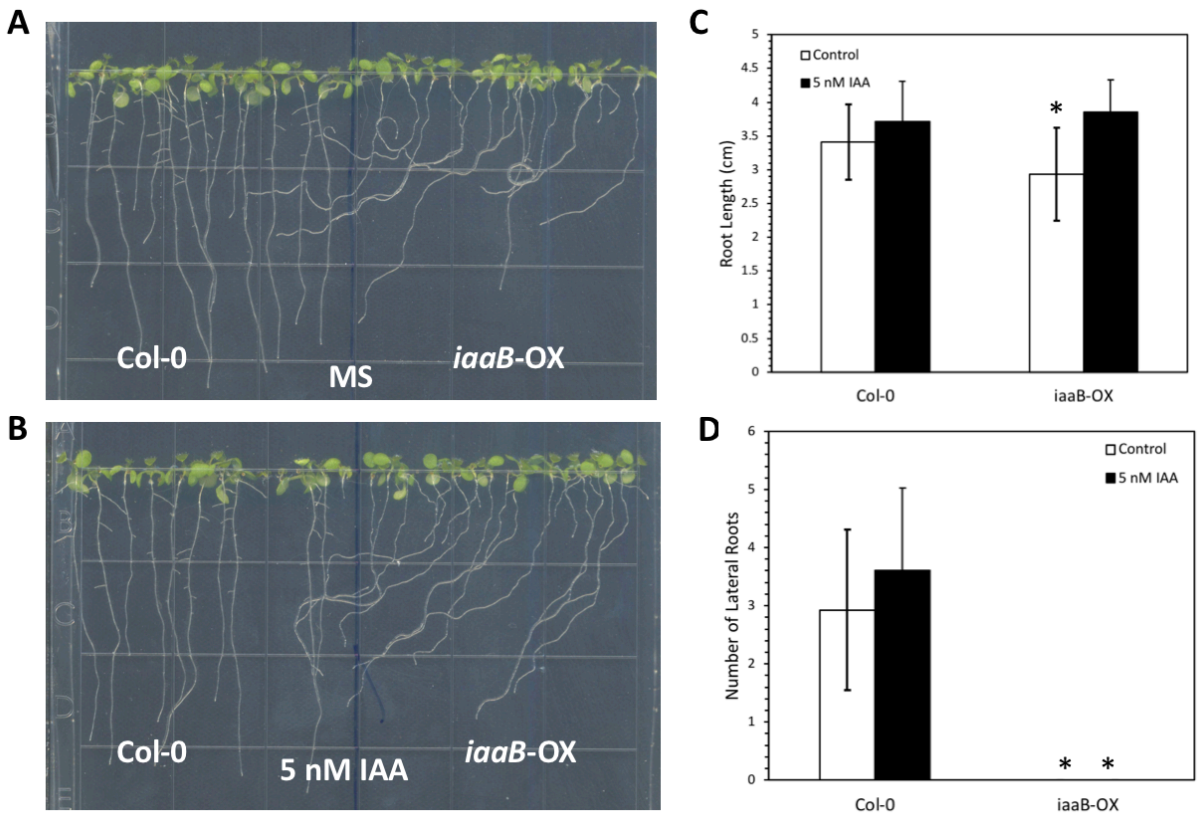

Figure 9. Exogenous IAA application partially rescues the root defects in *iaaB* overexpression plants. A) 10-day old Col-0 (wild-type) and *iaaB*-OX (iaaB-overexpression) plants on ½ MS plate. B) 10-day old Col-0 and *iaaB*-OX plants on ½ MS plate with 5 nM exogenous IAA application. B) Root length of 10-day old Col-0 and *iaaB*-OX plants. N=15 for *iaaB*-OX on MS plate, N = 14 for *iaaB*-OX on 5 nM IAA plate, N = 13 for Col-0 on MS plate, N = 10 for Col-0 on 5 nM IAA plate. C) Number of lateral roots of 10-day old Col-0 and *iaaB*-OX plants. N=15 for *iaaB*-OX on MS plate, N = 14 for *iaaB*-OX on 5 nM IAA plate, N = 13 for Col-0 on MS plate, N = 10 for Col-0 on 5 nM IAA plate. *significantly different from Col-0, p-value < 0.05.

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