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RESEARCH PAPER

Characterization of root response to phosphorus supply from morphology to gene analysis in field-grown wheat

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Abstract

The adaptations of root morphology, physiology, and biochemistry to phosphorus supply have been characterized intensively. However, characterizing these adaptations at molecular level is largely neglected under field conditions. Here, two consecutive field experiments were carried out to investigate the agronomic traits and root traits of wheat (*Triticum aestivum* L.) at six P-fertilizer rates. Root samples were collected at flowering to investigate root dry weight, root length density, arbusular-mycorrhizal colonization rate, acid phosphatase activity in rhizosphere soil, and expression levels of genes encoding phosphate transporter, phosphatase, ribonucleases, and expansin. These root traits exhibited inducible, inhibitory, or combined responses to P deficiency, and the change point for responses to P supply was at or near the optimal P supply for maximum grain yield. This research improves the understanding of mechanisms of plant adaptation to soil P in intensive agriculture and provides useful information for optimizing P management based on the interactions between soil P dynamics and root processes.

Key words: Agronomic trait, phosphatase, phosphate-starvation response, phosphate transporter, phosphorus fertilizer, root morphology, *Triticum aestivum* L.

Introduction

Phosphorus is one of the most important macronutrients that significantly affect plant growth and metabolism. Although total P content in soils is high, P availability is usually the lowest of the macronutrients (Cordell *et al*., 2009), and therefore plants often encounter a scarcity of phosphate (Pi) in soils of both agricultural and natural systems (Raghothama, 2000). In intensive agriculture, large amounts of inorganic P fertilizers and organic manures are applied to overcome soil P deficiency. However, because rock phosphate is a non-renewable resource and most P applied to soil is immobilized and becomes unavailable for plants and the recovery of applied P by crops in one growing season is often low (Vance *et al*., 2003), improving the efficiency of P use is therefore important in sustainable agriculture.

Soil-based P management, which manipulates soil P according to yield response to P fertilizer, has been long and widely used in optimizing P use (Kirkby and Johnston, 2008). Considering the vital roles of rhizosphere processes in P use, scientists have developed approaches to modify rhizosphere processes in increasing the efficiency of P use. In response to low availability of P in the rhizosphere, plants have developed highly specialized morphological, physiological, and biochemical adaptive mechanisms to modify the rhizosphere and, hence, increase the ability of their root systems

to utilize Pi from soils (Vance *et al*., 2003; Lynch and Brown, 2008; Hinsinger *et al*., 2009; Zhang *et al*., 2010; George *et al*., 2011). The conserved responses include: (1) investment of a greater proportion of photosynthates in the roots, alterations in root morphology, and establishment of symbiotic relations with arbusular-mycorrhizal (AM) fungi to increase exploration of the soil volume (Hermans *et al*., 2006; Bucher, 2007; Hammond and White, 2008; Lynch and Brown, 2008; Péret *et al*., 2011; Smith and Smith, 2011); (2) increased proton release and secretion of organic anions and phosphatase enzymes into the soil to mobilize Pi from inorganic and organic P sources in the rhizosphere (Marschner, 1995; Hinsinger, 2001; Jones *et al*., 2003; Jain *et al*., 2007; George and Richardson, 2008); and (3) enhancing the capacity of root cells to take up Pi by increasing the employment of high-affinity Pi transporters (Liu *et al*., 1998; Raghothama, 1999; Vance *et al*., 2003). Understanding these responses provides useful information to increase mobilization and acquisition of P by crops through both modifying rhizosphere processes and the so-called rhizosphere-based P management (Zhang *et al*, 2010; Li *et al*., 2011a). Rhizosphere-based P management aims to modify rhizosphere processes by localized supply of nutrients, intercropping (e.g. maize/faba bean intercropping), and exploitation of plant genetic potential through conventional and molecular breeding (Li *et al*., 2011a). For example, non-uniform supplies of nutrients have been long known to stimulate root branching (Robinson, 1994). A recent study in maize showed that localized application of P plus ammonium stimulated root branching and rhizosphere acidification and thus significantly increased P uptake, compared with broadcast application (Jing *et al*., 2010). Recent advances in marker-assisted selection and gene transformation have greatly increased the efficiency of breeding crops with improved root traits (Tian *et al*., 2012), which has been shown effective in improving P uptake by manipulating genes regulating root architecture (Guo *et al*., 2011; Li *et al*., 2011b), Pi mobilization from insoluble P pools in rhizosphere (Lopez-Bucio *et al*., 2000; Richardson *et al*., 2001; Wang *et al*., 2009; Liang *et al*., 2010), and Pi transportation (Seo *et al*., 2008; Ai *et al*., 2009).

As the ability of roots to utilize soil P is affected by soil P supply and rhizosphere processes, effective strategies for P management should involve multidisciplinary approaches based on the soil and the rhizosphere processes (Li *et al*., 2011a; Shen *et al*., 2011). The development of effective strategies requires better understanding of the root response to soil P supply in at least the following two aspects. First, estimation of the critical soil P concentration to trigger root responses and its relation with the optimal soil P level for crop production is needed. Previous studies showed that there is a critical P supply that triggers P-starvation response. For example, the transcripts of the P-starvation marker gene *Mt4* in the roots of *Medicago truncatula* were abundant when the plants grown in nutrient solution containing no Pi but were reduced with 0.02 and 0.1 mM Pi and became undetectable with 1 and 5 mM Pi (Burleigh and Harrison, 1998). Hill *et al*. (2006) compared the root morphological parameters of various pasture species grown in pots fertilized with six P concentrations and found that root adaptations were triggered at or near the critical soil P supply for 90% of maximum shoot mass. In intensive agriculture, although optimal P supplies for crop production have been estimated worldwide in a number of crops (Kirkby and Johnston, 2008), estimation of the critical P supply that initializes the morphological, physiological and biochemical responses of underground roots is largely neglected. Second, a better understanding of the morphological, physiological, and biochemical properties of the roots grown in P deficient, optimal, and excessive soils is needed. Numerous publications have described the regulation of P supply on root traits at the morphological and physiological levels and, more recently, at the molecular level; however, the majority of the researches focused on short-term responses under controlled conditions, and on-farm field-scale and systematic research to characterize the long-term responses of roots to P supply from the morphological and physiological to the biochemical aspects is still lacking. Taking these two aspects into account will enable the prediction of root processes and will be useful for the development of effective management strategies targeting both high yield and efficient P use.

Wheat is one of the most important food crops in the world and consumes much more P fertilizer than rice (*Oryza sativa* L.) and maize, both in terms of annual total consumption and in terms of consumption per unit area (Food and Agriculture Organization, 2006). Therefore, improving the efficiency of P uptake in wheat is important to the sustainable use of P resources. This study investigates the responses of agronomic performance and root traits to P supply from deficiency to excess in two field experiments in the main wheat area of China. The results show that the optimal soil P supply for maximum yield is at or near the critical P supply for triggering the P-starvation response of root, including morphology, AM colonization rate, acid phosphatase activity in rhizosphere soil, and expression levels of genes encoding Pi transporter, phosphatase, ribonucleases, and expansin. This research provides useful information for developing soil-based and rhizosphere-based P management with the aims of improving both high yield and efficient P use.

Materials and methods

Field experiments

Two consecutive field experiments were carried out during the growing seasons of 2009–2010 (2010 experiment) and 2010–2011 (2011 experiment) at Quzhou experimental station (36.5° N 115.0° E, 40 m above sea level) of China Agricultural University. The climate in the region is warm and subhumid and consists of summer rainfall and dry cold winters. The average annual temperature is 13.2 °C. According to data from 1980 to 2011, annual precipitation ranges from 213 to 840 mm (mean 494 mm). The precipitation during the wheat-growing season was 149.3 and 62.5 mm in the 2010 and 2011 experiments, respectively. The soil at the study site is a silt fluvo-aquic soil. The basic soil properties in 0–30 cm layer are given in Supplementary Table S1 (available at *JXB* online). *Triticum aestivum* L. cv. Kenong 9204 was used in both 2010 and 2011, sown on 8 October 2009 and 7 October 2010 and harvested on 14 June 2010 and 11 June 2011, respectively.

Both experiments were treated with six P supplies, 0, 25, 50, 100, 200, and 400 kg ha^{-1} of P as calcium superphosphate (referred as

P0, P25, P50, P100, P200, and P400, respectively). All treatments received 225 kg ha^{-1} of N as urea split into 75 kg ha^{-1} before sowing and 150 kg ha^{-1} at stem elongation (Feekes6.0). P fertilizer and 60 kg ha^{-1} of K_2O as potassium sulphate were added to the topsoil before sowing by broadcast application and then mixed by conventional tillage (0–30 cm depth). Each P treatment was replicated four times in a randomized complete block design with plot size 43.2 m^2 (5.4 × 8 m). Seeds were sown at a rate of 375 seeds m^{-2} with 20-cm row spaces in the 2010 experiment and 15-cm row spaces in the 2011 experiment. For both experiments, the sowing depth was 3–4 cm and irrigation was with ~90 mm underground water per irrigation before winter, during stem elongation, and near anthesis. Weeds were well controlled by manual removal. Pests and diseases were controlled by spraying insecticide (cypermethrin) and fungicide (carbendazim) before stem elongation and after flowering.

At flowering (Feekes10.5.2), shoot biomass yield, shoot P concentration, root dry weight (RDW), root length density (RLD), AM colonization rate, acid phosphatase activity in rhizosphere soil (RS-APase), and gene expression in shoots and roots were investigated. Shoot biomass yield was recorded by sampling the aboveground parts of two rows (0.5 m long per row) and drying to a constant weight at 60 °C.

After the aboveground parts were removed, the underground parts were used to determine RDW, RLD, and root AM colonization rate. To measure these root traits, soil volumes of 40 × 20 cm to a total depth of 60 cm with 10-cm increments in each plot of all six P treatments were dug out in the 2010 experiment, and a soil volume of 30 × 10 cm to a total depth of 60 cm with 10-cm increments in each plot of four P treatments (P0, P50, P100, and P400) were dug out in the 2011 experiment. Thus, there were six soil blocks of 40 20 10 cm in each plot in 2010 and six soil blocks of 30 10 10 cm in each plot in 2011. All visible roots in each soil block were picked out in the field by hand and placed in individual, marked plastic bags. These roots were washed free of soil after transfer to the laboratory and then frozen at –20 °C until RLD and AM colonization analysis.

To measure gene expression levels and RS-APase, 10 randomly selected plants with roots 30 cm in depth were collected at flowering in each plot. The flag leaves of nine other plants were sampled together and stored in liquid N_2 for gene expression analysis. Each root sample was randomly divided into two subsamples. One subsample was used for measurement of APase activity in the rhizosphere soil. The other subsample was quickly washed free of soil and both adventitious root and seminal roots were collected and stored in liquid N_2 until gene expression analysis.

At maturity (Feekes11.4), the whole plot was harvested to measure biomass yield, grain yield, and total P in the straw and grain.

Soil Olsen P and $CaCl_2$-P concentrations were also monitored at flowering and maturity. Five subsamples per plot were collected from the topsoil (0–30 cm) between wheat rows. The subsamples were then thoroughly mixed and air dried before being ground to analyse soil Olsen P and $CaCl_2$-P.

Determination of plant total P and soil Olsen P and $CaCl_2$-P

To determine plant total P, dried samples were milled and subsequently digested with concentrated H_2SO_4 and H_2O_2 for determining total P using the molybdate-blue colorimetric method (Murphy and Riley, 1962). Soil Olsen P was determined using the molybdovanadophosphatase method based on extraction from air-dried soil with 0.5 M $NaHCO_3$ at pH 8.5 (180 rpm, 25 °C) (Westerman, 1990). $CaCl_2$-P was extracted using 0.01 M $CaCl_2$ at a soil/solution ratio of 1:5 (Schofield, 1955) and determined by the molybdate-blue colorimetric method. Absorbance was recorded on a 4802 UV/VIS double beam spectrophotometer (UNICO, Shanghai, China).

Scanning and image analysis of roots

Cleaned root samples were dispersed in water in a transparent array (30 × 20 × 3 cm) and scanned (Epson Expression 1600, Seiko Epson, Nagano, Japan) at a resolution of 400 dpi. To evaluate root length, the images were analysed using WinRHIZO software (Regent Instrument, Quebec, Canada). After scanning, the root samples were oven dried and weighed. After calculation of the total root length and soil volume, the RLD was obtained.

Estimation of root AM colonization

To investigate root AM colonization, subsamples of root systems were randomly selected from the root samples collected at 0–60 cm soil depth at flowering and treated and stained with nonvital trypan blue (Phillips and Hayman, 1970) as described by Feng *et al.* (2003) with some modifications. Stained roots were observed with a microscope and the intensity of root cortex colonization by AM fungi was determined as described by Trouvelot *et al.* (1986) using MYCOCALC software (www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

Determination of acid phosphatases in rhizosphere soil

To measure RS-APase activity in the rhizosphere soil, roots were lifted out of the soil and shaken gently to remove loosely adhering soil, only leaving a proportion of tightly adhering soil, which was considered as rhizosphere soil. The roots with adhering rhizosphere soil were immersed into 50 ml of 0.2 mM $CaCl_2$ and shaken carefully. RS-APase activities in the rhizosphere soil suspensions were determined by the method of Neumann (2006).

RNA extraction and quantitative real-time PCR

Total RNA in the plant samples was extracted using Trizol reagent (15596018, Invitrogen, USA), treated with RNase-free DNase (79254, Qiagen, Germany), and further purified with a RNeasy Plant Mini kit (74904, Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed using a PrimeScript RT Perfect Real Time reagent kit (DRR037A, Takara, Dalian) according to the manufacturer's protocol. Quantitative real-time PCR was performed on a Mastercycler Realplex4 Real Time PCR System (Eppendorf, Germany) using SYBR Premix EX Taq (DRR041A, Takara) in 20-µl reaction volumes, which contained 10 µl SYBR Green PCR mix, 0.4 µM each forward and reverse primers, 0.4 µg diluted cDNA template, and the appropriate amounts of sterile double-distilled water. The primer sequences are listed in Supplementary Table S2. Levels of transcription were calculated with the $2^{-\Delta\Delta Ct}$ method using the wheat housekeeping gene *Actin* as an internal control. All reactions were set up using four biological replicates.

Statistical analysis

All data were calculated using Excel 2003 (Microsoft, USA), and results are presented as mean ± standard error. Analysis of variance was performed using the one-way analysis of variance (ANOVA) model in the SAS statistical software (SAS Institute, Cary, NC, USA). Comparisons of means were performed using Duncan's multiple range analysis test ($\alpha = 0.05$).

Results

Plant growth and P uptake

P fertilizer significantly increased Olsen P in the bulk soils sampled at flowering and maturity in both experiments (Tables 1 and 2). $CaCl_2$-P in bulk soil was only investigated at flowering. In both experiments, $CaCl_2$-P was low when P-fertilizer rate was lower than P100 and then increased when P-fertilizer rate increased (Table 1). Biomass yield at

Table 1. Soil Olsen-P, $CaCl_2$-P, shoot biomass, P concentration, P uptake, and root traits under different P supplies at flowering in the field experiments. Data are means of four biological replicates. AM, arbusular-mycorrhizal; APase, acid phosphatase. Statistical differences ($P < 0.05$) between P supplies are indicated by different superscript letters.

Trait	P supply (kg P ha^{-1})					
	0	25	50	100	200	400
2010 experiment						
Olsen-P (mg kg^{-1})	13.3^{d}	13.9^{d}	17.9^{d}	25.8^{c}	45.6^{b}	83.8^{a}
$CaCl_2$-P (mg kg^{-1})	0.07^{b}	0.08^{b}	0.04^{b}	0.12^{b}	0.45^{b}	2.41^{a}
Shoot biomass yield (Mg ha^{-1})	3.89^{c}	$4.60^{b,c}$	5.48^{b}	6.89^{a}	7.21^{a}	7.01^{a}
Shoot P (mg g^{-1})	2.28^{c}	2.26^{c}	2.31^{c}	$2.42^{b,c}$	$2.76^{a,b}$	2.80^{a}
P uptake (kg ha^{-1})	8.96^{b}	10.36^{b}	12.63^{b}	16.53^{a}	19.94^{a}	19.68^{a}
Root dry weight (g m^{-3})	105.2^{b}	106.6^{b}	$121.7^{a,b}$	152.5^{a}	$133.9^{a,b}$	$121.2^{a,b}$
Root length density (cm cm^{-3})	2.38^{b}	$2.71^{a,b}$	$2.68^{a,b}$	3.62^{a}	$3.21^{a,b}$	$2.80^{a,b}$
AM colonization rate (%)	36.51^{a}	33.22^{a}	14.94^{b}	8.40^{c}	6.59^{c}	2.43^{c}
2011 experiment						
Olsen-P (mg kg^{-1})	3.8^{d}	$7.4^{c,d}$	$8.9^{c,d}$	16.5^{c}	29.0^{b}	46.6^{a}
$CaCl_2$-P (mg kg^{-1})	0.09^{b}	0.08^{b}	0.10^{b}	$0.16^{a,b}$	$0.42^{a,b}$	0.53^{a}
Shoot biomass yield (Mg ha^{-1})	5.22^{d}	$7.20^{c,d}$	$7.88^{b,c,d}$	10.91^{a}	$10.29^{a,b}$	$9.64^{a,b,c}$
Shoot P (mg g^{-1})	1.46^{d}	$1.65^{c,d}$	$1.82^{b,c}$	$2.04^{a,b}$	$2.13^{a,b}$	2.30^{a}
P uptake (kg ha^{-1})	5.49^{c}	11.93^{b}	14.25^{b}	24.90^{a}	24.10^{a}	22.13^{a}
Root dry weight (g m^{-3})	143.1^{a}	–	155.5^{a}	175.3^{a}	–	160.3^{a}
Root length density (cm cm^{-3})	2.34^{a}	–	2.59^{a}	3.09^{a}	–	2.98^{a}
Rhizosphere APase (μg PNP g^{-1} soil h^{-1})	$343.1^{a,b}$	325.0^{b}	$338.9^{a,b}$	427.60^{a}	225.7^{c}	163.7^{c}
AM colonization rate (%)	52.68^{a}	42.29^{b}	37.73^{b}	21.51^{c}	10.87^{d}	11.42^{d}

Table 2. Soil Olsen-P, shoot biomass, grain yield, P concentration, and P uptake under different P supplies at maturity in the field experiments. Data are means of four biological replicates. Statistical differences ($P < 0.05$) between P supplies are indicated by different superscript letters.

Trait	P supply (kg P ha^{-1})					
	0	25	50	100	200	400
2010 experiment						
Olsen-P (mg kg^{-1})	6.7^{c}	11.5^{c}	12.0^{c}	$19.5^{b,c}$	$44.1^{a,b}$	55.8^{a}
Grain yield (Mg ha^{-1})	3.18^{d}	4.12^{c}	$4.42^{b,c}$	$5.24^{a,b}$	5.92^{a}	5.66^{a}
Shoot biomass yield (Mg ha^{-1})	7.36^{d}	$8.24^{c,d}$	$9.81^{b,c}$	$10.90^{a,b}$	12.54^{a}	12.61^{a}
Straw P (mg g^{-1})	0.35^{d}	$0.40^{b,c,d}$	$0.37^{c,d}$	$0.48^{a,b,c}$	$0.49^{a,b}$	0.55^{a}
Grain P (mg g^{-1})	3.29^{c}	3.40^{c}	$3.52^{b,c}$	$3.56^{b,c}$	$3.68^{a,b}$	3.87^{a}
P uptake (kg ha^{-1})	12.88^{d}	$15.71^{c,d}$	$19.15^{b,c}$	$21.41^{a,b}$	25.17^{a}	25.68^{a}
2011 experiment						
Olsen-P (mg kg^{-1})	4.7^{e}	6.5^{e}	9.6^{d}	17.2^{c}	32.7^{b}	53.6^{a}
Grain yield (Mg ha^{-1})	3.43^{c}	4.86^{b}	$5.77^{a,b}$	$5.89^{a,b}$	6.33^{a}	6.80^{a}
Shoot biomass yield (Mg ha^{-1})	6.80^{d}	9.35^{c}	$10.77^{b,c}$	$11.68^{a,b}$	$12.23^{a,b}$	13.41^{a}
Straw P (mg g^{-1})	0.22^{b}	0.26^{b}	0.30^{b}	0.39^{a}	0.42^{a}	0.43^{a}
Grain P (mg g^{-1})	2.68^{c}	$2.97^{b,c}$	$3.14^{a,b}$	3.38^{a}	3.45^{a}	3.37^{a}
P uptake (kg ha^{-1})	11.75^{d}	$15.63^{c,d}$	$18.13^{b,c}$	$22.16^{a,b}$	24.28^{a}	25.72^{a}

flowering and maturity increased with P-fertilizer rate at first, but did not significantly increase further when P-fertilizer rate exceeded P100 (Tables 1 and 2). P fertilizer significantly increased shoot P at flowering and grain P at maturity in both experiments (Tables 1 and 2). The increasing effect of P fertilizer on straw P at maturity was observed in both experiments with P-fertilizer rate increased to P100 but not significantly at higher P supplies (Table 2). P uptake in terms of total P accumulated in the aboveground parts at flowering and maturity was calculated. At flowering and maturity, P uptake increased with P-fertilizer rate at first, and then did not significantly increase further when P-fertilizer rate was higher than P100 in both experiments (Tables 1 and 2). These results show that P100 is a critical P supply for most agronomic and P-uptake-related traits under these experimental conditions.

Estimation of optimal P supply for maximum grain yield

This study observed a statistically significant response of grain yield to P-fertilizer rate in both experiments (Table 2). To estimate the optimal P supply for maximum grain yield, grain yield was plotted against soil Olsen P measured at maturity. Regression analysis revealed that the response of grain yield to soil Olsen P significantly fitted to a linear-plateau model in both experiments (Fig. 1). The regression equations clearly showed that the grain yield reached its highest at Olsen P 19.5 mg kg^{-1} in the 2010 experiment (Fig. 1A) and 20.17 mg kg^{-1} in the 2011 experiment (Fig. 1B).

Estimation of critical P supply for P-starvation response based on P-starvation marker gene expression analysis

Ta*IPS1.1* belongs to the MT4/TPS1 family, members of which have been used as molecular indicators of plant Pi status (Shin *et al*., 2006; Huang *et al*., 2011). Here Ta*IPS1.1* was used as an indicator of Pi status of wheat plants. In both roots and shoots in the 2011 experiment, the change point for the response of Ta*IPS1.1* to P supply was approximately soil Olsen P 20 mg kg^{-1} (Fig. 2).

The response of both grain yield and Ta*IPS1.1* to P supply changed at approximately Olsen P 20 mg kg^{-1} (Figs. 1 and 2), which corresponded to P100 in both experiments (Tables 1 and 2). In order to subsequently conveniently describe the responses of root traits to P supply, P0, P25, and P50 were classified as deficiency, P100 as optimum, and P200 and P400 as excess.

Responses of root morphology and physiology to P supply

RDW, RLD, and root AM colonization rate was investigated in 0–60 cm depth soil and RS-APase activity was investigated in the 30-cm soil layer at flowering. In both experiments, RDW and RLD increased with P-fertilizer rate at first, peaked at P100, and then declined (Table 1). However, the statistically significant effects of P fertilizer on RDW and RLD were observed only in the 2010 experiment. P fertilizer decreased root AM colonization rate in both experiments. In the 2010 experiment, the root AM colonization rate was maintained below 9% at the sufficient P-fertilizer rates (optimum and excess) and significantly increased when P-fertilizer rate decreased from sufficiency to deficiency (Table 1). In the 2011 experiment, the AM colonization rate was maintained at about 10% at the excessive P-fertilizer rate and increased steadily when P-fertilizer rate decreased from excess to optimum and deficiency (Table 1). RS-APase activity was measured in the 2011 experiment and the two excessive P-fertilizer rates had lower RS-APase activity than the deficient and optimal P-fertilizer rates (Table 1).

Responses of P-starvation response genes to P supply

In the 2011 experiment, the expression levels of 11 PSI genes were investigated in the roots at flowering, including six PHT1

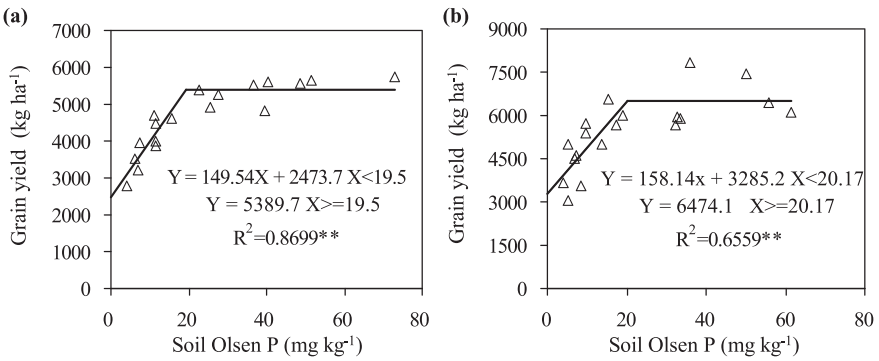

Fig. 1. Grain yield as a function of increasing soil Olsen P in the 2010 (A) and 2011 (B) field experiments.** $P < 0.01$.

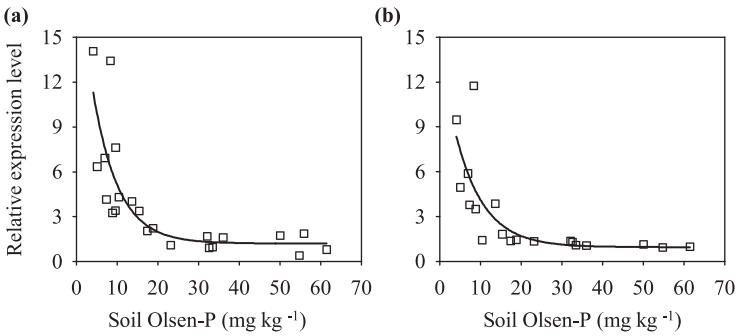

Fig. 2. Response of Ta*IPS1.1* relative expression level to soil Olsen P in shoots (A) and roots (B) in the 2011 experiment.

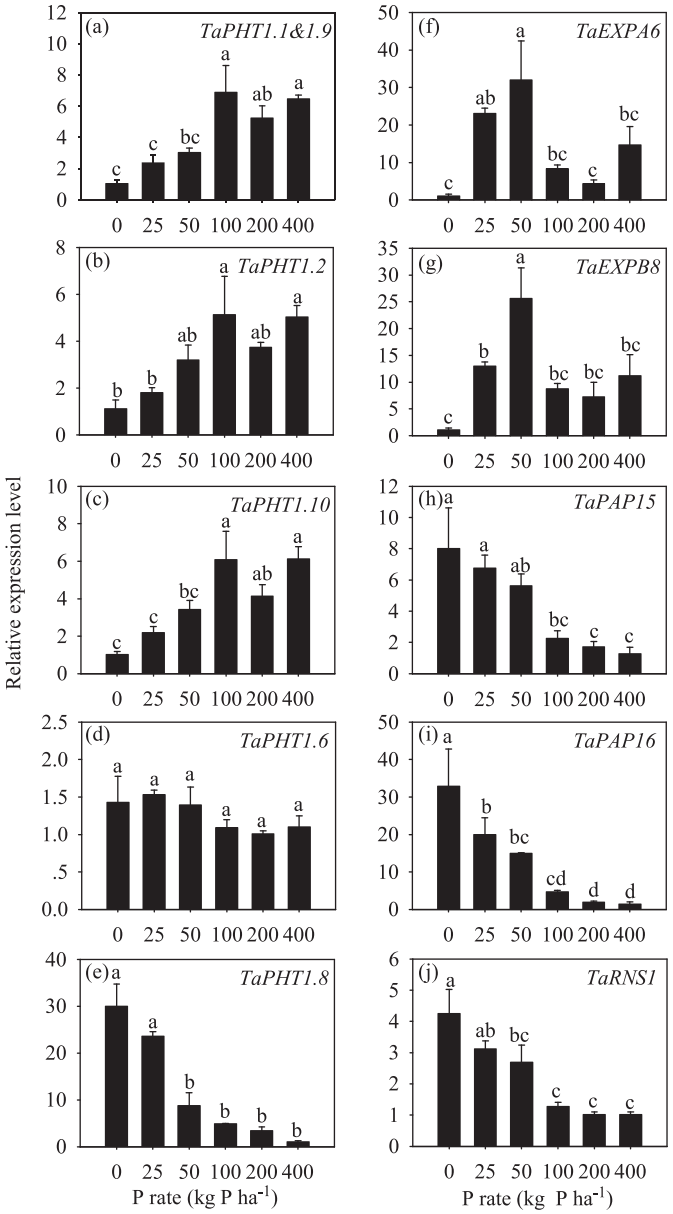

Fig. 3. Relative expression levels at flowering of P-starvation response genes in roots of wheat grown under different P supplies in 2011 field experiment. (A–E) PHT1 Pi transporters *PHT1.1* and *1.9*, *PHT1.2*, *PHT1.6*, *PHT1.8*, and *PHT1.10*, respectively; (F) α-expansin *EXPA6*; (G) β-expansin *EXPB8*; (H and I) purple acid phosphatase genes *PAP15* and *PAP16*, respectively; (J) ribonuclease gene *RNS1*. Data are mean ± standard error of four replicates. Different letters indicate significant differences between different P supplies ($P = 0.05$).

transporters, two expansin genes (Ta*EXPA6* and Ta*EXPB8*), two purple phosphatase genes (Ta*PAP15* and Ta*PAP16*), and one ribonuclease gene (Ta*RNS1*) (Fig. 3). The expression levels of Ta*PHT1.1* and *-1.9*, Ta*PHT1.2*, and Ta*PHT1.10* showed inhibitory responses to P deficiency, as they were maintained relatively high at the sufficient P-fertilizer rates and were downregulated steadily when P-fertilizer rate was reduced to deficiency (Fig. 3A–C). The expression of Ta*PHT1.6* did not show a significant response to P supply (Fig. 3D). The expression levels of Ta*PHT1.8*, Ta*PAP15*, Ta*PAP16*, and Ta*RNS1* exhibited inducible responses to P deficiency, as they were kept at a low level at the sufficient P-fertilizer rates and were upregulated steadily when P-fertilizer rate was reduced to deficiency (Fig. 3E, H–J). The expression levels of Ta*EXPA6* and Ta*EXPB8* showed combined inducible and inhibitory responses to P deficiency, as they were upregulated when P-fertilizer rate decreased from sufficiency to P50 and were downregulated when P-fertilizer rate decreased from to P25 and P0 (Fig. 3F, G).

Discussion

P fertilizer promotes plant growth and P uptake

P fertilizer significantly increased biomass yield, grain yield, total P in the investigated plant tissues, and P uptake in both field experiments, but the values of most of these traits did not significantly increase further when P-fertilizer rate exceeded P100 (Tables 1 and 2, Fig. 1). These results suggested that these two experiments were suitable for estimating the responses of the above- and underground parts to P supply covering deficiency, optimum, and excess. Soil Olsen P increased with P-fertilizer rate, while $CaCl_2$-P increased only when P-fertilizer rate exceeded P100 (Table 1), suggesting that P-fertilizer rate higher than 100 kg ha^{-1} may environmental risks.

The optimal P supply for maximum yield coincides with the change point for the response of P-starvation marker gene to P supply

This study estimated the optimal P supply for maximum yield based on the yield responses to soil Olsen P measured at maturity. Soil Olsen P about 20 mg kg^{-1} was optimal for achieving the highest grain yield in both experiments (Fig. 1). This is in the range 4.9–20.0 mg kg^{-1} that has been reported for wheat previously (Jackson *et al.*, 1997; Bollons and Barraclough 1999; Tang *et al.*, 2009) and is also in the range of target soil Olsen P (14–30 mg kg^{-1}) for winter wheat in the north China Plain based on the P management strategy 'building-up and maintenance method' (Li *et al.*, 2011a).

To determine the critical P supply for P-starvation response, this study then analysed the expression levels of Ta*IPS1.1* in shoots and roots in the 2011 experiment. The response pattern of Ta*IPS1.1* to P supply was similar in shoots and roots and had the change point Olsen P 20 mg kg^{-1} (Fig. 2), which corresponds to P100 (Tables 1 and 2). As Ta*IPS1.1* belongs to the MT4/TPS1 family, members of which are used as P-starvation marker genes to reflect plant Pi status (Shin *et al.*, 2006; Huang *et al.*, 2011), Olsen P 20 mg kg^{-1} also indicated the critical P supply for wheat plants to absorb sufficient P under these experimental conditions. This was supported by the fact that shoot P at flowering increased with increasing P-fertilizer rate at first and then did not increase further when P-fertilizer rate increased from P100 to higher rates (Table 1).

As already discussed, the optimal P supply for maximum yield coincided with the critical P concentration for P-starvation marker gene response in roots and shoots. Since

P-deficiency-induced changes in root morphology, physiology, and biochemistry are coordinated by a small number of regulatory systems controlled by both plant P status and soil Pi availability (George *et al*., 2011), this study then checked whether P100, which produced soil Olsen P approximately 20 mg kg^{-1}, was critical for the P-starvation responses. As shown in detail in the following discussion, the responses of most root traits to P supply changed at or near P100 (Table 1, Fig. 3). These results are supported by previous studies. For instance, a study on pasture species including grasses and dicots also observed that root morphological adaptations were triggered at or near the critical P supply for shoot mass, although it was carried out under controlled conditions (Hill *et al*., 2006).

Optimal P supply for crop production has been estimated worldwide in a number of crops. Thus, the coincidence between the optimal P supply for yield performance and the change points for the responses of root traits to P supply is that the numerous information about optimal P supply is significant because it could be used to predict the root processes related with P acquisition according to soil P supply, although the coincidence is needed to be investigated in more soil types and crop genotypes. Considering the difficulty in observing root processes in soil, the prediction of root processes is very useful for optimizing soil-based and rhizosphere-based P managements targeting both high yield and efficient P use.

Root processes respond differentially to P supply

In response to low Pi availability, plant roots adaptively change their morphology, physiology, and biochemistry, including enhanced adventitious rooting and lateral root branching, greater root hair and cluster root formation, increased mobilization of sparse soluble soil/fertilizer P by root exudates such as phosphatase and ribonuclease enzymes, upregulated activity of Pi transporters, and increased symbiotic association with AM fungi (Vance *et al*., 2003; Lambers *et al*., 2006; Shen *et al*., 2011). However, the investigated root traits responded to P supply in contrasting patterns under the current experimental conditions. These root traits included RDW, RLD, root AM colonization rate, RS-APase activity, and expression levels of 11 PSI genes encoding PHT1 Pi transporter, purple acid phosphatase, ribonuclease, and expansin, and therefore reflected the morphological, physiological, and biochemical properties of roots.

The traits that were induced by P deficiency included root AM colonization rate (Table 1), RS-APase activity (Table 1), and expression levels of Ta*PHT1.8*, Ta*PAP15*, Ta*PAP16*, and Ta*RNS1* (Fig. 3E, H–J). The critical P supply for P-deficiency response was P100 for root AM colonization rate in the 2010 experiment, and was P100 for Ta*PAP15*, Ta*PAP16*, and Ta*RNS1* expression, P50 for Ta*PHT1.8*, and P200 for root AM colonization rate and RS-APase activity in the 2011 experiment. Ta*PHT1.8* (known as Ta*PHT;myc*) has been shown to be specifically upregulated in roots colonized by AM fungi, indicating its function in Pi transport via a mycorrhizal-dependent pathway (Glassop *et al*., 2005). Therefore, upregulation of Ta*PHT1.8* by P deficiency would be the result of increased root AM colonization rate under P deficiency. This study observed that RS-APase activity was higher under P-deficient than under P-excessive conditions (Table 1), implying that genes encoding phosphatase were upregulated in the roots under P deficiency. This was supported by the upregulation of two purple acid phosphatase genes (Ta*PAP15*, Ta*PAP16*) in the roots by P deficiency (Fig. 3H, I). However, roots had relatively high RS-APase activity but relatively low levels of Ta*PAP15* and Ta*PAP16* mRNA at P100 (Table 1, Fig. 3H, I); this conflict might have been caused by the method of measuring RS-APase, which could not avoid contamination with organic compounds from damaged roots and microorganisms.

The traits that exhibited inhibitory response patterns to P deficiency included the expression of Ta*PHT1.1* and *-1.9*, Ta*PHT1.2*, and Ta*PHT1.10* (Fig. 3A–C). However, these four genes have been shown to be induced by P deficiency in a previous study (Davies *et al*., 2002) and a preliminary study (data not shown), and their orthologues in a close relative of wheat, barley (*Hordeum vulgare* L.), are also induced by P deficiency (Huang *et al*., 2011). The inhibition of these four wheat genes by P deficiency could be, at least partially, explained by the upregulated AM colonization under P deficiency (Table 1). Phylogenetic analysis of PHT1 proteins showed that these four genes are closely related to barley *Hv*PHT1.1 and *Hv*PHT1.9 (Supplementary Fig. S1). In barley, AM colonization has been found to inhibit the response of Hv*PHT1.1* and Hv*PHT1.2* to P deficiency in roots (Glassop *et al*., 2005). Ta*PHT1.6* showed a poor response to P supply and was not inhibited by P deficiency (Fig. 3D). Ta*PHT1.6* has been found to respond poorly to P deficiency in the roots of some wheat genotypes (Davies *et al*., 2002).

The two expansin genes Ta*EXPA6* and Ta*EXPB8* demonstrated combined inducible and inhibitory responses to P deficiency, as their transcripts increased when P-fertilizer rate decreased from sufficiency to P50 and then declined when P-fertilizer rate decreased from P50 to lower concentrations (Fig. 3F, G). Expansins are proteins that promote cell-wall loosening and extension and they function in diverse aspects of plant growth and development, including root development (Choi *et al*., 2006). For example, a root-specific and P-deficiency-inducible β-expansin, Gm*EXPB2*, was found to promote root development and enhance plant growth and P uptake under both low and high P (Guo *et al*., 2011). The α-expansin gene Ta*EXPA6* and β-expansin gene Ta*EXPB8* were found to predominantly expressed in roots in a previous study (Lin *et al*., 2005) and were induced by P deficiency in a preliminary study (data not shown), implying their possible functions in P-deficiency-induced root morphological changes. The response patterns of Ta*EXPA6* and Ta*EXPB8* to P supply were similar to, and could be at least partially explained by, the response patterns of RDW and RLD to P supply in that RDW and RLD peaked at P100 in the 2010 experiment (Table 1), while Ta*EXPA6* and Ta*EXPB8* reached their highest mRNA levels at P50 (Fig. 3F, G). In the 2011 experiment, RDW and RLD did not exhibit a significant response to P supply, but RDW and RLD at P0 were much lower than at P100 (Table 1), reflecting the inhibition of P0 on the expression levels of Ta*EXPA6* and Ta*EXPB8* (Fig. 3F,

G). The inhibition of P deficiency on root development has been shown for both hydroponically grown and field-grown wheat plants (Sun and Zhang *et al*., 2000; Hu *et al*., 2010).

In summary, this study characterized the root processes at six P supplies by investigating RDW, RLD, AM colonization rate, RS-APase activity, and expression levels of genes regulating Pi transporter, phosphatase, and root development. Although these processes are thought to increase the ability of the root systems to utilize Pi from soils in the published literature, several of these root traits were inhibited by P deficiency under the field conditions in this study. Therefore, more detailed research is needed to better understand the mechanisms of plant adaptation to soil P dynamics under field conditions. This study also observed that the optimal P supply for maximum grain yield coincided with the critical P supply for the response of P-starvation response marker genes to P deficiency and was at or near the change points for response of root processes to P supply. These results improve understanding of the molecular significance of optimal P supply for maximum yield and also provide useful information for optimizing P-management strategies based on the soil and the plant rhizosphere.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Basic soil properties in 0–30 cm soil layer before the field experiments.

Supplementary Table S2. Quantitative real-time PCR primers.

Supplementary Fig. S1. Phylogenetic analysis of PHT1 proteins from wheat, barley, rice, and maize.

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