Comparative transcriptome analysis provides key insights into gene expression pattern during the formation of nodule-like structures in *Brachypodium*

Jacklyn Thomas[1], **Megan J. Bowman**[2], **Andres Vega**[1], **Ha Ram Kim**[1], and **Arijit Mukherjee**[1]

[1]Department of Biology, University of Central Arkansas, Conway, AR 72035, USA

[2]Bioinformatics & Biostatistics Core, Van Andel Research Institute, Grand Rapids, MI 49503, USA

Abstract

Auxins can induce the formation of nodule-like structures (NLS) in plant roots even in the absence of rhizobia and nitrogen-fixing bacteria can colonize these structures. Interestingly, NLS can be induced in roots of both legumes and non-legumes. However, our understanding of NLS formation in non-legumes at a molecular level is limited. This study aims to investigate NLS formation at a developmental and molecular level in *Brachypodium distachyon*. We treated *Brachypodium* roots with the synthetic auxin, *2,4-D*, to induce NLS at a high frequency (> 80%) under controlled conditions. A broad base and a diffuse meristem characterized these structures. Next, we performed a comprehensive RNA-sequencing experiment to identify differentially expressed genes (DEGs) in *Brachypodium* roots during NLS formation. We identified 618 DEGs; several of which are promising candidates for control of NLS based on their biological and molecular functions. We validated the expression pattern of several genes via RT-PCR. Next, we compared the expression profile of *Brachypodium* roots with rice roots during NLS formation. We identified 76 single-copy ortholog pairs in rice and *Brachypodium* that are both differentially expressed during this process. Some of these genes are involved in auxin signaling, root development, and legume-rhizobia symbiosis. We established an experimental system to study NLS formation in *Brachypodium* at a developmental and genetic level, and used RNA-sequencing analysis to understand the molecular mechanisms controlling this root organogenesis program. Furthermore, our comparative transcriptome analysis in *Brachypodium* and rice identified a key set of genes for further investigating this genetic pathway in grasses.

Introduction

The Poaceae family, also known as grasses, includes important tropical and temperate crops such as rice, maize, and wheat. Global population increases and extreme weather conditions are driving the need for higher productivity of these agronomically important crops. One of the major challenges facing productivity is the uptake of nutrients, nitrogen in particular. This has led to an excessive dependence on nitrogen-based fertilizers. However, excess fertilizer usage has caused several adverse consequences at the economic, health, and environmental levels. Taking better advantage of nitrogen-fixing associations between plants and microbes seems an obvious solution to these problems. The primary source of biologically fixed nitrogen is facilitated by nitrogen-fixing bacteria that reduce dinitrogen to ammonium via nitrogenase activity. The most evolved plant-microbe nitrogen-fixing association is the root nodule symbiosis (RNS). In this symbiosis, the host plant forms a mutualistic symbiotic relationship with soil bacteria called rhizobia that result in the formation of specialized structures called nodules on the roots of the host plant. The nodules provide an ideal environment for nitrogen fixation to occur. However, this highly efficient nitrogen-fixing association is mostly limited to plants from the legume family. Therefore, non-legume crops still depend on fertilizers for their nitrogen needs. Our current knowledge of RNS places us in a position to extend it to non-nodulating crops such as cereals. Engineering nitrogen-fixing cereals are required for sustainable food production to feed the anticipated 9 billion people (Charpentier and Oldroyd 2010; Mus et al. 2016; Rogers and Oldroyd 2014).

Plant hormones such as auxins and cytokinins play key roles during different stages of nodule organogenesis in legume roots (Bensmihen 2015; Ding and Oldroyd 2009; Mukherjee and Ané 2011; Ryu et al. 2012). Genetic studies have improved our understanding of how hormones regulate this symbiosis. For instance, identification of gain-of-function mutants in the cytokinin receptors (*LjLHK1/MtCRE1*) has shown that bacterial infection is not required to form spontaneous nodules or nodule-like structures (NLS) (Murray et al. 2007; Tirichine et al. 2007). In other words, triggering of cortical cell divisions is sufficient to induce these structures in the absence of bacteria. A broad base, a diffuse meristem, and vascular tissue differentiation characterize NLS. Exogenous application of auxins can also induce NLS on plant roots (Hiltenbrand et al. 2016; Hirsch et al. 1989; Rightmyer and Long 2011; Wu et al. 1996). These findings show that bacterial infection is not required for organogenesis. Also, NLS can be induced on roots of legumes as well as nonlegumes (Christiansen-Weniger 1998; Hiltenbrand et al. 2016; Narula et al. 2006; Ridge et al. 1993; Senthilkumar et al. 2009). Furthermore, nitrogen-fixing bacteria such as *Azorhizobium caulinodans* and *Azospirillum brasilense* can colonize these structures (Christiansen-Weniger 1998; Hiltenbrand et al. 2016; Sriskandarajah et al. 1993). Therefore, identifying the genes controlling this root organogenesis program in cereals will have tremendous agricultural potential. While several studies have investigated hormone-induced

changes in plant root transcriptomes, very few studies have specifically looked into gene expression changes occurring during NLS formation in plant roots. At this point, not much is known about the molecular mechanisms underlying NLS formation in non-legumes. So far, only one study has investigated gene expression changes in roots of the tropical grass, rice during NLS organogenesis (Hiltenbrand et al. 2016). Further studies will provide more insights into the molecular mechanisms controlling NLS formation in grasses.

The wild grass *Brachypodium distachyon* (subsequently *Brachypodium*) with its high-quality genome sequence, small size, rapid life cycle, and available genetic resources has evolved into an important model system for new energy and food crops (Brkljacic et al. 2011; Budak et al. 2014; Initiative 2010; Ozdemir et al. 2008). While rice is an important model plant for tropical species, *Brachypodium* is an ideal model for temperate species that includes grain crops such as wheat, barley, and oats and forage grasses. Increasingly, *Brachypodium* is used with other grasses in comparative genomics studies to accelerate the process of gene discovery in grasses (Davidson et al. 2012; Huan et al. 2013; Sharma et al. 2017; Sibout et al. 2017). Comparative genomics facilitates identification of conserved and species-specific gene expression patterns between closely and distantly related species. These comparative approaches can also facilitate the extrapolation of knowledge obtained in model plants such as *Brachypodium* and rice to other crop species (Davidson et al. 2012; Jansen et al. 2013). Due to these reasons, *Brachypodium* has the potential to be an excellent model system for studying NLS organogenesis in grasses. In the current study, we investigated the formation of NLS in *Brachypodium* at a developmental level and also determined transcriptional responses in *Brachypodium* during NLS formation by RNA-seq. Next, we compared the transcriptomic profile of *Brachypodium* roots with rice roots during NLS formation and identified similarities of key gene expression signatures in these plants. This information provides valuable insights into the regulation of gene expression in two different kinds of grass during NLS development and can serve as a valuable resource for the understanding of detailed molecular mechanisms underlying this process.

Results

Upon auxin treatment, *Brachypodium* roots form nodule-like structures at a high frequency, and their numbers increase over time

We studied NLS formation in *Brachypodium distachyon* (Bd21) roots upon treatment with synthetic auxin, 2,4-Dichlorophenoxyacetic acid (2,4-D) under controlled experimental conditions. NLS were visible as early as 7 days post treatment (dpt) with auxin and appeared along the primary roots both independently and in clusters. The NLS had a broad base, were spherical at the distal end with a smooth epidermis, and a diffused meristem (Fig. 1a, b). Compared to NLS, lateral roots had narrow base and well-defined apical meristem (Fig. 1c). We measured the efficiency of the hormone treatment by determining the frequency of NLS formation over time. The NLS frequency was 84.4% at 7 dpt and 88.9% at 14 dpt (Fig. 1d). The average number of NLS per plant was significantly higher at 14 than 7 dpt (Student's *t* test, $P < 0.001$) (Fig. 1e). We used 2,4-D at a concentration of 50 μM for all the experiments in this study. So, we evaluated other root phenotypes induced by this treatment. Lateral root formation was reduced at 7 dpt (Student's *t* test, $P < 0.001$) and 14 dpt (Student's *t* test, $P <$

0.01) (Supplementary Fig. 1). Besides that, we did not observe any other significant changes in root phenotypes. We also wanted to investigate if nitrogen-fixing bacteria could colonize the NLS induced under these conditions. We used *Azorhizobium caulinodans* ORS571 (pXLGD4) containing the *lacZ* reporter gene for these studies (Gopalaswamy et al. 2000). Using histochemical staining with X-gal, we observed a blue coloration throughout NLS indicating *lacZ* expression (Fig. 1f). We performed the histochemical staining on surface-sterilized roots and observed this pattern for all the NLS indicating that nitrogen-fixing bacteria can colonize these structures under our experimental conditions.

Transcriptome analysis of gene expression in *Brachypodium* roots during NLS formation

We performed a comprehensive transcriptome analysis of gene expression in *Brachypodium* (Bd21) roots during auxin-induced NLS formation. Specifically, we studied the transcript profile of *Brachypodium* roots containing NLS 7 dpt with 2,4-D. The treatment groups for this study included (1) Bd21 roots + 2,4-D treatment and (2) Bd21 roots + mock treatment. There were three biological replicates for each treatment group and we used RNA samples only from plants forming NLS for treatment group 1. We prepared Illumina TruSeq libraries from these RNA samples and sequenced these in a PE100 format on an Illumina HiSeq2500. An average of 28 million reads was generated per sample with an average mapping rate of 70% to the coding regions of the *Brachypodium* genome. Our results show that there was a good correlation between the biological replicates for each sample (Fig. 2a). More than 22,065 transcripts were evaluated and we identified 618 differentially expressed genes (DEGs) (Supplementary Table 1). We identified the DEGs using an FDR-adjusted *P* value less than 0.05 and a twofold change cutoff ($|Log_2 (FC)|$ 1) (Fig. 2b). In this dataset, 470 genes were upregulated in expression, and 148 genes were downregulated in expression. Next, we performed a gene ontology (GO) analysis to investigate the biological significance of the DEGs based on the biological processes, molecular functions, and cellular location of the gene products. Using singular enrichment analysis (SEA) with agriGO, we identified the 62 GO terms that were significantly enriched (Du et al. 2010). These included 31 in biological processes, 22 in molecular functions, and 9 in the cellular component (Fig. 2c, d).

In this study, we focused on the following categories of genes: transcription factors (TFs), receptor kinases, transporters, and hormone-related genes. We identified 94 TFs, 77 protein kinases, and 147 transporters in our dataset of 618 DEGs (Supplementary Tables 2, 3, and 4). Some of the major TFs were auxin response factors, AP2/B3-like TFs, NAC domain-containing proteins, GRAS family TFs, lateral organ boundaries (LOB), WUSCHEL, and WRKY among others. Among protein kinases, the major ones were cysteine-rich receptor-like kinases (RLK), leucine-rich repeat RLKs, and serine-threonine protein kinases. Major transporters identified were auxin efflux and influx carriers, ABC-2 type transporters, MATE efflux carriers, and several nodulins (*MtN21*, *Nod26-like intrinsic proteins*, and *Major facilitator superfamily*). Several hormone-related genes, such as the auxin-responsive *GH3* family, *SAUR* gene, and hormone biosynthesis, were also identified (Supplementary Table 1).

Gene expression validation

We examined the expression pattern of ten genes via reverse transcription polymerase chain reaction (RT-PCR) to confirm the gene expression patterns identified by the RNA-seq experiment (Fig. 3). The primers used in these experiments were designed based on the JGI Phytozome database (Supplementary Table 5). Our RT-PCR results showed that these genes were expressed in *Brachypodium* roots, confirming the gene expression patterns identified via RNA-seq (Fig. 3).

Identification of single-copy ortholog gene pairs differentially expressed in *Brachypodium* and rice during NLS formation

To evaluate conservation of expression across species during NLS formation, we compared the DEGs in *Brachypodium* to the DEGs in rice from our earlier study (Hiltenbrand et al. 2016). We identified 76 single-copy ortholog gene pairs from rice and *Brachypodium* that were both differentially expressed during NLS formation (Supplementary Table 6). We compared the $\log_2$fold change of the differentially expressed *Brachypodium* genes to the $\log_2$fold change of the corresponding differentially expressed single-copy ortholog in rice. The linear regression value indicates a high correlation in the expression patterns (Fig. 4a). To assess the functional significance of the genes in this list, we determined the enriched GO terms via singular enrichment analysis (SEA) with agriGO (Du et al. 2010). Fourteen GO terms were significantly enriched. These included 9 in biological processes, 3 in molecular function, and 2 in the cellular component. GO terms in biological processes were enriched in anatomical structure development, anatomical structure morphogenesis, cell growth, and regulation of anatomical structure size among others. The 3 GO terms enriched in molecular function included transcription regulator activity, transcription factor activity, and DNA binding. The 2 GO terms enriched in cellular component were external encapsulating structure and cell wall (Fig. 4b).

Discussion

Brachypodium distachyon has emerged as an excellent model for temperate grasses including important cereals such as barley, wheat, and oats. In fact, it is more closely related to wheat and rye than rice and maize are, making it a convenient model system for analysis of diverse grasses. It has a relatively small and well-sequenced genome, and small stature (Brkljacic et al. 2011; Budak et al. 2014; Initiative 2010; Ozdemir et al. 2008). Also, the *Brachypodium* root system shows considerable similarity to a monocotyledonous root system, but less complicated than most monocot crops (Watt et al. 2009). This makes it a tractable model to investigate grass roots. Also, *Brachypodium* is increasingly used in comparative transcriptomics studies to compare and contrast genome wide expression patterns during important biological processes (Davidson et al. 2012; Huan et al. 2013; Sharma et al. 2017; Sibout et al. 2017). One of the challenging steps in agricultural biotechnology application is to transfer findings obtained in model species such as *Brachypodium* and rice to crop plants without sequenced genomes. A comparative transcriptomics study helps in identification of similarities and differences in gene expression patterns in closely or distantly related species and facilitates the selection of the right candidate genes for future genetic studies (Jansen et al. 2013). In this study, we

investigated the formation of NLS on *Brachypodium* roots and identified differentially expressed genes in *Brachypodium* during NLS formation. To the best of our knowledge, this is the first study to report on NLS formation in *Brachypodium*. We believe *Brachypodium* can serve as a great model to study NLS organogenesis in grasses.

In this study, first, we used auxin, *2,4-D*, to induce NLS on *Brachypodium* roots at a high frequency. This was similar to frequency observed in rice and *M. truncatula* under similar experimental conditions (Hiltenbrand et al. 2016). The distribution of NLS and their appearance on *Brachypodium* roots was analogous to those observed on rice and *M. truncatula* roots. This suggests the mechanism of NLS development from how the plant perceives auxin to regulation of its transport is likely conserved across land plants. In addition, we observed that lateral root numbers were reduced on *Brachypodium* roots. This pattern was also observed in rice and *M. truncatula* (Hiltenbrand et al. 2016). However, further studies are required to determine when or how the plant adjusts its developmental program. We also showed that nitrogen-fixing bacteria, *A. caulinodans*, could colonize the *Brachypodium* NLS. While further studies are required to determine the precise mechanism of colonization, our results show that similar to rice, the *Brachypodium* NLS can be colonized by nitrogen-fixing bacteria.

Our next aim was to identify the differentially expressed genes in *Brachypodium* roots during NLS formation. Using whole transcriptome profiling with RNA-seq, we identified hundreds of upregulated and downregulated genes in NLS-containing *Brachypodium* roots. Among these was a diverse array of genes encoding protein kinases, transporters, and transcription factors that are likely involved in the signaling pathway leading to NLS organogenesis (Supplementary Table 1). We were specifically interested to identify genes that displayed similar expression patterns in rice and *Brachypodium* roots during NLS formation. So, we compared gene expression levels in *Brachypodium* with those in rice during NLS formation and identified several orthologous genes that exhibited conserved expression patterns. Interestingly, we identified 76 single-copy (1-to-1) orthologs whose expression was well conserved during NLS formation in both kinds of grass (Supplementary Table 6). The single-copy status of such genes along with their expression patterns suggests their significance in this biological process. This was further supported by GO analysis of this group of commonly expressed genes (Fig. 4). In the following sections, we focus on selected genes belonging to the family of transcription factors, protein kinases, and transporters that are promising candidates for future studies. We specifically concentrate on the genes that are expressed in *Brachypodium* and rice.

Transcription factors (TFs) are important for root development and can be regulated by plant hormones, small signaling molecules, and miRNAs. In this analysis, we identified 94 TFs that are differentially expressed upon NLS formation. AP2/ERF proteins have significant functions in a variety of biological processes from growth and development to responses to environmental stimuli (Licausi et al. 2013). We identified several upregulated genes (Bradi3g50508.1, Bradi2g16442.1, Bradi1g14560.5, Bradi2g60554.7, etc.) in this category, including two *PLETHORA2* genes (Bradi5g14960.1 and Bradi3g48697.1). The *PLT* genes have been shown to mediate auxin distribution via expression of *PIN* auxin efflux carriers (Aida et al. 2004; Benjamins and Scheres 2008). A *PLT* gene (LOC_Os02g40070.1) was

also differentially expressed during NLS formation in rice (Hiltenbrand et al. 2016). Interestingly, *PLETHORA* genes are expressed in *M. truncatula* root nodules during symbiosis with rhizobia. Genetic studies have shown that *PLT* genes are required for maintenance of rhizobia-induced nodule meristem (Franssen et al. 2015). These findings suggest a possible role of the *PLT* genes in NLS formation in grasses. *WUSCHEL* (*WUS*)-related Homeobox (*WOX*) genes have been suggested to play an important role coordinating gene expression involved in both shoot and root meristem function and development (Haecker et al. 2004). In this study, we found *WOX11* (Bradi1g17420.1) to be upregulated in expression. Past studies have shown that *WOX11* is expressed in cell division regions in roots and shoots and that it may coordinate both auxin and cytokinin signaling to stimulate cell division during root development (Zhao et al. 2009). The rice orthologs of *WOX11* (LOC_Os03g20910.2 and LOC_Os07g48560.1) were also upregulated in their expression during NLS formation (Hiltenbrand et al. 2016). This implies that this gene might be a key regulator of NLS organogenesis in grasses. Another important class of TFs identified was the NAC TFs. While *NAC102* (Bradi1g04150.1), *NAC070* (Bradi5g11247.1), and *NAC094* (Bradi3g59380.1) were upregulated in their expression, *NAC1* (Bradi3g17287.1), *NAC046* (Bradi3g36670.2), and *NAC075* (Bradi2g05700.2) were downregulated in their expression. Several NAC TFs were also differentially expressed in rice roots during NLS formation. For instance, single-copy rice orthologs (LOC_Os01g60020.1 and LOC_Os02g15340.1) of *Brachypodium* NAC genes, Bradi2g5360.1 and Bradi3g09520.2 respectively, were also upregulated in expression (Hiltenbrand et al. 2016). The NAC TFs have been shown to be involved in both auxin signaling and root development (Nuruzzaman et al. 2013; Xie et al. 2000). The expression pattern of these TFs in *Brachypodium* and rice suggest a possible involvement in NLS development. The *Lateral Organ Boundaries Domain family* of TFs (e.g., Bradi1g68170.1, Bradi2g57380.2, Bradi3g55680.1, Bradi3g12550.1, and Bradi1g75120.1) was also well represented in our dataset. *LBD* genes have been shown to be involved in auxin signaling leading to root development (Lee et al. 2015; Liu et al. 2005; Okushima et al. 2007). In fact, single-copy orthologs of *LBD16* (Bradi3g55680.1) and *LBD29* (Bradi3g12550.1) were also upregulated in their expression in rice (LOC_Os02g57490.1 and LOC_Os08g44940.1 respectively) during NLS formation making these excellent candidates for further investigation (Hiltenbrand et al. 2016). A few of the *LOB* gene family members have been shown to be putative targets of Auxin Response Factors (ARFs) in rice and Arabidopsis (Inukai et al. 2005; Okushima et al. 2007). Several genetic studies have revealed that ARFs play significant roles in plant growth and development, including root development (Guilfoyle and Hagen 2007; Li et al. 2016). In our analysis, we identified several ARFs including *ARF3* (Bradi2g16610.4), *ARF5* (Bradi5g25157.1), *ARF10* (Bradi5g15904.1), and *ARF16* (Bradi3g49320.1) that were upregulated in expression during NLS formation. However, in rice, only *ARF16* (LOC_Os04g43910.1) and *ARF19* (LOC_Os06g48950.1) were differentially expressed (Hiltenbrand et al. 2016). This suggests that these are excellent candidates for future studies. ARFs have been known to interact with each other and also been reported to regulate and be regulated by other transcription factors (Wang and Estelle 2014). For instance, the *Arabidopsis* BREVIS RADIX (BRX) transcriptional coregulator has been shown to interact with ARF5 and increase the transcriptional activation potential of the ARF (Guilfoyle and Hagen 2012). The plant-specific *BRX* gene family has been identified as a regulator of root

growth (Briggs et al. 2006). In our study, we identified a *BRX-like 1* gene (Bradi3g52537.1) to be upregulated in expression. Whether the ARF5-BRX interaction occurs in *Brachypodium* requires further studies. Another important category of TFs identified in our dataset that plays a crucial role in plant shoot and root development is the GRAS family TFs (Hirsch and Oldroyd 2009). We identified two GRAS TFs, SCARECROW (Bradi4g44090.2) and Short-root (Bradi1g23060.1), to be differentially expressed in our dataset. These GRAS family TFs are examples of well-characterized regulators of a broad range of root-related developmental processes. Four GRAS TFs (LOC_Os06g40780.1, LOC_Os03g51330.1, LOC_Os07g40020.1, and LOC_Os11g04570.1) were differentially expressed in NLS-containing rice roots as well (Hiltenbrand et al. 2016). Importantly, the GRAS family TFs (NSP1 and NSP2) plays a significant role in nodule organogenesis (Heckmann et al. 2006; Kalo et al. 2005; Smit et al. 2005). It remains to be seen if any of these GRAS TFs identified in our analysis play a role in NLS organogenesis.

In our study, we identified several transporters whose expressions were differentially regulated. The ABC type transporters have been shown to play important roles in multiple processes including auxin transport (Geiser and Murphy 2005; Verriereal. 2008). We identified several genes (Bradi3g35377.1, Bradi3g12627.1, Bradi5g12307.1, Bradi2g47410.1, etc.) in this category whose expression was affected during NLS formation. The ortholog of Bradi3g35377. 1 gene in rice (LOC_Os08g30780.1) was also expressed differentially during NLS formation (Hiltenbrandetal. 2016). In addition to this ABC transporter, several others were expressed differentially in rice suggesting an important role this gene family might play in auxin transport leading to NLS formation. As expected, auxin efflux and influx carriers were also differentially expressed. For instance, auxin efflux carriers (Bradi2g48170.1, Bradi1g45020.1, and Bradi4g26300.2) were all upregulated in expression. Interestingly, thesingle-copy ortholog of Bradi2g48170.1inrice (*OsPIN8*; LOC_Os01g51780.1) was also upregulated in expression during NLS formation in rice suggesting an important role in this process (Hiltenbrand et al. 2016).Past studies have suggested that different PIN members have different biological functions and different auxin transport activities. For example, auxin transport and auxin content measurements suggest that PIN5 and PIN8 are antagonistic in nature (Adamowski and Friml 2015). In rice, *OsPIN5* (LOC_Os09g32770.1) was downregulated in expression while *OsPIN8* (LOC_Os01g51780.1) was upregulated in expression during NLS formation (Hiltenbrandetal.2016). However, in *Brachypodium*, we did not observe *PIN5* to be differentially expressed in our dataset. Further studies are required to understand the role of these *PIN* genes in NLS development. Besides these auxin efflux carriers, we also identified two auxin influx carriers in our study. Both these auxin influx carriers (Bradi1g68350.1 and Bradi3g21090.1) were upregulated in expression. Incidentally, the ortholog of Bradi1g68350.1 in rice (*OsLAX1*; LOC_Os03g14080.1) was also differentially expressed in NLS-containing roots. Several studies have shown that these auxin exporters and importers play important roles during plant growth, development, and even legume-rhizobia symbiosis (Adamowski and Friml 2015; Huo et al. 2006; Roy et al. 2017; Swarup and Peret 2012). It was quite encouraging to find that we identified these auxin carriers to be upregulated in expression in both rice and *Brachypodium*. We also identified several nodulin-like genes from different families: *MtN21* (Bradi3g57830.1 and Bradi2g21310.1), *Nod26-like intrinsic*

proteins (Bradi3g59390.1), and *Major facilitator superfamily* (Bradi1g43970.1, Bradi4g29150.1, Bradi4g29110.1, etc.). Several nodulin genes were differentially expressed in rice during NLS formation as well (Hiltenbrand et al. 2016). These genes were initially considered to be legume-specific as they are expressed during symbiosis with rhizobia (Mukherjee and Ané 2011; Oldroyd and Downie 2008). While some studies have shown that nodulins are involved in transporting nutrients, amino acids, and hormones during plant development, their roles in non-legumes are still not clear. Whether these genes play a role during NLS development will require further studies.

Several studies on root development have identified a network of peptides and receptor systems mediating short-and long-range communication (Stahl and Simon 2012; Yamada and Sawa 2013). The CLV3/EMBRYO SURROUNDING REGION (CLE) family of peptides plays important roles in most pathways involved in root development including root nodule organogenesis. In most cases, these peptides are recognized by leucine-rich-repeat receptor-like kinases (LRR-RLKs). In rice FON2, a close relative of CLV3 controls meristem size and requires FON1 (ortholog of CLV1) (Suzaki et al. 2006). During NLS formation in rice, a FON2 SPARE1 (FOS1; LOC_Os02g21890.1) and CLV1-like LRRs (LOC_Os07g04190.1 and LOC_Os06g50340.1) were found to be differentially expressed (Hiltenbrand et al. 2016). In *Brachypodium*, we identified the single-copy ortholog of the rice *FON2* gene (Bradi3g10697.1) to be differentially expressed in NLS-containing roots. Another class of peptide receptors identified in our dataset was the phytosulfokine receptor 2 (Bradi1g59360.1). The ortholog of this receptor (LOC_Os07g01710.1) was also upregulated in expression in rice roots containing NLS (Hiltenbrand et al. 2016). Recently, it was shown in *Lotus japonicus* that phytosulfokine receptor ligands regulate nodulation (Wang et al. 2015). It will be interesting to determine if these peptides and their receptors are also required for NLS organogenesis in grasses. Studies have shown that plant AGC kinases modulate auxin responses and auxin transport in plant developmental processes (Robert and Offringa 2008). Interestingly, the PIN efflux carriers are one of the primary targets of these plant-specific kinases (Robert and Offringa 2008). While more studies are reporting the roles of these kinases in different processes, an AGC kinase is required during legume-rhizobia symbiosis (Pislariu and Dickstein 2007). We identified two AGC kinases (Bradi4g33610.1 and Bradi4g42600.2) to be upregulated in expression in *Brachypodium* roots during NLS formation. The *Brachypodium* AGC kinase (Bradi4g42600.2) is a singlecopy ortholog of the rice gene (LOC_Os11g05320.1) whose expression was also increased during NLS formation (Hiltenbrand et al. 2016). Further genetic studies will offer valuable insights into the role of these key genes in controlling this important root developmental process.

Conclusions

Our results showed that we could induce NLS on *Brachypodium* roots upon auxin treatment at a high frequency under controlled conditions. A broad base and a diffuse meristem characterized these structures. While the frequency of NLS formation did not change over time, the average number of NLS formed per plant increased over time. We performed an RNA-seq experiment and identified 618 differentially expressed genes in *Brachypodium* roots during NLS formation. We identified a diverse array of transcription factors, protein kinases, and transporters in our dataset. Some of these genes (*PLETHORA, PSKR, LAX,*

PIN, etc.) have been implicated to play important roles during lateral root formation and root nodulation. Whether these are required for NLS formation will need additional studies. In this study, we analyzed the transcriptome of whole plant roots containing NLS. Since, lateral root formation is diminished in roots containing NLS, it is possible that gene expression changes occurring in these roots also correspond to fewer lateral root initiation. Future transcriptomics studies on isolated NLS alone will offer more insights. Comparative transcriptome analysis of *Brachypodium* roots with rice roots during NLS formation showed that there are overlapping gene expression patterns between these two species. Furthermore, we identified a core set of single-copy orthologs with similar expression pattern in these two model grasses. GO analysis and functional annotation support that these genes are excellent candidates for future genetic studies. This study can serve as a valuable resource for the understanding of detailed genetic mechanisms underlying this process.

Materials and methods

Plant material and growth conditions

We used the sequenced reference line Bd21 of *Brachypodium distachyon* for this study. Prior to germination, *Brachypodium* seeds were surface-sterilized in 2% (*v/v*) sodium hypochlorite solution for 15 min, rinsed several times with sterile water, and imbibed for 24 h in sterile water. Prior to hormone treatment, the germinated seedlings were grown for 7 days on low-nitrogen Fahraeus medium inside a Percival growth chamber (#CU-22L, Iowa, USA) as described in Hiltenbrand et al. (2016). The growth chamber was maintained at 16-h, 22 °C day and 8-h, 24 °C night cycle and 150 to 200 μmol m^{-2} s^{-1} light intensity, and relative humidity of 65%.

Hormonal treatment and plant phenotype scoring

Seven-day-old *Brachypodium* seedlings, grown as mentioned earlier, were treated with auxin, 2,4-Dichlorophenoxyacetic acid (2,4-D) (CAS# 94–75-7, Caisson Laboratories Inc., Utah, USA) as described in Hiltenbrand et al. (2016). Following treatment, the plants were grown on plates containing a low-nitrogen Fahraeus medium inside a Percival growth chamber (Model #: CU-22L, Iowa, USA) as mentioned earlier. Non-treated (mock) plants were grown as described above except the treatment solution did not include 2,4-D. In this study, water was used to dissolve 2,4-D. The plant phenotype scoring was performed using a Leica MZ6 stereomicroscope (Leica Microsystems Inc., Illinois, USA). For statistical analysis of data, we used JMP® 12 (SAS Institute Inc., North Carolina, USA).

Scanning electron microscopy

We performed scanning electron microscopy as previously described by Hiltenbrand et al. (2016) with minor modifications. The plant roots were dehydrated in a series of ethanol solutions: 25% for 5 min, 50% for 5 min, 75% for 5 min, and 95% for 30 min. The root samples were stored in 75% ethanol at 4 °C prior to SEM analysis. Drying of the root samples to a critical point (Pelco CPD2 Critical Point Dryer, Ted Pella, Inc., Redding, CA, USA) and sputter-coating of the sections using a Denton Vacuum Desk IV sputter coater (Denton Vacuum, LLC, Moorestown, NJ, USA) were performed as described in Hiltenbrand

et al. (2016). We used a JSM-IT100LA scanning electron microscope (JEOL USA, Inc., Peabody, MA, USA) to obtain the images.

Bacterial inoculation and X-gal staining

The bacterial inoculation of the *Brachypodium* roots was performed as described by Hiltenbrand et al. (2016). During post hormone treatment, the *Brachypodium* seedlings were inoculated with or without the *lacZ*-marked *A. caulinodans* ORS571 (pXLGD4). Bacteria were grown on LB/Tetracycline (10 μg/ml) plates at 28 °C until they reached an optical density of 0.6. The seedlings were inoculated with 10^8 cells/ml with *A. caulinodans* and allowed to grow in the plant growth chamber.

The histochemical staining of the *Brachypodium* roots was performed as described by Hiltenbrand et al. (2016) with minor modifications. The *Brachypodium* seedlings were sampled 7 days post-inoculation and the roots were washed with sterile water to remove loosely attached bacteria. Next surface sterilization of these roots was performed as described by Hiltenbrand et al. (2016). The efficiency of surface sterilization was determined by culturing the wash from the last root rinse on LB/Tetracycline plates. We did not observe any bacterial growth from the last wash indicating successful surface sterilization. Finally, the visualization of bacterial colonization via X-gal staining was performed on surface-sterilized roots as described by Hiltenbrand et al. (2016).

RNA extraction and RNA sequencing

We extracted total RNA from the plant roots using Qiagen RNeasy® Plant Mini Kit (Cat #74904, California, USA) as described in Hiltenbrand et al. (2016). For hormone-treated plants, RNA was extracted only from plant roots that had NLS. We performed RNA quantification, library preparation, and RNA sequencing at the Research Technology Support Facility (RTSF), Michigan State University, East Lansing, MI. Before library preparation and sequencing, the RNA integrity was checked using a Bioanalyzer (Agilent Technologies). We used the Illumina TruSeq Stranded mRNA Library Prep Kit LT to prepare multiplex sequencing libraries. These six libraries were combined into a single pool, loaded on one lane of an Illumina HiSeq 2500 Rapid Run flow cell (v2), and sequenced in a PE100 format with HiSeq Rapid SBS reagents. We used Illumina Real Time Analysis (RTA) v1.18.64 for base calling and used Illumina Bcl2fastq v1.8.4 to de-multiplex the output of RTA and convert to Fastq format.

RNA-sequencing data analysis

We assessed read quality using FASTQC v. 0.11.3 (Andrews 2010) and aligned to the *B. distachyon* v2.1 genome (Goodstein et al. 2012) using Subread v. 1.4.3. (Liao et al. 2013) with default parameters. Raw read counts to known exons were obtained using FeatureCounts v 1.4.3 (Liao et al. 2014) using strand-specific read counting. Counts per million (CPM) were calculated and log2 transformed using voom (Law et al. 2014). Transformation and differential expression analyses were conducted using the limma package v 3.28.7 in R (Ritchie et al. 2015).

OrthoMCL analyses

Single-copy orthologs between *Brachypodium* and rice were determined using OrthoMCL v 1.4 (Li et al. 2003). Spearman correlation was used to compare $\log_2$ fold change of the differentially expressed *Brachypodium* genes to the $\log_2$ fold change of the corresponding differentially expressed single-copy ortholog in rice. Correlation and linear regression analysis were completed using R 3.2.3.

Gene expression validation

We validated the results of RNA-sequencing for some genes via reverse transcriptase PCR (RT-PCR) as described in Hiltenbrand et al. 2016. Before cDNA synthesis, we treated the RNA samples with the Ambion® DNA-free™ DNase Treatment and Removal kit (Cat #AM1906, California, USA). We synthesized first strand cDNA from 300 ng of RNA using a Thermo Scientific RevertAid RT Kit (Cat #K1691, Delaware, USA) with Oligo(dT)$_{18}$ primers according to manufacturer's instructions. We used *Ubi18* and *Ubi10* as internal reference genes for RT-PCR analysis. The list of genes and their corresponding primer sequences are listed in Supplementary Table 5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

NLS	nodule-like structures
RNS	root nodule symbiosis
LHK1	Lotus histidine kinase 1
CRE1	cytokinin response 1
2,4-D	2,4-dichlorophenoxyacetic acid
dpt	days post treatment
DEGs	differentially expressed genes
FDR	false discovery rate
FC	fold change

GO	gene ontology
SEA	singular enrichment analysis
TFs	transcription factors
AP2/ERF	APETALA2/ethylene responsive factor
NAC	NAM (for no apical meristem), ATAF1 and −2, and CUC2 (for cup-shaped cotyledon)
ARFs	auxin response factors
bHLH	basic helix-loop-helix
GRAS	gibberellic-acid insensitive (GAI), REPRESSOR of GAI (RGA), and SCARECROW (SCR)
LOB	lateral organ boundaries
WUS	WUSCHEL
RLKs	receptor-like kinases
LRR	leucine-rich repeat
ABC	ATP-binding cassette
MATE	multi-antimicrobial extrusion protein
SAUR	small auxin upregulated RNA
RT-PCR	reverse transcriptase polymerase chain reaction
PLT	PLETHORA
WOX11	WUSCHEL-RELATED HOMEOBOX 11
BRX	BREVIS RADIX
SCR	SCARECROW
SHR	SHORT ROOT
NSP1	NODULATION SIGNALING PATHWAY1
NSP2	NODULATION SIGNALING PATHWAY2
PIN	PIN-FORMED
LAX	LIKE AUXIN1
CLV	CLAVATA
CLE	CLAVAT3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR)

FON	FLORAL ORGAN NUMBER
FOS1	FON2 SPARE1
CPM	counts per million

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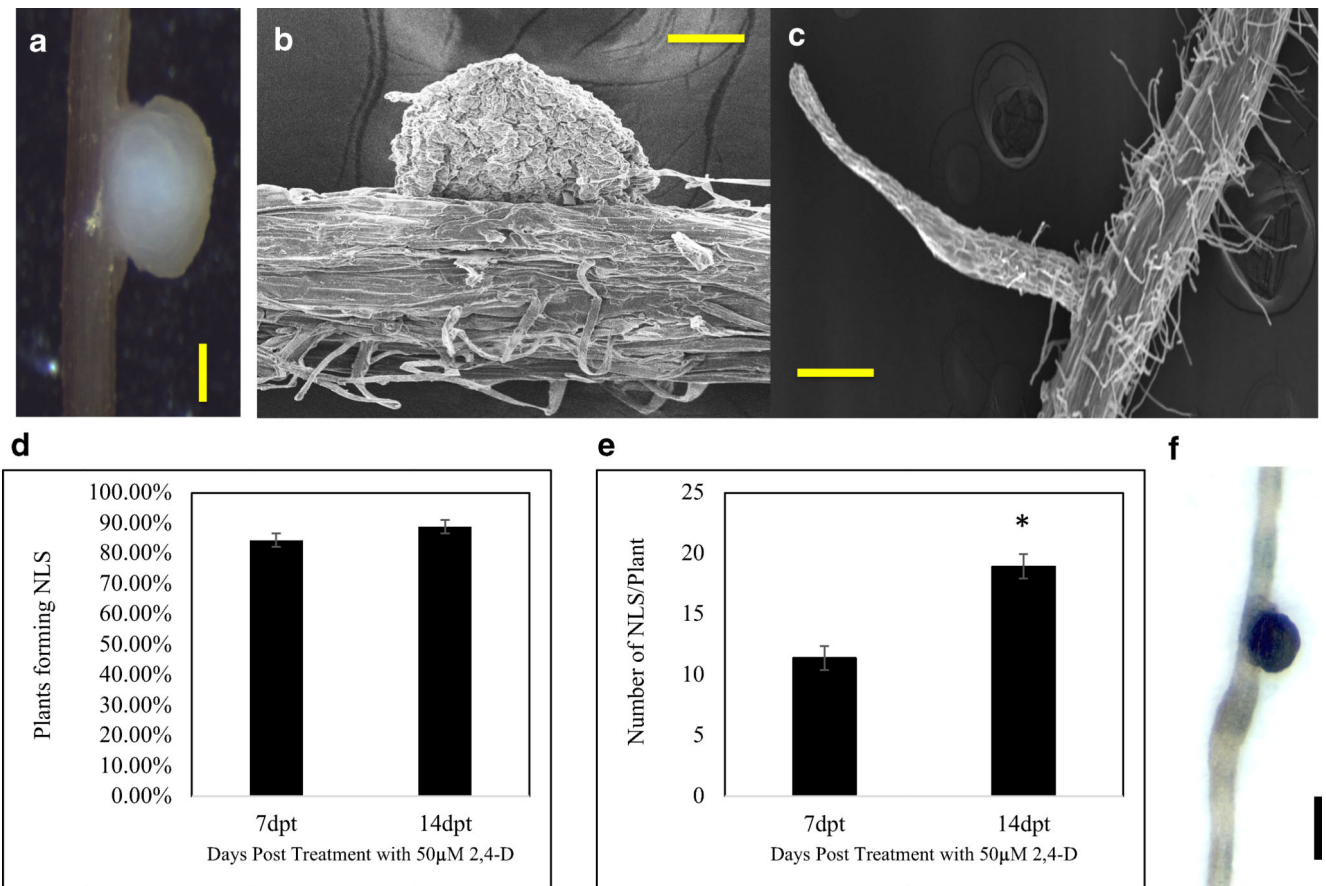
Fig. 1.

NLS formation in *Brachypodium* roots. **a, b** Images of *Brachypodium* NLS 14dpt with 50 μM 2,4-D under controlled conditions. The image in **a** was obtained using a Leica MZ6 stereomicroscope while the image in **b** was obtained using a scanning electron microscope (SEM). **c** The image of a lateral root obtained by SEM. Bar in microscope image equal 0.5 mm. Bars in SEM images equal 100 μm. **d** Frequency of NLS formed per plant upon treatment with 50 μM 2,4-D at 7 and 14 dpt in *Brachypodium*. Data represents the average of 5 experimental replications ($n = 10$–15) ± SE. **e** The average number of NLS increased significantly between 7 and 14 dpt in *Brachypodium* roots. The asterisk (*) shows a significant difference between the two time points by *t* test ($P < 0.001$). Data represents the average of 5 experimental replications ($n = 10$–15) ± SE. **f** Colonization of *A. caulinodans* ORS571 inside *Brachypodium* NLS via histochemical staining with X-gal. The staining was performed on surface-sterilized roots. Bar in image equal 2 mm
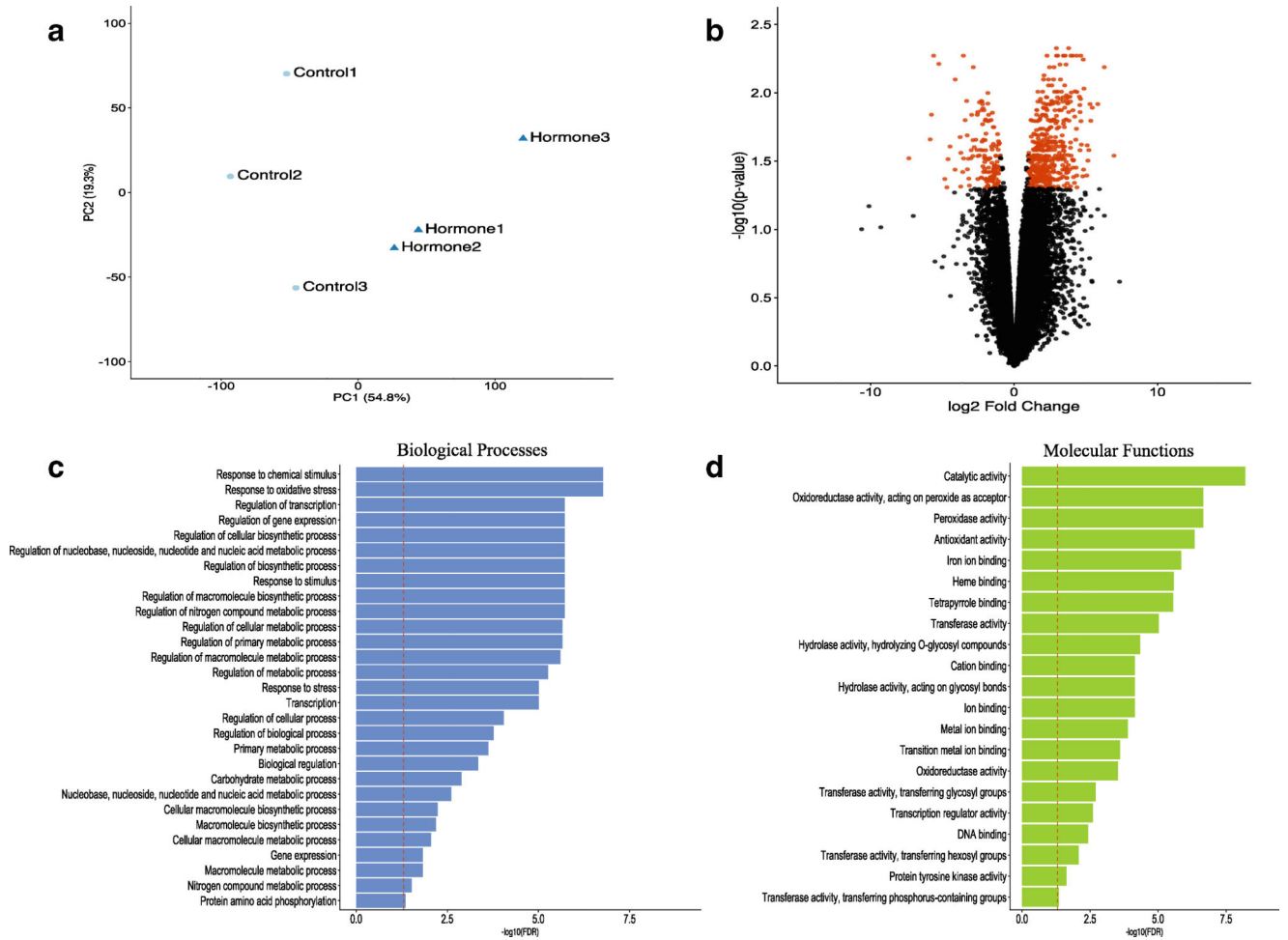
Fig. 2.

Transcriptome profile of *Brachypodium* roots during NLS formation. **a** Principal component analysis of transcriptome profiles for each biological replicate of control and NLS-containing *Brachypodium* roots. **b** Volcano plot of *P* values (FDR adjusted) and fold changes for all genes assessed in this study. Red dots indicate genes with an FDR-adjusted *P* value of < 0.05 and fold change of > 2. Gene ontology (GO) enrichment analysis of the differentially expressed genes between control and NLS-containing *Brachypodium* root samples. Bar charts of significantly enriched **c** biological processes and **d** molecular functions. In these charts, the x-axes indicate the −log10 of the FDR-adjusted *P* value, and the dotted red line denotes an FDR-adjusted *P* value of 0.05

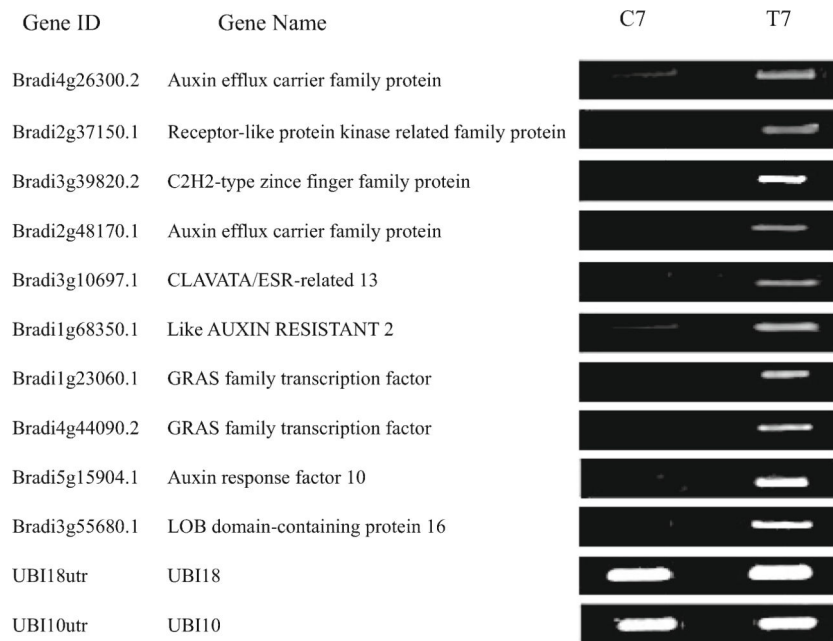| Gene ID	Gene Name	C7	T7
Bradi4g26300.2	Auxin efflux carrier family protein		
Bradi2g37150.1	Receptor-like protein kinase related family protein		
Bradi3g39820.2	C2H2-type zince finger family protein		
Bradi2g48170.1	Auxin efflux carrier family protein		
Bradi3g10697.1	CLAVATA/ESR-related 13		
Bradi1g68350.1	Like AUXIN RESISTANT 2		
Bradi1g23060.1	GRAS family transcription factor		
Bradi4g44090.2	GRAS family transcription factor		
Bradi5g15904.1	Auxin response factor 10		
Bradi3g55680.1	LOB domain-containing protein 16		
UBI18utr	UBI18		
UBI10utr	UBI10		

Fig. 3.

RT-PCR validation of differentially expressed genes identified by RNA-Seq. Expression pattern of ten DEGs was validated by RT-PCR. For the RT-PCR experiments, C7 and T7 represent cDNA templates synthesized from RNA samples at 7 dpt from control and NLS-containing root samples respectively. We performed these RT-PCR experiments in at least three biological replicates for all the samples. *Ubi18* and *Ubi10* were used as internal reference genes
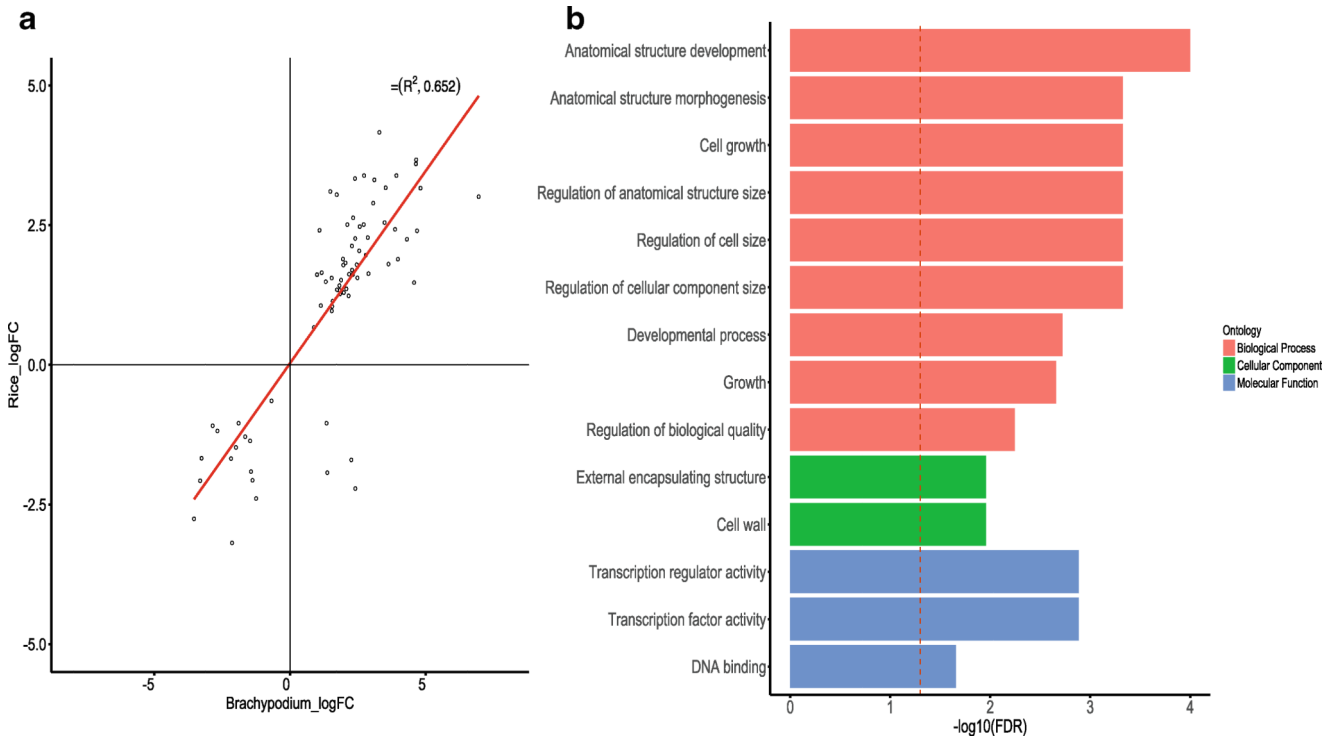
Fig. 4.

Linear regression of expression patterns and gene ontology enrichment of single-copy orthologs between *Brachypodium* and rice. **a** Linear regression analysis of the log fold change of single-copy ortholog pairs of *Brachypodium* (*x*-axis) and rice (*y*-axis) under 2,4-D treatment relative to their corresponding control root sample. **b** AgriGO gene ontology enrichment of the corresponding rice single-copy ortholog of genes differentially expressed in *Brachypodium* under 2,4-D treatment. The *x*-axis indicates the −log10 of the FDR-adjusted *P* value, and the dotted red line denotes an FDR-adjusted *P* value of 0.05