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Understanding the basis of differential auxin response in selected plant species

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Abstract

Auxin plays a pivotal role in regulating root development and the molecular and cellular basis of this process has been well described in model plant *Arabidopsis thaliana*. However, our understanding of these mechanisms in other plant species remains limited. To explore the conservation of auxin responses across species, we treated three plant species - Brachypodium, rice, and foxtail millet - with varying concentrations of auxin and analysed their primary root growth response. Additionally, we conducted RNA sequencing to identify differentially expressed genes (DEGs) under one optimised auxin concentration. Comparative DEG analysis and promoter analysis revealed the conservation of the classic auxin signaling pathway while highlighting potential species-specific variations in auxin sensitivity and response.

Background

Food Security and Sustainable Agriculture:

Food security is a global challenge that threatens millions of lives. Sustainable agriculture aims to minimize environmental disruption while maintaining grain production to ensure food security (Rehman et al., 2022). Maintaining soil health and avoiding soil degradation are key challenges for improving agricultural production. The health and distribution of a plant's root system are critical for sustainable and successful agriculture. Roots help prevent soil erosion, maintain soil nutrients, and improve soil water retention, contributing to soil quality and preventing degradation (Vannoppen et al., 2017). Roots play a vital role in crop growth by absorbing nutrients and water from the soil to support the entire plant's growth and development. Genetic data of root traits are becoming popular while phenotypic data is gradually facilitating plant breeding. Many automated phenotyping platforms were developed to describe root phenotyping and provide direction for breeding strategies (Kuijken et al., 2015). Some root traits have been described and applied in crop breeding to improve soil nutrient acquisition to solve some key challenges like yield in infertile soil, and symbioses with bacteria or fungi (Lynch et al., 2012).

Primary root growth is a key trait in breeding and agriculture, referring to root lengthening along its longitudinal axis, which contributes to root form function and character. Auxin and auxin response pathway is necessary for these processes (Svolacchia et al., 2020). Auxin and synthetic auxin analogues like NAA and 2,4-D are widely used in agriculture. Auxin contributes to fruit setting rate, promoting seed germination and crop yield. The acid growth hypothesis explains how auxin stimulates cell expansion. Recent research has demonstrated that auxin can induce interactions between transmembrane kinase auxin-signaling proteins and plasma membrane H-ATPases, leading to their phosphorylation, stimulating cell-wall acidification, and cell elongation in Arabidopsis (Lin et al., 2021). Auxin also plays a key role in plant defense mechanisms, providing a pathway to increase crop yield and stress resistance while reducing reliance on traditional chemical interventions (Ali et al., 2024). As the primary step of agriculture, Auxin is widely used to promote seed rooting, root growth of plants when transplanting, and rooting of cuttings in agriculture.

The role of auxin in root growth:

Root development is a complicated biological process that contains cells arising near the quiescent center, following elongation and differentiation (Overvoorde et al., 2010). Plant growth, including root growth, is primarily regulated by the plant hormone auxin. Auxin, also known as indole-3-acetic acid (IAA), is the first plant hormone to be discovered and studied. It is widely distributed in almost all plant tissues, with higher concentrations typically found in areas of vigorous growth. While auxin generally promotes organism growth and development, high concentrations can inhibit root elongation (Woodward et al., 2005). Auxin is primarily synthesized in the apical meristem of the plant and then transported to various parts of the plant body. Generating and maintaining auxin gradients are crucial for root development. Young leaves and cotyledons can synthesize auxin, which can be facilitated by long-distance pathways towards the root tips (Overvoorde et al., 2010). Additionally, "auxin polar transport" is another mechanism that directs auxin distribution through active transport led by integral membrane transport proteins. Maintaining auxin gradients is essential for root pattern and structure, while cell fate and differentiation rely on cellular auxin gradients, contributing to root hair and lateral root formation (Jones et al., 2009).

Auxin biosynthesis processes have also been found in roots, with some auxin synthesis genes expressed in the root, helping to maintain auxin gradients for normal root development (Ljung et al., 2005). Auxin underpins both primary root and lateral root development which contributes to establishing the root apical meristem (Roychoudhry et al., 2022). Primary root development relies on auxin response. Many evidences show that auxin signaling is the primary regulator of lateral root forming and contributes to its initiation and primordium development (Fukaki et al., 2009). Above all, auxin synthesized, transported, and maintaining gradients play important roles in root development, including primary root growth and lateral root forming. Auxin normally controls root development through the auxin signaling pathway.

Auxin signaling pathway

Genes typically respond to auxin through the TIR/AFBs-AUX/IAA-ARF signal pathway in roots. Auxin influences gene transcription by binding to and changing the conformations of TIR1/AFB receptors, promoting their interaction with AUX/IAA proteins. These proteins form heterodimers with ARFs to inactivate them in the absence of auxin. In the presence of auxin, the interaction of TIR1/AFB receptors and AUX/IAA triggers ubiquitination of AUX/IAA

followed by their proteasomal degradation. This frees ARFs from their co-repressors, allowing them to activate or repress transcription of their target genes (Roychoudhry et al., 2022). TIR1/AFB proteins family including TIR1 and five AFB members. Three pairs of paralogs in the Arabidopsis thaliana genome encode them and an amino-terminal F-Box followed by eighteen leucine-rich repeats (LRRs) is their character. TIR/AFB proteins cooperate with AUX/IAA to perceive auxin signal and mediate multiple auxin responses. AFB1 is crucial in inhibition of root growth and the early phase of root gravitropism which depend on auxin (Prigge, et al., 2020). AUX/IAA proteins are short-live nuclear proteins that repress ARF activate genes expression levels. AUX/IAA functions exclusively decide auxin-mediated transcriptional regulation and different auxin-sensing effects usually depend on different distinct auxin-binding affinities based on different TIR1/AFB-Aux/IAA protein combinations (Luo at al., 2018). AUX/IAA proteins and ARF proteins contribute to root development and are involved in lateral root formation.

ARFs are plant-specific transcript factors (TFs) which is conserved among almost all plants. Most of the ARFs shared a C-terminal Phox and Bem1p (PB1) domain, Middle Region (MR) domain, and an N-terminal DNA-binding domain (DBD). ARF-DBDs and their DNA binding properties decide which gene are regulated by auxin and this interaction occurs via the B3 subdomain (Cancé et al., 2022). ARFs bind to specific sites called AuxREs (Auxin Response Elements) in target promoters, with the TGTCNN consensus core sequences (most frequently TGTCTC and recently TGTCGG). This regulation of auxin response genes promotes plant growth. Loss of function in ARF proteins can lead to the inability to form lateral roots. For example, the double arf7/arf19 mutant may delay or even prevent lateral root formation and exhibit abnormal gravitropism in the root (Okushima et al., 2005). Exogenous auxin can partially restore low numbers of lateral root formation in these double mutants (Wilmoth et al., 2005). While ARFs can act as activators, they can also be repressors. For instance, arf10/arf16 double knockdown mutants show increased lateral root production, uncontrolled cell division, and tumor-like root apex (Wang et al., 2005). Previous research already found that ARF family can be classified in multiple clade in evolution which belong to several branches in phylogenetics tree. Similar clade usually have similar response and mechanisms to auxin, and ARF proteins in the same clade of ARF normally have similar

binding preference (Galli et al., 2018;Wang et al., 2007; Song et al., 2023). Different species treated by auxin may interfere ARF genes expression and different species from the same genus but different species have different sensitivity to auxin (Cancino-García et al., 2020).

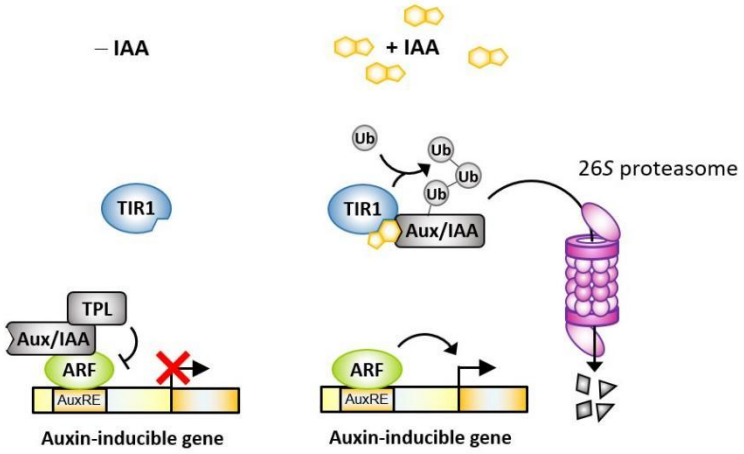


Figure1 TIR/AFBs-AUX/IAA-ARF signal pathway. TIR1/AFB receptor receive auxin signal, lead AUX/IAA degradation, then free ARF to regulate target genes

Background for plant species used in this work:

A. **Brachypodium (*Brachypodium distachyon*)** is a new model plant for genetics analysis. It has a small genome, similar in size to Arabidopsis, making it easier to study and compare with Arabidopsis. Phylogenetically, Brachypodium is closer to important cereals such as wheat and barley. Some crops are difficult to study due to their growing environment or genome. Therefore, Brachypodium was chosen for its phylogenetic position and characteristics as a model system (Kellogg et al., 2015). Studying the auxin response pathway in Brachypodium can provide insights into these mechanisms in other cereals. Although the ARF gene family, including their structural features and expression profiles, have been studied in Brachypodium, the details of the auxin signaling pathway and auxin response mechanisms remain unclear (Liu et al., 2018).

B. **Foxtail millet (*Setaria italica*)** is an early domesticated crop and a new model for C4 plant species. It is a valuable model plant due to its small and diploid genome, making it more tractable and conserved to the ancestor grass lineage. Foxtail millet also has

similar C4 photosynthesis to other C4 plants, which is useful for understanding this important trait. Exogenous auxin has been shown to improve photosynthesis efficiency and photochemical utilization, leading to increased grain yield in foxtail millet (Feng et al., 2023). Discovering the auxin response pathway in foxtail millet can help us understand how auxin works in C4 plants and apply this knowledge to agricultural production.

C. **Rice (*Oryza sativa ssp. Kitaake*)** is a widely used crop model plant cultivated worldwide. Plant hormones have been used for many years to increase yield and stress resistance. Auxin has been proven to play a key role in promoting root foraging in rice, enhancing its ability to acquire nutrients (Giri et al., 2018). Therefore, exploring the details of auxin response in rice roots is significant. We selected these three species as auxin treatment groups to represent the diversity of crops and key crops in agricultural production. We also added the MSU7 (Oryza sativa subsp. Japonica) genome as reference data in our experiment because Phytozome attempted to remove genes identified as likely Transposable elements (TE), which is crucial for proper gene family construction. Additionally, MSU7 has more annotation information after refiltering, resulting in well-annotated gene models (Ouyang et al., 2007).

Rationale, Aims and objectives

Auxin controls almost all aspects of root development by generating and maintaining appropriate concentration gradients. Auxin metabolism, transport, signaling pathways, and the molecular mechanisms controlling auxin-mediated root growth have been extensively studied in the model plant Arabidopsis thaliana. Arabidopsis is commonly used to characterize important mechanisms like the acid growth hypothesis (Lin et al., 2021). And auxin signaling pathway and components, including the function and structure, have been well described through Arabidopsis thaliana in many aspects. While previous research has been applied to crop production, this knowledge is limited for many plant species, especially some important crops. It is unclear whether these mechanisms are conserved across plant species or if there are species-specific differences. This needs to be investigated, especially

considering that previous genome-wide identification studies of auxin signaling components revealed gene family expansions and diversifications (Overvoorde et al., 2010).

A. Auxin response among different species

Our aim is to study how roots respond to auxin treatment and which genes are regulated by auxin in different species and the genes shared by them. We will also investigate the auxin regulation network and auxin response elements in response to auxin, particularly focusing on similarities and differences among multiple species.

B. Compare different auxin response

We will conduct different auxin treatments on Brachypodium, Rice, and Foxtail millet and perform RNA sequencing to identify differentially expressed genes under auxin treatment. The functions and phylogenetics relationship of these genes will be studied. Then we will do research of the promoters of up regulated genes, especially the motifs in promoters to understand how different plants respond to auxin and which motifs mainly contribute to auxin response.

Method and Materials

Sample collection and RNA-sequence

We selected three species, Brachypodium, Foxtail millet, and Rice (*Oryza sativa Kitaake*), and did surface sterilization for all three species seeds for agar cultivation. We have transferred cultivated seeds to a treated media because they were cultivated on a non-treated media. After 48h chilling for Brachypodium and 48h pre-germinate on filter paper for foxtail millet and rice, we planted them on 1/2 MS. Waiting for 3-4 days growing, we transferred them to auxin treatment media as five groups (0uM NAA, 0.05uM NAA, 0.1uM NAA, 0.5uM NAA, 1uM NAA). All media were previously supplemented with auxin at designed concentrations. We took pictures 24h after auxin treatment for all five groups of all three species. We measured the root tip position before auxin treatments and current root tip position when taking pictures to compare root growth change as phenotype analysis. Based on the phenotype analysis, we find 0.1 uM NAA treatment group is the most effective group, and higher than 0.5uM NAA treatment will almost completely suppress root growth. So, we selected 0.1uM NAA treatment groups to do RNA-sequence three hours later after auxin

treatment. We used root tip (around 6-7 millimeter) as RNA-seq samples and they were sent to sequencing company to purify and sequence after collection. Given absorbing external auxin abilities of different species, we did another experiment that we tried IAA, 2,4-D, and NAA treatment (mock, 0.1uM, 0.5uM) for all three species to see if the plants are not sensitive to auxin or hard to absorb external auxin.

DEGs analysis

RNA-sequencing is widely used to study the transcriptome of a genome to characterize and compare gene expression profiles of biological samples. Generated dataset can be analyzed for differentially expressed genes (DEG), which indicate quantitative change in expression levels between experimental groups. According to RNA-sequence data, we got fpkm (Fragments Per Kilobase Million) data for the whole genome of all samples, and both groups (auxin treatment groups and control groups) have four samples. Foldchange was calculated by fpkm data of two groups for each species. Then we defined a significantly differentially expressed gene (DEG) as one which was significantly differentially expressed compared to control at an adjusted p-value <0.05 level and foldchange>1.5 (log2 foldchange>0.5849) as up regulated and foldchange<-1.5 (log2 foldchange<-0.5849) as down regulated after auxin treatment. And we based on the Ensembl plants database (*Brachypodium, Setaria italica*) and the Phytozome database (*Oryza sativa Kitaake)* to collect the transcript sequence of all differentially expressed genes to do the phylogenomics analysis. The data set we referred were Brachypodium_distachyon_v3.0 from ensembl plants, Setaria_italica_v2.0 from Ensembl plants, Oryza sativa Kitaake v3.1 from Phytozome.

Phylogenetic analysis

We used three DEGs list files to identify orthologous relationships among all three species and other model plants (*Arabidopsis thaliana* and *Oryza sativa subsp. Japonica*). We created a BLAST database for reference and selected the longest sequence for each gene if they have multiple transcripts. For each species, we input transcript sequence files which were differentially expressed and used BLAST to compare to *Brachypodium distachyon, Setaria italica, Oryza sativa Kitaake, Oryza sativa subsp. Japonica, and Arabidopsis thaliana.* Because kitaake has the largest number, we mapped Brachypodium and foxtail millet to rice

and searched the genes which shared by all three species. Based on results, we also mapped three experiment species genome to MSU7 ID for following research.

GO enrichment analysis

GO enrichment is widely used to search and summarize which genes participate in the same pathway together in the input gene list. There are multiple GO enrichment tools and we tried goseq and PLAZA to do this. We chose to use PLAZA Monocots 5.0 to do GO enrichment for all genes which differentially expressed. For each species, we separated three groups as all regulated genes, up regulated genes, and down regulated genes, and genes of each groups were submitted respectively. Because Monocots cannot identify some genes ID, we mapped Brachypodium and foxtail millet to new ID which can be identified by it. For Brachypodium, we downloaded transcript sequences from Ensembl plants and mapped to Brachypodium distachyon v3.2 through Phytozome BLAST. For foxtail millet, we downloaded transcript sequences from Ensembl plants and mapped them to Setaria italica v2.2 through Phytozome BLAST. According to phylogenomics analysis results, we mapped all genes in list to MSU7 ID and used MSU7 genes which are orthologous to these genes of each three species to do GO enrichment. For each group, we summarized GO enrichment analysis results of three species and used -log10(P-value) as enrichment significance level to create table and heat-map through R. Then determined common and species-specific processes and their relevance with the observed physiological responses according to the heat-map.

Auxin signaling components expression

Auxin normally regulate genes expression through TIR/AFB-AUX/IAA-ARF pathway. Based on previous research, we collected the genes which contribute to auxin response pathway which include TIR/AFB proteins, AUX/IAA proteins, and ARF proteins of three species (Prigge et al., 2020). We searched and collected the expression level (fpkm) of all genes in the lists in RNA-sequencing results for each species. Then we downloaded sequences of these genes for each species, and they were mapped to MSU7 by Phytozome BLAST separately. We also referred the phylogenetics results to construct the expression level table. MSU7 IDs were used as label to create three bar charts to compared expression level and degree of expression change for each species by R. A few genes in the auxin signaling components expression data cannot be found in our RNA-sequence results and

they were deleted or not shown in the chart. Some genes of Brachpodium and foxtail millet were mapped to one same MSU7 gene, and we set two same MSU7 label and remarks original ID for distinction.

Auxin response elements search

In general, a motif is a short DNA sequence involved in a specific biological function, for example, the sequence that tends to bind by a specific factor can be defined as a transcription factor binding site. Find Individual Motif Occurrences (FIMO) is an online tool used to scan DNA sequences with motifs described as position-specific scoring matrices (Grant,C.E., 2011). PlantTFDB is a database that provides resources on plant transcript factors and the TF binding motifs that they interact with (Jin, J., 2016). We collected 1.5kb, 2kb, and 2.5kb promoter sequences of upregulated genes of the three species from the Ensembl Plants BioMart (Brachypodium, Setaria italica) and the Phytozome BioMart (Oryza sativa Kitaake). Promoter sequences are the upstream flank of the coding region (gene). Motif matrices files of all three species were respectively downloaded from PlantRegmap (PlantTFDB) and Jasper was used as a reference. We used MEME FIMO to predict all possible transcript binding sites in the promoter sequences and calculated the scores and P-values for each possible binding site. Then we referred to motif IDs that bind to ARF specifically through PlantTFDB to collect all significant ARF binding sites in promoter sequences. We then calculated the proportion of promoters containing ARF binding sites for three promoter lengths of the three species.

Auxin response elements enrichment

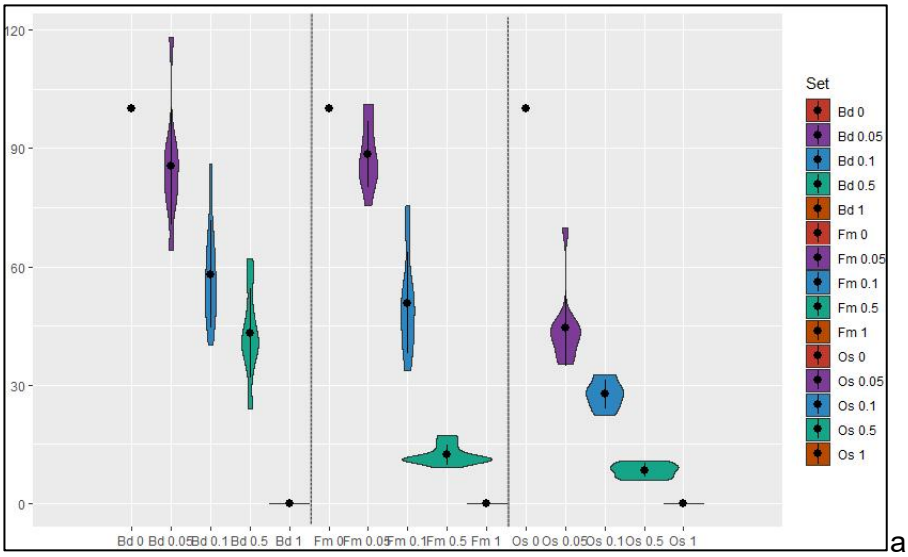
To discover the set of genes controlled by ARF, we applied Motif Enrichment Analysis (MEA) through MEME AME to detect binding motif enrichment (McLeay, R.C., 2010). We used the known DNA-binding models for ARFs through PlantTFDB to determine if ARF may be direct regulators of the differentially expressed genes and whether any other factors may regulate these genes directly. MEME AME was used to find which motifs are significantly enriched in the promoter sequences. It requires control group and test group to identify known motifs are relatively enriched in the test sequences compare with control sequences. We used the promoter sequences of the whole genome as control groups when we input the upregulated genes' promoter sequences as test groups. For each species, we

tested three promoter sequences of different lengths, and the lengths of the test groups and control groups were the same when we input (1.5kb, 2kb, 2.5kb).

Result

Phenotype and DEGs identification

FPKM method was used to calculate the expression level change between auxin treatment groups and control groups (zhao et al., 2021). We collected and calculated transcription data, there are 27713 genes in Brachypodium, 28759 genes in Foxtail millet, 30793 genes in Rice. Some genes may be silence that we didn't collect their transcription data. According Transcriptome data, we found 121 genes differentially expressed in the Brachypodium, 19 genes up regulated and 102 genes down regulated; 865 genes differentially expressed in the Foxtail millet, 513 genes up regulated and 352 genes down regulated; 7137 genes differentially expressed in the Rice, 3054 genes up regulated and 4083 genes down regulated. DEGs numbers and percentage in three species corresponding to the phenotype that under NAA, IAA, and 2,4-D treatments, Brachypodium show minimal change under all three treatments, rice is the most significant, and foxtail millet in the middle. And this phenotype show in all concentration of three kinds of auxin treatment. Compared with current versions of the genomes in phytozome and ensemble datasets, some genes cannot be identified and they were deleted in phylogenetics, GO enrichment and promoter analysis. Applied phylogenetics analysis, for up regulated genes, we found 4 genes shared by Brachypodium and rice, 1 gene shared by Brachypodium and foxtail millet, 169 genes shared by rice and foxtail millet, 3 genes shared by three species. For down regulated genes, we found we found 22 genes shared by Brachypodium and rice, 3 genes shared by Brachypodium and foxtail millet, 188 genes shared by rice and foxtail millet, none shared by three species. For 169 genes shared by rice up regulated genes list and foxtail millet up regulated genes list, we applied GO enrichment analysis separately.



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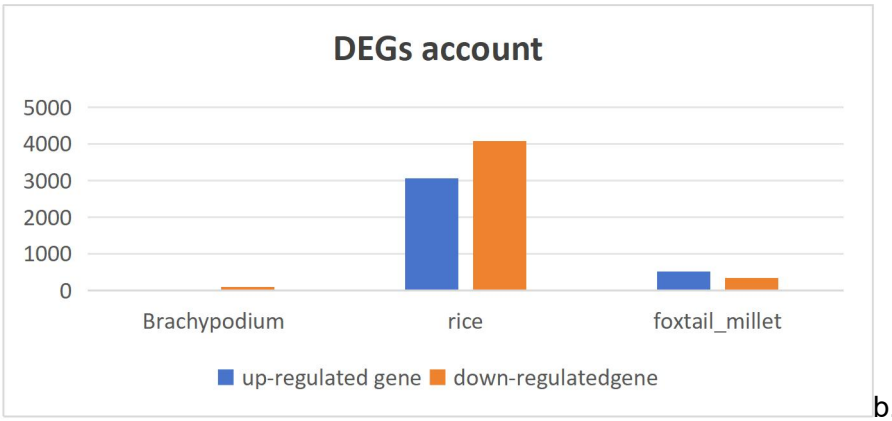

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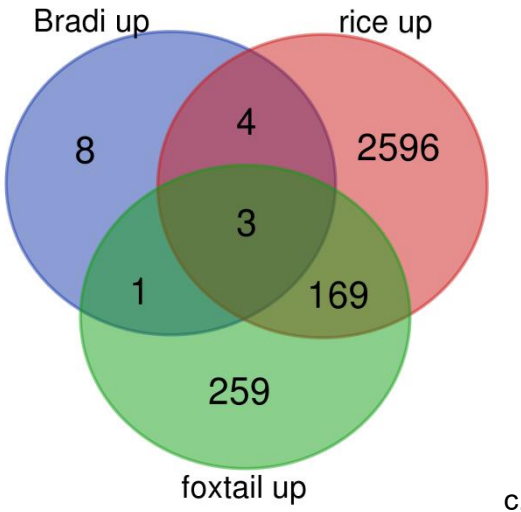

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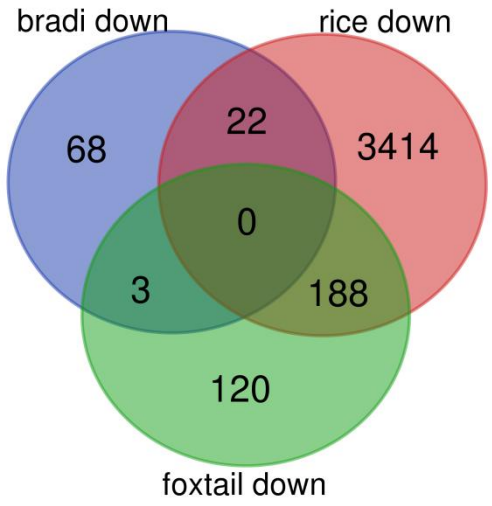

d.

Figure2 2a, root growth change under NAA treatment; 2b, DEG number in three species; 2c, phylogenomics analysis for up regulated genes; 2d, phylogenomics analysis for down regulated genes

GO enrichment analysis

To characterized auxin response coordination mechanisms more precisely, we tried two methods to do GO enrichment, one is using species original species genes ID, and the other one is using MSU7 genes ID which orthologs to these genes of three species. There no significant enrichment results for Brachypodium up regulated genes. For the other two species, the “response to auxin” GO term is the most common one which can be found in rice up regulated genes and foxtail millet up regulated genes (both original ID input and MSU7 ID input). “auxin homeostasis” GO term was enriched in foxtail millet up regulated genes and “auxin-activated signaling pathway” GO term was found in both foxtail millet and rice up regulated genes basing on MSU7 ID. “response to karrikin”, “response to chemical”, and “response to hormone” GO term were also found in the results. We also did GO enrichment for genes shared by foxtail millet and rice, and we tried three different gene IDs for this list (foxtail millet, rice, and MSU7). “response to auxin” GO term was found in all three results, “auxin homeostasis” GO term and “auxin-activated signaling pathway” GO term also can be found. “cell growth”, “root development” and some other related GO terms were found when we applied GO enrichment for down regulated genes for three species.

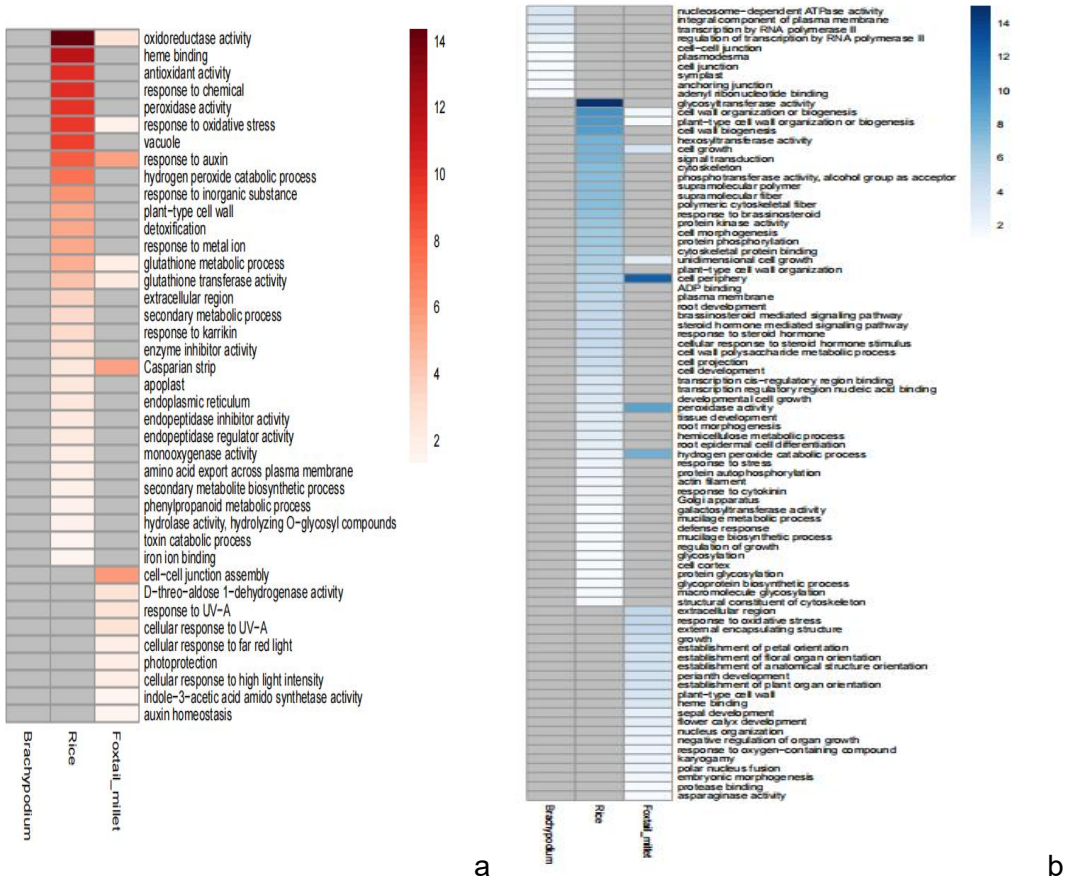

Figure3 a.GO enrichment for up regulated genes; b.GO enrichment for down regulated genes

Auxin signaling components expression

There are 6 TIR-AFB proteins in rice and 4 down regulated, Brachypodium and foxtail millet have 5 TIR-AFB proteins and none of them have significant expression change. There are 28 AUX/IAA proteins in rice, and 13 genes up regulated. There are 26 IAAs genes in foxtail millet, and 12 genes up regulated. There are 24 ARF genes in rice and foxtail millet, 5 ARFs genes in rice down regulated and 4 up regulated, and only one ARF genes in foxtail millet up regulated and also up regulated in rice. Compared to OsARF accession number from Rice Genome Annotation Project (RGAP), OsARF23, OsARF5, OsARF11, OsARF14 were identified as up regulated in rice, and SETIT_025988mg (Seita.8G135700) which orthologs to OsARF23 in MSU7 and orthologs to ARF2 in Arabidopsis up regulated in foxtail millet. Meanwhile, OsARF7, OsARF17, OsARF18, OsARF22, OsARF8 were identified as down regulated in rice.

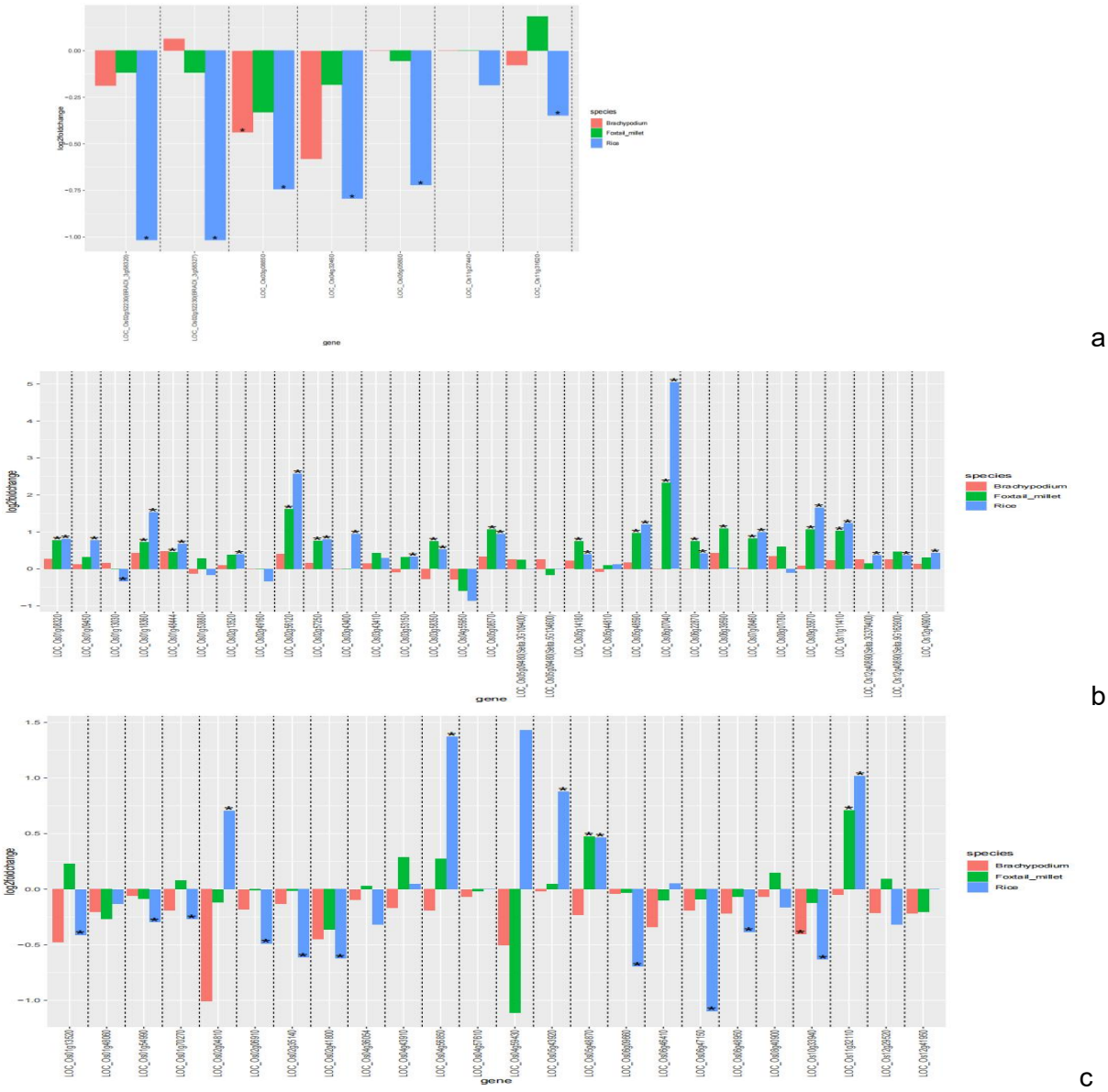

Figure4 foldchange for auxin signaling components, significant one marked as star;

a.TIR/AFB; b.AUX/IAA; c.ARF

DEGs promoters analysis

In promoter analysis, we tried three different lengths of upstream sequences as promoters for all up regulated genes for each species (1.5kb, 2kb, 2.5kb). We used MEME FIMO to predict any possible transcript factor binding sites. And we referred to PlantRegmap data to select ARF binding sites and calculated proportion of promoters containing ARF binding sites. For 1.5kb promoters, 69% of promoters in rice and foxtail millet have ARF binding sites. For 2kb promoters, 79% of promoters in rice and 78% of promoters in foxtail

millet have ARF binding sites. For 2.5kb promoter, 86% of promoters in rice and 84% of promoters in foxtail millet have ARF binding sites. For 19 Brachypodium up regulated genes, 1.5kb promoters have 16 with ARF binding sites, 2kb and 2.5kb have 17 with ARF binding sites. To know which motifs may play important roles in these promoters and which genes may be regulated by auxin, we applied motif enrichment. We used promoters of whole genome as control group to searched the motif which is significantly enriched in the promoters of provided genes list. MP00097 is ARF binding family which was significantly enriched in 1.5kb promoter of foxtail millet, and MP00059 (NAC) also shown in 1.5kb and 2kb promoters of foxtail millet. The AME results show that 26.2% 1.5kb promoters of up regulated genes have ARF binding sites and 17.5% promoters of whole genome have. SBP, ERF, and bHLH were found in the 2.5kb promoter of foxtail millet. B3 and C2H2 were found in 1.5kb and 2kb promoters of rice. There are no significant enrichment results were found for the 2.5kb promoter of rice and it could because too much background noise. And there are no significant enrichment results for Brachypodium for three different promoters. Then, as comparing, we also did motif enrichment for down regulated genes' promoters for three species. There are no ARF binding motif were found in the results.

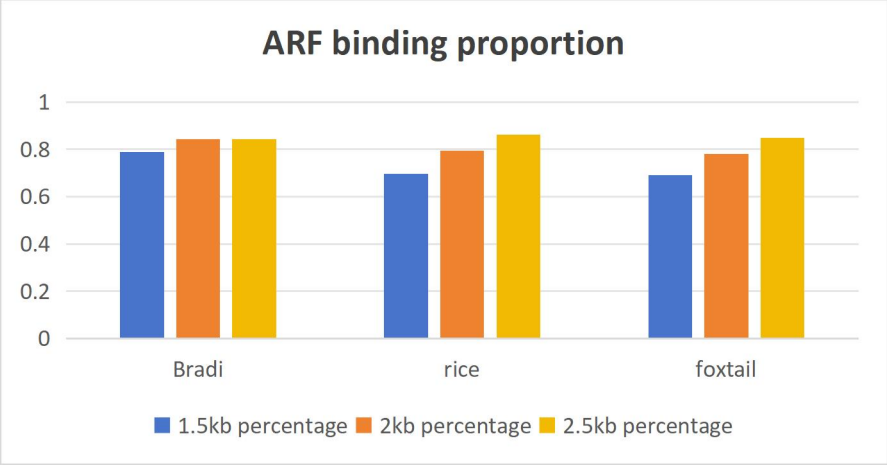

Figure5 the proportion of promoters with ARF binding sites

Conclusion

Auxin response genes show phylogenetics similarity among different species

According to phylogenetics analysis results and GO enrichment analysis, the genes response to auxin show similar sequences and functions among different species. Nearly half

up regulated genes in each rice are orthologs to foxtail and Brachypodium also shared some up regulated genes with the other two species. GO enrichment results show that both foxtail millet and rice up regulated genes shared some GO terms and were mainly related to auxin or hormone response. Down regulated genes also show that phenomena. We also did BLAST for auxin signaling components among three species and MSU7, it shows that even in different species, same auxin signaling components still maintain conserved sequence and functional subdomain. The genes in different species may have conserved sequences or similar functions which contribute to similar auxin responses among different species. We also found some genes like AUX/IAA proteins and ARF proteins show similar expression methods in foxtail millet and rice. Above all, different species show conserved auxin signaling components and classic TIR1/AFB-AUX/IAA-ARF response pathways.

Auxin signaling pathways show different responses in different species

Brachypodium was not sensitive to the concentration of auxin used at the time-point samples were collected and only a few genes differentially expressed and there are no signaling pathway components in the list. Rice is the most sensitive and almost 10% of genes in the whole genome up regulated. Foxtail millet is the medium. To identify if the differences are auxin response or external auxin absorb, we tried NAA, IAA ,and 2,4-D treatments for three species and all groups show the same phenotype result. Under same auxin concentration treatment, Brachypodium root has the least change, rice changes most, and foxtail millet is medium. For all differentially expressed genes, We collected species specific genes and GO terms. There are no GO terms shared by Brachypodium and the other two species. On the one hand, Brachypodium has low number differentially expression genes which enriched limited GO term. On the other hand, Brachypodium seems not sensitive to auxin that most related genes may not regulated and enriched, meanwhile, many auxin response genes in the other two species show significant expression change which also reflected in GO enrichment. GO enrichment analysis also shows that rice and foxtail millet show some different responses between them. We also collected some important genes shared by three species and the expression level. Auxin signaling components expression results show that the same gene may have different expression levels among different species. There are no genes significantly regulated in Brachypodium, and some genes are

only up regulated in rice. In a nutshell, Brachypodium seems have different auxin response compare to rice and foxtail millet. Rice and foxtail millet also have some differences auxin response methods while they have some similar response.

ARF proteins play important roles in regulating auxin response genes

We also discovered that AUX/IAA proteins only have up regulated genes among foxtail millet and rice, they may coordinate when response to auxin. OsARF5, OsARF11, OsARF14, OsARF23 were identified as up regulated in rice, ARF2(OsARF23) was up regulated in foxtail millet. OsARF5 (class IIa), OsARF11 (class IIa), OsARF14 (class IIb), OsARF23 (class Ia). Meanwhile, OsARF7, OsARF8, OsARF17, OsARF18, OsARF22 were identified as down regulated in rice. OsARF7 (class Ia), OsARF8 (class III), OsARF17 (class IIa), OsARF18 (class III), OsARF22(class III). OsARF8/10/18/22 are targeted by miR160 and miR167 to control different developmental pathways and stress responses in rice. It shows that ARF proteins seem to coordinate to respond auxin, and ARF in similar class may have similar response (Wang, D., 2007; Song, X., 2023).

AuxRE palys an important role in auxin response

We found most differentially expressed genes promoters have ARF binding sites in three species, and three different length promoters (1.5kb, 2kb, 2.5kb) contain binding sites in roughly the same proportion among three species. Not all ARF binding sites we identified will work in the auxin signaling pathway, but the result still show the potential of these genes to be regulated by ARF. We also applied motif enrichment analysis, and the result show that ARF and ERF binding motif were significantly enriched in foxtail millet up regulated genes promoters. It shows that ARF may prefer to bind with these genes which show the possible signaling pathway. Some other plant-specific motifs like C2H2 and B3 families were identified in the regulated genes list, which provides the possibility of them cooperate with auxin in regulating root development.

Discussion

We explored the similarities and difference auxin response among different species and collected genes shared by each other. We also studied the molecular basis of them to learn the auxin signaling pathway. The result show the classic TIR/AFB-AUX/IAA-ARF signaling

pathway and components are conserved between different species. And we also studied the different response among three species and explore the promoters of DEGs. The research provides the information and potential of species-specific auxin response. We will try the different auxin treatments for these three species and study how ARF regulate auxin response genes through promoter analysis deeper. We will also try to understand why Brachypodium is not sensitive to auxin in the future.

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