

Arbuscular mycorrhizal colonization outcompetes root hairs in maize under low phosphorus availability

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- **Background and Aims** An increase in root hair length and density and the development of arbuscular mycorrhiza symbiosis are two alternative strategies of most plants to increase the root–soil surface area under phosphorus (P) deficiency. Across many plant species, root hair length and mycorrhization density are inversely correlated. Root architecture, rooting density and physiology also differ between species. This study aims to understand the relationship among root hairs, arbuscular mycorrhizal fungi (AMF) colonization, plant growth, P acquisition and mycorrhizal-specific P_i transporter gene expression in maize.
- **Methods** Using nearly isogenic maize lines, the B73 wild type and the *rth3* root hairless mutant, we quantified the effect of root hairs and AMF infection in a calcareous soil under P deficiency through a combined analysis of morphological, physiological and molecular factors.
- **Key Results** Wild-type root hairs extended the rhizosphere for acid phosphatase activity by 0.5 mm compared with the *rth3* hairless mutant, as measured by *in situ* zymography. Total root length of the wild type was longer than that of *rth3* under P deficiency. Higher AMF colonization and mycorrhiza-induced phosphate transporter gene expression were identified in the mutant under P deficiency, but plant growth and P acquisition were similar between mutant and the wild type. The mycorrhizal dependency of maize was 33 % higher than the root hair dependency.
- **Conclusions** The results identified larger mycorrhizal dependency than root hair dependency under P deficiency in maize. Root hairs and AMF inoculation are two alternative ways to increase P_i acquisition under P deficiency, but these two strategies compete with each other.

Key words: *Zea mays*, rhizosphere, arbuscular mycorrhiza, phosphate acquisition, roots, exudates.

INTRODUCTION

Phosphorus (P), a crucial plant macronutrient (Bielecki, 1973; Raghothama and Karthikeyan, 2005) that participates in various metabolic pathways, is a key component of nucleic acids, ATP and phospholipids (Vance *et al.*, 2003; Abel, 2017). Phosphorus deficiency is a major limiting factor for crop production in many regions of the world (Vitousek *et al.*, 2010; Johnston *et al.*, 2014), because of its low mobility and low availability in most soils (Schachtman *et al.*, 1998). Root morphological adaptations, physiological changes that include organic compound exudation, and microbial cooperation are major strategies used by plants to overcome P limitation and increase P acquisition (Lambers *et al.*, 2006; Smith and Read, 2008). Crop species differ in their relative investment in each of these strategies; maize, with its fibrous root system, invests heavily in root morphological adaptations and efficiently establishes arbuscular mycorrhizal fungal symbioses, but exudes carboxylates poorly (Lyu *et al.*, 2016; Wen *et al.*, 2019).

Long total root length, high specific root length, vigorous root branching and long, dense root hairs (especially in the top soil layer) are typical root morphology adaptations, which

greatly increase the root surface area and soil volume exploration (Vance *et al.*, 2003; Lynch, 2005; Péret *et al.*, 2014). Root hairs (the tubular-shaped outgrowths from root epidermal cells) are one of the most important root morphological adaptations (Peterson and Farquhar, 1996); they greatly increase the root surface area and play an essential role in inorganic phosphate (P_i) acquisition (Gilroy and Jones, 2000). When plants were P-deficient, maize root hairs were responsible for a >30 % increase in biomass and shoot P content (but did not affect the shoot P concentration), and were even more important under drought (Klamer *et al.*, 2019). Because of the limited solubility and mobility of P_i in soils, the soil volume next to roots and the overall root surface area are most important for P_i acquisition. The rhizosphere, the soil that is in close contact with the root surface and that is strongly influenced by exudates from the plant, is massively extended in the root hair regions compared with hairless regions (X. Ma *et al.*, 2018). Various microorganisms, such as arbuscular mycorrhiza, ectomycorrhiza and ericoid mycorrhizas, enlarge the exploited soil and exude enzymes to mobilize P_i (Smith and Read, 2008; Collavino *et al.*, 2010; van der Heijden *et al.*, 2015), while beneficial

plant growth-promoting bacteria often indirectly stimulate plant growth (Ludewig *et al.*, 2019). The majority (probably 70–80 %) of terrestrial plant species are capable of interacting with arbuscular mycorrhizal fungi (AMF) in nature (Bonfante, 2018; Brundrett and Tedersoo, 2018). AMF connect intimately with the root and transfer nutrients into cortical cell layers and can extend up to several centimetres away from the root and form a dense hyphal network (Miller *et al.*, 1995; Smith *et al.*, 2011). The hyphae greatly increase the surface area and soil volume exploited by the root and play a vital role in nutrient acquisition, especially for the sparingly soluble and poorly mobile P_i (Finlay, 2008). Arbuscular mycorrhizal plants have two pathways for the uptake of P_i from the soil solution: the direct P_i uptake pathway via the root epidermis, including root hairs; and the indirect arbuscular mycorrhizal (AM) pathway, where P_i is initially taken up by external AM hyphae (Grace *et al.*, 2009). These different pathways are associated with distinct molecular P_i uptake transporters of the PHT1 P_i transporter family, which play roles specific to the two pathways (Benedetto *et al.*, 2005; Javot *et al.*, 2007). When mycorrhizal fungi colonize plants, the expression of mycorrhiza-specific P_i transporter genes (*ZmPht1;6* in maize and variously named orthologues in other species) was greatly enhanced compared with non-colonized roots (Nagy *et al.*, 2006; Sawers *et al.*, 2017).

Both root morphological adaptation and microbial co-operation require plants to allocate photosynthetic carbon below ground to competing sinks, either to promote cellular hair growth or for transfer to the symbiotic partner (Lynch, 2015). The formation and maintenance of root hairs appear to be relatively cheap processes with respect to carbon and energy demand (Jungk, 2001; Bailey *et al.*, 2002; Brown *et al.*, 2013b), while AM colonization is associated with a costly delivery of up to 15–20 % photosynthetic carbon to fungi in exchange for nutrients (Jakobsen and Rosendahl, 1990; Wright *et al.*, 1998; Jakobsen *et al.*, 2005; Ryan *et al.*, 2012). ‘Mycorrhizal dependency’ refers to the difference in plant growth when mycorrhizal and non-mycorrhizal plants are compared (van der Heijden, 2003; Tawaraya, 2003). This dependence differs among plant species and cultivars; for example, mycorrhizal dependency in barley was much lower than in maize (Kaepler *et al.*, 2000; Tawaraya, 2003). There is compelling evidence of a general trade-off between root hairs and mycorrhizal symbiosis: plant species and genotypes with long and dense root hairs rely less on mycorrhizal fungi for P acquisition (Chen *et al.*, 2005; Brown *et al.*, 2012, 2013a). Comparison of various plant species with different root hair length and mycorrhizal dependency demonstrated that root hairs and mycorrhiza are typically inversely correlated. These alternative, but functionally similar strategies are used by plants to increase phosphate acquisition by increasing the root surface area and broaden the depletion zone around the root (Schweiger *et al.*, 1995).

Maize (*Zea mays*) is one of the most widely cultivated crops for production of both food and fodder in tropical and temperate soils worldwide (Gore *et al.*, 2009). Two maize genotypes with contrasting root hair morphology, the root hairless mutant 3 (*rth3*) (Wen and Schnable, 1994) and its corresponding wild type (B73), were used in this study. The wild-type and *rth3* roots had similar diameter but contrasting root

hair morphology: the wild type’s roots were covered by dense root hairs around 1 mm long, while the root epidermis of *rth3* had only very short bumps in the root hair zone and appeared completely bald (Fig. 1). Previous studies showed that there was no growth difference between the wild type and *rth3* in terms of shoot biomass, root biomass, mycorrhizal colonization and shoot P content under P-sufficient conditions (Kumar *et al.*, 2019), and *rth3* can grow vigorously under standard conditions with sufficient nutrition (Wen and Schnable, 1994; Hochholdinger *et al.*, 2008). In this study we aimed to understand the effects of the presence of root hairs, AM colonization and its interaction with root hairs for P acquisition and plant growth under P deficiency. We hypothesized that (1) the wild type is more adaptive to P deficiency than *rth3*, because of the presence of root hairs; (2) the contribution of AMF to plant growth and P_i acquisition is generally larger (especially large in *rth3*) and more critical than that of root hairs, and cannot be compensated by root hairs.

MATERIALS AND METHODS

Plant material and cultivation

Maize seed inbred line B73 (wild type) and root hairless mutant *rth3* were surface-sterilized by rinsing them in 10 % (v/v) H_2O_2 solution for 20 min and stored in 10 mM $CaSO_4$ overnight. Seeds were placed for 3 d between filter papers soaked in a 4 mM $CaSO_4$ solution for germination.

The nutrient-poor subsoil used in the study was stored before use for >12 years. Mycorrhization of this soil was checked with classical trypan blue staining methods and was confirmed to be essentially absent (Neumann, 2007; Klammer *et al.*, 2019). The soil had the following properties: pH 7.6, C_{org} <0.3 %, $CaCO_3$ 30 %, mineral concentrations ($mg\ kg^{-1}$) 7.9 CAL-P, 59.9 Ca, 11.3 Mg, 15 Mn, 7.8 Fe, 0.6 Zn, 0.2 B and 0.7 Cu. Thirty percentage (w/w) of quartz sand was mixed with the soil for the optimization of soil structure. After mixing, dissolved fertilizers were added ($mg\ kg^{-1}$ soil): N (200) as NH_4NO_3 , K (200) as K_2SO_4 , P (32) as $Ca(H_2PO_4)_2 \cdot H_2O$, Mg (100) as $MgSO_4 \cdot 7H_2O$, Fe (2) as EDTA iron (III) sodium salt, Zn (2.6) as $ZnSO_4 \cdot 7H_2O$ and Cu (1) as $CuSO_4 \cdot 5H_2O$. The P concentration in the final substrate was measured, which was 9.8 $mg\ kg^{-1}$ CAL-P and 6.6 $mg\ kg^{-1}$ Olsen-P. PVC cylinders (height 48 cm; diameter 10 cm) were longitudinally cut into two halves. Every half-cylinder is referred as a rhizobox. Each rhizobox was filled with mixed soil to reach a final density of 1.3 $g\ cm^{-3}$. On the sowing date, six maize

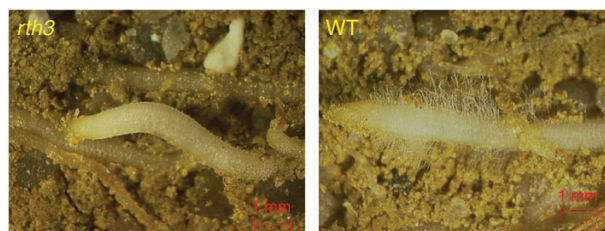


FIG. 1. Microscopic images of maize root hairless mutant *rth3* (left) and wild type (WT, right) grown in soil in rhizoboxes.

seedlings (three wild type, three mutant) were inoculated with *Rhizophagus irregularis* MUCL41833 (produced by Université Catholique de Louvain Croix du Sud, Louvain-la-Neuve, Belgium) with 8 g of beads per kilogram of soil (Loján *et al.*, 2017). Non-inoculated pots served as the control. The germinated seedling was planted at a depth of 2 cm in each rhizobox. After transplanting, the open part of the half-tube was covered with a transparent plexiglass observation window and secured with tape. Each treatment had three replicates, and a total of 12 rhizoboxes were prepared in the experiment (3 replicates × 2 treatments × 2 genotypes). The experiment used a randomized design and was conducted in the greenhouse (48°42'41.04" N, 9°12'34.20" E) in the summer of 2019. During the growth period, the boxes were kept inclined at an angle of 60° so that the roots grew along the lower side of the plexiglass cover. The rhizoboxes were weighed and irrigated from the top with distilled water every 2 d to maintain moisture at 60 % of water-holding capacity. Plants were harvested at 40 d after sowing.

Root hair length and organic acids in the root exudates

At harvest, pictures from the root hair zone were taken with a Stemi 2000-C video microscope equipped with Axio Vision 3.1 software (Zeiss, Oberkochen, Germany). From these pictures, root hair length was determined by taking the average length of ten root hairs per plant (Weber *et al.*, 2018). Afterwards, according to the method described by Neumann *et al.* (2014), root exudates were collected from a 1-cm subapical root area, visible on the observation window, by applying special sorption filter papers (MN156050, Macherey-Nagel, Munich, Germany). Sampling was conducted with five replicates for each minirhizotron, and after 2 h of collection the sorption filter papers were collected. The collected samples were re-extracted with 1 mL of 80 % (v/v) methanol and centrifuged at 18 000 g for 5 min. The supernatants (around 900 µL) were evaporated using a SpeedVac Concentrator (Savant, Farmington, USA) until dryness at 30 °C and re-dissolved in HPLC elution buffer (18 mM KH₂PO₄, pH 2.2 adjusted with H₃PO₄). The organic acids were determined by reversed-phase HPLC in the ion suppression mode with direct UV detection at 210 nm according to the method described by Haase *et al.* (2007). Isocratic elution was performed with 18 mM KH₂PO₄, pH 2.2, on a reversed-phase C-18 column (290 × 4.6 mm, GROM-SIL 120 ODS ST, 5 µm particle size), which was equipped with a 20 × 4.6 mm guard column with the same stationary phase (Dr Maisch, Ammerbuch, Germany). Identification and quantitative determination were conducted by comparison with known standards.

Rhizosphere acid phosphatase activities determined by zymography and image analysis

To visualize the acid phosphatase activity in the rhizosphere, direct zymography was used after the collection of the root exudates (Sanaullah *et al.*, 2016). Methylumbelliferyl (MUF) phosphate substrate was dissolved in MES buffer (pH 6.5) to obtain a working solution with a final concentration of 1 mM. Thin polyamide membrane filters (Tao Yuan, China)

with a pore size of 0.45 mm were saturated with substrate by soaking in the working solution. The membrane saturated with the substrate was applied directly to the rooted soil surface and incubated for 1 h. Fluorescent substrates in the membranes diffused into the soil and were hydrolysed by enzymes to release fluorescent MUF, which diffused back and was visualized under UV light (Spohn and Kuzyakov, 2014). After incubation the membranes were carefully lifted off the soil surface and all attached soil particles were gently removed using a soft brush. The membranes were placed under UV light (excitation, 365 nm) in a GeneFlash Documentation System (Syngene, Cambridge, UK) in a dark room and photographs of the membranes were taken. The imaging equipment, the camera (DSLR-A300, Sony, Japan) settings, exposure time (5 s) and positions of the camera and the membrane were fixed and constant for all samples. A calibration line was set up using membrane squares (4 cm²) that were soaked in solutions of increasing MUF concentration (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mM). The amount of MUF per unit area was calculated by dividing the volume of the solution absorbed by the membrane by the size of the membrane (Ma *et al.*, 2019). The membranes used for the calibration line were imaged in the same way as the samples.

The open-source software ImageJ was used for image processing and analysis. Images of zymograms were converted to 8-bit grey values. Linear regression was used to test the correlation between the grey values of the calibration membranes and the MUF concentrations, and consequently, the correlation with enzyme activities. The calibration point where the MUF concentration was zero was taken as the background signal and subtracted from all images. Five randomly drawn 50-pixel-wide lines across the root on each plant were used to calculate the rhizosphere extent of acid phosphatase activity. The rhizosphere extent of enzyme activity was defined as the distance from the root centre to the position with activity at least 20 % higher than that of bulk soil (Ma *et al.*, 2019).

Shoot dry weight and P analysis

The maize shoots were oven-dried at 60 °C for 3 d to determine shoot dry weight. Dried shoot material was ground to a fine powder. Two hundred and fifty milligrams of shoot dry matter was incubated with 65 % HNO₃, 10 mM H₂O₂ and distilled water in a microwave (MLS Maxi 44, Germany) at a maximum of 210 °C and 1400 W for 65 min. This solution was adjusted to 20 mL and filtered over activated charcoal and through 90-µm mesh filter paper. Phosphorus was measured spectrophotometrically by orthophosphate determination after the addition of molybdate-vanadate reagent (Gericke and Kurmies, 1952) using a spectrophotometer (U-3300, Hitachi, Tokyo, Japan).

Root analyses and mycorrhizal colonization

After shoot excision, roots were vigorously washed with sterilized deionized water in order to remove all soil from

the root surface. Root samples were gently dried with clean soft tissue; 0.3 g of fresh root was immediately frozen in liquid nitrogen and stored at -30°C for RNA extraction. The remaining root was used to calculate the total root length and was determined by flat-bed scanning (Epson Expression 1000 XL, Tokyo, Japan) and analysed with WinRHIZO (Regent Instruments, Canada) software. Root dry biomass was determined and specific root length was calculated as total root length divided by dry mass.

The roots were stained according to Brundrett *et al.* (1994) with slight modifications. Briefly, the roots were cut into segments 1 cm long, cleared with 10 % KOH at 90°C for 1 h, rinsed three times with tap water, acidified in 2 M HCl for 2 min and stained with 5 % ink–acetic acid for 30 min at 60°C . Root pieces were then soaked in tap water acidified with drops of acetic acid overnight to get rid of excess ink (Koske and Gemma, 1989; Vierheilig *et al.*, 1998). Finally, 30 randomly selected root pieces (1 cm long) from each replicate were placed on microscope slides (ten per slide) and observed for AMF colonization under a bright-field light microscope (Axioskop 2, Zeiss, Germany). As in the method described by Trouvelot *et al.* (1986), the mycorrhizal intensity of colonization (MIC) was determined using colonization classes, which were estimated for each root piece depending on the percentage of the root length colonized by AMF (#0 = 0 %, #1 \leq 1 %, #2 = 2–10 %, #3 = 11–50 %, #4 = 51–90 %, #5 > 90 %). The MIC was calculated as an average for 30 root pieces using the formula $\text{MIC} = (95n_5 + 70n_4 + 30n_3 + 5n_2 + n_1)/N \times 100\%$, where n_5, n_4, n_3, n_2, n_1 are the numbers of root pieces scaled as 5, 4, 3, 2 and 1, respectively, and N is the total number of fragments observed. The total colonized root length was calculated as total root length multiplied by MIC.

Mycorrhizal and root hair dependency

‘Mycorrhizal dependency’ refers to the difference in plant growth between mycorrhizal and non-mycorrhizal treatment. It was calculated by the following formula (van der Heijden, 2003; Tawaraya, 2003):

$$\text{Mycorrhizal dependency} = \left[1 - \left(\frac{bn}{\sum_1^1 a} \right) \right] \times 100\%$$

where a is plant dry mass of the inoculated genotype, n is the replicate number inoculated with AMF and b is the mean plant dry mass of the non-inoculated genotype.

‘Root hair dependency’ refers to the difference in plant growth between the hairless mutant and the wild type. It was calculated by the following equation:

$$\text{Root hair dependency} = \left[1 - \left(\frac{BN}{\sum_1^1 A} \right) \right] \times 100\%$$

where A is plant dry mass of the non-inoculated wild type, N is the number of replicates and B is the mean plant dry mass of non-inoculated *rth3*.

Gene expression analysis

Total RNA was extracted from ground root tissues with an innuPREP Plant RNA Kit (Analytik Jena, Jena, Germany) following the manufacturer’s instructions. The RNA concentration was determined with a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA). RNA (0.5 g) was reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

The AM-induced P_i transporter genes *ZmPt1;6* and *ZmPt1;11* and the AM-specific marker gene *ZmAm3* were quantified by relative real-time PCR on a CFX384 (Bio-Rad, Hercules, CA, USA). qPCRs were carried out in a 20- μL volume with 10 μL of Green Master Mix (2 \times) without ROX passive reference dye (Genaxxon Bioscience, Ulm, Germany), 1 μL of each primer (10 mmol L^{-1}) and 15 ng of template DNA. The *GAPDH* and β -actin genes were used as reference genes to normalize the expression data (Gutjahr *et al.*, 2008). All primers are listed in Supplementary Data Table S1. Thermal cycling included an initial denaturation step at 95°C for 20 min, followed by 50 cycles of denaturation at 95°C for 15 s and another 20 s at 60°C . A final melting curve was recorded from 65 to 95°C with increments of 0.5°C . Each biological sample was analysed as three technical replicates. For each replication, autoclaved water was used as a negative control. Gene expression was calculated using the Bio-Rad CFX Manager 3.1 software.

Statistical analyses

Normality and homogeneity of the variance for shoot dry weight, root dry weight, total root length, specific root length, P concentration and P content were assessed using the Shapiro–Wilk test and Levene’s test. One-way ANOVA followed by the Duncan test was used to test the significance of differences of these variables in response to AM inoculation, root hairs and total colonized root length. The effect of mycorrhizal inoculation, genotypes and their interactions on shoot dry weight, root dry weight, P content, P concentration, specific root length, total root length and mycorrhizal colonization were tested by two-way ANOVA. Pearson correlation coefficients were checked to determine relationships among AMF colonization, P_i transporter expression parameters, maize growth variables and P acquisition. All statistical analyses were done using the software SPSS v23.0.

RESULTS

Rhizosphere extent, plant growth, P_i acquisition and release of organic acid anions of wild type and *rth3*

The root hair length was on average 0.9 mm for the wild type while *rth3* essentially lacked root hairs completely and was totally bald (Fig. 1). We localized and quantified acid phosphatase activity in

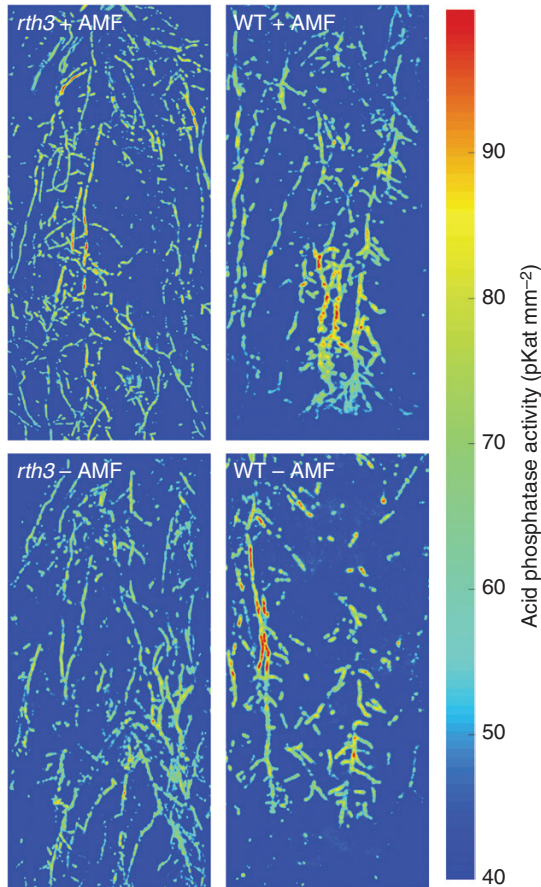


FIG. 2. Spatial distribution of acid phosphatase activity in the rhizosphere of maize root hairless mutant *rth3* (left) and wild type (WT, right). Representative examples are shown with AMF (top panels) and without inoculation (lower panels). The colour scale on the right shows linear enzyme activities (pKat mm⁻²). Top panels show *rth3* (left) and wild type (right) with inoculated arbuscular mycorrhizal fungi. Bottom panels show *rth3* (left), wild type (right) without inoculation of arbuscular mycorrhizal fungi.

both genotypes by *in situ* zymography (Fig. 2). Visual differences were immediately apparent between *rth3* and wild type regardless of AMF inoculation: root-associated acid phosphatase activities were higher and in thicker stretches along the roots, suggesting that the root hairs extended the activity zone of acid phosphatases, and these patterns were not affected by AMF inoculation (Fig. 2). The radial rhizosphere extension of acid phosphatase activity by root hairs was ~0.5 mm, which doubled the radial rhizosphere area for this enzymatic activity (Fig. 3). Malate, citrate, succinate and *trans*-aconitate were detected in root exudates, malate being the dominant organic acid anion. However, there was no difference between *rth3* and the wild type in terms of organic acid anion release from the roots close to the root tips (Supplementary Data Table S2).

Shoot and root dry weight, total root length and specific root length differed between the two genotypes when grown under limited P_i supply (Fig. 4). The nutritional P status (measured as shoot P concentration, which was around 0.1 %) indicated severely P-deficient conditions (Fig. 1). The non-inoculated *rth3* had the lowest shoot and root biomass, shortest total root length and lowest P content. Values for these traits in the wild type were 1.9- to 3.6-fold of those of the root hairless mutant *rth3*

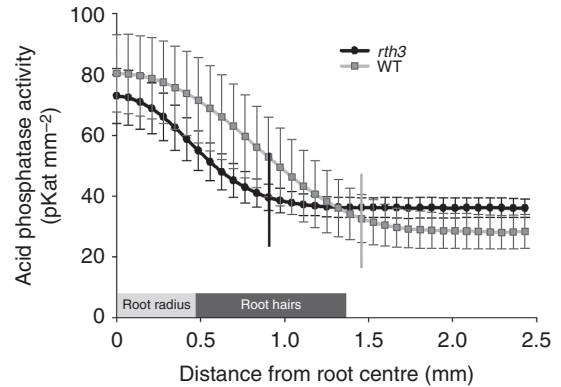


FIG. 3. Profiles of acid phosphatase activity as a function of distance from the maize root centre in the root-hairless mutant *rth3* and the wild type (WT). The root radius (0.45 mm) is shown as a light grey rectangle. The root hair length of wild type (0.9 mm) is shown as a dark grey rectangle. Vertical lines on the curves indicate the distance to which the rhizosphere acid phosphatase activity extended for the root-hairless mutant *rth3* and the wild type.

(Fig. 4A–C, F and Supplementary Data Fig. S1). The specific root length of the wild type was ~25 % higher than that of *rth3* (Fig. 4D). The P concentration in the shoot was very low, at around 0.1 %, indicating severe P deficiency, but it was not different between the two genotypes (Fig. 4E and Supplementary Data Fig. S1).

Effects of AMF inoculation on plant growth, P_i acquisition, mycorrhizal colonization, release of organic acid anions and related P_i transporter gene expression of wild type and *rth3*

In AMF-inoculated plants, fine extraradical hyphae were visible across the entire root, but these were absent in non-inoculated conditions (Supplementary Data Fig. S2). Inoculation with AMF significantly improved plant growth and P_i assimilation, and the enhancement was greater than that of root hairs (Table 1, Fig. 4A–C, E, F). The shoot and root biomass of AMF inoculated plants, as well as P content, were 4.0- to 7.4-fold greater those of non-inoculated plants in *rth3*, while only 1.8- to 4.6-fold greater in wild type (Fig. 4A–C, F). Stimulation of plant growth and P_i acquisition in *rth3* by AMF was 1.5- to 3.9-fold greater than in wild type (Fig. 4A–C, E, F), but both AMF-colonized genotypes ultimately had almost the same biomass. The total root length of AMF-inoculated wild-type plants was double that of non-inoculated wild-type plants and was 5-fold increased in *rth3*, while specific root length was almost not affected (Fig. 4D, Table 1). AMF colonization strongly improved the nutritional status of the plants and increased the internal P concentration ~2-fold to ~0.2 %, but these plants still experienced P deficiency, as the critical sufficiency level for maize is commonly around 0.3 %. Inoculation of AMF in the presence and absence of root hairs did not affect organic acid anion release from roots (Supplementary Data Table S2).

There was significant interaction between mycorrhizal colonization and the presence of root hairs ($P = 0.002$) (Table 1). The average mycorrhizal colonization of non-inoculated roots was <2 %, but AMF inoculation increased colonization to 46 % for the wild type and to 80 % for *rth3* (Fig. 5A, B). However,

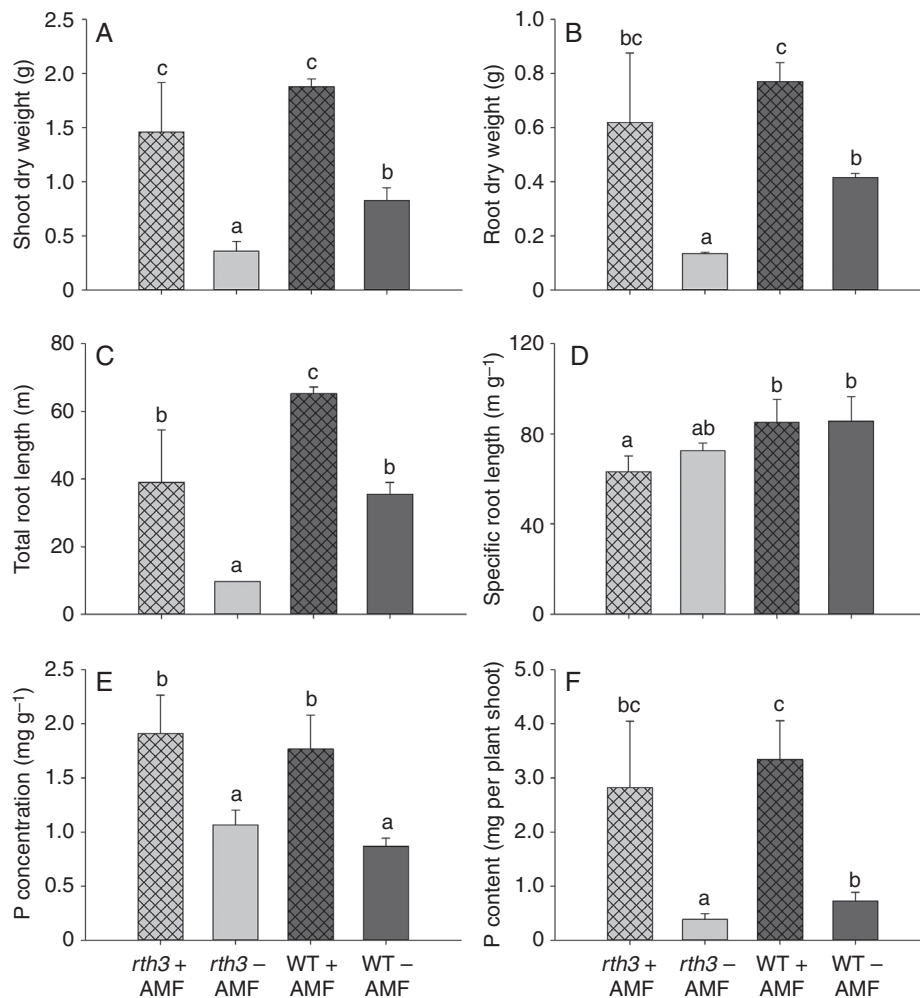


FIG. 4. The growth defect of the root hairless mutant *rth3* on P-deficient soil is compensated by AMF inoculation. Shoot dry weight (A), root dry weight (B), total root length (C), specific root length (D), P concentration (E) and P content (F) of maize *rth3* and wild type (WT) inoculated with AMF (+AMF) or not inoculated with AMF (-AMF). Different letters indicate significant differences ($P < 0.05$, Duncan's test) between treatments and genotypes.

the total colonized root length was similar in the two genotypes when inoculated (Supplementary Data Fig. S3). Mycorrhizal growth dependency (76 %) was higher than root hair dependency (57 %) (Fig. 5C).

ZmPht1;6, the primary mycorrhiza-specific P_i transporter gene, is exclusively found in AMF-colonized roots and is expressed at a high level. In agreement with the greater infection of *rth3* roots, *ZmPht1;6* gene expression was higher in the hairless roots. The less expressed, but also mycorrhiza-induced P_i transporter gene *ZmPht1;11* and the AMF colonization marker gene *Am3* were also induced by mycorrhization, both in AMF-colonized roots of *rth3* and in the wild type (Fig. 6).

Relationships among AM colonization, maize growth, P acquisition and P_i transporter gene expression

Mycorrhizal colonization was positively correlated with *ZmPht1;6* and *Am3* gene expression, shoot and root biomass,

as well as with P concentration and P content (Table 2). The expression of *ZmPht1;6* was positively correlated with shoot dry weight, P concentration and P content, indicating the importance of this gene in P_i uptake in maize (Table 2). Both shoot P concentration and shoot P content were also positively correlated with total root length, root and shoot biomass, as well as with each other.

DISCUSSION

Maize root hairs improved growth and P_i acquisition

As root hairs play an important role in P_i acquisition (Jungk, 2001; Lynch, 2005; Lambers et al., 2006), it was expected that the hairless mutant would perform much worse than the wild type under P-deficient conditions. The primary role of root hairs is to extend the root surface area and to increase the radial rhizosphere diameter to explore a larger soil volume (Ma et al., 2001;

TABLE 1. Results of two-way ANOVA showing the statistical significance of the effects of mycorrhizal inoculation, genotype and their interaction on shoot dry weight, root dry weight, P content, P concentration, specific root length, total root length and mycorrhizal colonization

Effect	Shoot dry weight	Root dry weight	Total root length	Specific root length	Shoot P content	Shoot P concentration	Mycorrhizal colonization	Exudation of organic acids
Genotype (G)	0.014*	0.023*	0.001**	0.006**	0.328	0.265	0.003**	0.179
Inoculation (I)	0.000**	0.001**	0.000**	0.344	0.000**	0.000**	0.000**	0.396
G × I interaction	0.882	0.422	0.952	0.389	0.832	0.848	0.002**	0.111

* $P < 0.05$; ** $P < 0.01$.

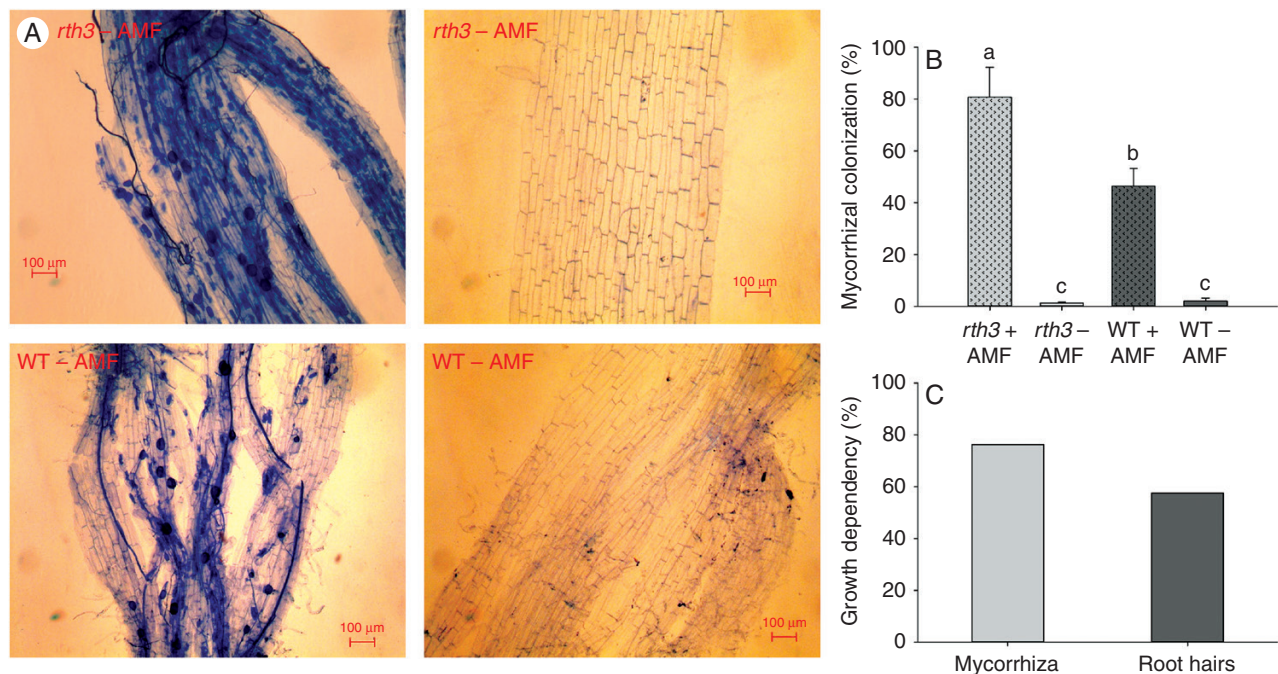


Fig. 5. Root hairs impair AMF infection. (A) Visualization of AMF colonization in maize roots of *rth3* and wild type (WT) inoculated with AMF (left panels) or not inoculated with AMF (right panels). (B) Mycorrhizal colonization and (C) growth dependence on mycorrhiza and root hairs.

Pang *et al.*, 2018). This was confirmed by up to 0.5 mm broader rhizosphere extent of acid phosphatase activity in the wild type compared with the hairless mutant (Figs 2 and 3), irrespective of AMF inoculation. This is in line with previous studies; root hairs greatly improved and extended rhizosphere acid phosphatase activity (Giles *et al.*, 2018) and may contribute 70–90 % of the total surface area relevant for nutrient absorption (Bates and Lynch, 1996; López-Arredondo *et al.*, 2014). Our study further verified that the root hair dependency of maize growth was 57 %. By contrast, the root hair dependency of barley was, according to results shown in Chen *et al.* (2005) and Jakobsen *et al.* (2005), around 75 %, which is notably higher than our results in maize, indicating that root hairs play more important roles in barley than in maize. Furthermore, the root hairs enhanced the P content by 44 %, but this increase was not significant and the P concentration was similar or tended to be even slightly decreased in plants with root hairs (Fig. 4E, F). This

is in line with previous research (Weber *et al.*, 2018; Klamer *et al.*, 2019; Ludewig *et al.*, 2019). However, root-hairless plants were severely depressed in biomass formation compared with the wild type, meaning that any additionally acquired P_i was immediately invested in producing more shoot and root biomass, leading to bigger shoots and longer roots (Fig. 4). Specific root length is also an important root morphology measure that can affect nutrient acquisition (Lambers *et al.*, 2006). Maize wild type had a higher specific root length than *rth3*, which indicated that the wild type has longer root length per unit invested in dry mass. This further allowed a greater soil volume to be explored per unit C invested and thus enhanced P uptake efficiency (Laliberté *et al.*, 2015). Therefore, maize root hairs not only improved P acquisition efficiency but also indirectly improved root morphology, with longer total root length and higher specific root length that further improved plant growth compared with the *rth3* hairless mutant.

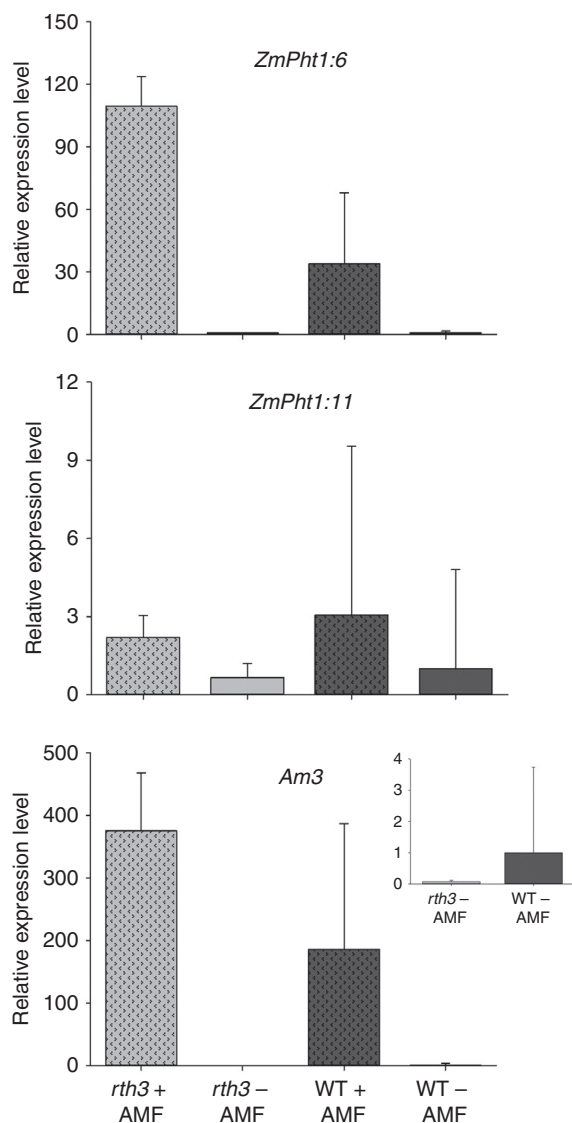


FIG. 6. Induction of mycorrhiza-induced *Pht* genes for phosphate transfer to the plant. Relative expression levels of *ZmPht1:6*, *ZmPht1:11* and *Am3* genes in *rth3* and wild-type (WT) maize root inoculated or not inoculated with AMF under P deficiency. Data points are means \pm s.e.m. ($n = 3$ biological replicates of three plants each).

Inoculation of AMF improved plant growth and P_i acquisition more effectively in *rth3* than in wild type

Inoculation of AMF increased plant growth and P_i acquisition of both the wild type and the *rth3* root hairless mutant 1.8- to 7.4-fold (Fig. 4). The P nutritional status, i.e. the shoot P concentration, was massively improved by AMF, facilitating maize growth and further P_i acquisition by AMF and by roots. Phosphorus uptake by the root and root hairs generates a radial depletion zone of up to 2 mm around the roots (Joner et al., 1995) due to the low mobility of P_i (Fontes and Weed, 1996). The hyphal network of AM fungi, by contrast, can extend up to 25 cm from the root, which is far beyond the depletion zone (Li et al., 1991; Jansa et al., 2003). Furthermore, root P_i assimilation

is mainly in the root hair zone (Smith et al., 2011), while the AMF also colonizes more mature regions of the root and supplies these regions with assimilated P_i (Richardson et al., 2011). Therefore, hyphae can greatly increase exploration of the soil volume for phosphate. Moreover, the diameter of extracellular hyphae is about one order of magnitude smaller than that of fine roots and often even thinner than that of root hairs; thus, hyphae can enter small soil pores that are inaccessible to thick roots (Drew et al., 2003; Smith and Read, 2008). Even when roots and hyphae have a similar volume, the surface area of hyphae in contact with soil is higher than that of the root due to its small diameter, which can greatly increase P_i assimilation (Jakobsen et al., 2005). All these factors probably contribute to the much larger biomass and greater P_i uptake of AMF-inoculated *rth3* compared with wild type under the non-inoculated condition (Fig. 4).

The root-hairless mutant *rth3* particularly profited more from the AMF inoculation than the wild type, which is in line with previous studies where root hair length negatively correlated with mycorrhizal dependency of various species, because root hair length contributed to plant P_i uptake (Tawarayaya, 2003). Thus, root hairs and AMF provide alternative, inversely correlated pathways for P_i foraging. Although root hairs and AMF provide alternative mechanisms to increase contact with the soil, the 33 % higher mycorrhizal growth dependency than root hair dependency strongly argues that AMF provide a more efficient way to acquire P_i even in young maize (Fig. 5C). Furthermore, the total colonized root length was similar in the two genotypes under the AMF inoculation condition and was accompanied by almost the same final biomass, indicating that AMF played a more important role than root hairs for P_i acquisition in maize. This is in line with previous studies showing that AMF play a critical role in nutrient acquisition, while root hairs are dispensable in maize (Wen and Schnable, 1994; Cozzolino et al., 2013). However, similar studies in barley showed that the root hairs play more important roles than AMF (Jakobsen et al., 2005; Chen et al., 2005; Li et al., 2014). This is probably due to the fact that the root hair length of barley wild type was almost doubled under the P-deficient condition compared with that under the higher P condition (Brown et al., 2012), while the root hair length in maize B73 wild type was not significantly different between P-deficient (this study, 0.90 mm) and P-sufficient conditions (0.83 mm) (Weber et al., 2018). This explanation is supported by observations in *Plantago lanceolata*, whose root hair length was not responsive to P availability but was highly dependent on AMF for P_i acquisition under P deficiency (Brown et al., 2013a). Other root morphological traits also influence plant dependency on AMF symbiosis; for example, species with thin roots rely less on AMF symbiosis for nutrient uptake than species with thick roots (Kong et al., 2014; Z. Ma et al., 2018). Similar phenomena exist among different root architectures, where the mycorrhizal colonization was lower in the thin first-order root than in the thick second-order roots (Eissenstat et al., 2015). This further indicated that root morphological properties (e.g. root hairs and root diameter) and mycorrhizal colonization act as alternative strategies in P_i uptake. It is also noteworthy that root hairs are already endogenously found

TABLE 2. Pearson correlations among mycorrhizal colonization, *ZmPht1;6*, *ZmPht1;11* and *Am3* expression, shoot dry weight, root dry weight total root length, P concentration and P content

	Mycorrhizal colonization	<i>ZmPht1;6</i>	<i>ZmPht1;11</i>	<i>Am3</i>	Shoot dry weight	Root dry weight	Total root length	Specific root length	Shoot P concentration
<i>ZmPht1;6</i>	0.945**								
<i>ZmPht1;11</i>	0.326	0.117							
<i>Am3</i>	0.887**	0.911**	0.065						
Shoot dry weight	0.732*	0.590*	0.564	0.682*					
Root dry weight	0.677*	0.530	0.637*	0.567	0.969**				
Total root length	0.520	0.352	0.547	0.511	0.941**	0.943**			
Specific root length	-0.375	-0.464	-0.158	-0.154	0.089	0.052	0.366		
Shoot P concentration	0.822**	0.692*	0.412	0.786*	0.955**	0.893**	0.851**	0.015	
Shoot P content	0.917**	0.824**	0.241	0.889**	0.766**	0.666*	0.591*	-0.166	0.903**

* $P < 0.05$; ** $P < 0.01$.

in young seedlings from the first days of growth, while AMF establishment requires several weeks to be functionally established (Smith and Read, 2008). Ultimately, AMF could compensate the loss of root hairs and even play a more critical role than root hairs for P_i acquisition under P deficiency even for juvenile maize.

Mycorrhizal colonization and mycorrhiza-specific P_i transporter *ZmPht1;6* expression were positively correlated with each other ($P < 0.01$) and both also positively correlated with plant growth, P status and P acquisition (Table 2). This indicated that the colonization rate and transporter gene expression are positively correlated with symbiotic function and activity. Mycorrhizal colonization promoted maize growth, P status, P_i acquisition and related P_i transporter gene expression in a plant genotype-dependent manner, in which the *rth3* hairless mutant benefited more than the wild type. As a consequence, plant biomass and P_i uptake were induced 1.6- to 2.5-fold by AMF colonization in *rth3* compared with the wild type (Fig. 4A, B, F). The mycorrhizal colonization of *rth3* was 1.7-fold that of the wild type (Fig. 5A, B), leading to higher AM-induced P_i transporter *ZmPht1;6* expression in *rth3* compared with the wild type (Fig. 6A). The following two mechanisms can explain the lower dependency of the wild type than the hairless mutant on AMF. Morphologically, the wild type, with long and dense root hairs, has a larger root surface area than the root-hairless mutant; thus, dependency on mycorrhiza for P_i acquisition was decreased. Physiologically, root hair growth and AM hypha establishment are both carbon- and energy-consuming and compete for resources (Kuzyakov and Domanski, 2000; Ryan et al., 2012). Hence, the abundance of AM colonization was not only induced by limited soil-available P, but also controlled by phytohormones and molecular mechanisms (Akiyama et al., 2005; Kobae et al., 2018). The phytohormone strigolactone plays an important role in the interaction of root establishment with AM symbiosis interaction (Akiyama et al., 2005; Besserer et al., 2006; Chagas et al., 2018). Colonization by AMF depends on the release of such signalling compounds into the rhizosphere to germinate spores and attract hyphae for root contact; a radially extended rhizosphere was expected to be beneficial for the number of AMF–root contact sites. However, root hairs expand the soil volume into which strigolactone is released, but had only a minor role in maize, as AMF colonization was higher in the hairless mutant than in the wild type

(Fig. 5). Molecular signals such as CLE (CLAVATA3/Embryo Surrounding Region-Related) peptides also play a key role in AMF colonization of various plants (Handa et al., 2015; Karlo et al., 2020). Future investigations of genetic and molecular pathways such as the relationship between the release of strigolactones and CLE peptides with AMF colonization are needed to reveal the contrast in mycorrhizal colonization between wild type and *rth3* in maize.

Conclusions

Our combined analysis of morphological, physiological and molecular factors and plant growth dependence on root hairs and AMF under P deficiency identified a larger mycorrhizal than root hair dependency. While both root hairs and arbuscular mycorrhizal inoculation can increase P_i uptake under P deficiency, the hairless mutant was more reliant on AMF inoculation than the wild type. Our data are consistent with the general assumption that root hairs and AMF inoculation are two alternative ways to increase P_i acquisition under P deficiency, but these two strategies compete with each other and root hairs cannot compensate for lack of AMF in maize.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Figure S1: examples of young *rth3* and wild-type maize plants inoculated or not inoculated with AMF and grown in rhizoboxes. Figure S2: examples of root-soil areas from maize *rth3* and wild type inoculated or not inoculated with AMF and grown in rhizoboxes. Figure S3: total colonized root length calculated as total root length multiplied by MIC. Table S1: primers used for qRT-PCR. Table S2: organic acids captured from *rth3* and wild-type maize roots inoculated or not inoculated with AMF.

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