Phosphorus acquisition efficiency in arbuscular mycorrhizal maize is correlated with the abundance of root-external hyphae and the accumulation of transcripts encoding PHT1 phosphate transporters

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Supporting Information as PDF; Fig.S1 – S7; Table S1 – S4; Analysis.
SUMMARY

- Plant interactions with arbuscular mycorrhizal fungi have long excited interest for their potential to promote more efficient use of mineral resources in agricultural production. Their use, however, remains limited, in part because of a lack of understanding of the factors that determine symbiotic outcome. In this work, variation in response to arbuscular mycorrhizal colonization was characterized in a panel of genetically diverse maize inbred lines.

- The parents of the maize Nested Association Mapping population were evaluated, with and without colonization, in early vegetative stages. Subsequently, six lines with contrasting phenotypes were selected for further characterization, including quantification of fungal colonization, mycorrhiza-mediated phosphorus uptake, and accumulation of transcripts encoding plant PHT1 family phosphate transporters.

- The relative growth of lines changed between non-inoculated and inoculated plants, indicative of variation in host capacity to profit from symbiosis. Patterns of Pht1 transcript accumulation varied among lines, and were correlated with outcome.

- Larger growth responses were correlated with more extensive development of root-external hyphae, increased accumulation of specific Pht1 transcripts and a high level of mycorrhiza-mediated phosphorus uptake. The data suggest that host genetic factors influence fungal growth strategy with subsequent impact on plant biomass production.

Key words: arbuscular mycorrhiza, maize, PHT1, phosphorus, root-external hyphae
INTRODUCTION

The rising cost of agricultural inputs and an increasing awareness of the negative environmental consequences have resulted in ever greater interest in beneficial crop-microbe interactions and their potential application (Perez-Montano et al., 2014; Vance, 2014). The most prevalent nutrient-delivering symbiosis is the association of plants with fungi of the phylum *Glomeromycota*, resulting in the formation of arbuscular mycorrhizas (Smith & Read, 2008). More than 80% of extant terrestrial plants establish arbuscular mycorrhizal (AM) symbioses, and this fundamental capacity has been retained in major crop species throughout the processes of domestication and improvement (e.g. Koide et al., 1988; Hetrick et al., 1992; Kaeppler et al., 2000; Sawers et al., 2008). Concomitantly, these same crops have retained a conserved molecular machinery required for symbiotic establishment and nutrient exchange (Paszkowski et al., 2002; Gutjahr et al., 2008; Yang et al., 2012; Willman et al., 2013).

AM fungi provide the plant host with greater access to soil nutrients and water through connection to a network of fungal hyphae more extensive than the plant's own root system (Bucher, 2007). In addition, AM symbioses have been implicated in enhanced tolerance to a range of abiotic and biotic stresses (Smith & Read, 2008). Such benefits are not provided without cost, however, and the plant host must provide carbohydrates to the fungus, which represents a diversion of photosynthetically fixed carbon away from primary productivity and yield. Ultimately, the outcome, which may be positive or negative, is dependent not only on the specific plant-fungus combination (Walder et al., 2012) but on the requirements and limitations imposed by any given environment (Janos, 2007). Indeed, in high-input modern agricultural systems, the benefit of the symbiosis to the plant may be...
marginal (Grace et al., 2009), and it has been hypothesized that conventional breeding practices may have promoted weakening of the mutualism (Hetrick et al., 1992, 1996).

Comparisons of mycorrhizal response, however, can be complicated by variation in overall plant adaptation to a given set of conditions - poorly adapted plants will typically show the greatest performance increase following AM colonization (e.g. Hetrick et al., 1992; Kaeppler et al., 2000), although such improvement need not indicate a superior capacity to benefit from colonization per se (Sawers et al., 2010). The maize mutant lrt1, which exhibits reduced lateral root development, illustrates an extreme case: high dependency under low P availability that can be largely compensated by the formation of AM symbiosis (Paszkowski & Boller, 2002). The question remains as to whether, and how, certain varieties derive greater benefit from AM symbioses than others and to what extent plant breeding can optimize these interactions for agricultural systems (Sawers et al., 2008; Fester & Sawers, 2011). A better understanding of the molecular and physiological impact of AM symbiosis has the potential to enhance greatly interpretation of outcome variation.

The best characterized benefit of AM symbiosis is enhanced plant phosphorus (P) nutrition. Given that limited P availability is a major check on global agricultural production and food security, assessment of AM outcome in terms of P nutrition is a justifiable approximation of this complex symbiotic trade-off. The efficiency with which crop plants convert P resources to yield (P Efficiency; PE) can be partitioned between the efficiency of uptake (P Acquisition Efficiency; PAE), and the efficiency of internal use (P Use Efficiency; PUE) (Rose et al., 2011; Veneklaas et al., 2012), AM symbiosis most directly impacting the former. Levels of P fertilizer uptake in agricultural systems are typically low (15-20%; Syers et al., 2008), largely as a result of the relative immobility of P in the soil and the ready
formation of a zone of P depletion around the root (Bucher, 2007). Optimization of the root
system architecture can contribute significantly to P foraging (Lynch, 2011), but, under a
given set of conditions, AM symbioses may present the greatest opportunity to access a
greater soil volume. Physiological studies have demonstrated that symbiotic phosphate
uptake is a distinct functional alternative to direct uptake by the plant (Smith et al., 2003;
Bucher, 2007), and in a field setting, the majority of the phosphate taken up by a plant may be
acquired via the symbiotic route (Schweiger and Jakobsen, 1999; Smith et al., 2003; Yang et
al., 2012).

Molecular analyses have supported the distinction between symbiotic and direct
phosphate uptake, identifying the various members of the plant PHT1 P transporter family to
play roles specific to the the two pathways. Where plants are competent to host AMF, there is
at least one family member (PT11 in rice and variously named orthologs in other species)
acting predominantly, or exclusively, during AM symbiosis (Rausch et al., 2001; Harrison et
al., 2002; Paszkowski et al., 2002; Glassop et al., 2005; Nagy et al., 2005; Maeda et al.,
2006; Caesar et al., 2014; Walder et al., 2015). Mutant analysis has demonstrated PT11
proteins to be essential for formation and maintenance of AM symbiosis (Maeda et al., 2006;
Javot et al., 2007; Yang et al., 2012), although this phenotype has been partially rescued by
nitrogen starvation in medic (Breuillin-Sessoms et al., 2015) and co-cultivation with wild-
type plants in maize (Willmann et al., 2013). In medic, the PT11 protein MtPT4 has been
localized to the peri-arbuscular membrane (Harrison et al., 2002; Kobae & Hata, 2010;
Pumplin et al., 2012), and the PT11 proteins are considered to provide the principal route of P
uptake from fungus to plant.
In this study, differences in mycorrhiza response among a panel of diverse maize lines are dissected to identify variation linked to a greater ability of the host to profit from the symbiosis. Selected lines were further characterized by quantification of AM-mediated and total P uptake, fungal colonization of roots and soil, and accumulation of transcripts encoding PHT1 family P transporters.

**MATERIALS AND METHODS**

**Evaluation of response to AMF in maize diversity panel**

A panel of 30 diverse maize lines, comprising the 26 diverse inbred founders of the maize NAM population (McMullen et al., 2009), Pa36 (a line tolerant of low P availability; Kaeppler et al., 2000), and the lines B73, W22 and W64A (a line used previously for study of AM symbiosis; Paszkowski et al., 2006), was evaluated in one litre pots, under conditions of low phosphorus availability, with or without inoculation with *Funneliformis mosseae* (isolate number 12, European Bank of Glomales, http://www.kent.ac.uk/bio/beg/), as previously described (Sawers et al., 2010). At 8 weeks after emergence, the aerial part of the plant was harvested, dried and weighed. Six experiments (A-F) were conducted in the greenhouse facility at the University of Lausanne, Switzerland, during the period 2007 - 2010. Each treatment was replicated three times in each experiment, with the exception of experiment D in which each treatment was replicated five times. Shoot dry weight data was analyzed without further transformation for clarity. Systematic variation among experiments was eliminated using linear estimation. The experiment effect was estimated separately for non-inoculated and inoculated plants. Mycorrhiza response was estimated for each genotyping by calculation of a $t$-interval for the difference of inoculated and non-inoculated means. All
analysis was performed using R statistics (www.r-project.org). See Supporting Information
for raw data and further details.

**Determination of elemental concentration by ICP-MS analysis**

Tissue samples were weighed then digested in 2.5mL concentrated nitric acid (AR Select
Grade, VWR) with internal standard added (20ppb In, BDH Aristar Plus). Sample digestion
and dilution was carried out as described in Ziegler *et al.*, 2013. Elemental concentrations of
B, Na, Mg, Al, P, S, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Mo, and Cd were measured
using an Elan 6000 DRC-e mass spectrometer (Perkin-Elmer SCIEX) connected to a PFA
microflow nebulizer (Elemental Scientific) and Apex HF desolvator (Elemental Scientific)
using the procedure described in Ziegler *et al.* To correct for machine drift both during a
single run and between runs, a control solution is run every tenth sample. All analysis was
performed using R statistics. See Supporting Information for raw data and full analysis.

**Characterization of AM phosphorus uptake in six selected lines**

Six maize lines with different low P tolerance were selected and evaluated at the Technical
University of Denmark. Plants were grown in 2.4 L PVC tubes in accordance with Smith *et
al.*, 2003. The growth medium (hereafter referred to as soil) contained 7.9 mg 0.5M
bicarbonate-extractable P kg⁻¹ (Olsen *et al.*, 1954) and was a 1:1 (w:w) mixture of sand and
irradiated soil (10 kGy, 10 MeV electron beam) that received basal nutrients (Pearson &
Jakobsen, 1993) and KH₂PO₄ at nil, 15 or 90 mg P kg⁻¹. All nutrients were added in solution
and carefully mixed into the soil. The root plus hyphal compartment (RHC) contained 2750 g
soil and the hyphal compartment (HC) was a small plastic vial placed in the middle of the
RHC. The HC contained 55 g of ³³P labeled soil (5 kBq g⁻¹) and lined with a 25 μm nylon
mesh at both ends to prevent root in-growth. Seven weeks later, bicarbonate extracts had a specific activity (SA = $^{33}$P/$^{31}$P) of 144.7, 79.9 or 29.4 kBq mg$^{-1}$ P in soil amended with nil, 15 or 90 mg P kg$^{-1}$. Each maize line was grown in 8 replicate pots in half of which 140 g dry soil-root inoculum of *Rhizophagus irregularis* BEG87 was thoroughly mixed into the growth medium. Filtered BEG87 inoculum leachings were added to all pots as an attempt to establish the same soil microbial community (Pearson & Jakobsen, 1993). Two pre-germinated seeds were planted in each pot and thinned to one at the two leaf stage. Plants were maintained under controlled conditions (12 hour day length at 500 $\mu$mol m$^{-2}$ sec$^{-1}$, 28/20°C day/night and 60 % relative humidity) and watered daily by weight to 70% of the water holding capacity. In addition to the initial basal nutrient dressing, supplemental N (NH$_4$NO$_3$), Mg and S (MgSO$_4$$^{2-}$) was added periodically to additionally provide 375 mg N, 15 mg Mg and 20 mg S per pot. Shoots were harvested at growth stage 51 (BBCH scale; tassel emergence at the top of the stem), oven dried to constant weight at 70°C and dry weights were recorded. Roots system was carefully washed clean using a pressurized water jet and a fine mesh to collect fine root pieces. Roots were blotted dry and total fresh weight (FW) was recorded. Subsamples were taken for root length/colonization measurement (1.5g FW, stored in 50% EtOH) and RNA extraction (1g, flash-frozen in liquid nitrogen). Dried and ground shoot and root samples were oxidized in a 4:1 mixture (v:v) of 65% nitric:70% perchloric acids, and total P was determined by the molybdate blue method using AutoAnalyzer 3 (Bran+Luebbe, Norderstedt, Germany). The $^{33}$P in shoot tissue was determined in the same digests in a Packard TR 1900 liquid scintillation counter (PerkinElmer, Waltham, MA, USA). Specific activities of $^{33}$P in shoots and in bicarbonate extracts of HC soil were used to estimate the relative contribution of the AM pathway to total shoot P uptake as described in Smith *et al.* (2004). Root length
was measured by image analysis using the Win-Rhizo software (Win-Rhizo version 2009b, Regent Instruments, Canada) and a scanner (Epson (ModelJ1221A), Seiko Epson Corp. Japan). Images were acquired by placing 1.5 g untangled roots (FWRL), from the RHC subsample in a water filled Plexiglas tray (17.5 x 23.9 cm). Total root length of each plant was calculated as RL x FWRoot x (FWRL)^{-1}. The abundance of total fungal structures (hyphae, arbuscules or vesicles) or arbuscules specifically was evaluated microscopically as percentage of root length using the grid-line intersect method (Newman, 1966) after clearing and staining (Kormanik & McGraw, 1982). Hyphal length was measured by a grid intersection method after wet-sieving of aqueous soil suspensions on membrane filters (Jakobsen et al., 1992). Where appropriate, mycorrhiza response was estimated for each genotyping by calculation of a t-interval for the difference of inoculated and non-inoculated means. All analysis was performed using R statistics. See Supporting Information for raw data and full analysis.

**Bioinformatic identification of maize Phl genes**

To identify a complete set of putative PHT1 encoding genes in maize, the *Saccharomyces cerevisiae* PHO84 protein (Uniprot id P25297) was used as a BlastP query (Altschul et al., 1990) to search the primary transcript predicted protein sequences from version 6a of the annotated B73 maize genome (Schnable et al., 2009), obtained from Phytozome 10 (Goodstein et al. 2012). Using a cut-off E-value of 1e^{-54}, 13 gene-models were retrieved and aligned using MUSCLE (Edgar, 2004). All 13 sequences contained the conserved GGDYPLSATIxSE motif in helix 4 reported previously to be present in PHT proteins (Karandashov & Bucher, 2005). The resulting block-alignment file was converted to Stockholm 1.0 format, and used as input to hmmbuild (HMMER suite version 3.1b2) to
search (hmmssearch) the maize primary transcript predicted protein sequences for additional PHT1 proteins. 35 new protein sequences were identified based on an inclusion threshold of E-value <0.01. None of these additional sequences, however, contained the conserved GGDYPLSATIxSE motif and consequently there were not considered to be authentic PHT1 proteins. The final list of 13 maize PHT1 encoding gene models is presented in Supporting Information.

Analysis of ZmPt transcript accumulation

A LightCycler 480 SYBR green I master mix kit (Roche; Mannheim, Germany) was used to prepare samples before analysis on a Roche 480 LightCycler. Each biological sample was analysed as three technical replicates. Three water controls were used for each gene tested. qRT-PCR expression and melting curves were calculated using the LightCycler 480 software (Roche, Version 1.5.9, Mannheim, Germany). Samples were normalized to the geometric mean of expression levels of 3 constitutive genes (GAPDH, Cyclophilin2, ß-actin) as described earlier (Gutjahr et al., 2008). In total 6 phosphate transporters were analysed together with an AM specific marker gene ZmAm3, ortholog of OsAM3 (Gutjahr et al., 2008) and a Rhizophagus irregularis elongation factor gene (Sokolski et al., 2010). Statistical analysis was performed using R statistics. See Supporting Information for full analysis.

Principal component analysis of combined growth, physiology and molecular data sets

Pairwise correlations were calculated for a matrix of growth, physiological and molecular data obtained from six selected lines as described above (shoot dry weight, shoot P, root dry weight, root P, total colonization, arbuscule abundance, length of root-external hyphae, P uptake from the hyphal compartment, transcript accumulation of Pt6, Pt8b, Pt11, Pt13a,
Pt13b, Am3 and RiEF) using rcorr::rcorr in the R statistics package and the results visualized using corrplot::corrplot. Principal component analysis (PCA) was performed with ade4::dudi.pca in the R statistics package using centered and scaled data, and the results visualized with ade4::scatter.

RESULTS

Mycorrhiza response is correlated with accumulation of phosphorus in the leaves

To define physiological and molecular patterns correlated with variation in the outcome of AM symbioses, a panel of thirty diverse maize lines (see Materials and Methods), was evaluated under P limiting conditions in the greenhouse, with (M) or without (NC) inoculation with the fungus Funneliformis mosseae. Plants were harvested eight weeks after emergence (V8 stage, before the onset of flowering; Counce et al., 2000) and shoot dry-weight (SDW; g) determined (Table 1). Collectively, the evaluated lines showed a positive outcome when inoculated with AMF (Fig. S1), with a significant (p < 0.001) increase in mean SDW from 1.05g in NC plants to 2.16g in M plants, equating to a panel-wide mycorrhiza response (MR = M - NC) of 1.1g ±0.08g (95% interval for difference in means).

Roots were harvested from a subset of plants, and the abundance of fungal structures quantified by microscopic inspection. NC plants were confirmed to be free of fungal structures. The greatest SDW in NC plants was seen in Pa36 (1.67g), and the lowest in Hp301 (0.44g). The level of colonization in M plants was generally high, with a mean of 57% ±0.7% (95% interval for proportion) of root positions examined containing at least one type of fungal structure (hyphae, arbuscules or vesicles), although a broad range of colonization was observed (5% - 98%). At the level of individual lines, all showed increased SDW following
inoculation (Fig. 1a; Table 1). In the panel evaluation, the most responsive line was Oh43 (MR = 1.85g), and the least responsive was Mo18W (MR = 0.72g). The contrast in outcome between Oh43 and Mo18W was reflected by rank-changing shifts in growth relative to other lines in the panel: similar and typical of the panel in the absence of fungus; different and outlying when inoculated (Fig. 1b). As such, Oh43 and Mo18W show similar dependence but a divergent capacity to profit from AM symbiosis.

Evaluation was conducted under P limiting conditions, suggesting that variation in performance would be driven largely by differences in P accumulation. To evaluate plant nutrition status, the accumulation of P and nineteen other elements was quantified in roots and leaves using inductively coupled plasma-mass spectroscopy (ICP-MS; Baxter et al., 2008). Ten elements (Na, Al, P, S, Mn, Fe, Co, Ni, Zn, Cd) were found to accumulate to different levels between M and NC plants, in either roots and/or leaves (p<0.05, adjusted for multiple tests; Table S1). Principal component analysis indicated variation in P content was the most important factor differentiating lines and NC and M plants (Fig. S2). Leaf P content was positively correlated with SDW, notably in AM plants (Table 1; Fig. 1; p<0.01, r²=0.24).

Leaf P content was similar in Mo18W and Oh43 when non-colonized (393ppm and 292ppm, respectively) but diverged when plants were inoculated (422ppm and 625ppm, respectively), mirroring SDW, and suggesting variation in P uptake efficiency to be driving the difference in MR (Fig. S3). On the basis of this initial screen, Mo18W and Oh43 were selected for more detailed physiological study. HP301 and Pa36 were selected also as they showed the lowest and highest dependence (i.e. NC SDW), along with the broadly used lines B73 and Mo17.

Mycorrhizal response is correlated with the abundance of root-external hyphae
As plant growth was correlated with P accumulation in the initial screen, it was decided to characterize in detail direct and AM P uptake across a range of P availability in the selected lines Oh43, Mo18W, Pa36, HP301, B73 and Mo17. Plants were grown in a previously described split-compartment system using $^{33}$P uptake and accumulation (Smith et al., 2003). SDW increased with greater P addition, irrespective of AMF inoculation (Table 2; Fig. 2). In contrast, average MR across lines ranged from positive to negative with increasing P availability (low P MR = 9.56g; medium P MR = 3.95g, high P MR = -1.57g). The relative MR among lines changed also with P availability. Growth at low P was strongly correlated with seed size (Fig. S4). Root dry weight (RDW) was greater in M than in NC plants at low P, was not significantly affected by colonization at medium P and was reduced in M relative to NC plants at high P (data not shown). As observed in the panel evaluation, SDW was correlated with total shoot P content, both among lines and between NC and M treatments (Fig. 2). At low P, MR (in terms of SDW) was higher in Oh43 than in the other lines, consistent with the results of the panel evaluation. Oh43 remained highly responsive at medium P, but exhibited a negative response at high P (Fig. 2). Although showing high MR at low P, Oh43 did not show a greater total root colonization than the other lines (Fig. 3a). Indeed, the proportion of root length containing arbuscules was marginally lower in Oh43 than other lines (Fig. 3a). In contrast, Oh43 supported a significantly greater development of extra-radical hyphae than the other lines at low P, and the greatest AM P uptake (Fig. 3b). Across all lines, the length of root-external hyphae decreased with increasing P availability, although P uptake from HC soil increased (Fig. 3b). B73 continued to supported abundant extra-radical hyphae even at high P availability with a concomitant high level of AM P uptake.
The maize genome encodes multiple AMF-responsive PHT1 phosphate transporters

To investigate further the balance of direct and AM P uptake, transcripts encoding Pht1 phosphate transporters were quantified. A previous report identifying 13 Pht1-encoding genes in the maize genome (Liu et al., 2016) was confirmed using a hidden Markov search (Table S3). Maize and rice PT protein sequences were aligned, along with a number of previously characterized mycorrhiza associated sequences from other species, and this alignment used to construct a maximum likelihood tree, identifying gene groups and providing the basis for the nomenclature used below (Fig. S5; maize genes are named by similarity to rice following a nomenclature reported in doi: http://dx.doi.org/10.1101/042028. A key relating the nomenclature used here to that of Liu et al. is provided in Table S3.)

ZmPT11 (previously reported as ZmPHT1;6. Nagy et al., 2006; Willmann et al., 2013) was the unique maize member of a group including the well-characterized mycorrhiza-associated proteins medic MtPT4 (Harrison et al., 2002), rice OsPT11 (Paszkowski et al., 2002), potato StPT4 (Nagy et al., 2005) and tomato LePT4 (Nagy et al., 2005). ZmPT14 was the only maize member of a second mycorrhiza-associated group that included barley HvPT8 and wheat TaPT1:myc (Glassop et al., 2005). ZmPT9 and ZmPT13a-d defined a further group with the mycorrhiza-associated rice protein OsPT13 (Yang et al., 2012). The remaining ZmPT proteins belonged to a larger group that contained a number of rice proteins characterized previously to play roles in direct P uptake and translocation.

To gain a general panorama, ZmPt transcript accumulation was investigated using two existing seedling transcriptome datasets (Wang et al., 2009; Li et al., 2010) and a third set profiling reproductive tissues (Davidson et al., 2011). Representative Pt transcripts were
found in all tissues and developmental stages examined, indicating the importance of the
family throughout the plant life cycle, while revealing specialization at the level of transcript
accumulation (Fig. S6). Ten Pt transcripts were selected and quantified directly in the roots
and shoots of B73 seedlings, grown under low (10μM), moderate (100μM) or high (1000μM)
P availability in the absence of AM colonization, or under moderate P with R. irregularis
inoculation. Seven of ten selected Pt transcripts (Pt6, Pt7, Pt9, Pt11, Pt13a, Pt13b and Pt14)
accumulated differentially between NC and M plants, in at least one of the tissues assayed
(Tukey HSD, α=0.05; Fig. 4). The transcripts Pt7, Pt9, Pt11, Pt13a and Pt14 accumulated to
significantly higher levels in the roots of M plants compared to NC plants. In the case of
ZmPt14, transcripts accumulated exclusively in the roots of M plants. Transcripts encoded by
ZmPt11 were the most abundant in colonized roots, although they were present also at lower
levels in roots and shoots of NC plants. The transcripts Pt6, Pt9, Pt11, Pt13a and Pt13b
accumulated to significantly lower levels in the leaves of M plants compared to NC plants.
With the exception of ZmPt14, all transcripts were detected in NC plants in at least one of the
tissues assayed.

Accumulation of ZmPt transcripts is correlated with mycorrhizal P uptake

To investigate the relationship between AM outcome and Pt function, accumulation of Pt6,
Pt8b, Pt11, Pt13a and Pt13b transcripts was quantified in root samples collected from the
characterization of six selected lines. In all cases, transcript accumulation broadly followed
the patterns previously observed in B73 with respect to inoculation status and P addition (Fig.
S7; Supporting Information). To synthesize growth, physiological and molecular data (Table
2), pairwise trait correlations were investigated across all treatments (Fig. 5a). Significant
positive correlations (p < 0.05) were observed between the abundance of fungal structures
and the accumulation of transcripts encoded by RiEF, AM3 (respectively, fungal and plant
marker transcripts) and Pt11, Pt13a, supporting the primary role of these two transporters in
AM symbiosis. Accumulation of Pt6 and Pt13b transcripts was significantly negatively
correlated (p < 0.05) with P accumulation and dry weight in both roots and shoots, indicating
induction under low-P stress. To investigate further patterns associated with AM outcome, the
analysis was repeated restricting the dataset to observations of M plants under low-P (Fig.
5b). A significant positive correlation (p < 0.05) was observed between the extent of root
external hyphae and P uptake from the hyphal compartment (PHC), which in turn was
positively correlated with shoot P and plant growth. Interestingly, the abundance of intra-
radical fungal structures was strongly, if not significantly, negatively correlated with both the
extent of root-external hyphae and PHC. At the molecular level, accumulation of Pt6 and
Pt8b transcripts was significantly positively correlated (p < 0.05) with root dry weight, and,
at lesser significance, with SDW, root and shoot P content and PHC. Accumulation of Pt13a
transcripts showed also a weakly significant (p < 0.1) positive correlation with shoot dry
weight. Trait correlation in M plants at low-P was investigated further using principal
component (PC) analysis. Collectively, the first two PCs captured 76% of the trait variation
and well separated the six lines (Fig. 6). The abundance of root-external hyphae, PHC and
shoot P were observed to increase together with root and shoot dry weight and accumulation
of Pt6 and Pt8b. Accumulation of the Pt6 and Pt8b transcripts was more tightly associated
with increasing root and shoot dry weight than with root-external hyphae or PHC. Oh43 was
characterized by high levels of root-external hyphae, PHC, shoot P and MR. The line Pa36
was associated more with higher dry weight than higher PHC. Total AM colonization and
Arbuscule abundance was antagonistic to the abundance of root-external hyphae, Hp301 and Mo18W exhibiting high levels of intra-radical fungal structures, but low levels of root-external hyphae, P accumulation, and root and shoot dry weight. Mo17 was distinct in expressing high levels of accumulation of AM associated transcripts, although with no associated increase in colonization, development of root-external hyphae or MR.

**DISCUSSION**

Data presented in this study reveal genetic variation in the capacity of maize varieties to profit from AM symbiosis, beyond differences in plant dependence (Janos 2007; Sawers et al., 2008, 2010). Evaluation of the relative growth of thirty highly diverse lines (McMullen et al., 2009) distinguished those that were highly responsive on the basis of poor performance in the absence of symbiosis (e.g. HP301) from those that benefited more from the symbiosis per se (e.g. Oh43). Support for this initial interpretation was obtained by detailed physiological characterization that provided a mechanistic explanation, and linked superior responsiveness to enhanced PAE on the basis of greater abundance of root-external hyphae.

P limitation of plant growth results primarily from the low mobility of P in the soil, especially at non-neutral pH, with AM fungi acting primarily to increase PAE through enhanced soil foraging (Smith & Read, 2008). Indeed, in this study, it was observed that increased plant growth in AM colonized plants was accompanied by greater P uptake. On reaching the root surface, or being delivered to the peri-arbuscular space, P will be rapidly taken up through the action of high-affinity PHT1 phosphate transporters, not limiting PAE. In contrast, movement of P into the vicinity of the root will be slow, resulting in the formation of a zone of P depletion surrounding the root (Bucher, 2007). As is consistent, it was not the extent of intra-radical colonization nor the accumulation of Pt11 transcripts that showed the
greatest correlation with P uptake, but the abundance of root-external hyphae. If anything, greater arbuscule abundance was negatively correlated with MR, HP301 and Mo18W exhibiting high levels of colonization but low levels of AM P uptake. This is in general support of previous studies (e.g. Schweiger & Jakobsen 1999, Jakobsen et al., 2001, Yao et al., 2001, Schnepf et al., 2008), including an evaluation of diverse fungal isolates with a common plant host, which again found a similar correlation between fungal P uptake and hyphal length (Munkvold et al., 2004). Although a further report characterizing AM response variation in four Chinese maize varieties (Chu et al., 2013) did not reveal a clear relationship between P uptake and the length of root-external hyphae, this may be a function of the specific genotypes evaluated, of the fact that the contribution of mycorrhizal P uptake was not directly quantified. Regarding the apparent antagonism between the abundance of intra-radical and root-external fungal structures, the data are consistent with a trade-off in fungal growth, the balance of which is influenced by plant genetic factors. Given the importance of hyphal abundance to PUE, the data suggests that quantification of intra-radical structures alone is not predictive of MR, nor an indication of the strength of mutualism.

Prior physiological characterization has demonstrated that AM P uptake is not a simple addition to the plant's direct uptake pathway, but may represent a functional alternative: in the extreme case, a colonized plant may obtain nearly all of its P requirement via the AM pathway, whether as a result of down regulation of the direct pathway or owing to a greater efficiency of fungal P foraging compared with that of plant roots (Smith et al., 2003; Schnepf et al., 2008). Furthermore, the AM pathway may remain important at higher P availability, even when MR itself is small, or even negative. In the evaluation of six selected lines, increasing the availability of P resulted in reduced abundance of both root-external and intra-
radical fungal structures, along with declining MR. Nonetheless, the absolute quantity of P obtained via the AM pathway was greater, presumably as the result of increased Pi in solution as the capacity of the soil to adsorb P was saturated. At highest availability, in the lines with the highest P uptake, the growth response to shoot P accumulation was apparently saturated. B73 and Pa36 attained maximum growth irrespective of AM inoculation, although in both M plants accumulated less shoot P in the shoots than NC plants, i.e. they exhibited greater PUE. Interestingly, although at high P B73 and Pa36 showed equivalent levels of intra-radical fungal structures, the abundance of the root-external hyphae was greater in B73. Indeed, B73 supported a high level of root-external hyphae, second only to Oh43 at low P and greater than all other lines at high P. Collectively, these data illustrate the complexity of determining symbiotic outcome, and the range of plasticity among even six lines, with respect to just a single environmental variable.

Previous transcriptome profiling has identified rice marker genes whose mRNA accumulation correlates well with the establishment and development of AM symbiosis, well differentiating colonized from non-colonized plants (e.g. Guimil et al., 2005; Gutjahr et al., 2015). In this study, transcripts encoding PHT1 phosphate transporters were quantified not only to generalize among NC and M plants, but also to investigate differences in AM outcome among lines. Overall, AM colonization was positively correlated with the accumulation of transcripts encoded by Pt11, and to a lesser extent those encoded by Pt13a, Pt13b and Pt14, consistent with previous reports (Nagy et al., 2006; Willmann et al., 2013; Liu et al., 2016). Arbuscule abundance was found to be a poor predictor of MR among the six lines evaluated in this study. It is therefore consistent that variation in ZmPt11 transcript accumulation was also largely independent of differences MR. These observations are
consistent with the physiological data suggesting P transfer at the peri-arbuscular interface to be non-limiting, either with respect to arbuscule abundance or to the concentration of PT11 proteins in the peri-arbuscular membrane. PT11 protein, however, has been reported also to regulate developmental responses to P limitation (Volpe et al., 2016), and high levels of Pt11 accumulation may have additional significance beyond P transfer. A number of additional maize Pt transcripts responded to AM inoculation, although they were less abundant than those encoded by Pt11. Significantly, at low P, a mild positive correlation was observed between accumulation of Pt13 transcripts and shoot biomass among colonized plants, indicating a role in the regulation of the symbiosis. Accumulation of Pt6 and Pt8b transcripts, although generally lower in M relative to NC plants, was positively correlated with root and shoot dry weight among M lines. Accumulation of Pt8b was positively correlated also with P uptake from the hyphal compartment. Interestingly, the correlation was stronger with dry weight than P content, indicating this to be more than a secondary effect of differences in P accumulation. These observations, along with previous characterization of pt11 and pt13 mutants in rice (Yang et al., 2012), suggest a role for PHT1 proteins not only in P uptake but in the fine tuning of cost-benefit in AM symbioses.

Previous characterization of variation in MR has placed an emphasis on the development of intra-radical fungal structures, and marker transcripts have been identified allowing molecular-based quantification of intra-radical colonization. In this study, it was observed that variation in the abundance of root-external hyphae was more significant than levels of intra-radical colonization in determining AM outcome. Although accumulation of the well characterized Pt11 transcript was not predictive of the abundance of extra-radical hyphae, correlations were observed between transcripts encoded by other Pt genes,
abundance of extra-radical hyphae and AM outcome. The identification of such variation, coupled with the availability of NAM populations for quantitative trait loci mapping (McMullen et al., 2009), opens up the possibility to characterize the genetic basis of host effects on the development of extra-radical hyphae, and potentially to develop molecular breeding strategies to target this important, but hard to evaluate, component of MR.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation ‘professeur boursier’ grants PP00A-110874, PP00P3-130704, by the Gatsby Charitable Foundation grant RG60824, by The Danish Council for Independent Research, Technology and Production Sciences grant 0602-01412B and by the Mexican National Council of Science and Technology (CONACYT) grant CB2012-151947. We thank Mesfin Nigussie Gebreselassie and Matthias Mueller for assistance with plant growth and evaluation.

AUTHOR CONTRIBUTION

Diversity screen: RS, BW, UP. Ionomic analysis: IB. Physiological characterization of phosphate uptake in selected lines: SS, MG, IJ. Identification of Pht1 genes and phylogenetic analysis: EGM, RCM, RS. Quantification of transcript accumulation: CQ, SS. Statistical analysis: JG, RS. Experimental design: UP, IJ, RS. All authors contributed to data interpretation and writing of the manuscript.
## Table 1. Mycorrhiza response in diverse maize lines

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Dry mass (g) and phosphorus (P) accumulation (ppm) in non-colonized (NC) and mycorrhizal (M) plants from the evaluation of 30 lines. Mycorrhiza response (MR) was calculated as M-NC, numbers in parentheses report a 95% confidence interval (CI). P accumulation reported +/- one standard error (SE).
### Table 2. Summary of growth, physiological and molecular data

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<th>RDW (g)</th>
<th>Root P (mg)</th>
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<th>Col Arb (%)</th>
<th>Hyp (m/g)</th>
<th>PHC (mg)</th>
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Characterization of colonized (M) and non-colonized (NC) plants of six selected lines grown under high (53.2 mgP/Kg) or low (7.9 mgP/Kg) phosphorus (P) availability. SDW, shoot dry weight; shoot P, P accumulation in the shoot; RDW, root dry weight, root P, P accumulation in the root; Col Tot, abundance of intra-radical fungal structures; Col Arb, arbuscule abundance; HYP, length density of root-external hyphae, PHC, P uptake from the hyphal compartment; Pt6, Pt8b, Pt11, Pt13a, Pt13b, Am3 and RiEF, transcript accumulation normalized to
**FIGURE LEGENDS**

**Fig. 1.** Mycorrhiza response is correlated with enhanced P uptake. (a) Shoot dry weight (SDW, g; normalized with respect to differences among replicates) of 30 diverse maize lines grown for 8 weeks, under greenhouse conditions, with (M; right box) or without (NC left box) inoculation with the fungus *Funneliformis mosseae*. Boxes show 1st quartile, median and 3rd quartile. Whiskers extend to the most extreme points within 1.5x box length; outlying values beyond this range are not shown. The mean values of NC (1.05g, n=540) and M (2.16g, n=552) groups are shown by vertical lines. Lines ordered by increasing mycorrhizal response from top to bottom. Box shading indicates mean accumulation of P$^{31}$ (ppm) in the shoot as determined by ionomic analysis, colour-key shown at top of panel. (b) Reaction norms for 30 diverse maize lines contrasting shoot dry-weight (R SDW, g; residual SDW with respect to group mean) of non-inoculated plants (NC) and plants inoculated with the fungus *Funneliformis mosseae* (M). Segments corresponding to six lines selected for further study are labeled and shown in bold. Point shading indicates accumulation of P31 (ppm) in the shoot as (a). (c) Accumulation of phosphate in leaves (P$^{31}$, ppm) and shoot dry weight (SDW, g) in the 30 maize lines (points correspond to mean values). Linear fit (yellow line) and associated 95% confidence interval (shaded area) shown.

**Fig. 2.** Shoot dry weight (Shoot DW; g) is correlated with total phosphorus (P) accumulation (Shoot P; mg) in six maize genotypes grown with (yellow circles) or without (open circles) inoculation with *Rhizophagus irregularis*, under three P regimes (7.9 mgP/kg, 15.5 mgP/kg, 53.2 mgP/kg). Points indicate the mean of n observations; whiskers extend +/- 1 standard error; trend lines based on a linear fit to individual observations for each treatment.
Fig. 3. Phosphorus (P) accumulation is correlated with the abundance of extra-radical hyphae. (a) percentage of total root length containing mycorrhizal structures (brown) and arbuscules (yellow) observed in inoculated seedlings of six maize inbred lines, grown under three different levels of phosphorus abundance (7.9, 15.5 and 53.2 mgP Kg$^{-1}$). Boxes show 1st quartile, median and 3rd quartile. Whiskers extend to the most extreme points within 1.5x box length. (b) Shoot phosphate acquired from $^{33}$P soil (mg) as a function of the length of external hyphal (m g$^{-1}$ soil).

Fig. 4. Accumulation of transcripts encoding PHT1 proteins responds to phosphorus availability and AM colonization. Heatmap representation of accumulation of selected ZmPt (Pt) transcripts quantified by real-time PCR relative to beta-actin. Accumulation of selected Pt transcripts was quantified in B73 seedling shoot or root tissue. Plants were grown across a range of increasing P availability at 10μM, 100μM, 1000μM P without inoculation with AMF (NC) and also at 100μM with inoculation with R. irregularis at 100μM (M). Mean accumulation was determined from three biological replicates, standardized (Z-score) within transcripts across experimental treatments and represented on a scale from white (below average accumulation) to brown (above average accumulation). Accumulation of the maize mycorrhizal marker transcript Am3 and the R. irregularis elongation factor RiEF is also shown. Transcripts responsive to inoculation with AMF marked with an asterisk.

Fig. 5. Accumulation of transcripts encoding PHT1 proteins is correlated with phosphorus accumulation and AMF inoculation across diverse maize lines. Selected ZmPt (Pt) transcripts, the maize mycorrhizal marker transcript Am3 and the R. irregularis elongation factor RiEF were quantified by real-time PCR in samples taken from the roots of plants described in Fig. 2. (a) Correlation matrix (Pearson) of transcript accumulation, shoot and root dry weight (DW, g), shoot and root phosphorus accumulation (P, mg), proportion of root length colonized (% Col, all structures) and proportion of root.
length containing arbuscules (% Arb). Red circles indicate positive correlation; blue circles indicate negative correlation; the size and intensity of
shading indicate magnitude. Correlations significant at p < 0.05 marked with an asterisk. Correlations significant at 0.05 < p < 0.1 marked with a point.
(b) as (a) with data restricted to observations of AM colonized plants grown under low P.

Fig. 6. AM P uptake is correlated with the extent of the root-external mycelium under low P. Principle component analysis (PCA) of plant-growth,
physiological and molecular observations of inoculated plants grown under low P. Biplot showing scores in the first two principal components (PC1,
PC2) for traits (black arrows: shoot dry weight (shoot DW), shoot P, root dry weight (root DW), root P, total colonization (%Col), arbuscule
abundance (%Arb), length of root-external hyphae (Hyphae), P uptake from the hyphal compartment (PHC), transcript accumulation of Pt6, Pt8b,
Pt11, Pt13a, Pt13b, Am3 and RiEF) and the individual genotypes (B73, Mo17, HP301, Pa36, Mo18W, Oh43). Points indicating the different
genotypes are colour-coded by mycorrhizal response (MR, g) calculated as the difference in shoot dry weight in colonized and non-colonized plants.
REFERENCES


Paszkowski, U, Boller, T. 2002. The growth defect of *lrl1*, a maize mutant lacking lateral roots, can be complemented by symbiotic fungi or high phosphate nutrition. *Planta* **214**: 584–590.


SUPPORTING INFORMATION

The following Supporting Information is available for this article:

Fig. S1 Association with mycorrhizal fungi promotes vegetative growth in diverse maize lines
Fig. S2 Inoculation with AMF is associated with changes in the ionome
Fig. S3 Oh43 and Mo18W differ in elemental profile
Fig. S4 Shoot dry weight is correlated with seed size
Fig. S5 Phylogeny of maize PHT1 proteins
Fig. S6 Maize PHT1 transcripts accumulate throughout the plant life-cycle
Fig. S7 Accumulation of six ZmPt (Pt) transcripts in root-samples
Table S1 Element accumulation in roots and leaves of non-inoculated and inoculated plants
Table S2 Phosphorus transfer data at low P
Table S3 Ph1 genes of maize (var. B73)
Table S4 Primers used in this study

Supplementary Analysis