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Diverse *Sorghum bicolor* accessions show marked variation in growth and transcriptional responses to arbuscular mycorrhizal fungi

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Abstract

Sorghum is an important crop grown worldwide for feed and fibre. Like most plants, it has the capacity to benefit from symbioses with arbuscular mycorrhizal (AM) fungi, and its diverse genotypes likely vary in their responses. Currently, the genetic basis of mycorrhiza-responsiveness is largely unknown. Here, we investigated transcriptional and physiological responses of sorghum accessions, founders of a bioenergy nested association mapping panel, for their responses to four species of AM fungi. Transcriptome comparisons across four accessions identified mycorrhiza-inducible genes; stringent filtering criteria revealed 278 genes that show mycorrhiza-inducible expression independent of genotype and 55 genes whose expression varies with genotype. The latter suggests variation in phosphate transport and defence across these accessions. The mycorrhiza growth and nutrient responses of 18 sorghum accessions varied tremendously, ranging from mycorrhiza-dependent to negatively mycorrhiza-responsive. Additionally, accessions varied in the number of AM fungi to which they showed positive responses, from one to several fungal species. Mycorrhiza growth and phosphorus responses were positively correlated, whereas expression of two mycorrhiza-inducible phosphate transporters, *SbPT8* and *SbPT9*, correlated negatively with mycorrhizal growth responses. AM fungi improve growth and mineral nutrition of sorghum, and the substantial variation between lines provides the potential to map loci influencing mycorrhiza responses.

KEYWORDS

arbuscular mycorrhiza, bioenergy, cereal, phosphate transporter, root, symbiosis, transcriptomes

1 | INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is a C_4 grass grown primarily for grain and animal feed across several continents with diverse climatic and environmental gradients. It is characterized by its tolerance to drought- and heat-stress, and its improved water and nutrient use efficiencies compared with other grasses (de Vries, van de Ven, van Ittersum, & Giller, 2010; Shakoor et al., 2014). Sorghum is grown as a staple cereal crop for millions of people in African countries and as a forage crop in other areas that are often characterized by harsh environments and marginal lands. More recently, sorghum has been targeted as a bioenergy feedstock crop due to the ability of sweet and cellulosic sorghum varieties to accumulate nonstructural (sugars) or structural carbohydrates (cellulose) in their stems (Mullet et al., 2014; Murray et al., 2008a, b). There are a number of comprehensive genetic resources available for sorghum, such as the sequenced

reference genome (Mace et al., 2013; Paterson et al., 2009), numerous diversity and association panels (Brenton et al., 2016; Casa et al., 2008; Deu, Rattunde, & Chantereau, 2006; Murray, Rooney, Hamblin, Mitchell, & Kresovich, 2009), an expression atlas (Shakoor et al., 2014), and germplasm databases (e.g., ICRISAT and GRIN), which are invaluable tools for research into improvement of sorghum both for grain and bioenergy production.

As with many crop species, achieving optimum yield of sorghum biofeedstocks relies on the plant's access to sufficient soil nutrients, particularly inorganic phosphate (Pi) and nitrogen (as NH_4^+ or NO_3^-; Epstein & Bloom, 2005). Although sorghum shows high nutrient-use efficiency, fertilization still results in increased yield (Anfinrud, Cihacek, Johnson, Ji, & Berti, 2013), and in most production environments, fertilizers account for 8–15% of sorghum production costs (Olson et al., 2013). Phosphate fertilizer consumption also comes at a sustainability cost, as the world's reserves of phosphate rock are rapidly decreasing, with no renewable alternative or other solution yet identified (Cooper, Lombardi, Boardman, & Carliell-Marquet, 2011; Herrera-Estrella & López-Arredondo, 2016). Therefore, development and research into potential agricultural management strategies that reduce fertilizer input without a significant detriment to yield or nutritional quality of the crop will be vital to the sustainability of both bioenergy and grain production (He & Nara, 2007; López-Arredondo, Leyva-González, González-Morales, López-Bucio, & Herrera-Estrella, 2014; Pieterse, de Jonge, & Berendsen, 2016).

Arbuscular mycorrhizal (AM) fungi are soil-borne, globally ubiquitous fungi that can colonize plant roots and provide an additional pathway for the uptake and transfer of mineral nutrients from the soil to the plant (Smith & Read, 2008). The hyphal network of AM fungi in the soil extends beyond the nutrient depletion zone of roots and allows AM fungi to access nutrients and transfer them to the plant. AM fungi have been shown to significantly improve the yield and nutrition of many plant species under abiotic stress such as nutrient deficiency and drought (Jayne & Quigley, 2014; Lehmann & Rillig, 2015; Lehmann, Veresoglou, Leifheit, & Rillig, 2014; Watts-Williams & Cavagnaro, 2014). There is potential for the symbiosis between AM fungi and sorghum to improve sorghum yield and nutrition on nutrient-depleted soils (Raju, Clark, Ellis, & Maranville, 1990). This could in turn lead to a reduction in the dependence on fertilizer application to reach competitive yields in developed countries, or an improvement on current yields without fertilizer input in developing countries (Bender & van der Heijden, 2015; Menge, 1983).

The genotype or cultivar of the host plant has been shown to influence the responsiveness (i.e., in growth or nutrition) of that plant to AM fungal inoculation (e.g., in wheat: Hetrick, Wilson, & Cox, 1992; maize: Sawers et al., 2017; barley: Baon, Smith, & Alston, 1993; *Medicago truncatula*: Schultz, Kochian, & Harrison, 2010; durum wheat: Ellouze et al., 2015; onion: Taylor et al., 2015), as has the species of colonizing AM fungus (Burleigh, Cavagnaro, & Jakobsen, 2002; Klironomos, 2003). A number of important crop species have shown potential in their ability to be bred for mycorrhizal responsiveness, that is, improvement in plant growth through the formation of arbuscular mycorrhiza (e.g., in maize: Kaeppler et al., 2000; wheat: Zhu, Smith, Barritt, & Smith, 2001; durum wheat: Singh, Hamel, DePauw, & Knox, 2012). Comparatively, our knowledge of AM symbiosis in sorghum is limited to a few studies (Bagayoko, George, Romheld, & Buerkert, 2000; Walder et al., 2012), and the effects of plant genetic diversity, which is important for breeding more responsive cereal crops (Sawers, Ramírez-Flores, Olalde-Portugal, & Paszkowski, 2018), has not been considered. At the molecular level, analyses are fairly limited. Genes encoding mycorrhiza-inducible phosphate transporters (PTs) and ammonium transporters (AMTs) in sorghum have been reported and their expression patterns studied (Koegel et al., 2013; Walder et al., 2015); however, a sorghum mycorrhizal root transcriptome has not been reported.

Sorghum is a diverse species with five major races (Bicolor, Caudatum, Durra, Guinea and Kafir) that show much phenotypic variation both between and within each race (Harlan & Stemler, 1976). To enable use of this wealth of diversity, various core collections have been established and genotyped (Brenton et al., 2016; Morris et al., 2013; Sukumaran et al., 2012) An association panel that focuses on bioenergy sorghum and comprises 390 sweet and cellulosic biomass sorghum accessions has been established (Brenton et al., 2016), and the parents of a nested association mapping (NAM) population were selected from this collection (S. Kresovich, personal communication, September 5, 2018).

In this study, we surveyed a subset of the parents of the sorghum bioenergy NAM population for their ability to develop AM symbiosis with *Glomus versiforme*. We then generated mycorrhizal root transcriptomes from which a core set of sorghum genes responsive to colonization was identified. Finally, we analysed all parents of the NAM population for their physiological responses to four diverse AM fungi. The combination of physiological and transcriptional data reveals considerable variation in symbiosis among these lines and may inform future use of recombinant inbred lines and the NAM population for mapping loci underlying symbiotic responses.

2 | MATERIALS AND METHODS

2.1 | Experiment 1: Evaluating mycorrhizal colonisation and expression of AM induced genes in diverse sorghum accessions

This experiment evaluated 14 *S. bicolor* bioenergy accessions and BTx623 for variation in the extent of mycorrhizal colonization and expression of AM fungal marker genes after inoculation with *G. versiforme*. It also confirmed AM fungal-induced phosphate transporter genes via RNA sequencing.

2.2 | Plant growth conditions

Seeds of 14 diverse *S. bicolor* accessions (three replicates per accession), the parent accessions of the Bioenergy Association Panel, and BTx623, the accession used for genome sequencing (kindly provided by Stephen Kresovich; Table S1), were surface-sterilized with 5% bleach for 10 min while shaking, before being rinsed thoroughly with deionized (DI) water, and planted directly into pots of autoclaved fine sand. Seeds were germinated in a growth chamber with a day/night temperature of 22°C/20°C and 16 hr/day length, for 5 days. Seedlings were then transplanted into Ray Leach Cone-tainers (Steuwe and

Sons) with 164 ml capacity containing a 16:4:1 (*v*/*v*/*v*) mix of fine sand (average particle size 200–300 μm), gravel (heterogeneous particle size 300–1,000 μm), and sterilized field soil (one plant per cone). Approximately 300 surface-sterilized *G. versiforme* spores were pipetted onto a thin sand layer about 5 cm from the rim of each Cone-tainer. Each treatment was replicated four times. The field soil used was collected from Cornell University's Homer C. Thompson Vegetable Research Farm near Freeville, New York (42.52149786436779, −76.33040097684062), from a field that had not received supplemental fertilization. The soil was a Phelps type and contained 6.85 mg P, 3.99 mg NO_{3}-, 104.96 mg K, 1578.53 mg Ca, 269.23 mg Mg, 15.17 mg Al, 1.78 mg Fe, 16.26 mg Mn, and 0.42 mg Zn kg soil^{-1}, had a pH of 6.2, and an organic matter content of 3.86%. The field soil was air-dried and sieved to <2 mm, before being autoclaved two times at 55 min per cycle, with at least 48 hr between cycles.

Following transplantation into cones, plants were grown in a greenhouse at the Boyce Thompson Institute in Ithaca, New York, between the months of December 2015 and January 2016, with 16 hr/day length, day/night temperature of 26°C/22°C, and supplemental lighting. Plants were watered twice daily with DI water and nutritionally supplemented twice weekly with 10 ml of modified ¼ strength Hoagland's solution (20 μM phosphate) and weekly with 2 ml of insoluble phosphate in the form of 0.5 M $CaHPO_4$ as a slow-release source of phosphate.

2.3 | Harvest and physiological analyses

Plants were destructively harvested 42 days post-transplantation into the Cone-tainers. Shoots were removed and dried in the oven at 65°C for at least 48 hr, before the shoot dry weight (SDW) was recorded. Roots were carefully washed out of the soil with DI water, blotted dry, and a subsample of the fresh roots was taken and flash frozen in liquid nitrogen for RNA isolation. Another subsample of fresh roots was placed into 70% EtOH for staining. Fresh root subsamples were cleared in 20% KOH at room temperature for 7 days, rinsed thoroughly with DI water, then stained using a 5% ink in vinegar solution (following Vierheilig, Coughlan, Wyss, & Piche, 1998) whereby roots were boiled for 3 min in the ink/vinegar solution and then rinsed thoroughly with DI water and allowed to destain for at least 24 hr in DI water acidified with vinegar added to 2% (*v*/*v*). Root samples were then analysed for total internal % root length colonized by AM fungi, following the gridline intersect method (Giovannetti & Mosse, 1980).

Flash frozen roots were ground and homogenized using a chilled mortar and pestle under liquid N_2, and a ~100 mg subsample of ground material was used to isolate total RNA (Clontech NucleoSpin Total Plant RNA kit), which included on-column DNase treatment (Clontech). First strand cDNA was synthesized from 500 ng of total RNA using dNTP mix and 50 U SuperScript III Reverse Transcriptase (Invitrogen) including RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen) and diluted with ddH_2O to 2.5 ng/μL.

To obtain gene expression data on the AM fungal marker genes, *G. versiforme* α-tubulin and *SbPT11*, quantitative RT-PCR was performed on an ABI PRISM 7900 HT sequence detection system (Applied Biosystems) using 10 ng (4 μl) of diluted cDNA in a 10 μl reaction using 0.3 μM of each gene-specific forward and reverse primer (Table S2) and SYBR Green PCR Master Mix (Applied Biosystems). PCR conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and finally a dissociation stage. Threshold cycle (CT) values were calculated automatically by the SDS 2.4 software (Applied Biosystems) and were normalized to the CT values of the *S. bicolor* housekeeping gene elongation factor-1α (*SbEF1α*). Relative expression values were then calculated and averaged over three biological replicates.

2.4 | Experiment 2: Exploring sorghum growth and nutrient responses to different AM fungal species

Having ascertained that variation for the AM symbiosis exists across the diverse sorghum accessions, we undertook an experiment to assess growth and nutrient responses of 18 accessions to four species of AM fungi. Experiment 1 did not provide a fair assessment of this parameter because it was a short experiment in small pot conditions not suited to observing biomass of large bioenergy sorghum plants.

2.5 | Plant growth conditions

Seeds of 18 diverse *S. bicolor* accessions (four replicates per accession) were germinated, as in Experiment 1. Seedlings were then transplanted into Ray Leach Cone-tainers (Steuwe and Sons) containing a 2:2:1 (*v*/*v*/*v*) mix of fine sand, black sand (heterogeneous particle size 200–5 mm), and gravel (one plant per cone). Each cone contained a 20 ml layer (equivalent to 35 g) of root-based mycorrhizal fungal inocula of one of *G. versiforme*, *Rhizophagus irregularis* (syn. *Glomus intraradices*), *Claroideoglomus claroideum*, *Gigaspora gigantea*, a 1:1 mix of *G. versiforme* and *C. claroideum*, or a mock root inoculum. The *Gi. gigantea* treatment was supplemented with 10 spores per cone, owing to the lower inoculum potential of the root-based inocula for this species compared with others. Each treatment was biologically replicated four times.

The root-based inocula were made by growing *Brachypodium distachyon* (Bd21) in cones (three plants per cone) filled with sand/gravel mix and inoculated with one of: 300 *G. versiforme* spores, 500 *R. irregularis* spores, 50 *C. claroideum* spores, 20 *Gi. gigantea* spores, or a mock treatment from the final wash of the *G. versiforme* spore extraction, and grown for 10 weeks in a growth chamber. Shoots were removed and the root ball, including sand/gravel substrate, was cut into 1 cm segments. An equivalent volume of sand was then added and mixed thoroughly with the roots. This mix was then dried at room temperature before being used as inoculant.

The root-based inocula of each AM fungal species were tested on *S. bicolor* (Rio) plants grown in sand/gravel mix and inoculated with either 10, 20, or 30 ml of the inocula in a layer placed one third of the way from the top of the cone. Plants were grown for 4 weeks in a temperature- and light-controlled greenhouse before being destructively harvested. Roots were washed free of the sand/gravel mix and placed into 70% EtOH for 24 hr before being cleared in 20% KOH and stained with AlexaFluor. Stained roots were observed under a compound microscope and assessed for inoculation by the gridline

intersect method (Giovannetti & Mosse, 1980). The plants inoculated with 20 ml of *R. irregularis*, *G. versiforme*, and *C. claroideum* root-based inocula achieved a similar colonisation rate. *Gi. gigantea* inoculum colonized plants to a lesser extent, so the root-based inoculum was supplemented with 20 *Gi. gigantea* spores in the main experiment.

Following transplantation into cones, plants were grown in a greenhouse at the Boyce Thompson Institute in Ithaca, New York, between the months of April and July 2016, with 16 hr/day length, day/night temperature of 26°C/22°C, and supplemental lighting. Plants were grown for 30 days in the Cone-tainers, were watered twice daily with DI water, and nutritionally supplemented twice weekly with 10 ml of modified ¼ strength Hoagland's solution (20 μM phosphate) and weekly with 2 ml of insoluble phosphate in the form of 0.5 M $CaHPO_4$. This growing period in the cones served to facilitate the effective colonization of the roots by AM fungi before plants were allowed a greater rooting volume.

After 30 days, the entire root ball and substrate were moved from the cone, into a Mini-Treepot (Steuwe and Sons) with 1.64 L capacity, containing a mix of 16:4:1 (*v*/*v*/*v*) play sand, gravel, and sterilized field soil. After transplantation to Mini-Treepots, plants were supplemented twice weekly with 20 ml of modified ¼ strength Hoagland's solution (20 uM phosphate) and once weekly with 4 ml of insoluble phosphate in the form of 0.5 M $CaHPO_4$. After 2 weeks in the Mini-Treepots, some plants developed visual signs of nutrient deficiency through leaf yellowing and necrosis, and all plants were supplemented with 20 ml of a modified ½ strength Hoagland's solution (1x N and 20 uM P), for two fertilization events. The visual signs of nutrient deficiency were ameliorated (yellowing) or halted (necrosis), and regular fertilization regime was resumed.

2.6 | Harvest and sample analyses

After growing for 49 days in the Mini-Treepots, plants were destructively harvested as follows: shoot height measurements were taken, shoot and root biomass were separated, shoot fresh weight was recorded, and the samples were placed in an oven at 65°C for at least 48 hr, before shoot dry weight (SDW) was recorded. Roots were carefully washed out from the sand/gravel/soil mix using DI water and the fresh root weight was recorded. Owing to the large size of the roots, for each plant, the top and bottom third of the root system were cut-off, the middle third was mixed by hand, and a weighed subsample of the fresh roots was taken and flash frozen in liquid nitrogen for RNA isolation. Another weighed subsample of fresh roots was taken from the mixed middle third of the root system and stored in 70% EtOH for staining. The remaining roots were dried in an oven at 65°C for at least 48 hr, before root dry weight was recorded; the ratio of fresh to dry root weight was used to extrapolate to total root dry weight (RDW).

For the mock-, *G. versiforme*-, and *R. irregularis*-inoculated plants, we conducted nutrient analyses. It was not possible to carry out this analysis for all samples and we selected those colonized by *G. versiforme* and *R. irregularis* because they showed the most consistent growth increases and because these fungi are widely used in the symbiosis community, so the data have the highest potential to be useful to other researchers. For nutrient analyses, dried leaves were separated from stems, weighed, and ground in a KitchenAid BCG111ER blade coffee grinder for 1 min each. A subsample of ~200 mg of ground leaf tissue was prepared for nutrient analysis by overnight digestion at room temperature in 4 ml of concentrated $HNO_3/HClO_4$ (40/60 *v*/*v*). The predigested samples were then heated to 120°C for 2 hr, then 190°C for 30 min before being allowed to cool to room temperature and diluted with 20 ml of ultra-pure (18 MΩ) water. Total mineral content of the leaf samples was then determined using inductively coupled plasma-emission spectrometry (ICP 61E trace analyzer, Thermo Jarrell Ash, Waltham, MA, USA). The detection limit for P was approximately 20 ppb.

Fresh root subsamples were cleared in 20% KOH at room temperature for 7 days, rinsed thoroughly with DI water, then stained using a 5% ink in vinegar solution (following Vierheilig et al., 1998). The roots were boiled for 3 min in the ink/vinegar solution and then rinsed thoroughly with DI water and allowed to destain for at least 24 hr in DI water acidified with approximately 200 μl vinegar. Roots were viewed under a compound microscope to confirm presence of internal structures, and representative photos of colonization were captured (Figure S1). All genotypes showed colonization, and fungal biomass was assessed based on α-tubulin transcript levels for a subset of samples.

For all the *G. versiforme*-inoculated plants, and a subset of *Gi. gigantea*-inoculated plants (six *S. bicolor* accessions), flash frozen root subsamples were homogenized for 30 s, two times, using a SPEX SamplePrep MiniG 1600 tissue lyser in stainless steel rack that had been precooled using liquid nitrogen. Total RNA was isolated from a ~100 mg subsample of the homogenized roots using TRIzol reagent (Invitrogen) and 20% sarkosyl (for method see Jordon-Thaden, Chanderbali, Gitzendanner, & Soltis, 2015, Appendix 4), including DNase treatment with RNase-free TurboDNaseI (Ambion). First strand cDNA was synthesized as in Experiment 1.

The genomic DNA sequence of SbEF1α and SbPT11 in 17 accessions (all except PI 508366) was cloned into the pGEM-T vector and subsequently sequenced by Sanger sequencing (Cornell University Institute of Biotechnology). A phylogenetic tree of the *Pht1* family of phosphate transporter genes was constructed including transporters mined from the genome sequences of sorghum, *Brachypodium distachyon*, rice (*Oryza sativa*), maize (*Zea mays*), and *Medicago truncatula* (Figure S2). The tree includes the 11 sorghum PT genes originally described in Walder et al. (2015), as well as two additional genes, *SbPT12* (Sobic.003G245200) and *SbPT13* (Sobic.10G133300), that we identified from the sorghum genome sequence. Oligo sequences for qPCR quantification of *SbEF1α* and *SbPT11* expression were designed based on the genomic sequence of 17 accessions, to ensure no SNPs were present and annealing efficiency was not compromised for these accessions. Oligo sequences used to amplify other genes-of-interest were designed using the BTx623 genome sequence and checked against the sequence of seven other accessions to avoid SNPs in either forward or reverse sequence. Orthologues of the characterized *Medicago truncatula* lipid biosynthesis genes (*MtFatM*, *MtRAM1*, *MtRAM2*, *MtSTR*, *MtSTR2*) in *S. bicolor* were identified by a BLAST search (https://blast.ncbi.nlm.nih.gov) of the genomic DNA sequence against the *S. bicolor* genome. *G. versiforme* α-tubulin and *G. gigantea* α-tubulin transcripts were assessed using primers shown

in Table S2. Threshold cycle (CT) values were normalized to the CT values of the *S. bicolor* housekeeping gene *SbEF1α*. Relative expression values were then calculated and averaged over four biological replicates.

2.7 | RNA sequencing

Strand specific RNA-seq libraries were prepared on three biological replicates per treatment (*G. versiforme* inoculated or mock-inoculated) from the Grassl, Wray, PI 562730, and PI 655972 accessions (Experiment 1) using the protocol described by Zhong et al. (2011) with modifications described in detail in the Supporting Information file 1. Raw RNA-seq reads were cleaned using Trimmomatic v0.35 (Bolger, Lohse, & Usadel, 2014). The cleaned reads were mapped to the *S. bicolor* genome v3.1.1 (https://phytozome.jgi.doe.gov) using STAR aligner with default parameters (Dobin et al., 2013). Read count for each gene was calculated by HTSeq v0.6.0 with the –m parameter set to intersection-nonempty (Anders, Pyl, & Huber, 2015). The raw data are available in the NCBI Sequence Read Archive under the accessions SRR8293498 to SRR8293520. A detailed list of accession and sample number is provided in the Supporting Information File 1 in the RNA-seq methods section.

Differential expression of transcripts in the RNA-seq dataset was calculated as log2-fold change (LFC) using the "DESeq2" package (Love, Huber, & Anders, 2014) in R version 3.4.3 (R Development Core Team, 2012; Supporting Information File 2). Differential expression between the mock and inoculated condition was tested against the null hypothesis LFC < 2 (Benjamini and Hochberg adjusted $p < 0.05$) separately for each genotype in pairwise contrasts. Additionally, 55 genes that showed differential expression in response to *G. versiforme* in at least one accession and also differentially expressed among the accessions when inoculated with *G. versiforme* (LFC $\geq$ 2; adjusted $p < 0.05$) were identified (Supporting Information File 2). A heatmap was created using rlog transformed counts from the DESeq object, centred on 0, and plotted using the package "pheatmap" (Kolde, 2015). Additionally presented in Supporting Information File 2 is differential expression analysis in which expression between the mock-inoculated and inoculated condition was tested against the null hypothesis that the treatment does not affect gene expression. This analysis therefore shows all differential gene expression.

2.8 | Calculations and statistical analyses (Experiments 1 and 2)

Mycorrhizal growth response (MGR):

$$MGR\ (\%) = \frac{SDW\ (\text{AM fungi} - \text{inoculated}) - mean\ SDW\ (\text{mock} - \text{inoculated})}{mean\ SDW\ (\text{mock} - \text{inoculated})} \times 100. \quad (1)$$

Mycorrhizal phosphorus response (MPR) was calculated in the same way, with values of shoot P content in the place of SDW in Equation 1.

To determine whether MGR (%) was significantly different from zero (in either positive or negative direction), 95% confidence intervals (CI) were calculated, and a treatment mean was deemed to be different where the 95% CI did not overlap zero. Following Shapiro–Wilk test, non-normal data were log-transformed to conform to the assumption of normal distribution for analysis of variance (ANOVA). For all physiological response variables, two-factor ANOVAs were performed, with *Accession* (18 levels) and *Mycorrhiza* (six levels [mock-inoculated, *G. versiforme*, *R. irregularis*, *C. claroideum*, *Gi. gigantea*, *G. versiforme/C. claroideum* mix]: growth data; three levels [mock-inoculated, *G. versiforme*, *R. irregularis*]: tissue nutrition data) as the factors. Following this, one-way ANOVAs were conducted on each accession, respectively, with *Mycorrhiza* treatment as the factor. For gene expression data, one-way ANOVAs were used to test the effect of *Accession* for each gene-of-interest respectively. Where a significant ($p < 0.05$) interaction or main effect was found, comparisons were made with Tukey's honestly significant difference (HSD). Principal components analysis (PCA) was performed on all the gene expression data (13 genes: six PTs, one AMT, five lipid biosynthesis, *G. versiforme* α-tubulin), MGR and MPR, in the 18 sorghum accessions (*G. versiforme*-inoculated plants only) in order to explore relationships between multiple variables simultaneously. A PCA biplot was constructed displaying a score for each individual plant and a loading for each variable included, on the first two principal components. All statistical analyses were performed using JMP Pro 12.0.1 (SAS Institute).

3 | RESULTS

3.1 | Variation in colonization levels and expression of an AM-induced phosphate transporter (*SbPT11*) across 14 sorghum bioenergy accessions inoculated with *G. versiforme*

In an initial experiment that aimed to survey colonization levels in a range of sorghum accessions, we transplanted 14 *S. bicolor* bioenergy accessions and BTx623 into substrate containing *G. versiforme* and assessed the AM colonization 42-day post-transplanting. All sorghum accessions were colonized by *G. versiforme* and colonization levels ranged from 21.7% (Atlas) to 53.2% (Chinese Amber) root length colonized and thus varied significantly across the accessions (Figure 1a and Table S3). *G. versiforme α-tubulin* transcript level which provides a useful proxy for living AM fungal biomass present in roots (Hause & Fester, 2005; Isayenkov, Fester, & Hause, 2004) likewise varied significantly, with a 2.6-fold difference in transcript levels between the lowest (Leoti) and the highest (Wray) accessions (Figure 1b). The expression of the phosphate transporter gene *SbPT11* varied significantly across the accessions, with a 5.6-fold difference between PI 297130 and Wray—the *G. versiforme* inoculated accessions with the lowest and highest *SbPT11* transcripts in the respectively (Figure 1c). SbPT11 is the orthologue of MtPT4/OsPT11, a Pi transporter that is required for a functional AM symbiosis (Javot, Penmetsa, Terzaghi, Cook, & Harrison, 2007; Yang et al., 2012; Figure S2). There was a weak positive correlation between expression of *α-tubulin* and *SbPT11* expression (R^2 = 0.16). As previous studies involving a single host genotype showed strong correlations between the fungal *α-tubulin* and *OsPT11/MtPT4* transcripts, the weak correlation observed here

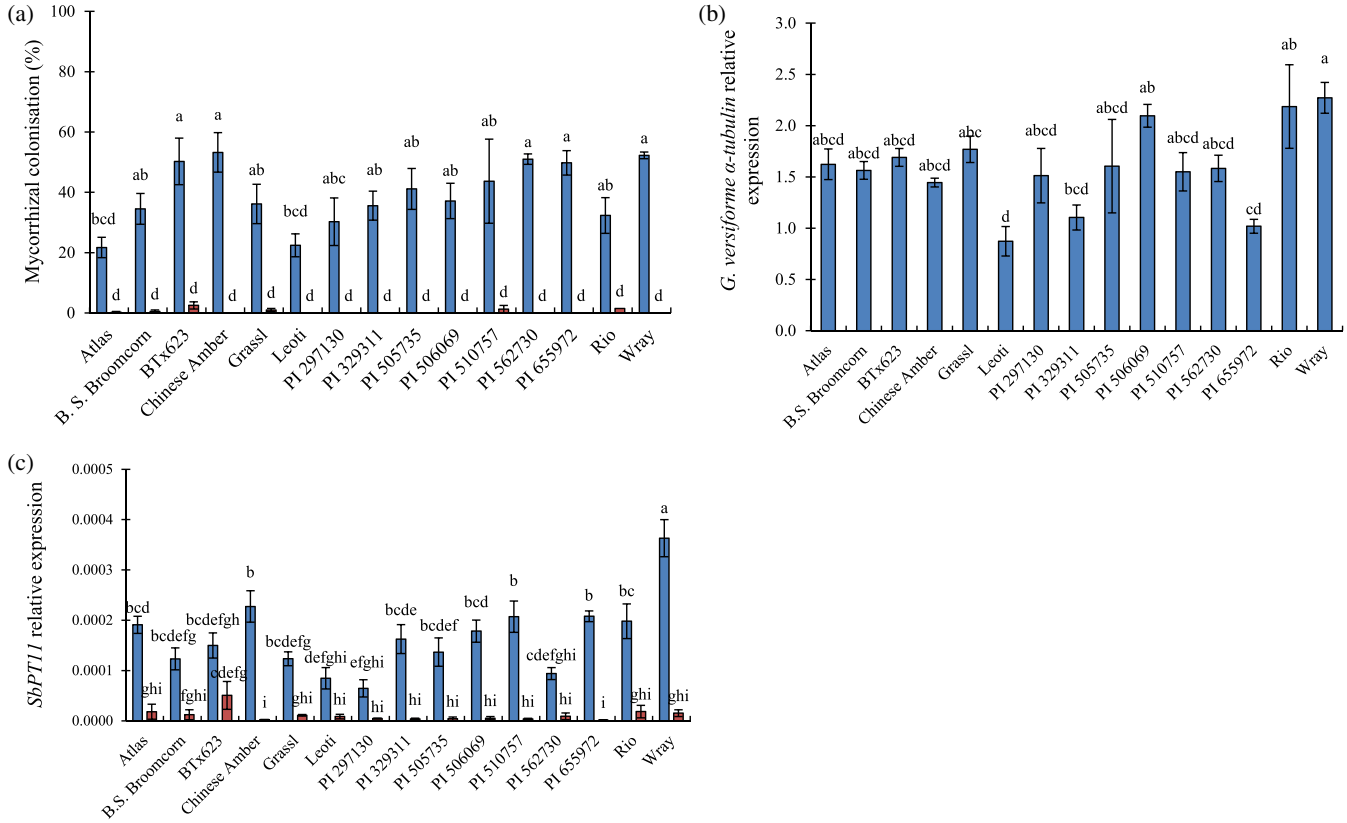

FIGURE 1 Mycorrhizal colonisation (a) and relative expression of *Glomus versiforme α-tubulin* (b) and *Sorghum bicolor SbPT11* (c) of 15 *S. bicolor* lines that have been mock-inoculated (red bars) or inoculated with the mycorrhizal fungus *G. versiforme* (blue). Mean ± *SEM*, $n = 3$. Bars with the same letters above them are not significantly different at the $p < 0.05$ level. BTx623 mock-inoculated samples showed minor colonisation (mean 2.5% root length colonized). There was no detected expression of *G. versiforme α-tubulin* in any mock-inoculated plants

suggests that the sorghum genotypes may vary in *SbPT11* expression and/or colonisation patterns.

3.2 | RNA-seq analyses reveal genotype-conserved and genotype-variable transcriptional responses to *G. versiforme* in the roots of four sorghum accessions

For transcriptome analysis, we chose four accessions, Wray, Grassl, PI 655972, and PI 562730, which showed comparable levels of colonization (Figure 1a) but which varied in their relative *G. versiforme α-tubulin* and *SbPT11* transcript levels (Figure 1b,c). RNA-seq data were then generated from both mock-inoculated and colonized roots. By using a stringent null hypothesis (logarithmic fold change [LFC] ≥ 2) to test for differential expression, we focused on sorghum genes that were strongly induced or down-regulated and therefore most likely to be robust across experiments. This test will not detect genes with low LFC or moderate LFC and high variance. Using this approach, between 363 and 470 genes were differentially expressed in colonized roots of each accession. This included changes in both low and high abundance transcripts (Figure 2a). The magnitude of the fold change was similar across accessions including LFCs ≥11 in all accessions. Sobic.003G317800 and Sobic.003G17900 predicted to code LysM domain proteins are among the most highly induced genes in all accession and are orthologues of OsAM3, whose induction during symbiosis has been noted previously (Güimil et al., 2005). Two hundred seventy-eight genes show an LFC ≥ 2 in transcript abundance in all four accessions and form a robust core set of symbiosis-induced genes (Supporting Information file 2) that can serve as a foundation for future molecular analyses. There is considerable overlap between genes in this set and the symbiosis-induced genes reported previously in rice (Güimil et al., 2005). Additionally, orthologues of most of the functionally characterized symbiosis-induced genes, including *STR* (Zhang, Blaylock, & Harrison, 2010; Gutjahr et al., 2012), *MtPT4/OsPT11* (Harrison, Dewbre, & Liu, 2002; Paszkowski, Kroken, Roux, & Briggs, 2002), *FatM* (Bravo, Brands, Wewer, Dörmann, & Harrison, 2017), *RAM1*, RAM2 (Gobbato et al., 2012), one sorghum orthologue of *OsPT13* (Walder et al., 2015), and *EXO70I* (Zhang, Pumplin, Ivanov and Harrison, 2015) are present in this set. Sorghum has 13 phosphate transporters within the Pht1 family (Supporting Information file 2, Figure S2), four of which are induced in mycorrhizal roots (Walder et al., 2015). Sobic.003G243400 (SbPT11) and Sobic.006G26800 (SbPT10) are present in the core symbiosis-induced gene set, whereas Sobic.002G116100 (SbPT8) and Sobic.006G026900 (SbPT9) show variable symbiosis-induced increases and did not pass the stringency threshold for inclusion in the core set (Supporting Information File 2).

Transcriptome data from four sorghum accessions grown simultaneously in a single experiment also provided an opportunity to examine the effects of host genotype on mycorrhiza-induced gene

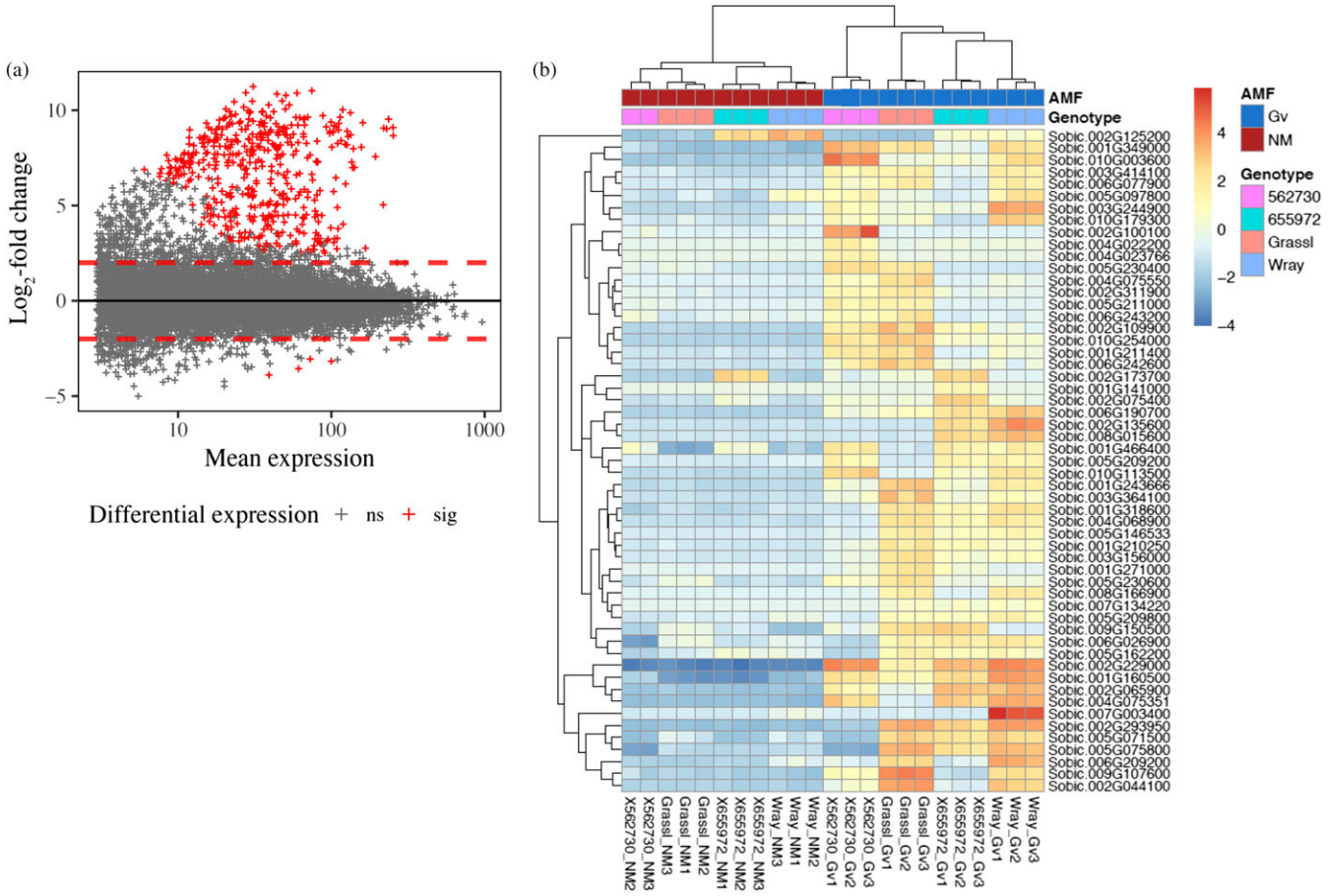

FIGURE 2 Differential gene expression in *Sorghum bicolor* cv. Grassl inoculated with *Glomus versiforme* (Gv) compared with the noninoculated condition (NM) (a; n = 3). Genes differentially expressed (|log2-fold change| ≥ 2; FDR < 0.05) shown in red. Heatmap of 55 genes differentially expressed among *S. bicolor* genotypes (b; DESeq2:|LFC| ≥ 2, FDR < 0.05) and differentially expressed during symbiosis (DESeq2:|LFC| ≥ 2, FDR < 0.05) for at least one genotype. Color and intensity indicates variance in regularized log2-transformed counts, centred on 0. Samples and genes are clustered by expression profiles (top and left trees, respectively) [Colour figure can be viewed at wileyonlinelibrary.com]

expression. Fifty-five genes show symbiosis-induced expression in at least one accession and differential expression across accessions. Of the 55 genes, 12 genes are predicted orthologues of symbiosis-regulated genes in rice (Güimil et al., 2005) including several highly symbiosis-induced genes; for example, Sobic.002G135600, predicted to encode a germin protein (orthologue OsAM4), is expressed at high levels in mycorrhizal roots of Wray but not in Grassl or PI 562730 (Figures 2b and S3, Supporting Information file 2). One of the mycorrhiza-inducible phosphate transporters, *SbPT9*, is likewise differentially expressed in a genotype-dependent manner: high in Wray and PI 655972 but barely detectable in PI 562730. Transcript levels of several groups of genes are higher in Grassl and Wray, relative to PI 655972 and PI 562730 (Figure 2b), but there are also modules shared by other combinations of accessions. In addition to the germin, which is a defence-associated protein, Wray shows high expression of a gene predicted to be involved in benzoxazinoid biosynthesis (Sobic.007G003400; Frey et al., 1997), a class of compounds with defensive properties, as well as antimicrobial peptides miAMP (Sobic.006G190700; McManus et al., 1999) and two gamma-thionins (defensins) (Sobic.004G075351, Sobic.004G075550; Bruix et al., 1993). Transcript levels for three chitinase genes (Sobic.002G109900, Sobic.003G156000, Sobic.003G364100), two genes annotated as ripening-related proteins (Sobic.001G210250, Sobic.006G242600), and an SLC-14L GRAS family transcription factor (Sobic.005G230600) predicted to regulate stress-related gene expression (Fode, Siemsen, Thurow, Weigel, & Gatz, 2008) are particularly high in Grassl. The chitinase and ripening-related genes are likely orthologs of genes whose expression is elevated during arbuscule degeneration in *M. truncatula* (Floss et al., 2017). Thus, the transcriptome analyses provide a core set of genes that show robust symbiosis-induced expression across genotypes. Additionally, a smaller set of genes provides evidence of genotype-dependent expression patterns.

3.3 | Eighteen sorghum accessions differ in their responses to colonization by four species of AM fungi

Having ascertained that the sorghum accessions show variation for colonization by *G. versiforme*, we undertook a larger experiment to assess growth and nutritional responses of 18 sorghum accessions to four diverse species of AM fungi, *G. versiforme*, *R. irregularis*, *G. gigantea* and *C. claroideum*. The 18 sorghum accessions (Table S1) were chosen because they are the parents of a bioenergy NAM population and are representative of the genetic and phenotypic diversity that is observed among the bioenergy sorghum accessions (Brenton et al.,

2016). The four species of AM fungi were selected because they span some of the diversity of the Glomeromycotina, and based on the pilot study (Figure 1), there is some variation in colonization level for at least one species across a subset of these sorghum genotypes. Each combination of sorghum accession and AM fungal species was grown for a period of 79 days at which time visible differences in the shoot phenotypes of the inoculated versus mock-inoculated plants were apparent. Shoot and root weight and shoot nutrient contents and concentrations were assessed. The different combinations of sorghum accession and colonizing AM fungal species resulted in considerable variation in a range of plant responses. This was indicated by a significant ($p < 0.05$) interaction between *Mycorrhiza* and *Accession* for shoot biomass, root biomass, shoot P content and concentration, and mycorrhizal growth response (Table 1; Figure S4).

There were nine sorghum accessions that had significantly greater shoot biomass than the respective mock-inoculated control plants when colonized with *C. claroideum*, eight that showed significant growth improvement with *G. versiforme*, five with *R. irregularis*, five with the *G. versiforme*/*C. claroideum* mix, and three accessions inoculated with *Gi. gigantea* that had significantly larger shoots than the respective mock-inoculated controls (Figure 3). Of the positively responding sorghum accessions, there were five that had shoot growth significantly enhanced by four or five of the AM fungal treatments (Leoti, PI 506069, PI 297155, PI 508366, and PI 562730). In contrast, there were eight sorghum accessions with no significant enhancement of shoot growth when inoculated with any AM fungal inoculation treatments. Furthermore, there were five accessions that displayed significant shoot growth depressions when inoculated with *Gi. gigantea* and two accessions when inoculated with *G. versiforme*.

TABLE 1 Statistical outcomes (p values) of the two-way analysis of variance performed between *Mycorrhiza* and *Accession* treatments for each response variable

	Mycorrhiza	Accession	Mycorrhiza * Accession
Shoot dry weight	<0.0001	<0.0001	<0.0001
Root dry weight	<0.0001	<0.0001	<0.0001
Shoot P concentration	0.0003	<0.0001	0.0003
Shoot P content	<0.0001	<0.0001	0.0002
Myc Growth Response	<0.0001	<0.0001	0.0008

The greatest benefit of AM fungal inoculation in terms of shoot biomass (i.e., exhibiting a large positive mycorrhizal growth response; Figure 4) was observed in the sorghum accessions that were the smallest in the mock-inoculated control treatment. Mycorrhizal growth responses (MGR) ranged from negative (−75%, *Gi. gigantea* colonizing BTx623) to highly positive (329%, *C. claroideum* colonizing PI 297155). For many accessions, the combination of inoculation with both *G. versiforme* and *C. claroideum* did not confer greater MGR than one or the other alone, and the dual inoculation was often actually less beneficial (Table S4). There was just one accession (Chinese Amber) where the growth response from the dual inoculation was greater than either of the respective single species inocula.

Shoot mineral nutrition was analysed for two of the AM fungal treatments (*G. versifome* and *R. irregularis*) and the mock-inoculated control. As for plant biomass, the effect of the colonizing AM fungal species on the shoot tissue concentrations and contents of many plant macronutrients (P, K, Ca, Mg, S) and micronutrients (Cu, Mn, Zn) was dependent on the sorghum accession in question (Supporting Information Table S5).

Of particular interest was the effect of AM fungal inoculation on shoot P nutrition; shoot P concentration was significantly higher in plants colonized by *R. irregularis* than the respective mock-inoculated controls in six accessions, and in plants colonized by *G. versiforme*, in three accessions (Figure 5a). Similarly, colonization by *R. irregularis* significantly increased shoot P content (a product of P concentration and shoot biomass) in seven accessions, and colonization by *G. versiforme* significantly improved P content in eight accessions (Figure 5b). Furthermore, seven sorghum accessions had significantly higher P contents than mock-inoculated controls with both of the AM fungal

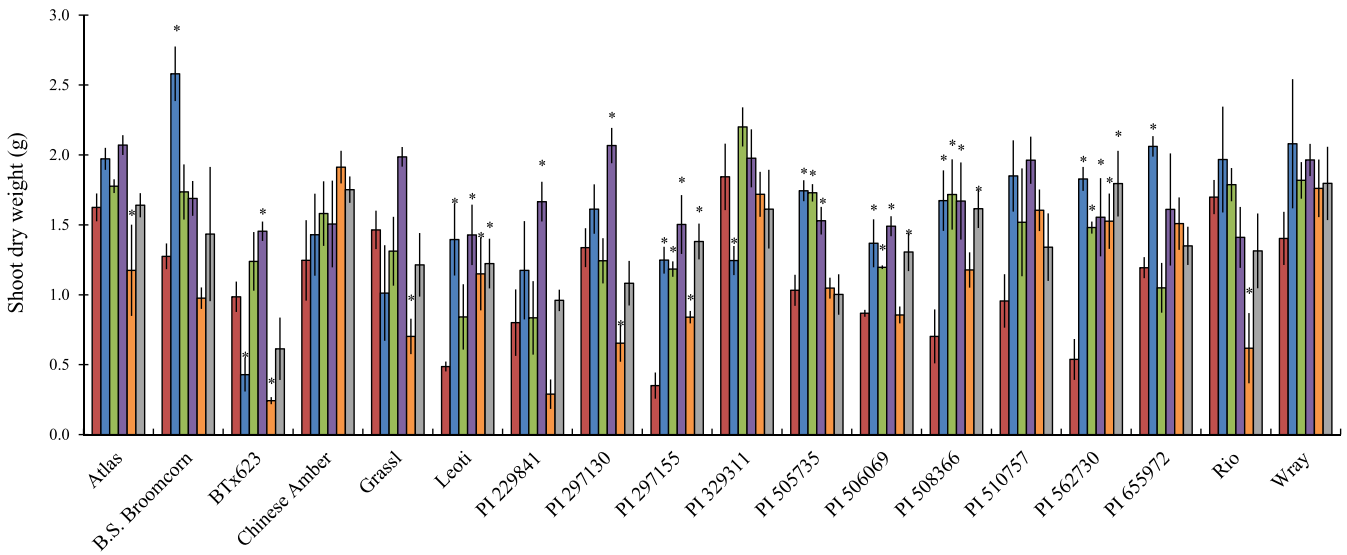

FIGURE 3 Shoot dry weights of 18 *Sorghum bicolor* lines that have been mock-inoculated (red bars) or inoculated with mycorrhizal fungi (*G. versiforme*: blue, *R. irregularis*: green, *C. claroideum*: purple, *Gi. gigantea*: orange, *G. versiforme*/*C. claroideum* mix: grey). Mean ± *SEM*, $n = 4$. Asterisks represent an inoculated treatment is significantly different (Tukey's HSD after one-way analysis of variance, $p < 0.05$) from that line's respective mock-inoculated control [Colour figure can be viewed at wileyonlinelibrary.com]

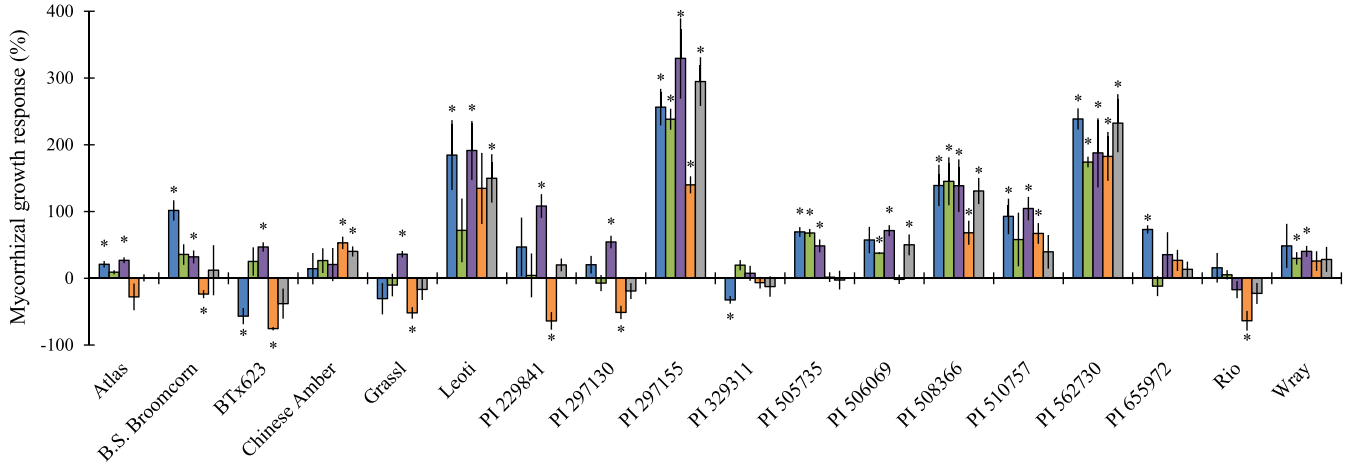

FIGURE 4 Mycorrhizal growth responses (see Eqn. in main text) of 18 *Sorghum bicolor* lines that have been inoculated with mycorrhizal fungi (*G. versiforme*: blue, *R. irregularis*: green, *C. claroideum*: purple, *Gi. gigantea*: orange, *G. versiforme*/*C. claroideum* mix: grey). Mean ± *SEM*, *n* = 4. Asterisks denote a treatment mean value that is significantly different from zero at the 95% confidence level [Colour figure can be viewed at wileyonlinelibrary.com]

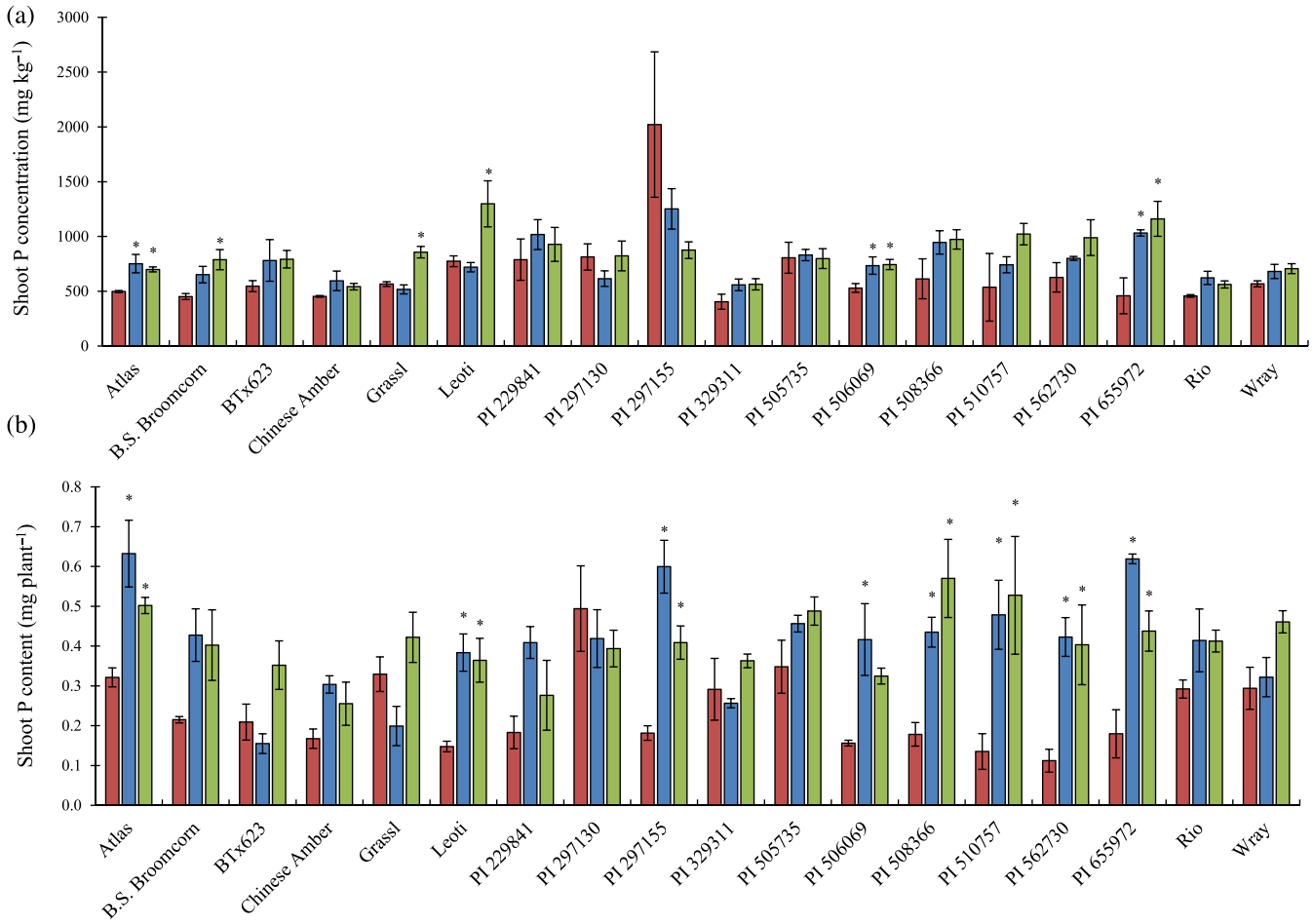

FIGURE 5 Leaf phosphorus concentrations (a) and contents (b) of 18 *Sorghum bicolor* lines that have been mock-inoculated (red bars) or inoculated with mycorrhizal fungi (*G. versiforme*: blue, *R. irregularis*: green). Mean ± *SEM*, *n* = 4. Asterisks represent an inoculated treatment is significantly different (Tukey's HSD after one-way analysis of variance, $p < 0.05$) from that line's respective mock-inoculated control [Colour figure can be viewed at wileyonlinelibrary.com]

species investigated. The greatest increase in shoot P content with *G. versiforme* inoculation was observed in PI 562730 (3.8-fold), and with *R. irregularis* was observed in PI 510757 (3.9-fold). The relationship between MGR and MPR was explored with regression analysis for *G. versiforme* and *R. irregularis*-inoculated plants, respectively (Figure 6); for both AM fungal treatments, the relationship was significant, positive, and moderate in strength (R^2 = 0.56 and 0.36 for *G. versiforme* and *R. irregularis*-inoculated plants, respectively).

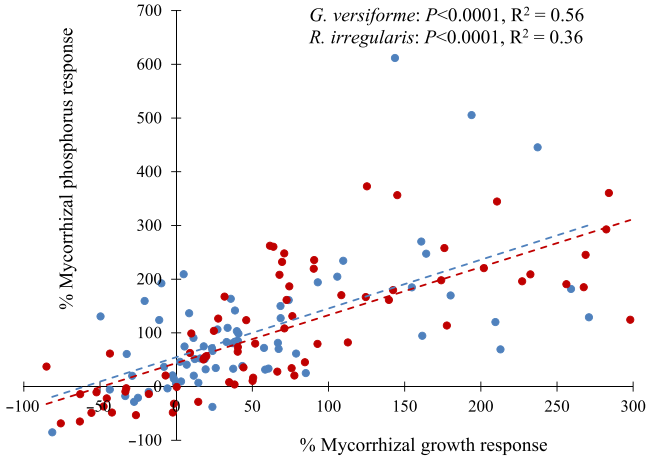

FIGURE 6 Scatterplot showing the relationship between mycorrhizal growth responses (%) and mycorrhizal phosphorus responses (%) in 18 *Sorghum bicolor* lines that have been inoculated either with the AMF *G. versiforme* (red dots) or *R. irregularis* (blue dots). Lines-of-best-fit were calculated for each AMF treatment, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

Shoot concentrations and contents of other plant macronutrients were significantly increased by inoculation with AM fungi (Figure S5). Potassium (K) nutrition was particularly improved, most commonly by inoculation with *R. irregularis*. The content of calcium (Ca), magnesium (Mg), and sulphur (S) in shoots was also generally improved by AM fungal inoculation. Of the micronutrients, the concentration and content of manganese (Mn) and copper (Cu) were the most increased by AM fungal inoculation.

3.4 | Expression of a selection of genes related to mineral nutrient transport and lipid biosynthesis and transport

Because phosphate transfer to the plant and the carbon allocation to the fungus are major factors that influence the outcome of the symbiosis for the plant, we analysed the expression of Pi transporter genes and genes involved in AM-induced lipid biosynthesis and transport. The RNA-seq data (Figure 1 and Supporting Information file 2) verified the symbiosis-induced expression of several Pi transporters as well as orthologues of genes involved in mycorrhiza-induced lipid biosynthesis and transport. It was not feasible to assess expression across the whole experiment, so we chose the sorghum-*G. versiforme* materials where the growth responses were largely positive (Figures 7 and S6)

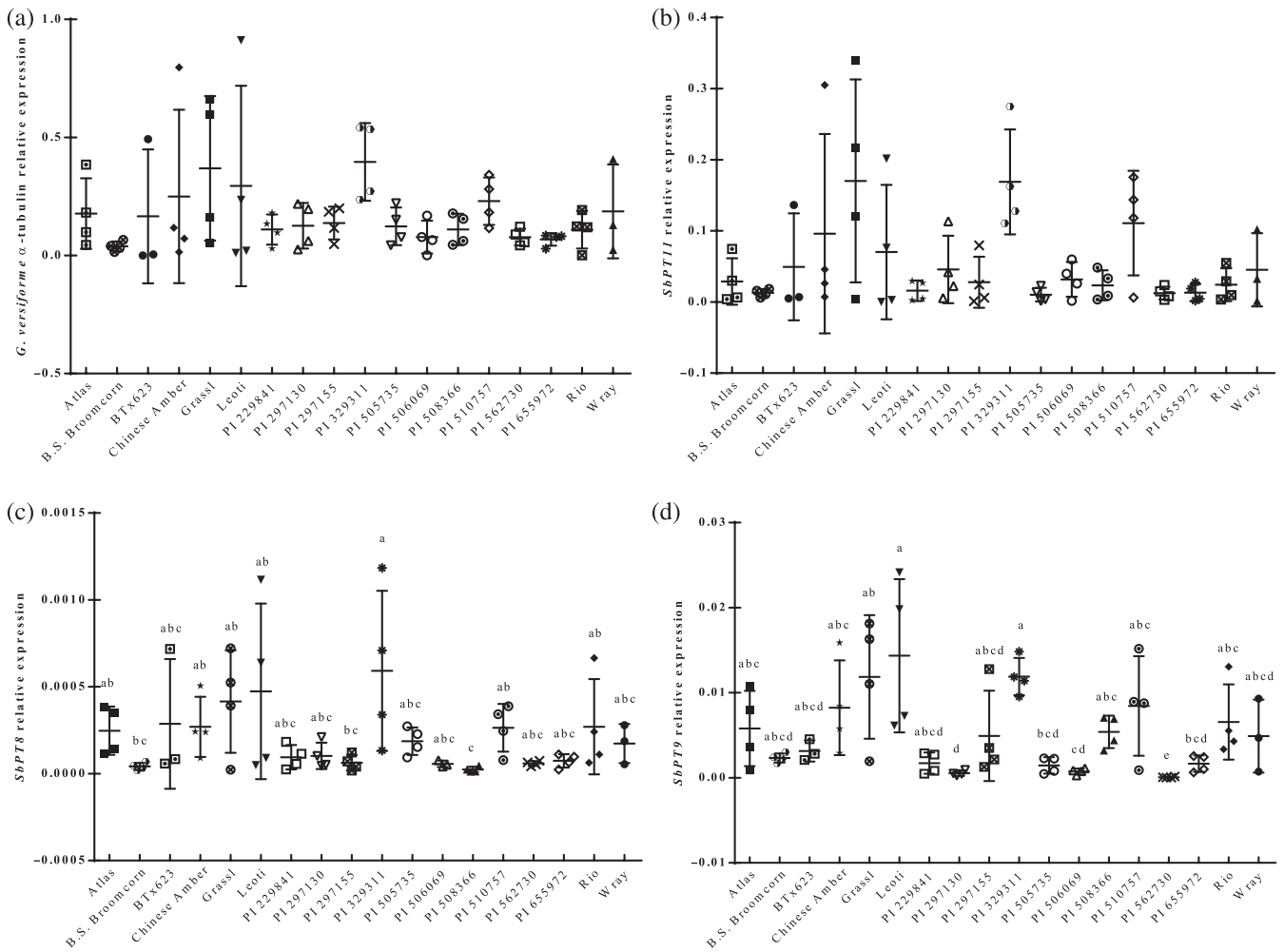

FIGURE 7 Expression of *G. versiforme* α-tubulin (a) and *S. bicolor* phosphate transporter genes (b–g) in the roots of 18 *S. bicolor* lines that have been inoculated with the mycorrhizal fungus *G. versiforme*. Mean ± *SD*, $n = 4$. Bars with the same letters above them are not significantly different at the $p < 0.05$ level. (a) *G. versiforme* α-*tubulin*, (b) *SbPT11*, (c) *SbPT8*, (d) *SbPT9*, (e) *SbPT10*, (f) *SbPT4*, (g) *SbPT7*

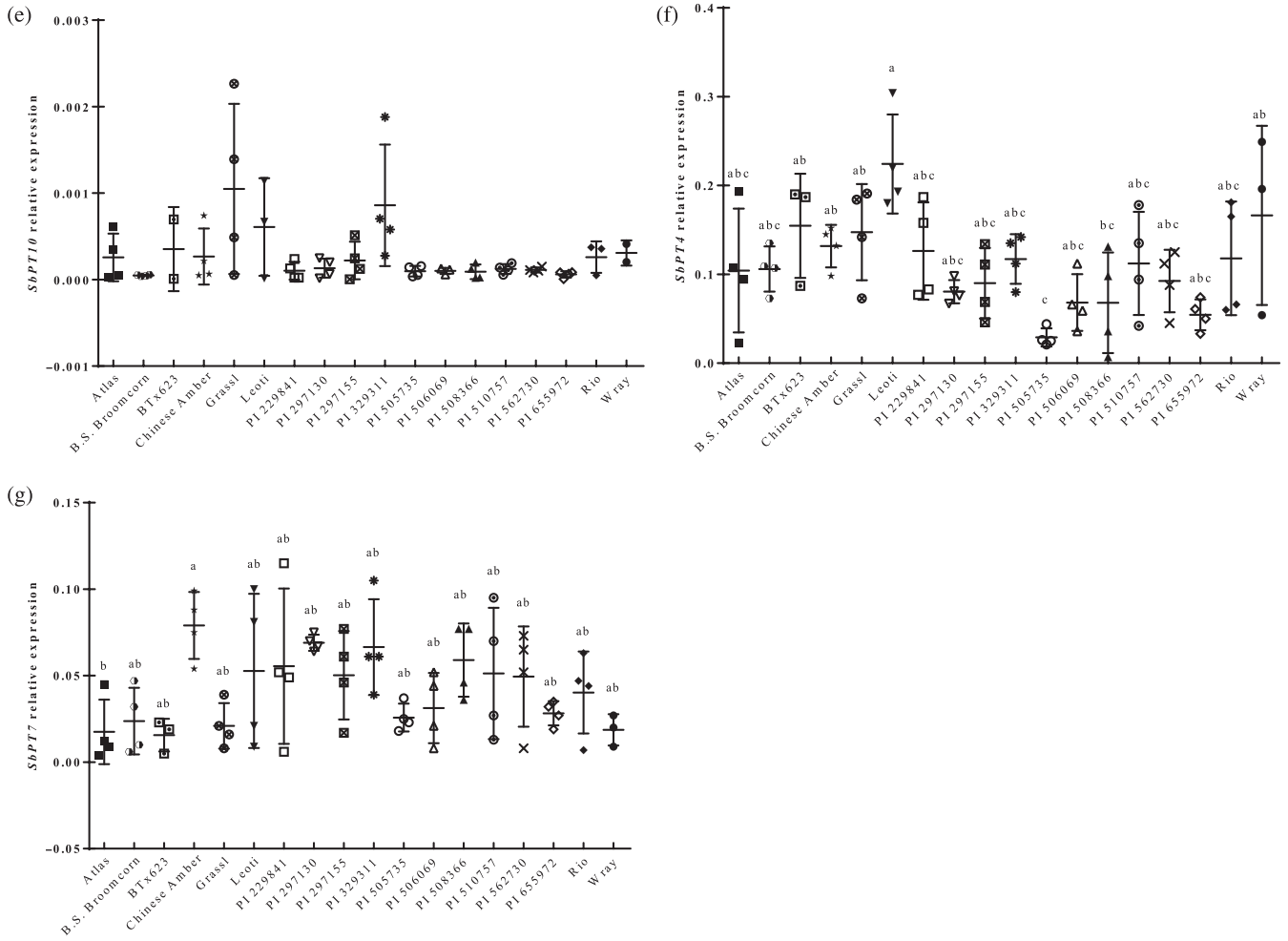

FIGURE 7 Continued.

as well as six sorghum accessions colonized by *Gi. gigantea* that displayed contrasting growth responses (Figure S7).

Expression of two constitutively expressed phosphate transporter genes (*SbPT4* and *SbPT7*; Walder et al., 2015), two symbiosis-inducible phosphate transporter genes (*SbPT8* and *SbPT9)* and an inducible ammonium transporter (*SbAMT3;1*; Koegel et al., 2013) varied significantly among the accessions (Table S6). Although ANOVA revealed no significant variation in *G. versiforme* α-tubulin or *SbPT11* transcript across the accessions, some accessions had high variability in expression of these genes between replicates (e.g., Grassl and Leoti), whereas others showed almost no variability (e.g., B.S. Broomcorn and PI 655972). The accessions with the highest expression of *G. versiforme* α-tubulin and *SbPT11* were PI 329311 and Grassl. The accessions with the lowest expression of these two genes were Black

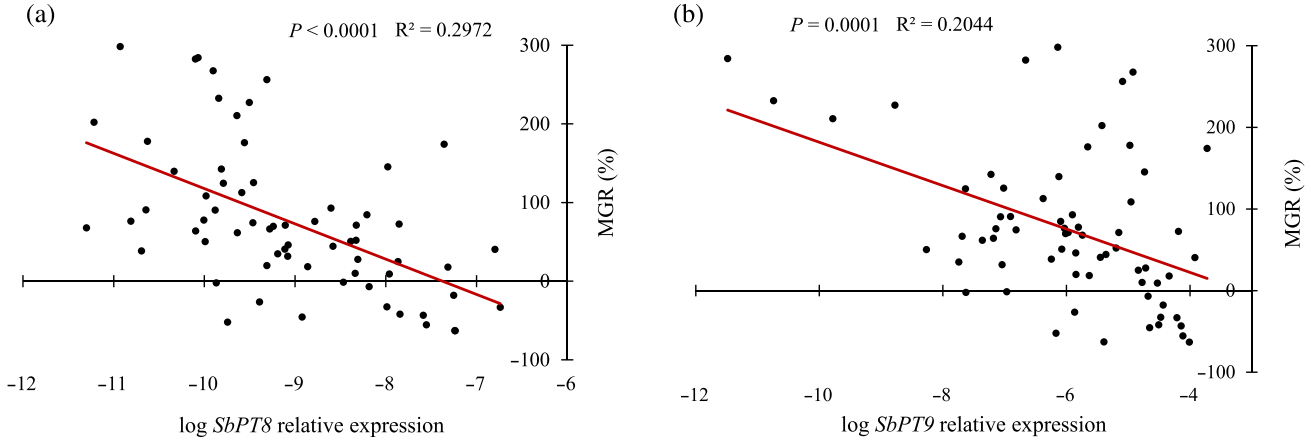

FIGURE 8 Scatterplots showing relationships between mycorrhizal growth responses (%) and expression of *Sorghum bicolor* phosphate transporter genes *SbPT8* (a) and *SbPT9* (b) in the roots of 18 *S. bicolor* lines that have been inoculated with *G. versiforme* [Colour figure can be viewed at wileyonlinelibrary.com]

Spanish Broomcorn, PI 655972 and PI 562730. Of the genes that showed variation across accessions, only *SbPT8* and *SbPT9* expression correlated with MGR and the correlation was negative (Figure 8). For the genes related to lipid biosynthesis and transport (*SbFatM*, *SbRAM1*, *SbRAM2*, *SbSTR*, *SbSTR2*), there was no significant effect of genotype. In the six sorghum accessions that displayed contrasting MGRs when colonized by *Gi. gigantea*, expression of four *PT* genes (all except *SbPT11* and *SbPT10*), and all the lipid-related genes except *SbSTR2*, varied significantly with *Accession* (Table S6 and Figure S7).

The relationships between the physiological measurements and gene expression data were further explored through a PCA. The first principal component (PC1) captured overall expression of symbiosis genes including all of the measured lipid biosynthesis and transport-related genes, *SbPT11*, and *G. versiforme* α-tubulin and explained 52% of the variation in the data (Figure 9). Mycorrhizal growth response (MGR) and mycorrhizal phosphate response (MPR) loaded weakly but negatively on PC1, but positively on PC2, an apparent decoupling of the physiological response to inoculation and symbiotic gene expression as measured at harvest. PC2 explained a further 15.2% of the variation and was also driven by the expression of *SbAMT3;1* and the constitutively expressed phosphate transporter genes, *SbPT4* and *SbPT7*. Among the AM-induced genes, *SbPT8*, *SbPT9* and *AMT3;1* were not as tightly coupled with *G. versiforme α-tubulin* and the lipid biosynthesis/transport-related genes, suggesting their expression is under differing and variable regulation compared with the major symbiosis-induced genes. The PCA reflected the negative relationship between MGR and *SbPT8* and *SbPT9* (Figure 8), as MGR loaded in the opposite directions on both PC1 and PC2 compared to *SbPT8* and *SbPT9*.

4 | DISCUSSION

4.1 | The sorghum mycorrhizal root transcriptomes

Several prior studies have established that AM symbiosis is accompanied by a dramatic increase in gene expression in roots (Güimil et al., 2005; Hogekamp & Kuster, 2013; Liu et al., 2007), and here, we show that this is true also of sorghum (Supporting Information file 2). However, we extended the transcriptome analyses with the inclusion of four plant genotypes. This enabled the identification of conserved, as well as variable, transcriptional responses to *G. versiforme* across these four *S. bicolor* accessions. We purposely used highly stringent criteria to identify genes that are induced in all accessions and this set of 278 genes includes orthologues of many symbiosis-induced rice (Güimil et al., 2005) and *M. truncatula* genes (Hohnjec, Vieweg, Pühler, Becker, & Küster, 2005; Liu et al., 2007). Likewise, stringent criteria enabled the identification of genes that are induced but whose expression varies across accessions. The latter set is perhaps the most unique as host-genotype effects on mycorrhiza-induced gene expression has not been examined at the whole transcriptome level. This variable set comprises 55 genes, many of which are predicted to encode enzymes involved

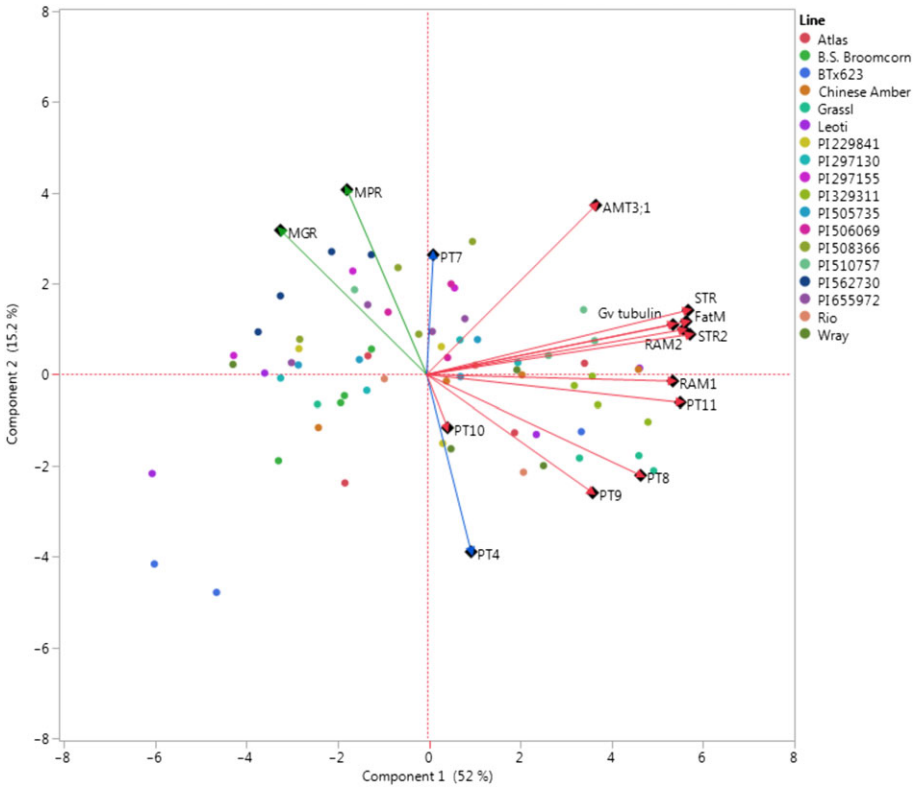

FIGURE 9 Principal components analysis (PCA) biplot displaying scores in the first two principal components (PC1: x-axis, PC2: y-axis) following PCA of 11 AMF-induced genes (red lines) including phosphate transporters (PTs) and lipid biosynthesis genes and transporters, two noninduced PT genes (blue lines), and two physiological measures of responses to AM fungi (mycorrhizal growth and phosphorus responses, respectively; MGR, MPR; green lines) in 18 *S. bicolor* accessions (individual colours indicated in legend) inoculated with the AM fungi *G. versiforme*. The sign and magnitude of the contribution of a given response variable is indicated by the arrows [Colour figure can be viewed at wileyonlinelibrary.com]

with stress- or defence-responses, whereas several others encode antimicrobial proteins. It is possible that these genes shed light on the enhanced resistance properties observed in some symbioses (Newsham, Fitter, & Watkinson, 1995), and with this in mind, it would be interesting to test mycorrhiza-induced resistance (Pieterse et al., 2014) in Wray, in which elevated expression of defence and antimicrobial genes was observed. In contrast, Grassl showed elevated expression of at least three genes whose orthologues are induced during arbuscular degeneration (Floss et al., 2017). Possibly, this reflects a higher proportion of degenerating arbuscules in Grassl which might be a factor contributing to its lack of positive MGR.

4.2 | Variation in mycorrhizal growth responsiveness across sorghum bioenergy accessions

An initial survey of colonization across a subset of the sorghum accessions provided evidence of variation in colonization levels and gene expression (Figures 1 and 2), but this experiment was a short-term experiment in spatially restricted growth containers and did not permit an assessment of mycorrhiza-induced growth responses. The second experiment, which used the full set of bioenergy accessions, aimed to assess mycorrhizal growth responses. Even without AM fungal inoculation, the accessions displayed enormous phenotypic diversity in terms of plant height, biomass, shoot nutrient accumulation, and expression of nutrient transporter genes. When inoculated with a given species of AM fungi, the ranking of biomass across the 18 accessions changed substantially. Generally, the accessions that were lower performing without inoculation were those that experienced the greatest benefit (as %MGR difference) with AM fungal inoculation, whereas those that ranked highest without AM fungal inoculation were nonresponsive to inoculation, or in some cases experienced a growth depression. For example, with *G. versiforme* inoculation, two accessions that had the smallest shoots without inoculation (PI 297155 and PI 562730) were able to increase their shoot biomass to a similar range as that of the highest performing, noninoculated accessions (Atlas and PI 329311). Given that all plants were supplied with very low amounts of phosphate, these accessions (PI 297155 and PI 562730) could be considered relatively dependent on AM fungi in comparison to the other accessions that were able to grow satisfactorily in the mock-inoculated treatment. In addition, this observation suggests that for some sorghum accessions their potential for biomass accumulation could be reached by inoculation with AM fungi, perhaps to a similar extent as would otherwise be achieved through application of inorganic P fertilizer.

There was a significant, positive correlation of MGR and MPR in plants inoculated with *G. versiforme* and *R. irregularis*. In the future, radiotracer studies would be useful for determining whether increased P transfer via symbiosis is the underlying cause for the large growth responses in PI 297155 and PI 562730, as performed in maize previously (Sawers et al., 2017). Alternatively, there may be other physiological factors interacting with the AM symbiosis, such as the plant's efficiency in utilizing P once it has been acquired (Veneklaas et al., 2012), or root architectural differences (Péret et al., 2014). It has been suggested previously that evidence of variation in mycorrhizal responsiveness (difference in growth or P nutrition between nonmycorrhizal and mycorrhizal plant) in different genotypes of the same species may indicate potential for that trait to be bred for (Ellouze et al., 2015; Kaeppler et al., 2000). Although, it has been argued that it would be more useful to identify factors that impact "mycorrhizal dependence," that is, the lowest nutrient concentration a plant can thrive without the mycorrhizal symbiosis (Janos, 2007) and target those factors for breeding in cereal crops (Sawers, Gutjahr, & Paszkowski, 2008).

As illustrated in the PCA (Figure 9), the growth and phosphorus responses (MGR and MPR) did not correlate with expression of *SbPT11*. Similar observations were made by (Walder et al., 2015), where *SbPT11* expression did not correlate with phosphorus responses in sorghum inoculated with *R. irregularis* or *F. mosseae*. Likewise, in comparison to maize genotypes, high mycorrhizal growth and P responses correlated most strongly with extraradical hyphal biomass and less so with *ZmPT6* transcript levels or with intraradical colonisation (Sawers et al., 2017). In all of these studies, it is important to consider that unlike shoot biomass or P content, which are cumulative over the entire experiment, the transcript measurements provide a single snapshot of transcript abundance at the end of the experiment and may not reflect the total activity. In our study, *SbPT11* transcripts and also *FatM*, *RAM1*, *RAM2*, *STR*, and *STR2* transcripts correlated well with colonization levels (as assessed by α-tubulin). In contrast, *SbPT8* and *SbPT9* transcripts were not as tightly coupled with *G. versiforme α-tubulin* transcripts and furthermore were negatively correlated with MGR (Figures 8 and 9). This is particularly interesting as *SbPT9* emerged from the transcriptome analysis as one of 55 genes to show robust differential expression across genotypes (Figure 2b). Furthermore, *SbPT9* is an orthologue of *OsPT13*, whose function influences arbuscule populations, although this seemed not to be the consequence of loss of phosphate transport per se (Yang et al., 2012). Thus, if *SbPT9* has a function similar to *OsPT13*, variation in *SbPT9* across genotypes might impact arbuscule population structure possibly influencing MGR. Regardless of the underlying mechanism, a link between variation in this inducible phosphate transporter and functional responses is intriguing and warrants further exploration.

4.3 | Variation in mycorrhizal growth responsiveness to diverse fungal species

Plant responsiveness to the mycorrhizal symbiosis in terms of growth, nutrition, and gene expression was highly dependent on the AM fungal species colonizing the plant. An example of extreme diversity in mycorrhizal responsiveness was observed in the sorghum accession PI 297130, which showed a significant positive growth response with *C. claroideum*, yet a negative growth response with *Gi. gigantea*, and was nonresponsive to the other three AM fungal treatments. Functional diversity within species and isolates of AM fungi has been demonstrated previously with other plant and fungal combinations at both the physiological (Mensah et al., 2015; Munkvold, Kjøller, Vestberg, Rosendahl, & Jakobsen, 2004; Smith, Smith, & Jakobsen, 2004) and molecular (Burleigh et al., 2002) level. Overall, *C. claroideum* was beneficial to the largest number of sorghum accessions in terms of shoot growth. Conversely, colonization with *Gi. gigantea* produced growth depressions in

five accessions. For many accessions, the combination of inoculation with both *G. versiforme* and *C. claroideum* did not confer greater MGR than one or the other alone. The dual inoculation was actually often less beneficial to sorghum growth than single inoculation, which suggests that these two AM fungal species cannot be "stacked" through simultaneous inoculation to produce greater growth responses.

Previous experiments with other plant species have demonstrated that AM fungi from the *Gigaspora* genus have also been poor performing symbionts in the AM symbiosis (Lendenmann et al., 2011; Li, Smith, Dickson, Holloway, & Smith, 2008; Smith et al., 2004; Taylor & Harrier, 2000). Smith et al. (2004) observed that the mycorrhizal P uptake pathway was almost nonfunctional in tomato colonized by *Gi. rosea*. Additionally, Li et al. (2008) postulated that the growth depressions in wheat plants colonized by *Gi. margarita* were not attributed solely to a C drain on the plant as fungal biomass was very low and were more likely due to AM fungal colonization preventing the direct P uptake pathway from working at its full capacity. Certainly, the low *Gi. gigantea α-tubulin* transcript levels in the six accessions analysed here, which included accessions that showed positive and negative MGRs, suggests that carbon drain was not likely responsible the growth depression. The outcome from these results for the purpose of maximizing and exploiting responsiveness of sorghum to AM fungi would be to focus on growing accessions that have been shown to benefit generally from AM colonization, such as PI 562730 and PI 297155, rather than those that have extremely contrasting responses to different AM fungi.

4.4 | Conclusions and implications

In summary, we have established four sorghum mycorrhizal root transcriptomes which enabled us to identify conserved and variable AM-regulated genes in sorghum accessions during AM symbiosis. Additionally, a comprehensive analysis of physiological responses of *S. bicolor* to inoculation with arbuscular mycorrhizal fungi has revealed substantial variation among the parents of the NAM bioenergy population. Additionally useful, the recurrent female parent Grassl is perhaps the least responsive accession which will aid the mapping of responsiveness either using recombinant inbred lines or possibly the NAM population. Mycorrhizal growth responsiveness across accessions was highly variable and also highly interactive with the diversity of AM fungal species. Furthermore, among the NAM parent accessions, there also appears to be variation in mycorrhizal dependence. This set of lines, encompassing diversity in both mycorrhizal responsiveness and dependence, could potentially be used to map loci underlying these traits in sorghum. In addition, identifying sorghum accessions that show a symbiotic benefit from the widest range of AM fungal species will be crucial for continuing investigation into how best to exploit the AM symbiosis to improve sorghum growth and nutrition on low nutrient soils, as they are likely to be the most able to respond positively to AM fungi in the field.

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AUTHOR CONTRIBUTIONS

S. J. W. W. conceived and conducted the majority of the experiments, analysed data, prepared the majority of the figures, and wrote the manuscript; B. E. conducted differential expression analyses (Supporting Information File 2) and analysed data and generated Figure 2 and interpreted PCA data, VLT generated RNA, and qPCR data in Figures 7, S5, and S6; A. M. M. and J. S. prepared RNA-seq libraries and wrote methods; X. S. and Z. F. assembled and analysed RNA-seq data (Supporting Information File 2) and provided bioinformatics advice; M. J. H. conceived the project and experiments, analysed data, and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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