Serendipita bescii promotes winter wheat growth and modulates the host root transcriptome under phosphorus and nitrogen starvation

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Summary

Serendipita vermifera ssp. bescii, hereafter referred to as S. bescii, is a root-associated fungus that promotes plant growth in both its native switchgrass host and a variety of monocots and dicots. Winter wheat (Triticum aestivum L.), a dual-purpose crop, used for both forage and grain production, significantly contributes to the agricultural economies of the Southern Great Plains, USA. In this study, we investigated the influence of S. bescii on growth and transcriptome regulation of nitrogen (N) and phosphorus (P) metabolism in winter wheat. Serendipita bescii significantly improved lateral root growth and forage biomass under a limited N or P regime. Further, S. bescii activated sets of host genes regulating N and P starvation responses. These genes include, root-specific auxin transport, strigolactone and gibberellin biosynthesis, degradation of phospholipids and biosynthesis of glycerolipid, downregulation of ammonium transport and nitrate assimilation, restriction of protein degradation by autophagy and subsequent N remobilization. All these genes are hypothesized to regulate acquisition, assimilation and remobilization of N and P. Based on transcriptional level gene regulation and physiological responses to N or P limitation, we suggest S. bescii plays a critical role in modulating stress imposed by limitation of these two critical nutrients in winter wheat.

Introduction

Wheat (Triticum aestivum L.) is a cereal grown on more land area than any other commercial food crop. Hard red winter wheat is a dominant dual-purpose (forage and grain) crop in the Southern Great Plains (Kansas, Oklahoma, and Texas), with approximately 8 million ha planted every year. In the state of Oklahoma, roughly 2 million ha of winter wheat are cultivated annually accounting for 75% of the state’s total cropland (Patrignani et al., 2014). NF101 is a hard red winter wheat cultivar developed by the Noble Research Institute, LLC and is a top performing dual-purpose cultivar in Oklahoma and North Texas.

Phosphorus (P) is an essential element for many fundamental processes in plant life, including photosynthesis, respiration, cellular membrane construction, and signal transduction, yet is limiting in most soils. Concentrations of P in the soil are typically 10 μM or less, and readily form sparingly soluble salts with Ca, Fe and Al (Piezynski and McDowell, 2005). Wheat consumes much more P fertilizer than rice (Oryza sativa L.) and maize (Zea mays L.) every year (Yuan et al., 2016), and the average yield gap of winter wheat production due to P limitation is estimated to be 22% worldwide (Kvakić et al., 2018).

Nitrogen (N) also plays a critical role in plant biochemistry, being an integral component of many compounds, including chlorophyll, enzymes, amino acids and proteins. Generally, N is considered to be the single most important factor for determining wheat productivity and grain quality around the world (Sinclair and Rufty, 2012; Ma et al., 2019). However, N use efficiency (NUE) in cereals is generally poor, where it is estimated that only 30%–40% of the total of N-fertilizers applied are actually harvested in the grain (Belete et al., 2018). More than 100 Tg of reactive N is produced annually, of which 50% is applied to three major cereals (wheat, 18%; maize, 16%; rice, 16%) that provide the bulk of human food calories and proteins consumed either directly as grain or indirectly through livestock products (Ladha et al., 2016).

One approach to improve nutrient acquisition, and potentially N and P use efficiency, is to utilize beneficial plant microbes. Mycorrhizal symbioses are ubiquitous associations occurring between soil fungi and the root systems of most plant species. Benefits imparted to the...
plant due to such associations include increased mineral nutrient absorption, water-use efficiency, disease resistance, and plant-to-plant communication (Behie and Bidochka, 2014; Rillig et al., 2016). Nutrient transfer from the mycorrhizal partner to the plant host is crucial, especially in nutrient limiting soils (Whiteside et al., 2019). Consequently, studies on such symbioses wherein nutrient transfer to plants has largely focused on limiting soil nutrients, such as N and P, are critical (Averill et al., 2019).

The Serendipitaceae (Oberwinkler et al., 2014) is a taxonomically, physiologically and ecologically diverse family of fungi in the Basidiomycota that include endophytes and lineages that appear to have repeatedly evolved ericoid, orchid and ectomycorrhizal lifestyles (Weiss et al., 2016). Members of this fungal group have been shown to exhibit plant growth-promoting properties in maize (Varma et al., 1999), tobacco (Nicotiana attenuate L.) (Sherameti et al., 2005), switchgrass (Panicum virgatum L.) (Ghimire et al., 2009; Ray et al., 2015), barley (Hordeum vulgare L.) (Waller et al., 2005) and wheat (Serfling et al., 2007; Ray and Craven, 2016). Like the more studied arbuscular mycorrhiza (AM), Serendipita symbionts often provide N and P to their host plant (Sherameti et al., 2005; Yadav et al., 2010), particularly in nutrient limiting soil (Nurfadilah et al., 2013). Approximately 95% of the N present in the soil is in the organic form, covalently bound to carbon (C) and requiring huge amounts of energy to release a biologically available form. Intriguingly, genome analysis of S. bescii and S. vermifera suggests that Serendipitaceae fungi have the metabolic capacity to assimilate N from organic forms of N-containing compounds (Ray et al., 2019). In light of their proven beneficial impact on plant growth and nutrient acquisition in winter wheat and numerous other important crops, Serendipitaceae fungi should be considered as an effective tool for enhancing plant productivity and stress tolerance in nutrient poor soils. However, wider implementation of the Serendipitaceae fungi in agricultural contexts is limited by a scarcity of suitable strains. Our group has previously identified a new strain of Serendipita, Serendipita vermifera ssp. bescii (hereafter referred to as S. bescii), which seems to be well adapted to the specific agro-climatic conditions of the Southern Great Plains, USA (Craven and Ray, 2017; Ray et al., 2018b).

In this study, we used a transcriptional approach combined with root phenotyping and estimation of forage biomass to characterize the physiological responses and regulation of N and P metabolism in NF101 winter wheat when colonized by S. bescii. Such an approach will improve our understanding of the role of S. bescii in modulating host plant stress imposed by limitation of these two critical nutrients.

Results

Serendipita bescii colonizes wheat root cells intracellularly

Confocal microscopic images of root cells of winter wheat cultivar NF101 colonized by S. bescii are shown in Fig. 1. Early stages of S. bescii colonization are marked by sparse hyphal growth between cells. At later stages, cortical root cells became completely packed with aggregates of moniloid (bulbous) fungal hyphae. These structures resemble hyphal protrusions bearing terminally swollen parts formed by some ericoid and orchid mycorrhiza (Vohník et al., 2012; Ray and Craven, 2016), as well as other Serendipitaceae fungi (Riess et al., 2014).

![Fig 1. Serendipita bescii colonized root cells of winter wheat cultivar NF101 14 dpi by confocal microscopy. Colonization is typically marked by cortical root cells packed with aggregates of swollen moniloid fungal hyphae (green cells, right panel). Fungal hyphae stained with WGA-AF 488 were visualized in the green channel. Plant cell walls stained with propidium iodide were visualized in the red channel. Images from these channels were overlaid to show fungal colonization in plant cells.](image-url)
Serendipita bescii colonization improves root growth and forage biomass production

Serendipita bescii significantly increased the number of lateral root numbers (Fig. 2) at the early stage (14 dpi) and forage biomass (Fig. 3) at later stages (74 dpi) of colonization in winter wheat under N or P-limitation, compared to uninoculated controls. The number of lateral root numbers in *S. bescii*-inoculated wheat seedlings at 14 dpi increased by 36% over uninoculated controls in either N or P limiting conditions, whereas shoot biomass at 74 dpi increased by 100% and 38% under N and P limiting conditions respectively. Importantly, these improvements were not observed when plants were treated with optimum levels of N and P. Increases in plant height due to *S. bescii* colonization at 74 dpi were numerically high, but statistically insignificant with an uninoculated plant (Fig. 3). Additionally, we did not find any significant impact on shoot nitrate or phosphate content due to *S. bescii* colonization under nutrient replete and limiting conditions.

Distribution of differentially expressed wheat genes due to *S. bescii* colonization

At 14 dpi, we identified 1516, 962 and 1159 differentially expressed (DE) wheat genes under nutrient replete, N-limitation and P-limitation conditions, respectively. Under the same conditions, at 74 dpi, we have identified 675, 149 and 165 DE genes respectively. Out of these, 534 and 447 genes at 14 dpi, 80 and 106 genes at 74 dpi were differentially expressed specifically due to *S. bescii* colonization under N- and P-limitation conditions (Supporting Information Table S1). Network analysis of all six sets of DE genes based on their expression profile is shown in Fig. 4. DE genes for all the three nutrient treatments were tightly clustered together, in a colonization stage-dependent manner (Fig. 4). Interestingly, N-limitation at the early stage is dominated by down-regulated genes, whereas at the late stage, both N- and P-limitations are dominated by upregulated genes. A hierarchical clustering heatmap of DE genes due to *S. bescii* colonization.

**Fig 2.** Effect of *S. bescii* colonization on root architecture (left) of winter wheat cultivar NF101at 14 days post inoculation *in vitro* under full N and P, N- and P-limitations. Significant increases in total number of root tips (right) were observed under N- or P-limiting conditions when colonized with *S. bescii* with respect to the uninoculated control. Blue dots denote mean values. Boxes with different letters denote significant difference between treatments at \( P < 0.05 \).
colonization under nutrient replete, N- and P-limitation conditions, and having an absolute value of log2 fold-change difference ≥ 4 is shown in the Supporting Information Fig. S1. The DE genes showed a discrete pattern for both stages of colonization within individual nutrient treatments.

**KEGG enrichment analysis of differentially expressed genes**

Under nutrient replete conditions, we identified 26 and 14 KEGG pathways at 14 and 74 dpi, respectively that were enriched according to Fisher’s exact test. Similarly, under the N-limitation condition, 11 and 14 pathways, and under the P-limitation condition, 27 and 20 pathways, were identified at 14 and 74 dpi, respectively (Supporting Information Table S3). Enrichment analysis of the top 10 pathways based on P-value, for all three treatments and at two different stages of colonization is shown in Fig. 5.

**Regulation of P and N metabolism in plants**

**Root architecture**

Genes encoding multi-copper oxidases have been found to inhibit primary root growth under low P (Svistoonoff et al., 2007). Under the P-limited conditions examined here, four multi-copper oxidase genes were upregulated due to *S. bescii* at early stage of colonization (Supporting Information Table S4). Of these four genes, two were co-induced under N limitation at the same stage. Auxin has been reported to play critical roles in both root development during P starvation (Müller and Schmidt, 2004), and a nitrate-mediated signalling pathway for lateral root elongation (Sun et al., 2017). Several auxin-responsive genes
were differentially expressed specifically due to *S. bescii* colonization both under nutrient replete and depleted conditions (Supporting Information Table S2). Of those, one known as Air 12 (auxin induced in roots) (Traes_5BL_AD9944DCC) and putatively involved in lateral root morphogenesis (Gibson and Todd, 2015) was downregulated both under N or P starvation conditions during early stages of colonization. In addition, three genes, encoding auxin efflux carrier proteins that are involved in root specific auxin transport were upregulated under nutrient replete and P starvation condition at the same stage (Supporting Information Table S4). In contrast, these auxin efflux carrier proteins were downregulated in the absence of *S. bescii* suggesting a role for the fungal symbiont in regulating auxin transport.

Ethylene modulates auxin biosynthesis, transport and signalling to fine-tune root growth and development (Qin and Huang, 2018). We found three ethylene biosynthesis genes that were specifically downregulated during the early stage of colonization under N and P starvation conditions (Supporting Information Table S4). One root hair-specific gene (Traes_3DL_2F30F7D2D) putatively involved in root hair elongation was specifically downregulated at the early stage of colonization due to either N or P starvation.

**Phosphate (Pi) transport and utilization**

Hydrolases such as phosphatases and ribonucleases are involved in releasing Pi from organic sources and are

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greatly induced by P deficiency in model plant Arabidopsis and rice (Plaxton and Tran, 2011). We found one (Traes_5BS_F389AB55C) ribonuclease H-like superfamily protein putatively involved in triacylglycerol degradation, and one purple acid phosphatase gene (Traes_2BL_859881F87) that were specifically downregulated at the early stage of colonization under P-starvation conditions (Supporting Information Table S4).

**N uptake and remobilization**

Plants take up nitrate as the preferred source of N when it is available in abundance (Crawford and Glass, 1998). When *S. bescii* was absent, three nitrate transporters were induced upon N-limitation. However, when the fungus was present, all of them were repressed in under nutrient replete condition, whereas none of them was differentially expressed under N-depleted condition (Supporting Information Table S2). Conversely, we identified two ammonium transporters in the plant (Traes_6AL_CCF2A8825, Traes_6DL_3286E5385) that were specifically downregulated at the early stage of colonization under N-starvation conditions, again when colonized by *S. bescii* (Supporting Information Table S4).

After entering the plant cell, ammonium is eventually transformed into the key N donor glutamine, via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway. We identified a SNF1-related protein kinase gene (Traes_3DL_6103E4919), putatively involved in nitrate assimilation, and an amidase gene (Traes_2DS_6B79F7E51) that acts on glutamine to free up cellular N to be up- and downregulated respectively under N-starvation, only when *S. bescii* was present (Supporting Information Table S4).

Autophagy and the ubiquitin-proteasome mechanism are the two major protein degradation pathways in plants, potentially liberating N as a consequence. In this study, we found three each of aspartyl proteases and cysteine proteinases genes putatively involved in the autophagy pathway that were specifically downregulated due to *S. bescii* colonization under N-starvation conditions (Supporting Information Table S4). After protein degradation, released amino acids are typically loaded into phloem vessels by amino acid permeases (AAP) and oligopeptide transporters (OPT). We found two AAPs and three OPTs to be downregulated due to *S. bescii* at the early stage of colonization under N-starvation (Supporting Information Table S4).

The MtN21 protein-encoding genes are reported to act as bidirectional transporters of amino acids such as glutamine and are able to export amino acids out of cells when necessary (Ladwig et al., 2012). We found, two MtN21 genes (Traes_3AS_3B1466678, Traes_3B_848719122) to be down- and one upregulated (Traes_5BL_CBB9D7E7F) under the N limitation condition at 14 and 74 dpi respectively (Supporting Information Table S4).

**Biosynthesis of phytohormones and secondary metabolites**

Strigolactones are carotenoid-derived plant hormones and are recognized as important local and systemic...
signals involved in the regulation of shoot architecture and acclimatization of plants to P-limiting conditions (Czarnecki et al., 2013; Kapulnik and Koltai, 2016). Additionally, abscisic acid (ABA) and gibberellic acid (GA) are involved in P starvation signalling in plants (Baek et al., 2017). ABA has also been implicated in the nitraremediated signalling pathway for lateral root elongation (Harris and Ondzighi-Assoume, 2017). In our study, two putative strigolactone esterase D14 genes (Traes_4BL_D05623E45, Traes_4BL_D62825306) involved in the strigolactone signalling pathway, and one GA 2-oxidase 6 gene (Traes_1DS_125A395C1) was upregulated under P starvation condition at the later stage of colonization (Supporting Information Table S4).

P starvation typically induces the regulation of secondary metabolites such as glucosinolates, flavonoids and phenylpropanoid compounds such as anthocyanins (Steyn et al., 2002; Jiang et al., 2007). A flavonoid biosynthesis gene (Traes_4AS_E7B757DEA) and an anthocyanin biosynthesis gene (Traes_4BL_8AA727112) were found to be downregulated specifically due to S. bescii at 14 dpi under both P- and N-limiting conditions. Additionally, three sulfotransferase genes that are putatively involved in biosynthesis of glucosinolates were upregulated when S. bescii was present at 74 dpi, under P-limitation conditions (Supporting Information Table S4).

Membrane lipid remodelling

Membrane lipid remodelling during P starvation consists of degradation of phospholipids, to release Pi and the synthesize glycolipids to substitute for phospholipids (Pant et al., 2015). We found, genes involved in degradation of phospholipids (Traes_3B_5BB189B41, Traes_7BL_11E10C066), glycerolipid (Traes_3B_8E820A790, Traes_3B_C5168A652) and fatty acid biosynthesis (Traes_4AS_080C79870, Traes_4DL_74F91F3BD) that were upregulated upon P-starvation when the fungal symbiont was present (Supporting Information Table S4).

Discussion

Serendipita bescii colonization promotes host plant growth

Serendipitaceae fungi often provide N and P to the host plant, particularly in nutrient-limited conditions and in orchids and ericaceous plants (Nurfadilah et al., 2013; Wu et al., 2019). In this study, we report the plant growth promoting potential of S. bescii, an endophyte we isolated from switchgrass roots and inoculated onto the roots of winter wheat under N- or P-limiting conditions. When S. bescii was present, we found a significant increase in both the number of lateral root numbers in young wheat seedlings and forage biomass in more mature plants, specifically under nutrient-depleted conditions in S. bescii colonized plants. This finding is consistent with the AM symbiosis, wherein the fungus promotes plant growth under suboptimal N and P, but incongruent with S. indica (a close relative of S. bescii), whose influence on plant growth promotion seems to be independent of N and P levels (Achatz et al., 2010). In this study, we did not record any S. bescii-induced plant growth promotion under optimum N and P conditions. Further, Barazani et al. (2005) and Achatz et al. (2010) showed that S. indica did not significantly increase shoot N and P content in tobacco and barley. We also did not find any significant impact on shoot nitrate or phosphate acquisition due to S. bescii colonization under nutrient replete and limiting conditions. This could be primarily due to the fact that, shoot nutrient acquisition and plant growth promotion are not necessarily positively correlated phenotypic traits.

Regulation of P starvation response in winter wheat by S. bescii

Plants respond to P-limitation with a variety of morphological, physiological, biochemical and molecular changes collectively called the phosphate starvation response (PSR), which may improve P acquisition and/or mobilization leading to improved P use efficiency. In this study, we propose that S. bescii modulates at least some of the PSR response in winter wheat, such as a reduction in primary root elongation and a corresponding increase in the number of lateral roots. This is a critical part of the PSR, trait employed by plants to increase their effective root surface area in search of available Pi (López-Bucio et al., 2002; Chevalier et al., 2003). Serendipita bescii colonization under P starvation upregulated four mult copper oxidase genes (Supporting Information Table S4) known to be involved in primary root inhibition, as well as increased lateral root numbers suggesting their role in modulating the PSR.

Genes encoding plant hormones not only play critical roles in plant growth and development but also in plant-microbe symbioses. P-starvation typically impact plant hormone levels, with auxin, ethylene and strigolactones categorized as low P-induced hormones that positively regulate the PSR, and cytokinins and gibberellins are known as low P-repressed hormones that negatively impact the PSR (Scheible and Rojas-Triana, 2018). Partially in support of this notion, we found that P starvation in plants colonized by S. bescii induced the upregulation of genes involved in root-specific auxin transport, and, the strigolactone signalling pathway, both positive PSR regulators, and GA 2-oxidase 6 gene responsible for inactivation of bioactive GA. Several reports suggest that
such inactivation of bioactive GA leads to increasing tillering in monocots such as rice and switchgrass (Lo et al., 2008; Wuddineh et al., 2015). While this is not the subject of the current study, but we have consistently seen increased tillering in switchgrass and winter wheat when colonized with S. bescii both in greenhouse and field conditions.

When Pi is unavailable, plants synthesize certain types of ribonucleases (RNAses) and purple acid phosphatases (PAPs) that can access and mobilize phosphate from RNA and other organic compounds during P starvation. We found that S. bescii colonization downregulated RNAses and PAPs under P-limiting conditions suggesting its role in host P remobilization.

Finally, phospholipids are fundamental components of plant membranes and, during phosphate starvation, phospholipids are degraded, and conversely, a non-phosphorus galactolipids accumulates in the root plasma membrane of plants (Nakamura et al., 2005) leading to the release of Pi needed by the plant cells. In our study, plants with S. bescii and grown under limiting P, upregulated genes involved in glycerolipid biosynthesis and genes responsible for degradation of phospholipids. This suggests that S. bescii may play a critical role in host lipid metabolism, at least under P-limiting conditions.

Serendipita bescii colonization modulates N metabolism in winter wheat

N deprivation has multiple consequences for plant fitness, including a decreased rate of photosynthesis, coupled to an increased translocation of photosynthate to roots, resulting in a decreased shoot:root ratio. Macroscopically, we observed yellowing of leaves and a severe reduction in forage biomass of uninoculated wheat plants grown under N- and P-limitation. Significantly, these phenotypes were largely alleviated when the fungal symbiont was present (Fig. 3).

In terms of gene expression, N starvation conditions in plants inoculated with S. bescii, exhibited a down-regulation of ammonium transporters and genes involved in nitrate assimilation in plant cells. To maintain essential cellular functions for surviving in an N-limiting environment, plants release N from leaves, grain, and other source tissues to meet its requirement (Kant et al., 2010). Amino acids released from protein degradation are eventually loaded into the phloem for remobilization from source to sink tissues. We found that under N-limited conditions, S. bescii colonization seemingly controlled the degradation of storage proteins by downregulating several genes belonging to aspartyl proteases and cysteine proteinases known involved in autophagy. Further, colonized plants repressed several amino acid and oligopeptide transporters involved in amino acid translocation, restricting N-remobilization. These findings along with improved biomass data (Fig. 3) support a role for S. bescii in dealing with N-limitation and improving plant growth through a distinct, as of yet unknown mechanism.

To conclude, we show that S. bescii colonization increases the number of lateral roots and improved forage biomass in winter wheat under a nutrient-limited regime. To alleviative host responses related to nutrient limitation, S. bescii activates plant genes involved in the adaptive P and/or N starvation response to help the host plant survive under nutrient limiting conditions. Based on this transcriptome regulation and the observed physiological responses to N or P limitation, we suggest that S. bescii has the potential to alleviate stress imposed by limitation of these two critical nutrients in winter wheat.

Experimental procedure

Fungal material and culture conditions

Serendipita bescii strain NFPB0129 was isolated from a switchgrass plot located in Oklahoma, USA (Ray et al., 2018b) and used in this study. The strain was routinely maintained in Modified Melin Norkan’s (MMN) media (Marx, 1969), with 1% glucose as a C source at pH 6.0 in 24°C.

Plant material

Seeds of hard red winter wheat cultivar NF101 were surface sterilized following the protocol modified after Xi et al. (2009). Briefly, seeds were washed with 50% Clorox® (8.25% sodium hypochlorite, Clorox, Oakland, CA, USA) containing 0.1% Tween® 20 (Amresco, Solon, OH, USA) for 30 min. After three washes with sterile water, seeds were soaked in sterile water and kept at 4°C overnight. Next day, seeds were treated one additional time with 50% Clorox® for 30 min, washed with sterile water for three times and air dried on sterile filter paper.

Colonization of wheat seedlings in vitro

In vitro colonization was performed using Magenta™ GA-7 plant culture boxes (7.5 × 7.5 × 10 cm) with lids (Magenta LLC, Lockport, IL, USA). The boxes were filled with 100 ml of M medium (Schultz, 2013) pH 5.5, containing 0.3% phytagel® and amended with 1% sucrose. N and P starvation were imposed by preparing M Media without any nitrate or phosphate respectively. M media supplemented with nitrate and phosphate were prepared as controls for direct comparison. Surface sterilized wheat seeds were plated onto these media.
Inoculum of S. bescii was prepared by following the protocol described in the study by Ray et al. (2018a). Briefly, starter culture for inoculation was prepared by growing S. bescii in 5 ml of standard M media for 2 weeks in 50 ml Falcon tube. After 2 weeks, mycelia was harvested by brief low speed centrifugation (5000g; 2 min), washed with sterile water for three times to get rid of residual media. Subsequently, the inoculum was prepared by grinding 50 mg of fresh, washed vegetative mycelia in 500 μl of sterile water. In vitro colonization was achieved by injecting 20 μl of aqueous mycelial suspension directly into the media with a sterile pipette (Ray et al., 2015). Seedlings were maintained in a TCR-180 Conviron® growth chamber at 24°C, 50% RH and 16 h photoperiod for 2 weeks (Controlled Environments Ltd, Winnipeg, MB, Canada). After 2 weeks, a subset of in vitro colonized and the corresponding uninoculated control plants were transplanted into one-gallon trade pots (2.68 l, Stuewe and Sons, Inc. Tangent, OR, USA) filled with Metro-Mix®360 (Sun Gro® Horticulture, Agawam, MA, USA) and transferred to the greenhouse. The Metro-Mix®360 was pre washed with clear water to get rid of residual N or P. The plants were maintained in the greenhouse for 60 days (24°C, 50% RH, 16 h photoperiod), i.e. 74 days post inoculation (dpi) and watered with either optimum, or N- or P-deficient ½ strength Hoagland’s solution (Caisson Labs, Smithfield, UT, USA) respectively. The entire experiment was conducted with three biological replicates for each treatment.

**Visualization of S. bescii colonization by confocal microscopy**

Colonization of wheat roots by S. bescii was confirmed by fluorescence staining and confocal microscopy following the protocol described in the study by Ray et al. (2018a). Briefly, colonized roots were fixed in 50% ethanol, cleared with 20% KOH, and stained with WGA-AF 488® (Life Technologies, Carlsbad, CA, USA), followed by counter staining with propidium iodide (PI: Biotium, Hayward, CA, USA) to visualize fungal mycelia and plant cell wall respectively. Stained root samples were visualized with a Leica TCS-SP8 point scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) (Fig. 1).

**Estimation of root and forage biomass production**

Roots were harvested at 14 dpi from in vitro culture boxes and the total number of root tips were counted using the WinRHIZO™ Pro (Regent Instruments Canada) root image analysis software following the manufacturer’s instructions. Shoot biomass was harvested from greenhouse grown plants at 74 dpi. Within each nutrient treatment, the data were analysed by one-way ANOVA using CoStat statistical software 6.4 (Cohort Berkeley, CA, USA). Treatment means were compared using LSD value at $P \leq 0.05$. The data were plotted graphically using the package ggplot2 (Wickham, 2016) in R studio® (R Studio, Boston, MA).

**RNA sequencing and data analysis**

At harvest (14 dpi, early stage; 74 dpi, late stage), total RNA was isolated from root tissues using an RNaseasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA-seq libraries were prepared using TruSeq Stranded mRNA sample preparation kit (Illumina, San Diego, CA). Individual libraries were uniquely indexed using TruSeq single indexes (Illumina), and pooled in equimolar ratio. The pooled libraries were sequenced on a NextSeq 500 Sequencing system (Illumina).

The reads for each sample were quality trimmed to remove any low quality bases and primer/adaptor sequences. Reads less than 30 bases long after trimming were discarded, along with their mate pair. The trimmed reads were then mapped to version 2.2 of the *T. aestivum* genome (Phytozome v12.1) using HISAT2 (Kim et al., 2015). The identified transcripts were compared with the reference genome’s set of transcripts using Stringtie (Pertea et al., 2015). Differential expression testing was performed using DESeq2 (Love et al., 2014). To identify genes whose expression was regulated in response to S. bescii colonization in the nutrient replete condition and either of the N- or P-depleted conditions, we performed pairwise comparisons and only considered genes having log2 fold change ≥2 and adjusted $P$-value <0.05 as biologically significant (Supporting Information Table S2). RNA-seq data have been submitted to the DDBJ/EMBL/GenBank databases under accession number PRJNA573046.

**Network analysis and hierarchical clustering of differentially expressed genes**

Visualization of biologically significant wheat genes based on their expression data across all the treatments and two time points were done in a force-directed graph to infer the relationship network using the web-based visualization tool Divenn (Sun et al., 2019). Up or downregulated genes were represented by red or blue nodes respectively. The yellow node denotes genes upregulated in one treatment but downregulated in the other (Fig. 4).

Hierarchical clustering of differentially expressed genes having an absolute value of log2 fold-change difference ≥ 4 was done to depict global changes of differentially expressed genes across all treatments and time points. The heat map representation of hierarchical...
clustering was generated using the function heatmap.2 in the package gplots (Warnes et al., 2016) in R studio® (R Studio) (Supporting Information Fig. S1).

**KEGG pathway enrichment analysis of wheat genes**

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of wheat genes was done using KOBAS 3.0 (Xie et al., 2011). Enrichment analysis was done based on Fisher’s exact test with FDR-corrected P-value < 0.05. The rich factor is calculated as the ratio of the numbers of DEGs enriched in this pathway, to the total number of genes annotated in the same pathway. Top 10 pathways based on FDR-corrected P-values, for all three treatments at two different stages of colonization were plotted graphically using the package ggplot2 (Wickham, 2016) in R studio® (R Studio) (Fig. 5).

**qRT-PCR validation of candidate genes**

Quantitative RT-PCR was performed with three replicates from independent biological experiments to verify differential transcription of the candidate genes following the protocol described in the study by Yi et al. (2009), using QuantiTect®RT-kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction (Supporting Information Fig. S2). Real-time PCR was run on the Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Normalization and comparison of mean CT (threshold cycle) values were calculated following the method by Livak and Schmittgen (2001). To compare relative abundance of transcripts of target genes, the mean CT of triplicate reactions was normalized by wheat actin 2 (wACT2), which is one of the most stably expressed genes (Tenea et al., 2011).

**Estimation of relative abundance of S. bescii in planta**

The relative abundance of *S. bescii* in the different nutrient treatments and across different time points was estimated by qPCR. The wheat ACT2 (wACT2) (Tenea et al., 2011) and *S. bescii* ACT1 (sACT1) genes were used as indicators for calculation of relative abundance. Based on haploid genome size of each organism (wheat: 34Gb, *S. bescii*: 37 Mb) and their ploidy as well as karyotype and C-value (Dolezel, 2003), we calculated that 23 ng of wheat gDNA and 0.3 ng of *S. bescii* gDNA will contain similar numbers of ACT gene templates (7953 copies and 7929 copies respectively). Based on this calculation, a dilution series of *S. bescii* gDNA (0.24, 0.18, 0.12, 0.06 ng) was prepared by mixing each with 23 ng of wheat gDNA, and the CT values were measured to generate a standard curve (*S. bescii*/wheat template ratio (%) = 98.03922 × 2^−ΔCt, R² = 0.09901). Finally, the relative abundance of *S. bescii* in wheat root was quantified by using this standard curve from the ΔCt value of the root DNA (Supporting Information Fig. S3).

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different stages of *S. bescii* colonization under nutrient replete, N-, and P-limitation conditions. The signal ratios are shown in a red-blue colour scale, where red represents up-regulation and blue represents down-regulation. Each column namely, Full N P, N limitation, P limitation for early and late stage represents log2 fold-change value and each row represents a differentially expressed gene. The hierarchical clustering between all the differentially expressed genes were shown on the left tree.

**Figure S2.** qRT-PCR validation of representative genes. Bars denote mean log2 fold change. Stacked bar denote representative gene is differentially expressed in more than one treatment.

**Figure S3.** Estimation of relative abundance of *S. bescii* in planta. (A) Standard curve (B) Relative abundance of *S. bescii* in wheat root across different nutrient treatments in two different stages of colonization

**Supplementary Table 1.** Number of differentially expressed wheat genes for all three nutrient treatments at two different stages of colonization

**Supplementary Table 2.** List of differentially expressed genes, for all three nutrient treatments at two different stages of colonization. Differentially expressed genes are represented as log2 transformed values. Differential expression testing was performed using DESeq2. Genes were considered significantly differentially expressed between nutrient-replete and -deplete conditions if it passed the significance threshold requiring the adjusted p-value <0.05 and an absolute value of log2 fold-change difference ≥ 2.

**Supplementary Table 3.** List of enriched KEGG pathways, for all three nutrients treatments at two different stages of colonization. The background number is the total number of genes in a certain pathway. The input number is the number of differentially expressed genes mapped to the indicated pathway. The Rich factor is the ratio of the number of differentially expressed genes to the total gene number in a certain pathway. FDR-corrected p-value <0.05 was considered as significantly enriched.

**Supplementary Table 4.** List of all the differentially expressed wheat genes induced by *S. bescii*, that plays critical role in fungal colonization, nitrogen and phosphorus metabolism.

**Supplementary Table 5.** List of all the primers used in the current study.