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A first glimpse at genes important to the *Azolla–Nostoc* symbiosis

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Abstract
Azolla is a small genus of diminutive aquatic ferns with a surprisingly vast potential to benefit the environment and agriculture, as well as to provide insight into the evolution of plant-cyanobacterial symbioses. This capability is derived from the unique relationship *Azolla* spp. have with their obligate, nitrogen-fixing cyanobacterial symbiont, *Nostoc azollae,* that resides in their leaves. Although previous work has specified the importance of the exchange of ammonium and sucrose metabolites between these two partners, we have yet to determine the underlying molecular mechanisms that make this symbiosis so successful. The newly sequenced and annotated reference genome of *Azolla filiculoides* has allowed us to investigate gene expression profiles of *A. filiculoides*—both with and without its obligate cyanobiont, *N. azollae*—revealing genes potentially essential to the *Azolla-Nostoc* symbiosis. We observed the absence of differentially expressed glutamine synthetase (GS) and glutamate synthase (GOGAT) genes, leading to questions about how *A. filiculoides* regulates the machinery it uses for nitrogen assimilation. Ushering *A. filiculoides* into the era of transcriptomics sets the stage to truly begin to understand the uniqueness of the *Azolla-Nostoc* symbiosis.

Keywords Ferns · Nitrogen-assimilation · Nitrogen-fixation · RNA-sequencing · Symbiosis

1 Introduction

Plant-microbial symbioses have long been of interest to biologists. With nitrogen (N) being a major limiting nutrient for plant development, many symbiotic strategies have evolved to fulfill this need. The arbuscular mycorrhizal (AM) mutualistic associations between almost all land plants and the glomeromycete fungi has been well-characterized (Parniske 2008; Paszkowski 2006; van Ooij 2011). Similarly, the nitrogen-fixing root nodule (RN) symbiosis restricted to a few angiosperm lineages (mostly legumes) that associate with various nitrogen-fixing bacterial symbionts (e.g., *Glycine max* and the soil bacterium, *Bradyrhizobium japonicum*) is well-known because of its agricultural significance. Together, these two distinct symbioses form the most common microbial interaction pathways seen in plants (Oldroyd 2013; Stracke et al. 2002) and they each require that a common symbiosis pathway (CSP) be established (Oldroyd 2013). Most of what we know about plant-microbial interactions (symbiotic and otherwise) comes from studying these symbioses.

Plant-cyanobacterial symbioses are also of critical importance to understanding the overall dynamics of N-fixing symbioses (Adams et al. 2013; Peters 1991). These have evolved in multiple lineages across land plants yet remain markedly understudied. The *Azolla-Nostoc* relationship is a remarkable example of such a symbiosis, and recently the genome of *Azolla filiculoides* was sequenced and annotated, providing a unique look into the molecular biology of plant-cyanobacterial symbioses (Li et al. 2018). It was discovered that *A. filiculoides* completely lacks the essential CSP genes of other N-fixing symbioses (Li et al. 2018), suggesting that the *Azolla–Nostoc* symbiosis uses unique signaling pathways to interact with its N-fixing symbiont. It is possible that because this is an extracellular symbiosis, *Azolla* spp. use mechanisms more similar to ectomycorrhizal symbioses (Werner 1992), but the genes these symbioses use to communicate are still being discovered (Flores-Monterroso et al. 2013; Martin et al. 2001; Martin et al. 2016). Similarly, understanding how *Azolla* spp. and *N. azollae* interact is ongoing.

Azolla is a small genus (ca. 8 species) of diminutive aquatic ferns that resides on the surface of still waters, such as ponds

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or ditches (Carrapiço 2010; Metzgar et al. 2007; Peters 1989; Raja 2014; Small and Darbyshire 2011; Van Hove and Lejeune 2002). The *Azolla* spp. sporophyte has two leaf types—non-photosynthetic leaves that skim the water surface, and photosynthetic leaves that each house a symbiotic cavity (Fig. 1a, i-iii). Each species of *Azolla* houses an obligate nitrogen-fixing cyanobacterial symbiont (*Nostoc azollae*) within the cavity in every photosynthetic leaf. These leaf cavities contain specialized hairs that engage in the exchange of nutrients, namely sucrose and ammonium, between the symbionts (Calvert et al. 1985; Calvert and Peters 1981; Hill 1977; Kaplan and Peters 1981; Kaplan and Peters 1988). It is thought that glutamine synthetase and glutamate synthase are used by the plant to assimilate ammonium from its cyanobiont (Ray et al. 1978). These partners have been co-evolving for over 100 million years (Metzgar et al. 2007). Together, they are capable of fixing more than three times the amount of nitrogen that the legume-*Rhizobium* symbioses produce (Hall et al. 1995). *Azolla-Nostoc* can fix so much nitrogen that it has been used as a "green fertilizer" in rice farming for over 1500 years in Asia (Lumpkin and Plucknett 1980), and has also been adopted in other parts of the globe (Khumairoh et al. 2012; Satapathy and Chand 2010).

Unlike other plant-microbial symbioses that are re-established from the environment with every new generation of plants, *N. azollae* is a permanent resident within *Azolla* spp. (Hill 1975; Perkins and Peters 1993; Peters and Calvert 1983; Ran et al. 2010). This suggests that the genes governing the interaction between these partners are different from other plant-microbial symbioses. The growth and development of *Azolla* spp. and *N. azollae* are precisely timed to one another, such that their life cycles are synchronized (Peters and Calvert 1983; Zheng et al. 2009b). *Nostoc azollae* exists as a colony of short, non-heterocyst-forming filaments at the apical shoot meristem of *Azolla* spp., and only develops into heterocyst-forming filaments once the leaf cavity has matured completely (Perkins and Peters 1993; Zheng et al., 2009a). *Nostoc azollae* is also passed on vertically to new generations of the fern (just as mitochondria are) during sexual reproduction in the form of akinetes within megaspores (Perkins and Peters 1993).

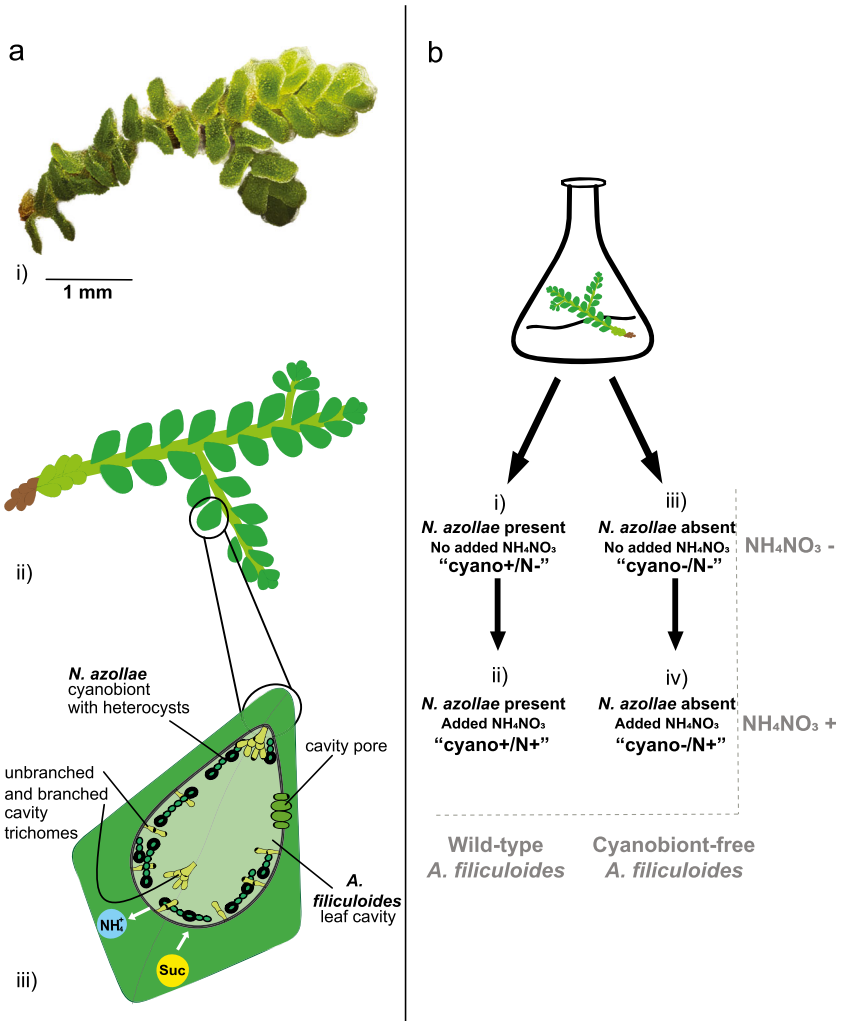


Fig. 1 *Azolla filiculoides*, plant details (**a**) and growth culture conditions (**b**). a. i. Close-up of plant, consisting of a primary stem with alternate leaves at regular intervals along the stem; secondary (branching) stems develop in the axil of certain leaves [image courtesy of Macroscopic Solutions (www.macroscopicsolutions.com). ii. Cartoon representation of *A. filiculoides*. Iii. Cartoon representation of an *A. filiculoides* leaf showing leaf cavity and contents; direction of sucrose and ammonium nutrient exchange between *N. azollae* and *A. filiculoides* is indicated. b. Growth-culture conditions for *A. filiculoides* prior to RNA-sequencing. i. Wild-type *A. filiculoides,* without added NH_4NO_3. ii. Wild-type *A. filiculoides,* with added NH_4NO_3. iii. Cyanobiont-free *A. filiculoides*, without added NH_4NO_3; iv. Cyanobiont-free *A. filiculoides*, with added NH_4NO_3. To obtain the putative symbiosis gene set, we focused on comparing gene expression differences between the optimal growth condition (i) and the three other growth conditions (ii-iv)

These two partners are inextricably linked, and it is believed that this partnership is obligate for both the plant and the cyanobacterium. About one third of the *N. azollae* genome is pseudogenized, including genes for processes such as DNA initiation (Ran et al. 2010). Though it still has genes for photosynthesis and maintains function of the photosystems when isolated (Ran et al. 2010; Ray et al. 1979), the photosynthetic capabilities of *N. azollae* are limited within the pocket, as exhibited by its reduced amounts of CO_2-fixation, Rubisco mRNA, and Rubisco and phosphorubulokinase protein compared to free-living *Nostoc* species (Braun-Howland and Nierzwicki-Bauer 1990; Ekman et al. 2008; Kaplan and Peters 1988).

Azolla spp. can be grown without their cyanobiont, but they are rarely seen in nature without *N. azollae* (Peters 1989). Cyanobiont-free *Azolla* spp. are still capable of developing the leaf pocket with its internal hair structures, but there are other components of the pocket missing, such as the mucilage that lines the cavity (Goff 1983). Experiments have also revealed that when grown without the cyanobiont, *A. filiculoides* needs exogenous nitrogen supplementation to grow at rates comparable to those observed in intact symbiosis (Hill 1975). However, the addition of external nitrogen sources to the intact symbiosis can disrupt it, resulting in decreased nitrogenase gene expression in the cyanobiont (Li et al. 2018). Together, these findings suggest that *N. azollae* still retains the ability to photosynthesize and *Azolla* spp. can still utilize external nitrogen sources, but when the symbiosis is intact and conditions optimal, they are reliant on one another.

Understanding this intricate symbiosis will provide new insight into how plants and microbes interact. There are many unique aspects of the *Azolla-Nostoc* symbiosis that we know little about, so we stand much to gain by investigating it. Of further importance, *Azolla* spp. have countless environmental benefits. In addition to their use as a green fertilizer, they are being studied as a source for biofuel production (Brouwer et al. 2014; Miranda et al. 2016; Muradov et al. 2014), use in bioremediation (Sood et al. 2012; Zazouli et al. 2014), and for their ability to remove carbon dioxide from the atmosphere to combat global climate change (Brinkhuis et al. 2006; Moran et al. 2006; Whaley 2007).

Despite its environmental potential, and the uniqueness of the symbiosis itself, the molecular underpinnings important to the *Azolla-Nostoc* symbiosis are unknown. The recent availability of reference genomes for both *A. filiculoides* (Li et al. 2018) and *N. azollae* (Ran et al. 2010) allows for an unprecedented new perspective. Here, we take a broad view of RNA-sequencing data that compares *A. filiculoides* both with and without its symbiont to obtain a sense of the potential key elements of this symbiosis to provide a foundation for future work.

2 Materials and methods

2.1 Plant materials

Whole plants of *Azolla filiculoides* were obtained, courtesy of Dr. Henriette Schluepmann, from The Netherlands, Utrecht, Galgenwaard ditch—the same *A. filiculoides* source that was used for genome and transcriptome sequencing in Li et al. (2018). All plants were surface sterilized prior to culturing. Some plants were cultured with the symbiosis intact (referred to as wild-type *A. filiculoides*), while others were cultured on erythromycin to remove the *N. azollae* cyanobiont (referred to as cyanobiont-free *A. filiculoides*, as described in Dijkhuizen et al. (2018)). Erythromycin is a bacteriostatic drug, limiting the growth of the cyanobiont, such that as *A. filiculoides* grows, the cyanobiont is depleted until it is cleared from the plant completely (Forni et al. 1991). The presence and absence of the cyanobiont was verified by examining intact leaves using fluorescence microscopy (Online Resource 1).

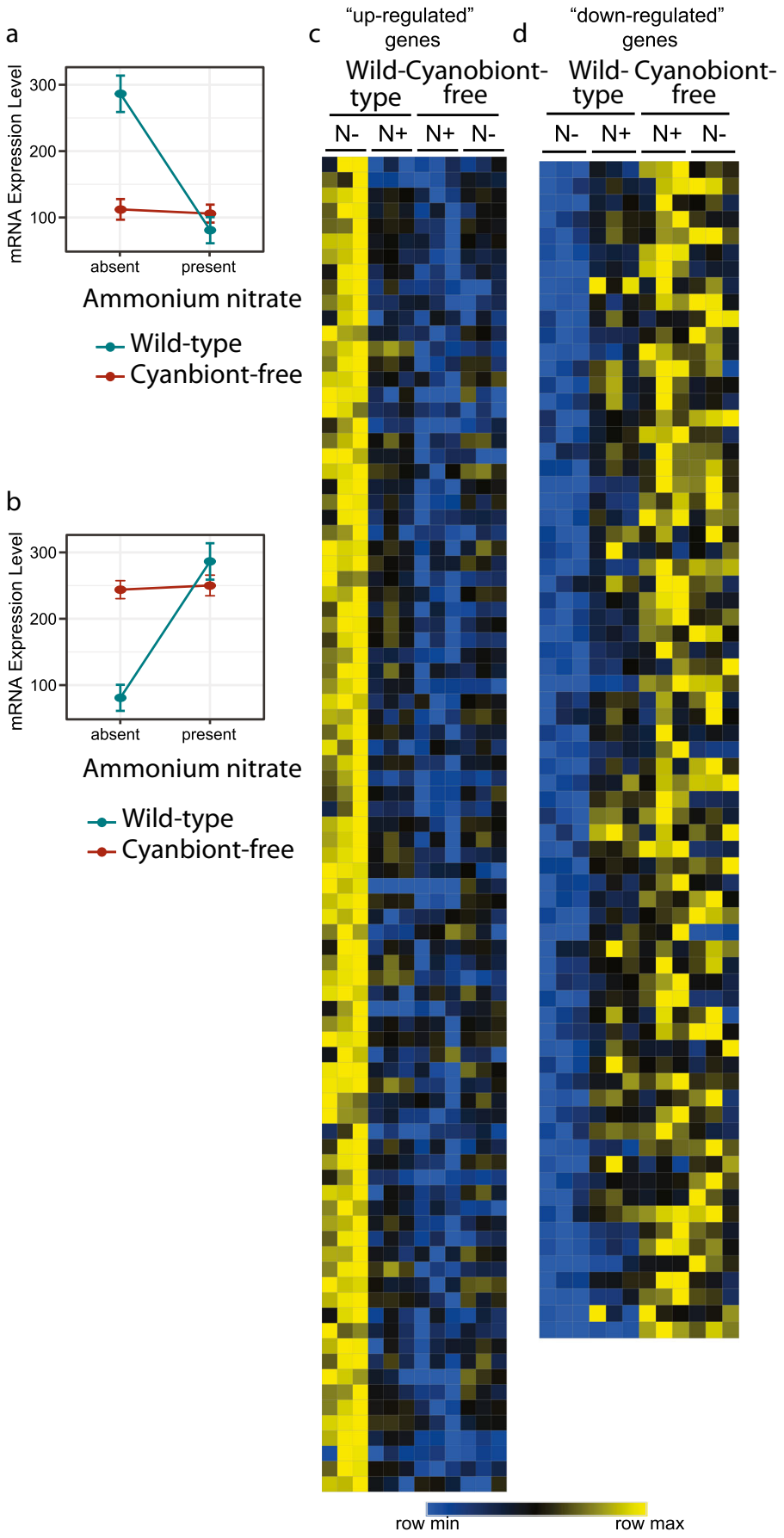
2.2 Plant culturing conditions

Cultures of wild-type *A. filiculoides* were maintained in IRRI 2 media, while cyanobiont-free *A. filiculoides* cultures were maintained in IRRI 2 media supplemented with ammonium nitrate (NH_4NO_3) prior to shifting them to experimental conditions (Watanabe et al. 1992).

Figure 1b (i-iv) depicts the four experimental conditions used for the *A. filiculoides* gene expression analysis. Plants were grown in three independent replicates in each experimental condition for two weeks prior to harvesting RNA for sequencing to allow them time to adapt to the experimental growth conditions. Wild-type *A. filiculoides* grown without NH_4NO_3 supplementation represents the natural/optimal growth conditions for this symbiosis (Fig. 1b, i). The other three conditions (wild-type *A. filiculoides* grown with NH_4NO_3; cyanobiont-free *A. filiculoides* grown without NH_4NO_3; and cyanobiont-free *A. filiculoides* grown with NH_4NO_3) are sub-optimal and address the loss of the cyanobiont (Fig. 1b, iii-iv); the response of *A. filiculoides* to external N supplementation (Fig. 1b, ii); and its response to N starvation (Fig. 1b, iii), respectively.

2.3 RNA-sequencing analysis and identification of putative symbiosis genes

Full details for RNA extraction and RNA-sequencing analysis of *A. filiculoides* are described in Li et al. (2018). Putative symbiosis genes were defined as genes whose mean mRNA expression levels were up-regulated or down-regulated in the optimal growth conditions for wild-type *A. filiculoides* compared to the other


a
mRNA Expression Level
300
200
100
absent
present
Ammonium nitrate
Wild-type
Cyanbiont-free
b
mRNA Expression Level
300
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absent
present
Ammonium nitrate
Wild-type
Cyanbiont-free
c
"up-regulated" genes
Wild-type
Cyanobiont-free
N-
N+
N+
N-
d
"down-regulated" genes
Wild-type
Cyanobiont-free
N-
N+
N+
N-
row min
row max

◀ **Fig. 2** Putative symbiosis genes. **a** Representative mRNA expression levels (number of transcripts) for an up-regulated symbiosis gene. **b** Representative mRNA expression levels (number of transcripts) for a down-regulated symbiosis gene. **c** Gene expression of the 88 up-regulated putative symbiosis genes. **d** Gene expression of the 72 down-regulated putative symbiosis genes. Each row is a gene, and its expression is shown as a log2-fold change normalized to that row. Yellow is maximum expression, blue is minimum expression. Information on the genes in each gene list can be found in Online Resources 2 and 3, respectively

experimental growth conditions (Fig. 1b, i vs. ii-iv; Fig. 2 a-b). Differential gene expression was analyzed in the following pairings (using an adjusted p value of 0.005):

(1) Wild-type vs. cyanobiont-free *A. filiculoides* (in each NH_4NO_3 condition)
(2) Wild-type *A. filiculoides* with vs. without ammonium nitrate
(3) Cyanobiont-free *A. filiculoides* with vs. without ammonium nitrate

Comparisons (2) and (3) identified genes differentially transcribed in response to *A. filiculoides* using external N sources or undergoing N starvation, respectively, and not to the intact symbiosis. These were used to trim the gene lists in comparison (1) to the putative symbiosis genes, defined as genes differentially transcribed in relation to the presence of the wild-type symbiosis (Online Resources 2 and 3). Data from the RNA-sequencing analyses can be accessed in the NCBI SRA under the BioProject PRJNA430527 and PRJNA430459, as well as through FernBase (www.fernbase.org). Expression data was visualized using Morpheus from the Broad Institute (https://software.broadinstitute.org/morpheus/).

2.4 Analysis of GS/GOGAT genes and proteins in *A. filiculoides*

Glutamine synthetase (GS) and glutamate synthase (GOGAT) were examined for gene expression differences and predicted post-translational modifications (PTMs). The expression levels for the GS/GOGAT genes were isolated from the larger RNA-sequencing dataset using their gene ids and visualized using Morpheus. The gene sequences were translated to amino acid sequences and analyzed for potential PTMs. Tyrosine nitration and serine phosphorylation (for 14–3-3 binding and proteolysis) were assessed by aligning the *A. filiculoides* GS amino acid sequences to those of *Medicago truncatula* (MtGS1 and MtGS2, using GenBank/EMBL accession numbers (Y10267 and AY225150, respectively) in AliView with Muscle (Edgar 2004; Larsson 2014; Online Resource 4). More general potential PTMs (particularly phosphorylation and ubiquitination) were assessed using the ModPred server (http://montana.informatics.indiana.edu/ModPred/index.html; Online Resources 5 and 6).

2.5 Predicted transcription factor binding enrichment in *A. filiculoides* symbiosis gene promoters

A cursory examination of transcriptional regulation in the *A. filiculoides-N. azollae* symbiosis was done by looking at predicted transcription factor binding. We compiled the DNA binding-site sequences for the transcription factors identified in our up-regulated symbiosis gene list (*WRKY*, *bHLH*, and *MADS*-box) using the JASPAR database (Sandelin et al. 2004). We gathered the promoter sequences for all the genes in the up- and down-regulated putative symbiosis gene lists, and from all the genes in the *A. filiculoides* genome (Li et al. 2018). Promoter sequences were obtained by selecting the 1000 base pairs upstream of each gene AUG start site (Ming et al. 2015). Custom scripts were used to find the DNA consensus sequence for each transcription factor within each gene promoter in our up- and down-regulated putative symbiosis gene lists, and in the whole genome as a control. A Fisher's exact test was used to evaluate the potential enrichment of predicted transcription factor binding sites in the promoters of the symbiosis gene lists compared to all the promoters in the genome. We used a subset of genes that showed the strongest putative symbiosis expression patterns for this analysis.

2.6 Gene ontology analysis of putative symbiosis genes

The lists of up- and down-regulated putative symbiosis genes were used for gene ontology enrichment analyses to identify and categorize the broad biological pathways and processes involved in the symbiosis between *A. filiculoides* and *N. azollae*, based on their annotations through the Gene Ontology Consortium (http://www.geneontology.org/). The goatools python script package (Klopfenstein et al. 2018), the annotated *A. filiculoides* genome (Li et al. 2018), and the go-basic.obo ontology file from the GO Consortium (Ashburner et al. 2000) were used to perform this analysis, which resulted in a table of different GO categories and the genes assigned to each (Online Resource 7). Custom python scripts and Rawgraphs (Mauri et al. 2017) were then used to summarize and visualize these results to see which GO categories were most relevant to the *A. filiculoides-N. azollae* symbiosis.

3 Results

3.1 Identification of putative symbiosis genes

RNA-sequencing analysis of wild-type *A. filiculoides* and cyanobiont-free *A. filiculoides* resulted in distinct expression patterns that involved the largest expression difference occurring between the optimal, wild-type *A. filiculoides* symbiosis growth conditions (Fig. 1b, i) and the three other experimental conditions (Fig. 1b, ii-iv). Genes with these expression patterns were considered "putative symbiosis genes" (Fig. 2 a-b). We found 88 transcriptionally "up-regulated" and 72 transcriptionally "down-regulated" putative symbiosis genes (Fig. 2 c-d) that were used to inform us about the symbiosis—particularly focusing on the main themes of the symbiosis: nitrogen transport and metabolism and sucrose transport.

Regarding nitrogen transport and metabolism, we observed up-regulation of an ammonium transporter and transporters of key metabolic co-factors, including a molybdate transporter and an iron transporter (gene ids *Azfi_s0034.g025227.AMT2*, *Azfi_s0167.g054529*, and *Azfi_s0018.g014823*, respectively, in Online Resource 2). However, we did not find differential expression of the ammonium assimilation enzymes glutamine synthetase (GS), glutamate synthase (GOGAT), or glutamate dehydrogenase (GDH) in our up-regulated putative symbiosis gene list (GS/GOGAT further examined in section 3.2).

One of the eight *A. filiculoides* GDH genes was observed in the down-regulated putative symbiosis gene list, along with one of the two *A. filiculoides* nitrate reductase gene (*Azfi_s0088.g042502* and *Azfi_s0006.g010439*, respectively, in Online Resource 3).

Additionally, no differentially-expressed sucrose transporters were observed, even though the *A. filiculoides* genome has three sucrose transporters and 15 SWEET transporters, which have been implied in the transport of sugars in nodules of leguminous plants (Kryvoruchko et al. 2016; Sugiyama et al. 2017).

3.2 Analysis of GS/GOGAT genes and proteins in *A. filiculoides*

The *A. filiculoides* genome has seven GS genes and 14 GOGAT genes that could be involved in nitrogen assimilation for the symbiosis (GS gene ids: *Azfi_s0033.g025083, Azfi_s0008.g011642, Azfi_s0002.g001200, Azfi_s0043.g026977, Azfi_s0092.g043135, Azfi_s0935.g093110, Azfi_s1095.g097334;* GOGAT gene ids: *Azfi_s0002.g001246 Azfi_s0002.g001983, Azfi_s0002.g002358, Azfi_s0025.g023211, Azfi_s0032.g024827, Azfi_s0044.g028329, Azfi_s0048.g030424, Azfi_s0135.g050516, Azfi_s0563.g077082, Azfi_s0563.g077084, Azfi_s0773.g086306, Azfi_s0773.g086307, Azfi_s2751.g112893, Azfi_s3593.g116342*).

Examining the gene expression patterns of the GS/GOGAT genes revealed a small subset that were differentially expressed across the experimental conditions (Fig. 3): five GS genes (gene ids: *Azfi_s0002.g001200, Azfi_s0008.g011642, Azfi_s0033.g025083, Azfi_s0043.g026977, Azfi_s0092.g043135*) and three GOGAT genes (gene ids: *Azfi_s002.g001246*, *Azfi_s0032.g024827*, and *Azfi_s048.g030424*). However, none of these differentially expressed GS or GOGAT genes had putative symbiosis expression patterns. No distinct expression patterns were observed that link these genes to any of the experimental conditions.

Given the lack of differential expression at the mRNA level of the GS/GOGAT genes, we examined the amino acid sequences of these genes for PTMs to explain how they may be regulated. We focused on the previously described tyrosine-167 nitration in *M. truncatula* GS1 (Melo et al. 2011) and the serine-97 phosphorylation in *M. truncatula* GS2 (Lima et al. 2005).

Aligning the *M. truncatula* and *A. filiculoides* GS sequences revealed that the *A. filiculoides* GS proteins do not have either the nitrated tyrosine-167 residue or the phosphorylated serine-97 residue, which is linked to proteolysis via 14–3-3 binding (Online Resource 4). Several of the *A. filiculoides* GS genes have other tyrosine residues that are distinct between *MtGS1* and *MtGS2*. The GS gene *Azfi_s0043.g26977* has tyrosine 108, and the GS genes *Azfi_s0008.g011642, Azfi_s0002.g001200,* and *Azfi_s0092.g043135* have tyrosine

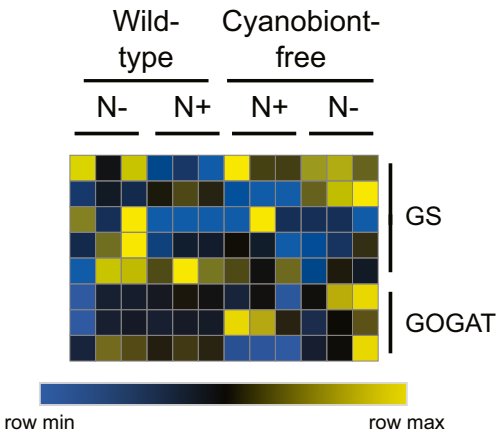

Fig. 3 GS/GOGAT gene expression in *A. filiculoides*. The gene expression pattern of the differentially expressed GS/GOGAT genes across the four experimental conditions. Only five GS and three GOGAT genes showed differential expression, but not in a putative symbiosis pattern. Each row is colored based on the maximum (yellow) and minimum (blue) of the log2 fold-change of gene expression for that gene. GS gene ids: *Azfi_s0002.g001200, Azfi_s0008.g011642, Azfi_s0033.g025083, Azfi_s0043.g026977, Azfi_s0092.g043135*. GOGAT gene ids: *Azfi_s002.g001246*, *Azfi_s0032.g024827*, and *Azfi_s048.g030424*

263 (Online Resource 4); however, these were shown to not be nitrated in *M. truncatula.* All of the *A. filiculoides* GS genes lack the N-terminal transit peptide and the C-terminal extension that are characteristic of the plastid-localized GS sequences, but they do appear to have the four conserved domains involved in the active site cylinder (Eisenberg et al. 1987).

The absence of these known PTMs led to us examine the sequences more broadly to identify other potentially modified sites. The GS protein sequences show several medium- and high-confidence phosphorylation and ubiquitination sites (Online Resource 5). The GOGAT large subunit protein sequences also had predicted phosphorylation and ubiquitination sites (Online Resource 6). For the GOGAT small subunit sequences, only two have high-confidence phosphorylation sites, but there are many predicted ubiquitination sites.

3.3 Predicted transcription factor binding enrichment in *A. filiculoides* symbiosis gene promoters

Four transcription factors—two *WRKY* transcription factors (*Azfi_s0250.g060244* and *Azfi_s0007.g011077,* Online Resource 2), a *bHLH* factor *(Azfi_s0319.g064443,* Online Resource 2), and a *MADS*-box factor (*Azfi_s0078.g038112,* Online Resource 2) were present in our set of up-regulated putative symbiosis genes (Fig. 4a). Computational analyses were done to examine the predicted binding enrichment of these transcription factors as a preliminary exploration of whether these up-regulated transcription factors may play a role in regulating the putative symbiosis gene set. Overall, their binding sites are not enriched in the promoters of the putative symbiosis genes.

The DNA consensus sequences for each transcription factor (Fig. 4 b-d) were highly represented in the promoters of the *A. filiculoides* genome. The *p* values for the *WRKY*, *MADS*-factor, and *bHLH* predicted transcription factor binding sites within the putative symbiosis gene promoters ranged from 0.134–0.247 and 0.004–0.341 in the up-regulated and down-regulated gene promoters, respectively (Table 1). Though the binding site sequences were prevalent in the promoters of the putative symbiotic genes (Fig. 5 a-b), only the *WRKY* transcription factor was significantly enriched (having a *p* value below the significance threshold of 0.005) in the down-regulated list (p value 0.004; Fig. 5b). No other transcription factor binding sites were significantly enriched in either putative symbiosis gene list; nor was the *WRKY* binding site enriched in the up-regulated gene list. *MADS*-box factor and *bHLH* binding sites were underrepresented, meaning there were fewer sites in the promoters of our putative symbiosis genes than in the promoters of the entire genome (Table 1).

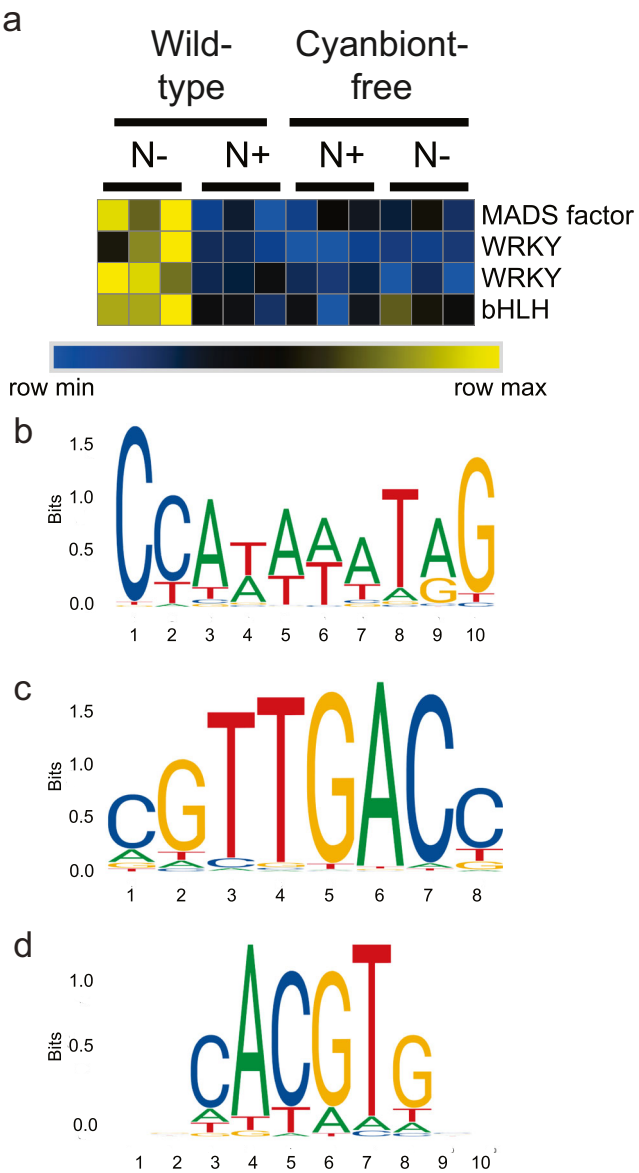

Fig. 4 Putative symbiosis transcription factors. **a** Gene expression pattern of up-regulated transcription factors in the putative symbiosis gene list between *A. filiculoides* sequencing conditions (see Fig. 1b). Each row is colored based on the maximum (yellow) and minimum (blue) of the log2 fold-change of gene expression for that gene. **b** DNA-consensus site for *MADS*-box factor transcription factor. **c** DNA-consensus sequence for the *WRKY* transcription factors. **d** DNA-consensus sequence for the basic helix-loop-helix (*bHLH*) transcription factor. Difference in letter size for bases indicates how conserved that base is at those consensus sites

3.4 Gene ontology analysis of putative symbiosis genes

To categorically examine the remainder of the putative symbiosis genes, gene ontology (GO) analysis was performed. The GO categories that were most enriched in our up-regulated putative symbiosis gene set were macromolecular metabolic processes, phosphorus metabolic processes,

Table 1 Transcription factor (TF) binding enrichment. Each transcription factor is shown with the number of genes that have binding sites (BS) in their promoters for genes in the up- or down-regulated putative symbiosis lists (Online Resources 2 and 3) compared with the number of genes that have binding sites (BS) in their promoters in the entire genome. The total number of genes with binding sites for each transcription factor in the entire genome is constant. A Fisher's exact test was used to calculate the *p* value

Putative symbiosis TFs	Genome-wide promoters with BS	Up-regulated putative symbiosis promoters with BS (p value)	Down-regulated putative symbiosis promoters with BS (p value)
WRKY	14,526	51 (0.134)	33 (0.004)
MADS box	3302	8 (0.247)	8 (0.341)
bHLH	5436	21 (0.193)	14 (0.330)

regulation of metabolic processes, and transport (Fig. 6a). The GO categories with the highest number of up-regulated genes are macromolecule and protein modification, protein phosphorylation, and protein metabolism—suggesting a possible important role for cell signaling and PTMs in mediating the *A. filiculoides-N. azollae* symbiosis. Many of these genes are kinases, such as calcium-dependent protein kinase (*Azfi_s0074.g037462*), leucine-rich receptor kinases (*Azfi_s0076.g037872*; *Azfi_s0078.g038285*; *Azfi_s0001.g001029*), and an ALE-2 kinase (*Azfi_s0078.g038258*; Online Resource 7). There are also different proteolytic enzymes, such as an asparaginase (*Azfi_s0179.g056280*) and cysteine protease (*Azfi_s0010.g012193*; Online Resource 7). Other enzymes present in this GO data include chalcone synthase (*Azfi_s0010.g012322*), which may be involved in communication between the two symbionts (see Discussion).

The down-regulated GO categories include ion transport, phosphorus metabolism, and genes involved in external structures (Fig. 6b). The transport category mainly entails ion transport, including zinc transport (*Azfi_s0065.g035730*), detoxification MAT-E efflux transporter (*Azfi_s0017.g014533*), and S-type anion channel (*Azfi_s0107.g045132*; Online Resource 7). The phosphorus metabolic processes are mostly involved in nucleotide synthesis or phosphate production (such as *Azfi_s0003.g008179* and *Azfi_s0241.g059717*; Online Resource 7). The full details of the gene ontology results are provided in Online Resource 7.

4 Discussion

4.1 Aspects of nitrogen metabolism in the *A. filiculoides-N. azollae* symbiosis

Our RNA-sequencing analysis of *Azolla filiculoides* revealed a broad swath of genes that are differentially expressed based on the presence or absence of *N. azollae* (Li et al. 2018). The list of putative symbiosis genes (Fig. 2) led to many questions about the *Azolla-Nostoc* symbiosis and highlighted its uniqueness. The two main nutrients exchanged in this symbiosis are ammonium and sucrose. Stable-isotope labeling studies have shown that *A. caroliniana* incorporates the ammonium it uptakes from *N. azollae* into glutamine and then glutamate for transport throughout the plant (Kaplan and Peters 1981; Meeks et al. 1987).

Previous research looking at the activity of nitrogen-assimilating enzymes in *A. caroliniana* also revealed high levels of glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH) activity in the intact wild-type symbiosis (Ray et al. 1978). In contrast, both GS/GOGAT activities were decreased in cyanobiont-free *A. caroliniana*, indicating their role in assimilating nitrogen from the symbiont. Additionally, there is a large body of work outlining the differential expression of these enzymes in crop plants such as alfalfa, soybean, rice, and wheat, including their expression in different tissues and cell-types and response to nitrogen source (Bernard et al. 2008; Goodall et al. 2013; Ishiyama et al. 2004; Ishiyama et al. 2003; Ortega et al. 1999; Ortega et al. 2001; Temple et al. 1998). There is also evidence that these genes are controlled at both the transcript and protein levels, and that changes in transcript level do not always correlate with protein levels, so both levels of regulation are important (Ortega et al. 1999; Temple et al. 1998).

We anticipated genes related to the metabolism and transport of ammonium to be differentially expressed or subject to PTMs to modify their activity or abundance—especially the *A. filiculoides* GS/GOGAT enzymes. While we did observe up-regulation of transporter genes important for this process (an ammonium transporter and co-factor transporters for molybdate and iron), we did not see putative symbiosis expression patterns in the ammonium assimilation enzymes (Fig. 3).

It is still possible that the GS/GOGAT genes are controlled transcriptionally, but our whole-plant approach to transcriptomics failed to capture the subtler tissue-specific or cell-type specific expression of these genes. To investigate whether this might be the case, in situ hybridization and protein localization would need to be carried out. These enzymes could also be regulated by unknown PTMs, since our data on the *A. filiculoides* GS/GOGAT protein sequences showed they lacked the PTMs shared with *Medicago* GS. It is also possible that binding of other proteins is involved, such as ARC11 or

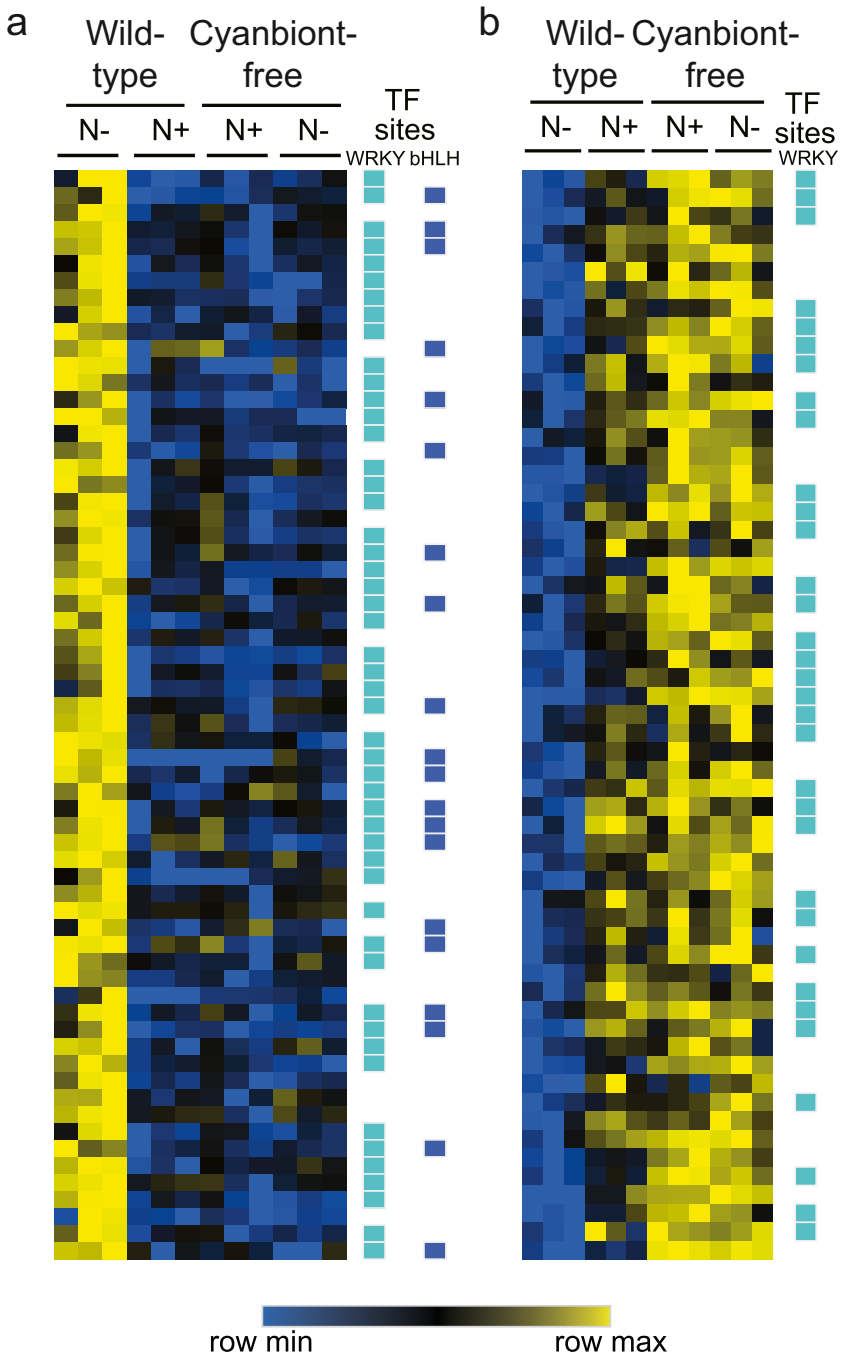

Fig. 5 Predicted transcription factor binding in *A. filiculoides*. **a** Gene expression of up-regulated putative symbiosis genes have a consistent pattern (high expression is yellow; low expression blue). Most have *WRKY* transcription factor binding sites (cyan); many have *bHLH* transcription factor binding sites; but there was no significant enrichment compared to the whole genome. **b** Down-regulated putative symbiosis genes have the opposite expression pattern compared to up-regulated putative symbiosis genes, and most still have *WRKY* transcription factor binding sites (cyan). Each row is colored based on the maximum (yellow) and minimum (blue) of the log2 fold-change of gene expression for that gene

14–3-3 proteins, because these can play a role in regulating the stability and activity of GS/GOGAT proteins (Finnemann and Schjoerring 2000; Lima et al. 2005; Lima et al. 2006; Osanai et al. 2017; Sung et al. 2011; Takabayashi et al. 2016). To this end, we observed two ACT-domain-containing proteins that were up-regulated in the putative symbiosis gene set, as well as one 14–3-3 protein (gene ids: *Azfi_s0261.g060759*, *Azfi_s001.g000807*, and *Azfi_s0055.g033895*, respectively; Online Resource 2), that might lead to understanding how the *A. filiculoides* GS/GOGAT proteins could be regulated. Work on the protein level is required to identify whether ACT-domain proteins or 14–3-3 proteins are binding to the *A. filiculoides* GS/GOGAT proteins.

Regarding GDH, the work in Ray et al. (1978) showed that GDH activity levels remain high regardless of the absence or presence of the symbiont. It could be a default means for nitrogen assimilation regardless of nitrogen source, or it could be functioning in a different way, such as maintaining nutrient balance or recycling cofactors. Of the eight GDH genes that *A. filiculoides* has, only one was expressed in a putative

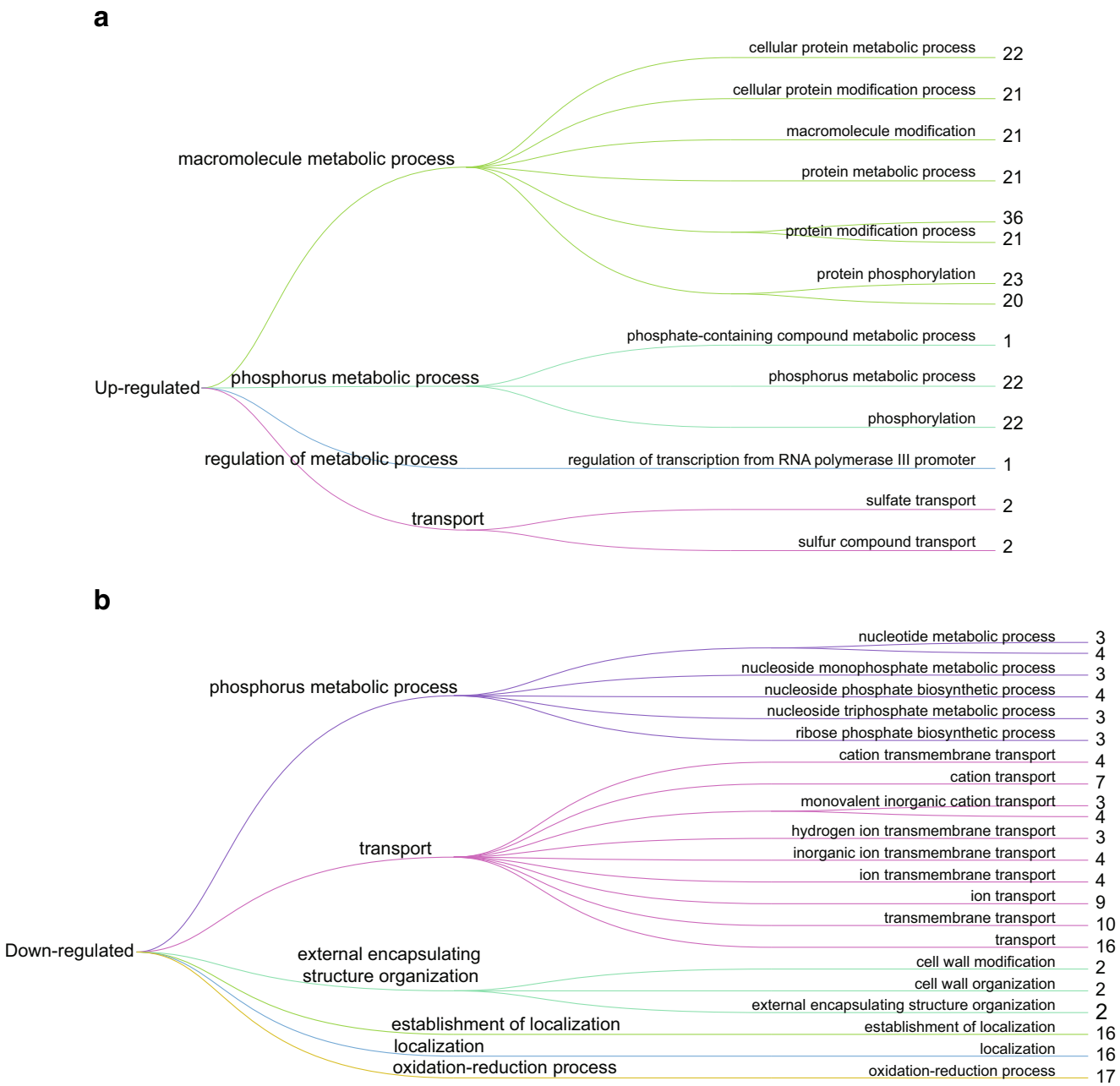

Fig. 6 Putative symbiosis gene ontology. **a** Top gene ontology categories from the up-regulated putative symbiosis gene list. **b** Top gene ontology categories from the down-regulated putative symbiosis gene list. Each category is denoted by line color, and the number of genes within the category is indicated at the end of each line. Gene IDs can be found in Online Resource 7

symbiosis gene expression pattern, but it was down-regulated. We do not know if any of them have cell-type specific expression or undergo post-translational modification.

The prevalence of protein modification genes in the putative symbiosis GO analysis (Fig. 6) might reflect the importance of this process in modulating the GS/GOGAT and GDH enzymes. One of the kinases present in our up-regulated putative symbiosis gene set in *A. filiculoides* was a calcium-dependent kinase (Fig. 6; Online Resource 7), and previous work in *M. truncatula* showed that their cytosolic GS proteins are differentially regulated by the activity of calcium-dependent kinases (Lima et al. 2006; Seabra and Carvalho 2015). The role of the calcium-dependent kinase could begin to be understood by examining the levels of phosphorylated GS/GOGAT proteins in *A. filiculoides,* and their activity, but this would only scratch the surface.

There is a lot to learn about nitrogen assimilation in the *Azolla-Nostoc* symbiosis. We now know how many different GS/GOGAT genes and GDH genes *A. filiculoides* has, but we still do not know their roles in this symbiosis because they did

not demonstrate differential expression patterns or contain PTMs identified in *Medicago*. Though this leads to more questions than answers, it indicates that further study of the nitrogen assimilation machinery in the *Azolla-Nostoc* symbiosis will result in new knowledge about how plants and microbes share nutrients. It is also possible that new discoveries will be made regarding sucrose transport, because our results showed the *A. filiculoides* sucrose transporters lacked a putative symbiosis expression pattern.

4.2 Communication and recognition between the two partners

Much remains to be learned about how *A. filiculoides* and *N. azollae* communicate with, and recognize, one another. The *Azolla-Nostoc* symbiosis shares some aspects observed in intracellular nitrogen-fixing symbioses, and other aspects observed in extracellular plant-microbe symbioses, but does not completely match either. Because the common symbiosis pathway (CSP) is one of the most completely understood networks for how plants interact with microbes, we used this as a starting point for comparison. In both plant-fungal (AM) and plant-bacterial (RN) symbioses, kinase signaling is important for triggering the expression of genes needed to form the symbiotic structures. This signaling cascade is reliant on kinases from different families that respond to Myc-factors (for AM symbioses) or Nod-factors (for RN symbioses), including a LysM receptor-like kinase (such as MtLYR1 for AM and MtNFP for RN), a leucine-rich-repeat receptor kinase (such as MtDMI2), and a calcium-calmodulin kinase (MtDMI3), which eventually modulates transcription for the formation of the structures needed for AM or RN symbioses (MacLean et al. 2017; Messinese et al. 2007; Oldroyd 2013).

Though the *A. filiculoides* genome lacks homologues for the CSP, we did find three leucine-rich receptor kinases in the up-regulated putative symbiosis gene set (Online Resource 2). However, because the symbiotic cavity and necessary structures for the *Azolla-Nostoc* symbiosis form even in cyanobiont-free *Azolla* spp., these kinases are likely doing something different than the leucine-rich receptor kinases in AM and RN symbioses. Overall, there are many kinases up-regulated in the GO results, and their role in this symbiosis remains to be determined.

Work in other extracellular symbioses, which could be more like the *Azolla-Nostoc* symbiosis, are beginning to show how these symbiotic partners communicate as well. Flavonoid compounds are among the factors that may be crucial signaling molecules for these symbioses (reviewed in Martin et al. 2001; Martin et al. 2016). We do see enzymes involved in flavonoid and phenolic compounds in the GO data for *A. filiculoides* (Online Resource 2). One such promising enzyme for how *A. filiculoides* may signal to *N. azollae* is chalcone synthase. This enzyme functions to produce the flavonoid naringenin chalcone, which is a precursor for many diverse flavonoids (Abdel-Lateif et al. 2013). As pointed out in Li et al. (2018), naringenin (a product made from naringenin chalcone) has been shown to inhibit hormogonia production in *Nostoc* species (Cohen and Yamasaki 2000). It is possible that naringenin is the signal that triggers *N. azollae* to begin developing from hormogonia into heterocyst-containing filaments to start fixing nitrogen in the leaf pocket. To test the effect of naringenin on the symbiosis between *A. filiculoides* and *N. azollae,* heterocyst formation could be measured upon application of exogenous naringenin.

Enzymes, such as chorismate synthase or dihydroflavonol 4-reductase (DFRA), that are important for the production of aromatic organic compounds were also seen up-regulated in the RNA-seq data from comparison 1 that looked holistically at the genes differentially expressed when the cyanobiont was present or absent (see Section 2.3; data not shown). DFRA is indicated in anthocyanin production, and previously deoxyanthocyanins from *Azolla* spp. have been shown to induce hormogonium-suppression in *Nostoc punctiforme* (Cohen et al. 2002). There is great potential for further investigation of these genes to help us outline the pathway for how cyanobacteria and plants communicate with each other. In general, plant-cyanobacterial symbioses are poorly understood, so this could be an important opportunity to address a large gap in our knowledge of plant-microbial symbioses.

4.3 Control of gene expression

Examining the differentially expressed genes related to the *Azolla-Nostoc* symbiosis is a necessary step for understanding how the symbiosis works. It is also important to understand how these genes are differentially expressed. Previous studies have examined the roles of transcription factors in plants, especially in legumes during the process of nodulation (Cerri et al. 2016; Kumar et al. 2016; Liu et al. 2015; Moreau et al. 2011). In *M. truncatula*, several transcription factors were important for nodule-specific gene expression including *WRKY* and *NAC*, among others. *WRKY* transcription factors have also been shown to be important components of the signal transduction pathway for bacterial recognition and immune response in plants (Bakshi and Oelmüller 2014; Kumar et al. 2016; Samad et al. 2017). Even though *WRKY*, *bHLH*, and *MADS*-box transcription factors are up-regulated in the *A. filiculoides-N. azollae* symbiosis (Fig. 4a; Table 1), we only found enrichment of putative *WRKY* transcription factor binding sites in the promoters of the down-regulated putative symbiosis genes. To fully understand the role of transcriptional regulation in this symbiosis, further experiments are needed such as ChIP-seq to determine which promoters these transcription factors bind.

4.4 New insights into plant-cyanobacterial symbiosis

This first pass at investigating the transcriptional profile of the *Azolla-Nostoc* symbiosis to identify putative symbiosis genes allows us to plan future experiments to better explore this relationship and understand its uniqueness. Our work has shown that the *A. filiculoides-N. azollae* symbiosis is likely using very different strategies than previously observed in other plant-microbial symbioses. Further analysis of the GS/GOGAT and GDH genes and proteins will be crucial for answering questions that get to the heart of the nutrient exchange in this symbiosis. The transcriptomics data will help us begin to unravel what signals are required for reliable communication between the partners, and provide guidance for further research to understand this aspect of the symbiosis. It is also important to note there are numerous genes that are not functionally annotated in the *A. filiculoides* genome that are present in the putative symbiotic gene set. As we begin to uncover more about this symbiosis, the functions of these genes could reveal unique ways of engagement between *Azolla* and *N. azollae*. Together, these results provide an exciting opportunity to discover new ways plants and microbes interact.

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Compliance with ethical standards

Conflict of interest Authors ANE, F.-W.L., and KMP declare that they have no conflicts of interest.

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