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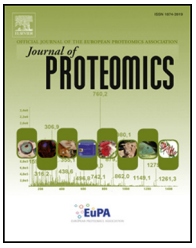

Proteomics insights into the basis of interspecific facilitation for maize (*Zea mays*) in faba bean (*Vicia faba*)/maize intercropping

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ABSTRACT

Faba bean/maize intercropping significantly promotes maize productivity in phosphorus-deficient soils. This has been attributed to the below-ground interactions including rhizosphere effects and spatial effects. Nevertheless, the molecular mechanisms underlying these interactions have been scarcely investigated. Here, three types of pots were used to distinguish the influences of rhizosphere effects vs. spatial effects. Phosphorus and nitrogen uptake of shoots, biomass, total root length, and root classification were evaluated between the three treatments. Quantitative RT-PCR and proteomics analyses were conducted to investigate the putative components in the molecular basis of these interactions. Quantitative RT-PCR results indicated that rhizosphere effects promoted maize phosphorus status at molecular levels. 66 differentially accumulated protein spots were successfully identified through proteomics analyses. Most of the protein species were found to be involved in phosphorus, nitrogen, and allelochemical metabolism, signal transduction, or stress resistance. The results suggest that rhizosphere effects promoted phosphorus and nitrogen assimilation in maize roots and thus enhanced maize growth and nutrient uptake. The reprogramming of proteome profiles suggests that rhizosphere effects can also enhance maize tolerance through regulating the metabolism of allelochemicals and eliciting systemic acquired resistance via the stimulation of a mitogen-activated protein kinase signal pathway.

Biological significance
The results obtained contribute to a comprehensive understanding of the response of maize to the changes of rhizosphere condition influenced by the below-ground interactions in faba bean/maize intercropping at molecular levels. The identified protein species involved in nutrient metabolisms and stress resistance reveal the molecular basis underlying the major advantages of effective nutrient utilization and higher stress tolerance in legume/cereal intercropping systems. This work provides essential new insights into the putative

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components in the molecular basis of interspecific facilitation for maize in faba bean/maize intercropping.

1. Introduction

Intercropping, the mixed growth of two or more crops, is an ancient and sustainable cropping practice that has been used in agroecosystems in China [1], India, Southeast Asia, Latin America, and Africa [2]. Intercropping is still widely used as one of the techniques for increasing crop yields in tropical and temperate zones [3]. Legume/cereal intercropping systems enable optimal distributions of space and resources [4,5] and offer several major advantages, such as effective utilization of nutrients [6–9], and greater resistance to weeds, pests, and diseases [10,11]. These advantages mainly result from the below-ground interactions between the intercropped species, including rhizosphere effects and spatial effects [12]. Rhizosphere effects include the energy transfer, matter cycling, and information transmission that enable the many interactions between plants, soils, microorganisms, and the larger environment. Spatial effects result from the distribution of the roots, which vary in different intercropping systems due to the characteristics of intercropped species in rooting depth and/or seasonality [7,12].

P is probably the most limiting mineral nutrient for plant growth in agroecosystems globally [13]. Faba bean/maize intercropping, a representative of legume/cereal intercropping system, is known to improve phosphorus (P) uptake in P-deficient soils [7]. Rhizosphere effects improve the availability of P in the rhizosphere of maize due to rhizosphere acidification that occurs via the release of organic acids and protons from faba bean roots [7]. P is transported from the external sources into root cells by specific transporter proteins that span the plasma membrane [14]. The identified plant P transporters have been classified into three families: PHT1, PHT2, and PHT3 [15]. P uptake is particularly dependent on the high-affinity transporters of the PHT1 family [15]. Five genes of the maize PHT1 family (*Pht1*;1, *Pht1*;2, *Pht1*;3, *Pht1*;4, *Pht1*;6) have been cloned and the expression of them can be detected in maize roots. It is also known that phosphate (Pi) starvation treatment causes an induction of the expression of these genes [16]. After being transported into root cells, inorganic P is assimilated and incorporated into general metabolism through oxidative phosphorylation in root tissues [17]. This P can then be used for the growth and development of maize plants.

Through the rhizosphere effects, faba bean/maize intercropping not only enhances the P nutrition of maize, but also alters the chemical and microbiological properties in the rhizosphere of maize [18,19]. Plants can reduce the growth of susceptible neighboring plants, herbivores, and pathogens by producing and releasing potent phytotoxins, thus reducing competition, pests and diseases [20]. 2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) are two important phytotoxins of this kind. DIMBOA is the predominant phytotoxin secreted by young maize plants [21]. Maize glucosyltransferases benzoxazinone synthesis8 (BX8) and UDP-glucosyltransferase (BX9) convert DIBOA and DIMBOA into non-toxic and stored forms as DIBOA-glucoside and DIMBOA-glucoside, respectively. Maize thus avoids the deleterious effects of these two phytotoxins [20,21]. Plant-growth-promoting rhizobacteria (PGPR) colonize the rhizosphere of many plant species and confer beneficial effects, such as increased plant growth and reduced susceptibility to diseases caused by plant pathogenic fungi, bacteria, viruses, and nematodes [22,23]. PGPR can elicit pathogenesis-related proteins (PRs) that lead to induced systemic acquired resistance (SAR) [24], which can result in abscisic acid (ABA) accumulation and reactive oxygen species (ROS) degradation in plants [25–27]. To date, however, few studies have attempted to evaluate the responses of maize to the changes of chemical and microbiological properties between faba bean/maize intercropping and maize monocropping.

Spatial effects are also important for interspecific facilitation in the faba bean/maize intercropping system. A previous study suggested that the compatibility of the spatial root distribution contributes to symmetric interspecific facilitation in faba bean/maize intercropping [4]. However, it is hard to distinguish whether the interspecific facilitation for maize in faba bean/maize intercropping is mainly derived from rhizosphere effects or from spatial effects.

Over the past decade, considerable progress has been made in our understanding of the physiological basis of the faba bean/ maize intercropping system [3,4,6,7,18,19]. However, to our knowledge, the putative components in the molecular basis of the interactions in this intercropping practice are little known. Proteomics strategies have become powerful tools that, when combined with complementary molecular genetic and physiological experimentation, can provide a framework for understanding the molecular basis of complex biological processes [28]. In this study, three types of pots [pots divided by plastic solid barriers (SB), divided by nylon mesh barriers (MB), not divided (no barrier, NB); Fig. S1] were used to distinguish the influences of rhizosphere effects vs. spatial effects in interspecific facilitation in faba bean/maize intercropping. The differences between MB and SB treatments were found to result from rhizosphere effects, while the differences between NB and SB treatments were influenced by both rhizosphere and spatial effects. To analyze the molecular basis of the higher P use efficiency of maize in faba bean/maize intercropping, the crops were planted in P-deficient soils. Quantitative RT-PCR (qRT-PCR) was used to detect the expression levels of several important P transporter genes in maize roots to monitor the differences of P nutrient status between the three treatments. We used high-resolution two-dimensional (2D) electrophoresis and MALDI-TOF/TOF tandem mass spectrometry to comprehensively investigate the putative components in the molecular basis of the interspecific facilitation for maize in faba bean/maize intercropping. Based on the data, we assume a protein reference map to predict the molecular mechanisms of interspecific facilitation for maize in faba bean/maize intercropping.

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2. Materials and methods

2.1. Plant materials and growth conditions

Faba bean (*Vicia faba* L. cv. Lincan NO. 5) and maize (*Zea mays* L. cv. Zhengdan958) were used in this study. Pot experiments were conducted using calcareous sandy soil, in the greenhouse (the temperature condition: 25–32 °C) of China Agriculture University (Beijing), from April to June. The soil was added with P-deficient basal fertilizers [composition (mg · kg^{-1} soil): N 200 (NH_4NO_3), P 50 and K 200 (KH_2PO_4 and K_2SO_4), Mg 50 ($MgSO_4 \cdot 7H_2O$), Fe 5 ($C_{10}H_{12}N_2O_8FeNa$), Mn 5 ($MnSO_4 \cdot 4H_2O$), Cu 5 ($CuSO_4 \cdot 5H_2O$), Zn 5 ($ZnSO_4 \cdot 7H_2O$), B 0.67 (H_3BO_3) Mo 0.122 ($(NH_4)_6MoO_{24} \cdot 4H_2O$)]. We used pots with two compartments to provide three types of root interactions (three treatments), including a plastic solid barrier (SB) to eliminate root contact and solute movement, a nylon mesh (37.5 μm) barrier (MB) to prevent root intermingling of the two species but permit the exchange of root exudates, and no root barrier (NB). Plastic pots were cut in the middle, separated with the appropriate material into two compartments, and then reconstructed. Each pot (22 cm in diameter and 28 cm in depth) contained 6 kg of fertilized soil (Fig. S1). The experiment consisted of three cropping treatments with ten biological replicates of each treatment. Five biological replicates were harvested at the first time, and the other five biological replicates were harvested at the second time (The schematic diagram for the experimental system can be seen in Fig. S4). The seeds of faba bean and maize were germinated for five and three days, respectively, in the dark at 25 °C. One faba bean and one maize uniform seeds were sown in a single pot at the same time. Plant samples were harvested for the first time before the jointing stage of maize after 44 days of growth (time point 1). Plant samples were harvested for the second time during the jointing stage of maize after 64 days of growth (time point 2). The shoots of both maize and faba bean at the both time points were kept at −20 °C for the measurement of the biomass and P or N concentrations. The roots of both maize and faba bean of time point 2 were kept at −20 °C for the measurement of biomass and total length and for the root classification. The maize roots of time point 1 were frozen in liquid nitrogen and kept at −80 °C for proteomics and qRT-PCR analyses.

2.2. Measurement of the P and N concentrations of shoots and the biomass of both shoots and roots

The shoots harvested at both time points (five shoots from maize and five shoots from faba bean at each time point) and the roots harvested at time point 2 (five roots from maize and five roots from faba bean) were weighed after being dried at 60 °C for 7 days. N and P concentrations were then measured for the shoots after digestion in a mixture of concentrated H_2SO_4 and H_2O_2. N was measured by the micro-Kjeldahl procedure. P was measured using the vanadomolybdate method [29].

2.3. Root analysis for total root length measurement and root classification

The roots harvested at time point 2 (five roots from maize and five roots from faba bean) were scanned using a root scanning instrument (WinRHIZO system). The root scan pictures were analyzed using WinRhizo 2005 software to generate the data including total root length and root classification. Root classification was conducted by counting the percentage of root length in different root diameter classes.

2.4. qRT-PCR analysis

Total RNA from maize root samples were isolated using TRIzol reagent (Invitrogen), digested with DNase I, and reverse-transcribed using Superscript-III Reverse Transcriptase (Invitrogen) into cDNA to be used as templates for subsequent qRT-PCR analyses. qRT-PCR analyses were performed using SYBR Premix Ex Taq Mix (Takara) on a Rotor-Gene 3000 (Corbett Research), according to the manufacturer's instructions. Thermal cycling programs were set as followed: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 15 s, 72 °C for 10 s; and then 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s for the dissociation stage. Relative expression levels were calculated using the $\Delta\Delta C_T$ method. *UBIQUITIN* was used as the reference gene. All reactions were performed in three biological replicates and a no-template control was included in each reaction (three biological replicates used for qRT-PCR analysis were randomly chosen from the five biological replicates harvested at time point 1). The primer sequences of the examined genes are listed in Table S1.

2.5. Preparation of protein extractions

The maize roots harvested at time point 1 were used for proteomics analyses (three biological replicates were randomly chosen from the five maize roots). The total protein contents were extracted using a denaturing protein extraction (Phenol extraction) procedure according to Saravanan and Rose [30], with minor modifications. 5 g maize root tissue was ground with liquid nitrogen in a mortar, and the homogenate was resuspended in four volumes of pre-cooled extraction buffer (1:4 wt/vol, 250 mM sucrose, 20 mM Tris–Hcl, pH 7.5, 10 mM EDTA, 1 mM PMSF, 1 mM DTT). After centrifugation (20 min, 15,000 ×*g*, 4 °C), the supernatant was collected and an equal volume of ice-cold Tris–HCl, pH 7.5, saturated phenol with the supernatant was added. The mixture was vortexed on ice for 1 h. After further centrifugation (20 min, 15,000 ×*g*, 4 °C), the phenol phase was collected. Proteins were precipitated from the phenol phase with three volumes of 100 mM ammonium acetate in methanol, overnight at −20 °C. After centrifugation (10 min, 15,000 ×*g*, 4 °C), the pellets were collected. The pellets were rinsed four times with ice-cold acetone containing 13 mM DTT, and then lyophilized. Approximate 40 mg lyophilized total protein was extracted from 5 g fresh roots of maize. The lyophilized pellets were then dissolved in sample buffer (7 M urea, 2 M thoiurea, 4%w/v CHAPS, 2% Ampholine, pH 3.5–10, 1% w/v DTT; 1 mg pellets for 0.1 mL buffer) by shaking at room temperature for 1 h. The protein concentration was determined with a Bradford assay using bovine serum albumin as the standard [31].

2.6. Two-dimensional electrophoresis (2-DE) and image analysis

First-dimension isoelectric focusing (IEF) separation was performed using ReadyStrip Linear IPG strips (pH 4–7, 24 cm;

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GE). The strips were loaded with 1.5 mg of maize root total protein. IEF was performed at 200, 500, and 2000 V for 1 h, 8000 V gradient for 30 min, 8000 V for 8 h, and 500 V for 9 h. For the second-dimension polyacrylamide gel electrophoresis (SDS-PAGE), IPG strips were placed onto 15% SDS-PAGE gels to separate. The gels were stained with Coomassie Brilliant Blue (CBB) R-250 (Sigma). To account for experimental variation, three biological replicate gels, which came from three independent experiments for all treatments, were run to obtain statistically reliable results.

The 2-DE gels were scanned at a 300 dpi resolution with a UMAX Power Look 2100XL scanner (Maxium Tech., Taipei, China). Image analysis was carried out with PDQuest software (version 8.0.1; BioRad). The built-in statistical module of a log transformation was used. After automated detection and matching, manual editing was carried out. Only those protein spots that could be detected in all of the three biological replicated gels were considered to be reliable protein spots. The synthetic gels were overlapped using the molecular marker as well as several protein spots present in all profiles as landmarks. Total quantity in valid spots was chosen as normalization parameters. Two comparison groups were carried out (MB vs SB group and NB vs SB group). Statistical significance was determined using Student's *t*-tests ($n = 3$, $p < 0.05$). Protein spots with at least 1.5-fold differences in accumulation values compared with the control ($p < 0.05$) were considered as differentially accumulated protein spots.

2.7. In-gel digestion, mass spectrometry, database searching and functional classification

In-gel digestion was performed according to a procedure described previously [32], with some modifications. Protein spots were excised from the 2D gels and destained with 50 mM NH_4HCO_3 in 50% (v/v) methanol for 1 h at 40 °C. After drying completely, the gel pieces were digested at 37 °C for 16 h with 10 ng/μl trypsin. Digested peptides were extracted three times with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile, lyophilized, and analyzed with MALDI-TOF/TOF tandem mass spectrometry.

For MALDI-TOF/TOF MS/MS analysis, the peptides were resuspended with 10 μL 70% ACN containing 0.1% TFA. 1 μL was spotted onto an AnchorChip™ MALDI target plate (Bruker Daltonics). 1 μL matrix solution (1 mg/mL, a-cyano-4-hydroxycinnamic acid in 70% acetonitrile containing 0.1% TFA) was spotted after the peptide solution dried. Mass spectra were acquired on a MALDI-TOF/TOF mass spectrometer (UltrafleXtreme, Bruker Daltonics, Billerica, MA, USA). The instrument was operated in the positive reflection mode and externally calibrated using a peptide calibration kit (Bruker Daltonics, Billerica, MA, USA). MS spectra were acquired with 400 laser shots per spectrum, whereas MS/MS spectra were obtained using 1500 laser shots per fragmentation spectrum. To acquire the MS/MS fragmentation spectra, the 15 strongest peaks of each MS spectra were selected as precursor ions (excluding trypsin autolytic peptides and other known background ions).

For database searching, MS data were uploaded with Biotools software (Ver. 3.2 Bruker Daltonics) to Mascot for database searching on the Matrix Science (London, U.K.) public web site (http://www.matrixscience.com) and searched against the NCBI nr protein database (version 20120707). Search parameters were set as: green plants; proteolytic enzyme, trypsin; max missed cleavages, 1; fix modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide mass tolerance, 100 ppm; fragment mass tolerance, 0.5 Da. Only significant hits as defined by Mascot probability analysis were considered in subsequent data analyses. The protein species were functionally categorized by UniProtKB (http://www.uniprot.org) and the Gene Ontology Tool (http://www.geneontology.org) combined with manual analysis. Subcellular locations of identified protein species were predicted using WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html), Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html) and UniprotKB (http://www.uniprot.org/) database programs.

2.8. Statistical analysis

The software of MS Excel and SAS was used for data analyses. Statistical significance of differences between treatments was determined by analysis of variance (ANOVA) and the LSD (least significant difference) multiple comparisons (SAS Institute). The degree of freedom in statistical analysis was the default "$n - 1$".

3. Results

3.1. Maize shoot P uptake, maize biomass, and maize root characteristics were significantly different between the three treatments after 64 days' intercropping

Faba bean and maize were planted in P-deficient soils to ensure the P nutrient was the most important limiting factor for the growth of crops in this study. At time point 1, there was no visible difference in maize shoots between the three different treatments (Fig. 1a). At time point 2, there were visible differences in maize shoots between the three different treatments (Fig. 1b). To explore the relationship between shoot P uptake and shoot biomass for both faba bean and maize, shoot P uptake and shoot biomass were measured from plant materials at both time points. Before the jointing stage of maize plants, there was no significant difference in shoot P uptake amount or shoot biomass of either maize or faba bean between the three treatments (Fig. 1c, d purple; Fig. S2a, b purple). However, there were significant differences in maize shoot P uptake and maize shoot biomass between the three treatments after 20 days' rapid growth of maize (Fig. 1c, d green). Following 64 days of intercropping, rhizosphere effects (MB vs SB) enhanced maize shoot P uptake and maize shoot biomass by 28.5% and 13.8%, respectively, while both rhizosphere and spatial effects together (NB vs SB) significantly enhanced these parameters by 61.2% and 21.0%, respectively. There was a significant difference in faba bean shoot P uptake, but no significant differences in faba bean shoot biomass between the three treatments after 64 days' intercropping (Fig. S2a, b). These results confirmed previous conclusion [7] that intercropping with faba bean can significantly improve maize growth resulting from the uptake of P

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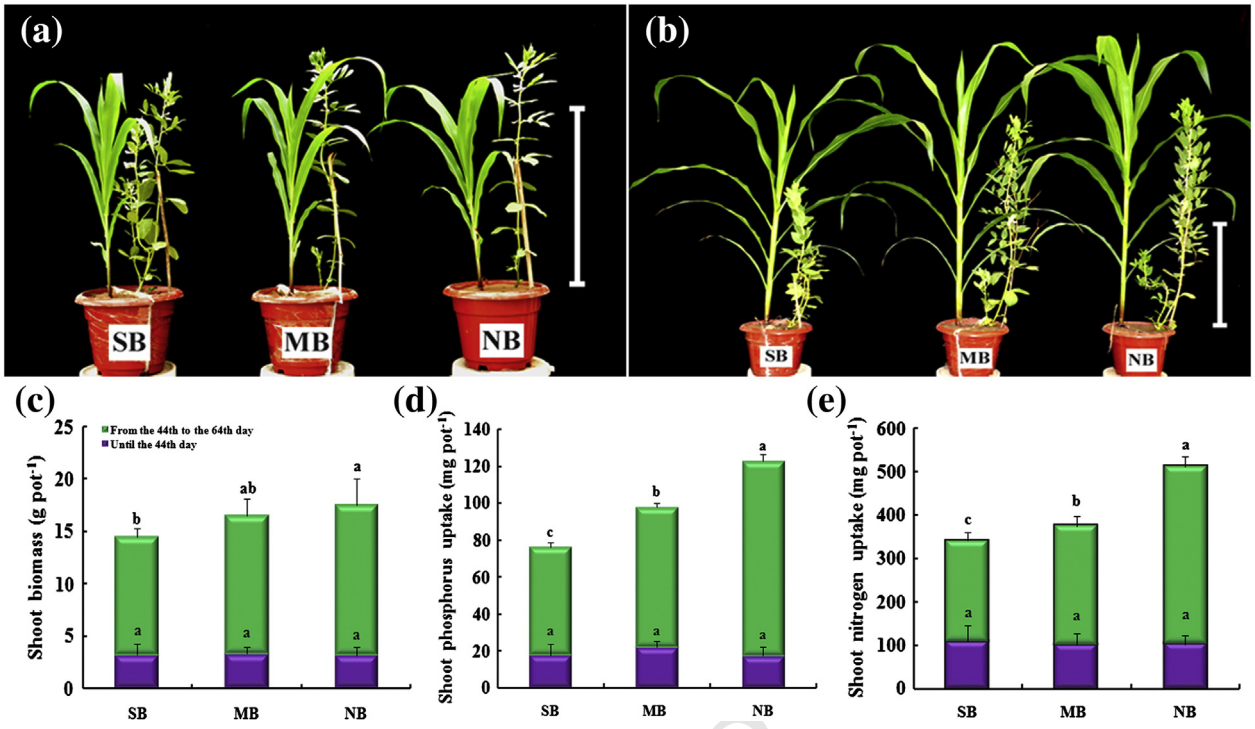

Fig. 1 – Phenotype analyses of maize (*Zea mays*) shoots. **(a) Representative picture of maize and faba bean (*Vicia faba*) after 44 days' growth. Bar = 50 cm. (b) Representative picture of maize and faba bean after 64 days' growth. Bar = 50 cm. (c) Shoot biomass of maize. (d) Shoot phosphorus uptake of maize. (e) Shoot nitrogen uptake of maize. SB, MB, and NB indicate three different treatments: SB, pots divided by plastic solid barriers; MB, pots divided by nylon mesh barriers; NB, pots with no barrier. Differences among the lowercase letters above the bars indicate a significant ($p < 0.05$) difference among the three treatments. The data are presented as means + SD ($n = 5$).**

mobilized by faba bean roots in P-deficient soils, and thus indicated that the plant materials were suitable for further analyses.

To investigate the relationship between P uptake and the growth and development of maize or faba bean roots, the biomass and total length of root samples at time point 2 were also measured. The results showed that there were significant differences for both the maize root biomass and the maize total root length between the MB and SB treatments. Our results indicated that rhizosphere effects significantly enhanced maize root biomass and total root length by 25.4% and 67.9%, respectively (Fig. 2a, b). There was a significant difference in faba bean total root length but no significant difference in faba bean root biomass between the three treatments after 64 days' intercropping (Fig. S3a, b). This indicated that rhizosphere effects improved the growth and development of maize roots in faba bean/maize intercropping. To further explore the maize root characteristics in the different treatments, root classification was conducted by counting the percentage of root length in different root diameter classes. The percentage of maize root

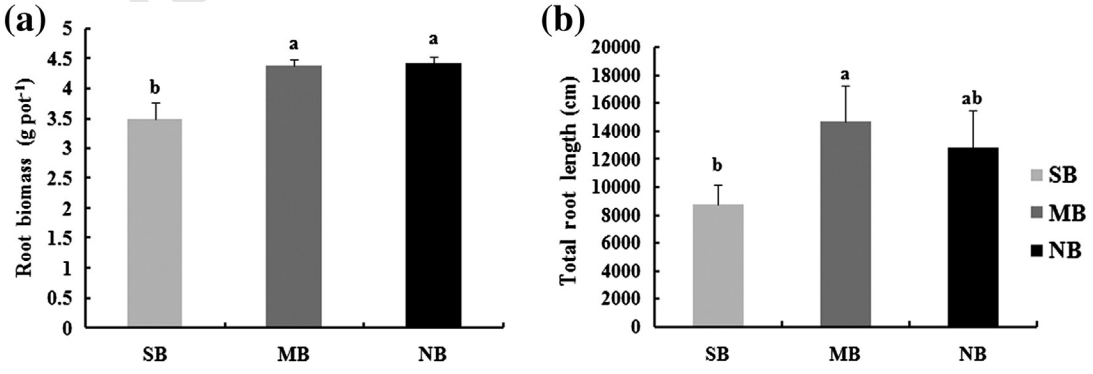

Fig. 2 – Phenotype analyses of maize (*Zea mays*) roots. **(a) Root biomass of maize after 64 days' growth. (b) Total root length of maize after 64 days' growth. SB, pots divided by plastic solid barriers; MB, pots divided by the nylon mesh barriers; NB, pots with no barrier. Differences among the lowercase letters above the bars indicate a significant ($p < 0.05$) difference among the three treatments. The data are presented as means + SD ($n = 5$).**

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Table 1 – The percentage of maize root length in different root diameter classes.

Treatments	0.000 < .L. < =0.300	0.300 < .L. < =0.500	0.500 < .L. < =0.800	.L. > 0.800
SB	47.60 ± 4.02b	24.56 ± 1.25a	14.53 ± 1.49a	13.31 ± 1.68a
MB	55.54 ± 3.08a	21.28 ± 0.64b	12.61 ± 1.98a	10.57 ± 1.62a
NB	48.83 ± 3.59ab	22.57 ± 0.56b	15.07 ± 2.36a	13.53 ± 1.66a

Note: Differences among the lowercase letters indicate a significant ($p < 0.05$) difference among three treatments in the same root diameter class. The data are presented as means ± SD (n = 5).

length in the "0.000 < .L. < =0.300 (mm)" class was significantly greater in the MB treatment (55.54%) than in the SB treatment (47.60%). The percentage of maize root lengths in the "0.300 < .L. < =0.500 (mm)" class was significantly lower in the MB treatment (21.28%) than in the SB treatment (24.56%) (Table 1). These results indicated that rhizosphere effects promoted the development of maize fine roots in faba bean/maize intercropping.

3.2. qRT-PCR analysis indicated that rhizosphere effects improved the P status of maize at the molecular genetic level after 44 days' intercropping

To obtain direct molecular genetic evidence to show whether or not the promotion of growth and development of intercropped maize resulted from the P mobilized by faba bean roots, qRT-PCR analysis was used to investigate the expression patterns of five P transporter genes that have already been characterized in maize. Expression of the five genes can be induced by phosphate-starvation treatment [16]. As such these five genes are appropriate markers for evaluation of P status in maize. The results showed that the expression levels of *Pht1;1*, *Pht1;2*, *Pht1;3*, *Pht1;4*, *Pht1;6* were downregulated in maize roots at time point 1 in the MB treatment compared to the NB treatment (Fig. 3a, b, c, d, e), indicating that rhizosphere effects had enhanced the P content in soils and thus improved the P status of maize after 44 days' intercropping. This confirmed that maize roots at time point 1 had been influenced by the belowground interactions and were thus suitable for further proteomics analysis.

3.3. Protein spots separation, image analysis, and protein species identification

The proteome profiles of maize roots following 44 days of intercropping are shown in Fig. 4a, b, c. After CBB R-250 staining, each gel contained approximately 1000 protein spots (Fig. 4a, b, c), which were distributed evenly in the range of 10–95 kDa. 9 representative gels (a total of 3 treatments, and 3 representative biological replicate gels for each treatment) were used for comparative analyses (PDQuest software, version 8.0.1; BioRad). After automatic detection, a total of 973 ± 58, 1063 ± 70, and 1012 ± 81 spots were detected in SB, MB, and NB treatments, respectively. Spot detection was refined by manual editing, removal, or addition of missing or

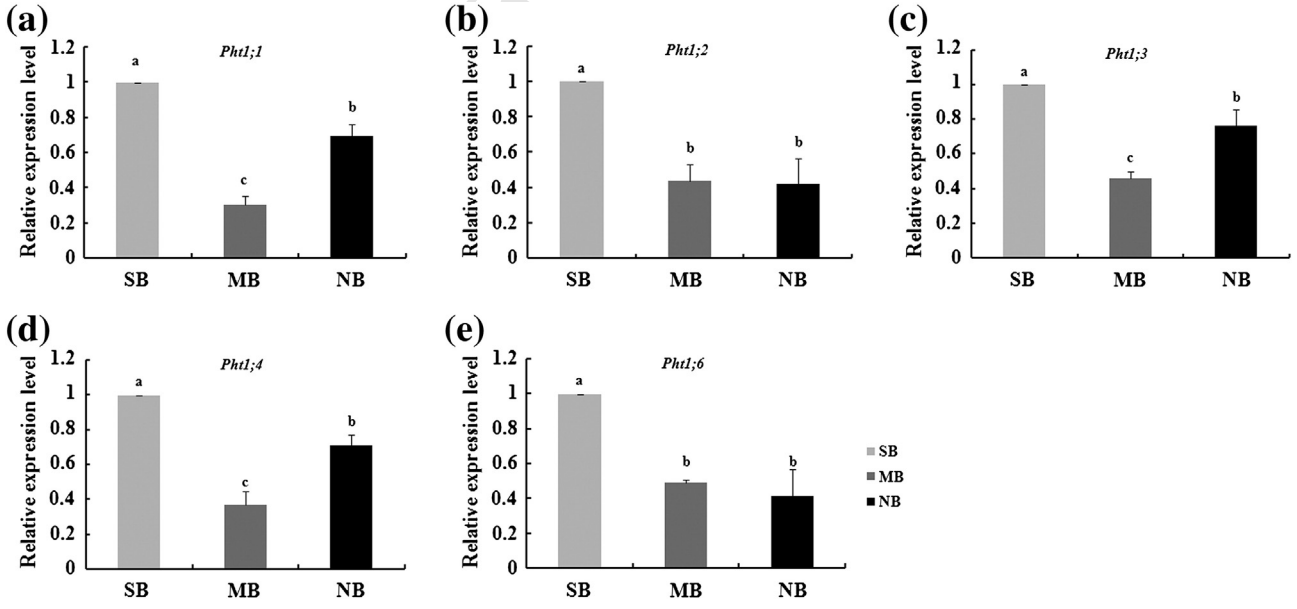

Fig. 3 – Expression levels of maize phosphorus transporter genes observed with qRT-PCR analysis. (a) *Pht1;1*. (b) *Pht1;2*. (c) *Pht1;3*. (d) *Pht1;4*. (e) *Pht1;6*. The samples were quantified by qRT-PCR using *UBIQUITIN* as a reference gene. SB, MB, and NB indicate three different treatments. SB, pots divided by plastic solid barriers; MB, pots divided by nylon mesh barriers; NB, pots with no barrier. Differences among the lowercase letters above the bars indicate a significant ($p < 0.05$) difference among the three treatments. The data are presented as means + SD ($n = 3$).

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undetected spots. Spots that appeared in all three biological replicate gels were identified as reliable protein spots. Total quantity in valid spots was chosen as the normalization parameters according to the instructions for PDQuest software (Bio-Rad). In total 853 spots were well-matched in all 9 gels. The comparative analysis was firstly carried out between the MB and SB treatments (MB vs SB comparison group), which indicates the influences derived from the rhizosphere effects. Then the comparative analysis was performed between the NB and SB treatments (NB vs SB comparison group), which indicates the influences derived from both rhizosphere and spatial effects. With 1.5-fold quantitative change and Student's t-test $p < 0.05$ set as the cut off criteria, a total of 68 differentially abundant protein spots were detected during the comparative analyses. No qualitative differences (newly appeared/disappeared) spots were detected in the comparative analyses. Of the 68 differentially accumulated protein spots, 66 (marked in Fig. 4a, b, c) were successfully identified using MALDI-TOF/TOF peptide spectral data and database searching. Of the 66 successfully identified protein spots, 45 (36 up and 9 down accumulated protein spots) were different in the MB vs SB comparison group (marked in Fig. 4a, b), 46 (25 up and 21 down accumulated protein spots) were different in the NB vs SB comparison group (marked in Fig. 4b, c), and 25 overlapped (marked in Fig. 4b; Venn diagram as seen in Fig. 4d).

3.4. *Functional classification and annotations of the differentially accumulated protein spots*

Table 2 summarizes various parameters about the differentially accumulated protein species and the protein spot IDs corresponding to protein spots shown in Fig. 4a, b, c. The differentially accumulated protein species in the MB vs SB comparison group were classified into eight categories, including P and energy metabolism (13.3%), N and C metabolism (11.1%), amino acid and protein metabolism (20%), secondary metabolism (13.3%), signal transduction (13.3%), disease and defense (15.6%), cell structure (4.4%), and unclassified (8.9%) (Fig. 5a). 80% of these protein species were up-accumulated in the MB treatment compared to the SB treatment. 100% of the protein species related to P and energy metabolism, 80% of the protein species related to N and C metabolism, 78% of the protein species related to amino acid and protein metabolism, 67% of the protein species related to secondary metabolism, 100% of the protein species related to signal transduction, and 100% of the protein species related to disease and defense were up-accumulated in the MB treatment compared to the SB treatment (Table 2).

The differentially accumulated protein species in the NB vs SB comparison group were also classified into eight categories, including P and energy metabolism (10.9%), N and C metabolism (8.7%), amino acid and protein metabolism (19.6%), secondary metabolism (17.4%), signal transduction (8.7%), disease and defense (19.6%), transporters (2.2%), and unclassified (13.0%) (Fig. 5b). 54% of these protein species were up-accumulated in the NB treatment compared to the SB treatment. 100% of the protein species related to P and energy metabolism, 50% of the protein species related to N and C metabolism, 56% of the protein species related to amino acid and protein metabolism, 38% of the protein species related to

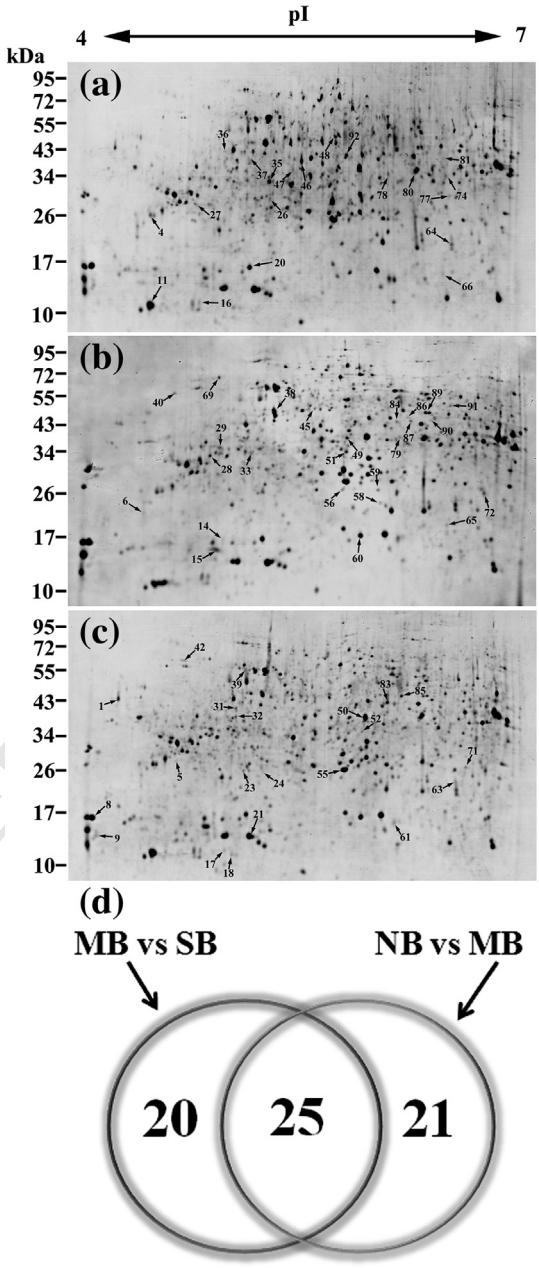

Fig. 4 – Representative two-dimensional gels and Venn diagram analyses. (a–c) Representative two-dimensional gels showing differentially accumulated protein spots in maize (*Zea mays*) roots in different treatments. (a) Representative two-dimensional gel of maize root total proteins in the SB treatment. (b) Representative two-dimensional gel of maize root total proteins in the MB treatment. (c) Representative two-dimensional gel of maize root total proteins in the NB treatment. (d) Venn diagram analysis of protein species that were differentially accumulated in the MB vs SB comparison group and the NB vs SB comparison group. 20 protein species that were only differentially accumulated in the MB vs SB comparison group are marked in (a), 25 overlapping protein species are marked in (b), and 21 protein species that were only differentially accumulated in the NB vs SB comparison group are marked in (c).

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Table 2 – Differentially accumulated protein species of maize roots involved in the MB vs SB comparison group and the NB vs SB comparison group, as identified by MALDI-TOF/TOF.

Category	Spot ID	Protein name	Score	Taxonomy	Theoretical Mr(kDa)/pI	Expermental Mr(kDa)/pI	M	SC	accession no.	Accumulated levels (+:up; –:down)	
										MB vs SB	NB vs SB
P and energy metabolism											
	37	pyruvate dehydrogenase2	248	*Zea mays*	40.072/5.54	40.16/5.29	5	14%	NP_001104914	1.54	N
	40	PREDICTED: apyrase 2-like	81	*Glycine max*	63.477/5.55	52.33/4.69	1	3%	XP_003548478	1.52	1.66
	50	malate dehydrogenase, cytoplasmic	376	*Zea mays*	35.909/5.77	34.01/5.94	5	21%	NP_001105603	N	1.55
	51	malate dehydrogenase	98	*Zea mays*	12.055/5.11	30.25/5.82	1	10%	AAK58078	1.96	1.71
	69	ATP synthase beta chain	89	*Zea mays*	59.057/5.90	55.82/4.93	3	7%	NP_001151807	3.58	2.16
	79	malate dehydrogenase	103	*Zea mays*	12.055/5.11	33.94/6.16	1	10%	AAK58078	1.58	1.51
	80	malate dehydrogenase	305	*Zea mays*	35.669/7.63	34.3/6.31	3	7%	ACG36184	1.5	N
N and C metabolism											
	24	fructose-bisphosphate aldolase, cytoplasmic isozyme	84	*Zea mays*	38.891/6.96	23.69/5.36	1	5%	NP_001150049	N	–2.84
	35	fructokinase-2	295	*Zea mays*	35.858/5.34	31.27/5.38	4	17%	NP_001105211	–1.82	N
	55	triosephosphate isomerase, cytosolic	260	*Zea mays*	27.278/5.53	24.33/5.81	3	15%	ACG24648	N	–1.71
	66	glutamine synthetase root isozyme 3	71	*Zea mays*	39.556/5.34	18.85/6.44	2	7%	NP_001105296	3.12	N
	78	Putative uncharacterized protein	123	*Zea mays*	34.244/5.92	32.65/6.13	2	7%	ACG36179	1.78	N
	84	alpha-galactosidase precursor	61	*Zea mays*	45.373/5.72	38.94/6.14	1	2%	NP_001147362	1.62	1.9
	89	glutamate dehydrogenase	363	*Zea mays*	44.285/5.96	41.94/6.33	5	13%	AAB51596	1.77	1.58
Amino acid and protein metabolism											
	1	Cysteine protease Mir1	47	*Zea mays*	43.054/5.05	39.15/4.38	1	3%	NP_001105571	N	1.59
	4	translationally-controlled tumor protein	212	*Zea mays*	18.787/4.53	23.94/4.63	2	14%	ACG24638	1.92	N
	18	translationally-controlled tumor protein	210	*Zea mays*	18.787/4.53	14.36/5.17	2	14%	ACG24638	N	–2.2
	29	aspartic proteinase oryzasin-1 precursor	53	*Zea mays*	55.263/5.43	32.1/5.02	1	2%	NP_001148782	2.02	2.03
	33	retrotransposon protein SINE subclass precursor	60	*Zea mays*	55.863/5.85	31/5.2	1	1%	NP_001152501	3.3	2.55
	36	adenosylhomocysteinase	122	*Zea mays*	53.898/5.63	39.36/5.1	2	4%	NP_001148534	1.71	N
	48	S-adenosylmethionine synthetase 1	500	*Zea mays*	43.418/5.57	46.85/5.81	6	21%	ACG42196	1.58	N
	60	eukaryotic translation initiation factor 5A	352	*Zea mays*	17.714/5.61	17.88/5.89	4	28%	NP_001105606	1.79	1.53
	71	Glutathione transferase III(a)	254	*Zea mays*	23.910/5.96	23.99/6.2	4	18%	CAB38118	N	–2.62
	86	aspartate aminotransferase	145	*Zea mays*	49.679/8.39	39.73/6.21	4	7%	ACG37512	–1.54	–1.82
	90	Uncharacterized protein	55	*Zea mays*	37.160/5.97	39.83/6.37	1	4%	ACF85494	–1.66	–2.47
	91	chorismate synthase 2	108	*Zea mays*	47.472/6.84	44.88/6.49	3	7%	NP_001148583	2.17	1.92
Secondary metabolism											
	5	chalcone-flavonone isomerase	83	*Zea mays*	23.715/4.65	24.61/4.77	2	17%	NP_001150388	N	–2.27
	31	unknown	182	*Zea mays*	35.470/5.09	37.14/5.17	4	8%	ACF83731	N	–1.65
	32	unknown	186	*Zea mays*	35.470/5.09	34.99/5.17	4	8%	ACF83731	N	–2.13
	38	UDP-glucosyltransferase BX9	287	*Zea mays*	50.559/5.22	43.06/5.35	4	11%	AAL57038	–1.8	–2.45
	39	hypothetical protein OsI_16194	47	*Oryza sativa Indica Group*	33.615/9.42	51.33/5.24	1	3%	EEC77416	N	1.88
	45	benzoxazinone synthesis8 /BX8	282	*Zea mays*	47.994/6.06	42.72/5.59	3	12%	NP_001144409	1.96	1.58
	46	O-methyltransferase	341	*Zea mays*	39.223/5.48	36.54/5.57	5	14%	AAQ24342	1.53	N
	47	isoflavone reductase homolog IRL	559	*Zea mays*	32.831/5.69	35.17/5.58	7	27%	NP_001105699	–2.63	N

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	52	glyoxylase1	531	*Zea mays*	32.450/5.59	30.98/5.9	5	26%	NP_001105217	N	−1.78
	74	IN2-2 protein	77	*Zea mays*	27.814/6.45	32.08/6.41	1	5%	ACG26195	1.71	N
	83	herbicide safener binding protein1	241	*Zea mays*	40.626/5.65	39.84/6.11	3	10%	NP_001106076	N	2.5
	92	alpha-1,4-glucan-protein synthase[UDP-forming]	183	*Zea mays*	41.691/5.75	40.94/5.71	4	8%	NP_001105598	2.28	N
Signal transduction											
	27	14-3-3-like protein	201	*Zea mays*	28.988/4.82	26.72/4.91	2	11%	NP_001105677	1.63	N
	28	DREPP4 protein	112	*Zea mays*	22.596/4.89	30.35/4.95	1	7%	ACG38669	2.6	2.51
	63	rhicadhesin receptor precursor	135	*Zea mays*	22.964/6.58	21.78/6.49	1	7%	ACG37538	N	1.84
	64	rhicadhesin receptor precursor	176	*Zea mays*	22.964/6.58	21.15/6.5	2	11%	ACG37538	1.7	N
	72	germin-like protein subfamily 1 member 17 precursor	138	*Zea mays*	24.688/6.41	24.32/6.59	1	7%	ACG41245	2.03	2.56
	81	osmotic and salt stimulation MAPK1	178	*Zea mays*	42.699/6.23	36.77/6.48	2	7%	ABD77415	2.86	N
	87	uncharacterized protein LOC100274292	519	*Zea mays*	33.566/5.96	37.95/6.25	5	25%	NP_001142128	1.61	1.85
Disease/defense											
	8	pathogenesis-related protein 1	244	*Zea mays*	17.669 /4.38	17.17/4.23	1	15%	ABA34055	N	−1.8
	14	Peroxiredoxin-5	192	*Zea mays*	23.918/7.74	18.13/5.04	2	15%	NP_001148437	3.7	4.52
	15	pathogenesis-related protein 5	263	*Zea mays subsp. Parviglumis*	18.203/4.87	16.9/4.98	2	19%	ABA34032	1.56	2.1
	17	major pollen allergen Car b 1 isoforms 1A and 1B	202	*Zea mays*	16.773/4.99	14.62/5.11	2	19%	NP_001147371	N	−2.78
	20	pathogenesis-related protein 10	341	*Zea mays*	17.130/5.13	18.15/5.28	4	25%	NP_001147373	1.51	N
	21	pathogenesis-related protein 1	199	*Zea mays*	17.074/5.39	15.84/5.29	2	21%	ACG29538	N	−2.24
	58	allene oxide cyclase1	336	*Zea mays*	25.932/9.05	21.58/6.02	4	20%	NP_001105245	2.14	2.09
	59	ABA-, stress-and fruit-ripening inducible-like protein	264	*Zea mays*	15.762/5.74	23.2/6.02	2	24%	CAA72998	8.06	11.43
	65	abscisic stress ripening protein 2	50	*Zea mays*	14.895/6.15	21.15/6.5	1	6%	NP_001147703	12.52	24.47
	77	Uncharacterized protein	517	*Zea mays*	34.284/7.75	29/6.52	6	27%	ACN27920	1.55	N
	85	peroxidase	53	*Zea mays*	38.869/6.49	41.98/6.2	1	4%	AAS75393	N	−1.64
Transporters											
	23	hemoglobin 2	76	*Zea mays*	20.690/5.02	23.54/5.23	1	6%	NP_001105819	N	−1.64
Cell structure											
	11	profilin-2	110	*Zea mays*	14.178/4.63	14.14/4.65	1	9%	ACG33212	−2.44	N
	26	actin, partial	185	*Zea mays*	37.273/5.28	27.2/5.38	2	8%	AAB40106	−1.69	N
Unclassified											
	6	hypothetical protein OsJ_04535	49	*Oryza sativa Japonica Group*	29.680/10.36	20.76/4.55	1	8%	EAZ14610	−1.63	−2.41
	9	hypothetical protein OsI_16194	47	*Oryza sativa Indica Group*	33.615/9.42	15.15/4.24	1	3%	EEC77416	N	−2.26
	16	ML domain protein	219	*Zea mays*	17.027/5.11	14.61/4.96	2	20%	ACG38215	1.9	N
	42	Uncharacterized protein	82	*Zea mays*	52.051/4.76	55.91/4.83	2	5%	ACN30693	N	2.36
	49	uncharacterized protein LOC100383880	534	*Zea mays*	38.233/5.66	33.54/5.81	6	22%	NP_001169979	1.51	−1.93
	56	hypothetical protein SORBIDRAFT_10g029090	55	*Sorghum bicolor*	26.507/5.76	23.22/5.79	1	3%	XP_002437541	−1.59	−2.13
	61	hypothetical protein	44	*Zea mays*	9.35/6.23	17.13/6.12	1	12%	ACG31129	N	−2.94

Notes: Spot ID, Spot number as shown in 2-DE gels in Fig. 4. Score, Protein score. Theoretical Mr(kDa)/pI, Theoretical molecular weight and pI. Expermental Mr(kDa)/pI, Experimental molecular weight and pI. M, the matched peptides. SC, sequence coverage. Accession no., Accession number of Uniprot and/or NCBI database. Accumulated levels MB vs SB (+:up; −:down), "+/−" indicates the up/down-accumulated folds of protein species in the MB treatment compared to the SB treatment. Accumulated levels NB vs SB (+:up; −:down), "+/−" indicates the up/down-accumulated folds of protein species in the NB treatment compared to the SB treatment. "N" indicates no significant difference ($p < 0.05$, $n = 3$).

secondary metabolism, 100% of the protein species related to signal transduction, and 56% of the protein species related to disease and defense were up-accumulated in the NB as compared to the SB treatment (Table 2).

3.5. *Rhizosphere effects promoted nitrogen (N) assimilation in maize roots and N uptake in maize shoots in P-deficient soils*

In order to exclude the influence of N fixation, the crops were planted in N-adequate but P-deficient soils (N 200, P 50 mg · kg^{-1} soil). Moreover, there was no significant difference in faba bean shoot N uptake (Fig. S2c), indicating that the N fixation of faba bean root nodules had not influenced the N concentration in rhizosphere prior to time point 2. Therefore the N in soils couldn't be the direct limiting factor for maize N metabolism in the present study. However, the proteomic results showed that glutamine synthetase (GS, spot 66) and glutamate dehydrogenase (GDH, spot 89), two key enzymes in N assimilation and metabolism, were up-accumulated in maize roots in the MB treatment compared to the SB treatment after 44 days' intercropping (Table 2). Consistently, our results show that there were significant differences after 64 days' intercropping but no significant difference after 44 days' intercropping in maize shoot N uptake between the three treatments (Fig. 1e). These results indicate that rhizosphere effects promoted N assimilation in maize roots and then enhanced N uptake in maize shoots grown in P-deficient soils.

4. Discussion

Effective utilization of nutrients and higher tolerance to stress are known as two major advantages in legume/cereal intercropping systems [6,10,11]. The advantages have been attributed to the below-ground interactions including rhizosphere effects and spatial effects [7,12]. In this study, the differentially accumulated protein species in the MB vs SB comparison group are derived from rhizosphere effects; while the differentially accumulated protein species in the NB vs SB comparison group are derived from rhizosphere effects together with spatial effects. Our results (Table 2) showed that key enzymes related to P and N metabolisms (spots 51, 69, 79, 89) were up-accumulated to the higher levels in the MB vs SB comparison group compared to the NB vs SB comparison group, and the GS (spot 66; the rate-limiting enzyme in N metabolism) was only up-accumulated in the MB vs SB comparison group. Moreover, 7 protein species (spots 14, 15, 20, 58, 59, 65, 77) putatively involved in disease and defense were up-accumulated in the MB vs SB comparison group while only 5 such protein species (spots 14, 15, 58, 59, 65) were observed in the NB vs SB comparison group. This suggests that the advantages in nutrient utilization and stress tolerance mainly result from the rhizosphere effects in faba bean/maize intercropping. Hence, our discussion will focus on the differentially accumulated protein species influenced by rhizosphere effects.

4.1. *Rhizosphere effects promote P and N assimilation in maize roots, and then enhanced maize growth and nutrient uptake*

The P use efficiency of plants includes several component traits such as P uptake, transport, and internal utilization [33]. In this study, our results show that rhizosphere effects significantly improved maize shoot P uptake and promoted the growth and development of maize shoots and roots after 64 days' intercropping (Figs. 1 and 2). qRT-PCR analysis indicated that rhizosphere effects had already enhanced the P status of maize at the molecular genetic level after 44 days' intercropping. These data suggest that the promotion for maize P uptake and maize growth results from the improvement of P metabolism processes that were directly triggered by mobilized P nutrient through rhizosphere effects.

P enters metabolic pathways primarily through the adenosine synthesis process, which occurs in mitochondria during respiration via oxidative phosphorylation in root tissues [17]. Malate dehydrogenase, the rate-limiting enzyme in the tricarboxylic acid (TCA) cycle, is important for providing the nicotinamide adenine dinucleotide hydrogen (NADH) and the reduced flavin adenine dinucleotide ($FADH_2$) to the electron transport chain of respiration. The adenosine triphosphate (ATP) synthase, which acts to catalyze Pi and adenosine diphosphate (ADP) to produce ATP, plays an important role in oxidative phosphorylation. In this study, both ATP synthase (spot 69) and several isoforms of malate dehydrogenase (spots 51, 79, 80) accumulated more in the MB treatment than in the NB treatment. Malate dehydrogenase occurred as multiple gel spots, in agreement with the presence of different protein isoforms and post-translational modifications (PTMs). This indicates that rhizosphere effects promote the respiration and oxidative phosphorylation through inducing the accumulation of malate dehydrogenase isoforms and ATP synthase, and thus enhancing the P assimilation in maize roots. Plant roots are known to secrete organic acids and thus can solubilize the insoluble inorganic P in soils [34]. Overexpression of the gene encoding malate dehydrogenase in transgenic alfalfa resulted in significantly enhanced organic acid synthesis and exudation [35]. Based on these data, we suppose that, in addition to benefiting from the organic acid exudation from faba bean roots [7], intercropped maize can secrete more organic acid to mobilize inorganic P nutrient in soils through the enrichment of malate dehydrogenase derived from the rhizosphere effect. Apyrase, a nucleoside-phosphatase, was reported to function in the mobilization of Pi from extracellular ATP that may originate from dead cells, efflux, or phage activity [36]. Thus, an attractive hypothesis is that the up-accumulation of an apyrase 2-like protein specie (spot 40) derived from rhizosphere effects might result in the mobilization of Pi through the catabolism of extracellular ATP. In addition, a recent study reported that a H^+-ATPase can energize P uptake during mycorrhizal symbioses in rice and *Medicago truncatula* [37]. Arbuscular mycorrhizal (AM) fungi are important components in intercropping agroecosystems [38]. This supported the implication of the results that the up-accumulation of ATP synthase and nucleoside-triphosphatase in maize roots plays important roles in the more active nutrient uptake of maize in faba bean/maize intercropping. Collectively, these data suggest that rhizosphere effects can promote P assimilation in intercropped maize roots and mobilize P in the rhizosphere environment through enhancing the abundances of ATP synthase, malate dehydrogenase and nucleoside-triphosphatase.

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N metabolism processes are associated with P and energy metabolism. Our results indicated that rhizosphere effects also promoted N metabolism in maize roots through enhancing the abundance of some N metabolism related protein species such as GS (spot 66) and GDH (spot 89) in P-deficient soils. GS and GDH are key enzymes in the N assimilation and metabolism pathways [39]. The role of GS in N management, growth rate, yield, and grain-filling has been suggested by the finding of co-localizations between quantitative trait loci (QTLs) for agronomic traits and GS activity [40,41]. Strong evidence using genetic analyses has also linked the gene encoding GS with grain filling in rice and maize [42,43]. The coordination and optimal functioning of nitrogen and carbon metabolism in plants are critical in plant growth and, ultimately, biomass accumulation [44]. GDH, which is able to convert amino acids into transport compounds with a low C/N ratio, plays a significant role in maintaining C/N balance [45,46]. Overexpression of the genes encoding GS or GDH in transgenic plants was shown to improve plant growth and productivity [47–50]. These data suggest that rhizosphere effects can also promote N assimilation in maize roots and thus improve maize growth and productivity on N-adequate but P-deficient soils.

Plant nutrition and growth are intrinsically linked at several levels of integration. Nutrient provision promotes growth, and growth generates 'demand' signals for nutrients [51]. Our results show that rhizosphere effects promoted P and N assimilation after 44 days of intercropping, and promoted maize shoot and root biomass, total root length, fine root amount, and shoot P and N uptake after 64 days of intercropping. This suggests that efficient P and N metabolism significantly enhanced maize shoot and root growth in faba bean/maize intercropping, and conversely, the vigorous growth of maize shoot and optimized maize root morphology are favorable for nutrient uptake to support efficient P and N assimilation.

4.2. *The reprogramming of protein species involved in stress resistance suggests that rhizosphere effects can enhance the tolerance of maize in faba bean/maize intercropping*

Many intercropping systems provide crops with higher resistance to weeds, pests and diseases as compared to monoculture systems [10,11,52]. However, few studies have reported the higher tolerance in faba bean/maize intercropping. In this study, several protein species related to allelochemical metabolism and stress resistance were differentially accumulated in maize roots between the MB treatment and the SB treatment. Allelopathy plays important roles in weed, pest and disease control [53]. DIMBOA and DIBOA are two important allelochemicals for biotic stress resistance in maize roots. Our results (Table 2) show that BX8 (spot 45) was up- accumulated while BX9 (spot 38) was down-accumulated in intercropped maize roots in the MB treatment compared to the SB treatment. DIMBOA and DIBOA are both accepted as substrates by BX8, while DIMBOA is the preferred substrate of BX9 [21]. Based on these biochemical characteristics of BX8 and BX9, we suggest that rhizosphere effects provide an ecological advantage in biotic stress resistance through regulating the ratio of BX8 and BX9. On one hand, the down-accumulation of BX9 stimulates the intercropped maize to secrete more DIMBOA to achieve a higher resistance for weeds, pests and diseases; on the other hand, the up-accumulation of BX8 provides maize a greater ability to avoid the toxic effects of DIBOA secreted by microorganisms in soils. Moreover, PGPR colonize the rhizosphere of many plant species and then elicit SAR that can provide plants higher stress resistance [24]. A previous study showed that intercropping with faba bean increased the diversity of the bacterial community in the rhizosphere of maize [18]. In this study, three protein species related to defense or pathogenesis (PRs) (peroxiredoxin-5, spot 14; pathogenesis-related protein 5, spot 15; pathogenesis-related protein 10, spot 20), two protein species related to ABA signal pathway (ABA-, stress-and fruit-ripening inducible-like protein, spot 59; abscisic stress ripening protein 2, spot 65), and two protein species responsive to oxidative stress (allene oxide cyclase1, spot 58; uncharacterized protein, spot 77) accumulated to higher levels in maize roots in the MB treatment compared to the SB treatment (Table 2). Since these differentially accumulated protein profiles are consistent with the performance of SAR induced by PGPR [24–27], we suggest that rhizosphere effects promote the growth of PGPR, then induce SAR that leads to PRs accumulation, ABA pathway activation, and ROS degradation in maize roots, and thus can improve the tolerance of maize in faba bean/maize intercropping.

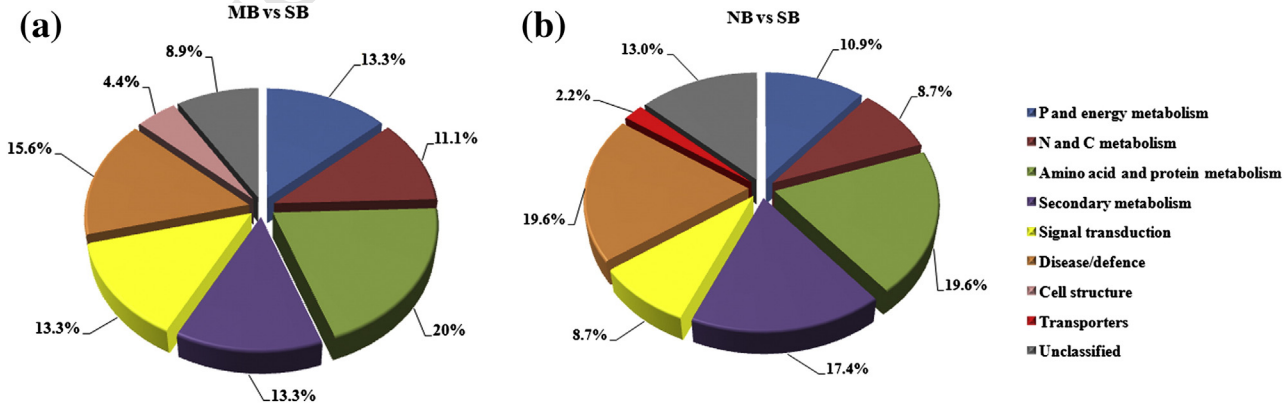

Fig. 5 – Classification of identified protein species according to putative molecular function. (a) Classification of the differentially accumulated protein species in the MB vs SB comparison group. (b) Classification of the differentially accumulated protein species in the NB vs SB comparison group.

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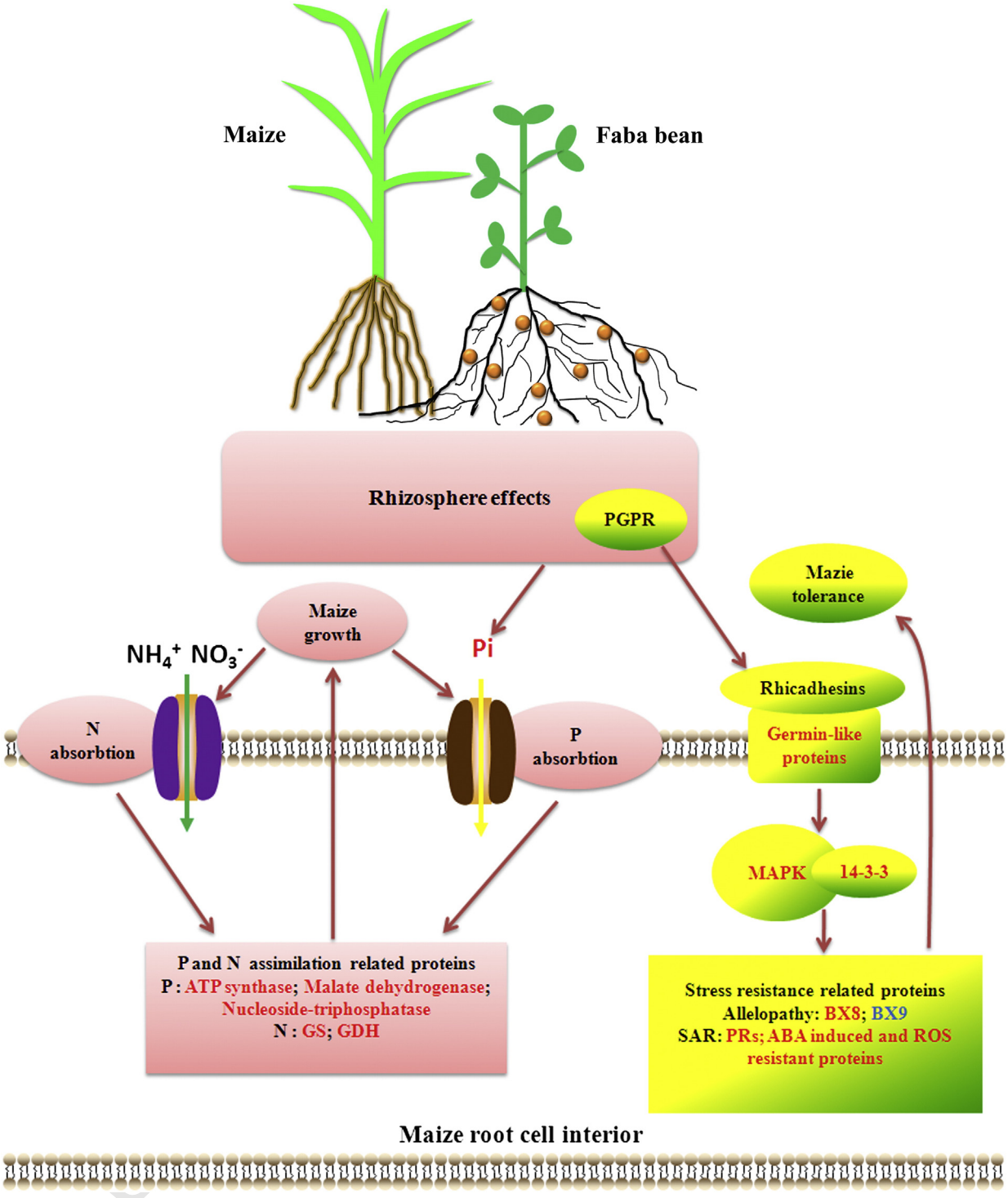

Fig. 6 – Working model of the molecular mechanisms underlying the interspecific facilitation in the faba bean/maize intercropping. The mobilized phosphate (Pi) and up-accumulated protein species are presented in red font. The down-accumulated protein species are presented in blue font.

Plants regulate their metabolic processes to adapt to minute changes in ecological environments through specific signal pathways. Our results showed that several protein species related to signal perception and transduction in plant defense responses were up-accumulated by rhizosphere effects (Table 2). Rhicadhesins are cell surface proteins from bacteria such as the genus *Agrobacterium* and *Rhizobium* and have been suggested to mediate the first step of the attachment of bacteria to root hairs [54]. Previous studies have shown that germin-like proteins can act as the receptors for rhicadhesins [55–57]. Mitogen-activated protein kinase (MAPK) cascades are major components downstream of receptors or sensors that transduce extracellular stimuli into intracellular defense responses in plants [58–61]. The 14-3-3 family proteins mediate signal transduction by

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binding to phosphoserine/phosphothreonine-containing proteins, and act as active cofactors in MAPK pathways [62,63]. In the present study, two germin-like protein species (rhicadhesin receptor precursor, spot 64; germin-like protein subfamily 1 member 17 precursor, spot 72), a mitogen-activated protein kinase (MAPK1, spot 81), and a 14-3-3-like protein (spot 27) were up-accumulated in maize roots in the MB treatment compared to the SB treatment. Based on these results, we suppose that the active bacteria in the rhizosphere of intercropped maize secrete more rhicadhesins, which are perceived by maize roots through the germin-like proteins, then induce the MAPK1 pathway to motivate intracellular defense reactions, and thus provide maize with higher tolerance to stress.

5. Conclusion

Our results suggest that the interspecific facilitation for maize in nutrients utilization and stress tolerance mainly result from the rhizosphere effects in faba bean/maize intercropping. A working model (Fig. 6) was proposed to predict the putative components in the molecular basis of interspecific facilitation for maize underlying the rhizosphere effects. In this model, rhizosphere effects mobilize Pi in soils and promote P and N assimilation in maize roots through enhancing the abundances of some protein species such as ATP synthase, malate dehydrogenase, nucleoside-triphosphatase, GS, and GDH, and then establish a virtuous cycle between nutrients provision and maize growth to provide maize higher yields. Rhizosphere effects can also provide maize with higher tolerance to stress through regulating the metabolism of allelochemicals and inducing SAR via the stimulation of a MAPK signal pathway by PGPR.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.06.027.

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