Enhancing phosphorus uptake efficiency through QTL-based selection for root system architecture in maize

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Abbreviations

ABL, advanced backcross line; CRL, crown root length; CRN, crown root number; LP, low phosphorus; MAS, marker-assisted selection; NP, normal phosphorus; PRL, primary root length; PUE, phosphorus use efficiency; PupE, phosphorus uptake efficiency; PutE, phosphorus utilization efficiency; QTL, quantitative trait locus; RDW, root dry weight; RIL, recombinant inbred line; RLD, root length density; RSA, root system architecture; SRL, seminal root length; SRN, seminal root number.

ABSTRACT

Root system architecture (RSA) plays an important role in phosphorus (P) acquisition, but enhancing P use efficiency (PUE) in maize *via* genetic manipulation of RSA has not yet been reported. Here, using a maize recombinant inbred line (RIL) population, we investigate the genetic relationships between PUE and RSA, and develop P-efficient lines by selection of quantitative trait loci (QTLs) that coincide for both traits. In low-P (LP) fields, P uptake efficiency (PupE) is more closely correlated with PUE ($r = 0.48\text{--}0.54$), and RSA in hydroponics is significantly related to PupE ($r = 0.25\text{--}0.30$) but not to P utilization efficiency (PutE). QTL analysis detected a chromosome region where two QTLs for PUE, three for PupE and three for RSA were assigned into two QTL clusters, *Cl-bin3.04a* and *Cl-bin3.04b*. These QTLs have favorable effects from alleles coming from the large-rooted and high-PupE parent. Marker-assisted selection (MAS) identified nine advanced backcross-derived lines carrying *Cl-bin3.04a* or *Cl-bin3.04b* that displayed mean increases of 22%–26% in PUE in LP fields. Furthermore, a line L224 pyramiding *Cl-bin3.04a* and *Cl-bin3.04b* shows enhanced PupE, relying mainly on changes in root morphology, rather than root physiology, under both hydroponic and field conditions. These results highlight the physiological and genetic contributions of RSA to maize PupE, and provide a successful study case of developing P-efficient crops through QTL-based selection.

KEYWORDS: Maize; Quantitative trait loci; Phosphorus; Root system architecture; Marker-assisted selection

1. Introduction

Phosphorus (P) is an essential macronutrient for crop growth and yield productivity (Marschner et al., 1995; Shen et al., 2011). Because of the poor mobility of P and its ability of binding organic components, the available P in many soils is often lower than that required by plants for optimal growth and development (Raghothama et al., 1999). P fertilizers applied in agriculture can ensure crop yields; however, they are produced from non-renewable rock-P sources (Cordell et al., 2009), and their excess application leads to water eutrophication (Dodds et al., 2009). Therefore, improving the efficiency of P fertilizers and decreasing residual P in environments are challenges for sustainable agricultural production. Maize (*Zea mays* L.) is one of the most widely cultivated crop plants, whose growth is sensitive to P deficiency (Usuda and kousuke, 1991). More than 30% of maize production in the world is affected by lack of P in the soil (Vance et al., 2003). Hence, the development of P-efficient maize varieties that can grow and yield better with low P supply should be a major breeding target to enable sustainable agricultural production (Vance et al., 2003; Li et al., 2010).

P use efficiency (PUE) refers to the ability of plants to produce biomass or yield under certain available P conditions, and is further divided into two components: P uptake efficiency (PupE) and P utilization efficiency (PutE) (Moll et al., 1982; Lynch, 1998; Wissuwa 2003; Wang et al., 2010a). PupE is the ability of plants to take up P from soils, and PutE is the ability to produce biomass or yield using the acquired P. Enhancing PUE in plants can be achieved by improving PupE or PutE or both. However, the relative importance of PupE and PutE to PUE varies with crop species and available P in soils (Shenoy and Kalagudi, 2005; Parentoni et al., 2010; Wang et al., 2010a; Veneklaas et al., 2012). Studies in wheat and soybean suggest that PupE could be more important than PutE under limited P supply (Manske et al., 2001; Zhao et al., 2004), whereas PutE could be the limiting factor for enhancing PUE under adequate P supply (Manske et al., 2001; Wang et al., 2010a). In maize, studies suggest that PupE makes a more critical contribution to improving whole-plant PUE than PutE under both P sufficient and deficient conditions: 1) high PUE in maize grown in low-P (LP) environments is mainly due to enhanced PupE (Corrales et al., 2007); 2) phenotypic correlations are closer between PUE and PupE than those between PUE and PutE (Mendes et al., 2014); 3) under normal-P (NP) conditions PUE of a set of maize genotypes is

also determined by PupE rather than PutE, because PUE variability is mainly explained by variations in PupE (Parentoni and Lopes de Souza, 2008). Given the essential role of PupE in maize PUE irrespective of P levels, maize breeders should pay great attention to PupE traits for developing high-PUE maize varieties.

Improvement of plant PupE could be achieved by physiological and morphological changes to roots. Root physiological responses to P deficiency involve increasing the secretion of acid phosphatase, ribonuclease and organic acids to release P from its conjugations, and increasing the expression of phosphate transporters (PHTs) to accelerate P uptake (Vance et al., 2003; Shen et al., 2006; Lamber et al., 2008; Rose et al., 2013). Since root secretion always increases coordinately with the upregulation of P uptake processes (Calderón-Vázquez et al., 2011), PupE improvement based on physiological alterations has mainly focused on genetic modification of *PHT* genes. The function of *PHT* genes in P acquisition in roots has been characterized in *Arabidopsis* and crop species (rice, wheat, and soybean) (Shin et al., 2004; Ai et al., 2009; Miao et al., 2009; Qin et al., 2012). In maize, four *PHT* homologs (*ZmPHT1;1*, *ZmPHT1;2*, *ZmPHT1;3*, and *ZmPHT1;4*) were identified with their high transcript abundance in roots that are upregulated by P starvation (Nagy et al., 2006; Calderon-Vazquez et al., 2008). Although Mitsukawa et al (1997) showed an increased rate of P uptake in transgenic *Arabidopsis* overexpressing *AtPHT1;1* (Mitsukawa et al., 1997), whether enhanced expression of these *PHT* genes can lead to improved overall PUE at the whole plant level has not been reported.

Root morphological changes refer to alterations of the root system architecture (RSA) in response to P status (White et al., 2013). As P has low mobility and its concentration tends to be highest in the upper soil horizons (Ma et al., 2009), P-efficient crop plants always have increased root topsoil foraging (Lynch and Brown, 2001), root hairs (Wang et al., 2004; Miguel et al., 2015), lateral roots (Zhu and Lynch, 2004; Postma et al., 2014), and root/shoot ratio (Hermans et al., 2006). Many attempts have been made to improve PupE through RSA modification in crops (Wang et al., 2010b; de Sousa et al., 2012; Gamuyao et al. 2012; Hufnagel et al., 2014; Pariasca-Tanaka et al., 2014; Azevedo et al., 2015). By selecting shallow root traits in a traditional breeding program, several P-efficient soybean varieties have been developed and commercially released in South China (Wang et al., 2010b). For

molecular breeding, some QTLs or genes have been identified involved in root morphological changes in response to P starvation (de Sousa et al., 2012; Gamuyao et al. 2012; Hufnagel et al., 2014; Azevedo et al., 2015). Among them, *Pup1/PSTOL1* (phosphorus-starvation tolerance 1), a serine/threonine kinase gene, is the most important one that enhances grain yield (GY) under LP conditions in both rice and sorghum (Gamuyao et al. 2012; Hufnagel et al., 2014). Marker-assisted selection (MAS) of *OsPSTOL1* allele could improve PupE of rice mega-varieties in Sub-Saharan Africa where P deficiency is a major problem (Pariasca-Tanaka et al., 2014). In maize, six *PSTOL1* homologs have been predicted, with four members co-localized with P-related QTLs (Azevedo et al., 2015). Although modifying root traits *via* genetic approaches enables the enhancement of PupE in several crops (e.g., soybean and rice), these attempts have not yet been made in maize.

In this study, using a maize recombinant inbred line (RIL) population derived from two lines Ye478 and Wu312 (Li et al., 2010; Liu et al., 2011), a QTL analysis was performed to identify the genetic basis of PUE and evaluate its genetic relationship to RSA. An advanced backcross line (L224) containing major QTLs for both PupE and RSA at chromosomal region *Bin3.04* was selected from the BC4F3 population. Evaluation of the physiological and morphological contribution of RSA to PupE in L224 is further reported.

2. Results

2.1. Evaluation of PUE-related traits in the field

PUE-related traits, including PUE, PupE and PutE (Table 1), were evaluated for the 218 RILs and two parental lines Ye478 and Wu312 in field trials across two years (2007 and 2008). Under both NP and LP conditions, Ye478 had a higher PUE increased by 22.9%–72.0% as compared with Wu312 (Table 2). Of the two main components of PUE, the PupE was 17.8%–61.1% higher in Ye478 than in Wu312, but the PutE was not significantly different between these two lines. For the RILs, the PUE and PupE under LP conditions separately increased 4.8–5.6 and 4.7–6.1 times as compared with those under NP conditions, whereas the PutE only increased slightly under LP conditions (Table 2).

Within the RIL population, the PUE-related traits were extensively segregated and normally distributed across all environments, and considerable phenotypic variation existed as

indicated by the coefficient of variation (CV) ranging from 23.3% to 66.6% (Table 2). Between the two field trials in 2007 and 2008, the mean values of all traits in RILs were similar and their correlations were moderate ($r = 0.25\text{--}0.61$). Significant effects were observed for genotypes, years, and their corresponding interaction (Table S1), indicating strong environmental influences on PUE-related traits. The heritability (h^2) for PUE-related traits was similar between the two P levels, with lower h^2 for PUE and PupE ranging from 39% to 51%, and higher h^2 for PutE from 67% to 73% (Table S1).

The phenotypic correlations among PUE-related traits were investigated within the RIL population. Under NP and LP conditions, PUE showed significant positive correlations with both PupE and PutE, while no correlation or even significant negative correlations were observed between PupE and PutE (Tables 3 and S2). In terms of correlations to PUE, those of PupE ($r = 0.48\text{--}0.54$) were higher than those of PutE ($r = 0.32\text{--}0.38$) under LP conditions, while they were similar under NP conditions (Table 3). This result suggests a relevant contribution of the P uptake process to the overall PUE when P supply is limited.

2.2. Phenotypic relationship between PUE- and RSA-related traits

To investigate the contribution of roots to PUE in maize, the phenotypic correlation between RSA- and PUE-related traits was determined. A hydroponic experiment was conducted with NP supply to evaluate the RSA-related traits of the RILs and two parental lines, including root biomass (root dry weight, RDW), root number (seminal root number, SRN; crown root number, CRN) and root length (primary root length, PRL; seminal root length, SRL; crown root length, CRL; lateral root length, LRL) (Table S3). Compared with Wu312, Ye478 had a larger root system with higher root biomass, number and length, except for lower PRL. Within the RIL population, considerable phenotypic variation existed for RSA-related traits (CV values ranged from 17.2% to 42.1%).

As indicated by the Pearson correlation coefficients (Table S4), under P deficiency in 2007 (07LP trial), PUE showed significant positive correlations to several RSA-related traits including SRL ($r = 0.21$), CRL ($r = 0.23$), and RDW ($r = 0.21$), and PupE showed significant correlations with SRN ($r = 0.30$), SRL ($r = 0.30$) and RDW ($r = 0.25$). In two other trials (07NP and 08NP), PUE and PupE showed significant positive relationships to SRL ($r = 0.18\text{--}$

0.30). By contrast, PutE showed no correlation or negative correlations to RSA-related traits in all trials. Principle component analysis (PCA) showed that RSA-related traits were all closely related and grouped together (Fig. 1). Only PupE from all trials was assigned into the RSA-related group. Thus, Pearson correlation coefficient and PCA suggest close phenotypic relationships of RSA with PupE, but not with PutE, in maize.

2.3. Detection of QTLs responsible for PUE- and RSA-related traits

In the RIL population, a total of 29 putative QTLs were identified for all investigated traits across all independent experiments, including 16 for PUE-related traits detected in the field and 13 for RSA-related traits detected in hydroponics (Tables S5 and S6). Phenotypic variation explained by each QTL was moderate for both PUE- and RSA-related traits, ranging from 6.2% to 21.2%. Among the QTLs identified for PUE-related traits, 10 (63%) carried the favorable alleles that originated from the P-efficient parental line Ye478.

The 29 identified QTLs were distributed in all ten maize chromosomes except chromosome 7 (Tables S5 and S6). Eight QTLs (28%) were located in chromosome region *Bin3.04* at positions of 79 cM and 120 cM and assigned to two QTL clusters, named *Cl-bin3.04a* and *Cl-bin3.04b*, respectively (Fig. 2). The alleles contributing to these QTLs were all from P-efficient Ye478 and explained moderate amounts of variance ranging from 6.2% to 14.1% (Tables S5 and S6). *Cl-bin3.04a* harbored one QTL for RDW, two for PupE and one for PUE; *Cl-bin3.04b* harbored four QTLs for SRL, SRN, PUE and PupE, respectively. Moreover, *Cl-bin3.04b* also harbored a QTL for PupE in response to LP (Table S7). These results revealed two highly relevant QTL clusters in which three QTLs for RSA-related traits and five QTLs for PUE-related traits, particularly for PupE, were co-localized.

2.4. Performance of advanced backcross lines (ABLs) containing *Cl-bin3.04a* and *Cl-bin3.04b*

To evaluate the effect of *Cl-bin3.04a* and *Cl-bin3.04b* on genetic improvement of PUE, we investigated the yield performance of an advanced backcross population (BC₄F₃) under NP and LP conditions across two years (Fig. 3). Based on the flanked simple sequence repeat (SSR) markers phi036 and umc1773 (Fig. 2), five and four ABLs containing *Cl-bin3.04a* and

Cl-bin3.04b were identified, respectively (Fig. 3). These ABLs showed 83.9%–97.2% genetic similarity to the recurrent parent Wu312, with an average of 88.1%. Compared with all ABLs within the population, *Cl-bin3.04b*-containing ABLs showed marked PUE increases (25.89%–29.78%) under LP and moderate PUE increases (6.11%–18.50%) under NP; *Cl-Bin3.04a*-containing ABLs also showed marked PUE increases (13.58%–29.78%) under LP, but no significant PUE change under NP (Fig. 3). Additionally, in terms of PUE, ABLs with *Cl-bin3.04a* or *Cl-bin3.04b* or both performed higher responses to LP compared with those without them (Fig. S1). For RSA-related traits evaluated in hydroponics, *Cl-bin3.04a*- and *Cl-bin3.04b*-containing ABLs gained average increases of 9.6% and 14.8% for CRN, 3.3% and 8.6% for CRL, 7.5% and 22.6% for LRL, and 4.5% and 20.2% for RWD, respectively (Fig. S2). These results indicate that the introgression of *Cl-bin3.04a* and *Cl-bin3.04b* from P-efficient Ye478 could improve the RSA and PUE of the recurrent line Wu312 simultaneously.

To pyramid the effects of these two QTL clusters, an ABL (L224) carrying both *Cl-bin3.04a* and *Cl-bin3.04b* was selected from the BC₄F₃ population. This line showed about 87.4% genetic similarity to the recurrent parent Wu312 (Fig. S3). PUE of L224 exhibited significant increases of 26.9%–54.3% and 53.6%–66.7% under NP and LP conditions, respectively, compared with that of Wu312 (Fig. 3). The improvement of PUE in L224 was likely double that of ABLs carrying each single QTL cluster. From a breeding point of view, the performance of L224 testcrosses with line 178 was also tested over two years under NP and LP conditions (Table 4). In contrast to the control hybrid Wu312 × 178, most traits for GY, Pup, PUE and PupE were significantly enhanced in hybrid L224 × 178 irrespective of P levels. Hybrid L224 × 178 gained up to 8.4%–31.2% increases in PupE, but no significant change in PutE, further revealing a specific improvement in P uptake process. These results implicate the application of these two QTL clusters at chromosomal region Bin3.04 as targets for MAS to breed P-efficient maize.

2.5. Root morphological changes confer improved PUE in L224 carrying *Cl-bin3.04a* and *Cl-bin3.04b*

To investigate the contribution of roots to PUE in L224, root physiological and morphological

characters were compared between L224 and Wu312 grown in hydroponics at two contrasting P levels. L224 had significantly higher P acquisition per plant than Wu312 under both NP and LP (Table 5). P acquisition per plant was usually determined by two main components: total root surface area (root morphological character) and P uptake activity per unit of root surface (root physiological character). Compared with Wu312, L224 had significantly higher RDW, PRL, and LRL irrespective of P levels, and higher SRL under NP condition, suggesting larger size and longer length of roots (Table 5). However, P acquisition per root length (representing P uptake capacity) in L224 was similar to or even significantly lower than that in Wu312. This result suggests that the higher PUE in L224 relies on improvements in root morphology rather than root physiology.

The expression of candidate genes involved in P acquisition in maize roots was further compared between L224 and Wu312 (Table 5). The *PSTOL* gene has been suggested as the key determinant for positively regulating P responsive root growth in rice, sorghum and maize (Gamuyao et al., 2012; Hufnagel et al., 2014; Azevedo et al. 2015). Among six maize *PSTOL* homologs, only *ZmPSTOL3.04*, located in chromosomal region *Bin3.04*, was found to present higher expression levels in L224 than in Wu312 under LP, but not under NP conditions. Other *ZmPSTOL* genes had similar or even lower expression levels in L224 than in Wu312. Interestingly, *ZmPSTOL3.04* coincided with the chromosomal region of *Cl-bin3.04a* (Fig. 2, Azevedo et al., 2015). Three root-expressed *PHT* genes (*ZmPHT1;1*, *ZmPHT1;2* and *ZmPHT1;4*), representing P uptake capacity, were also upregulated under LP, but no significant difference was observed between the two genotypes (Table 5). These results further support the contribution of root morphology to higher P acquisition in L224, which might be explained by the increased expression of *ZmPSTOL3.04* from the favorable allele of *Cl-bin3.04a* in roots.

To further verify the RSA-based improvement of PUE in L224 under field conditions, PUE-related traits (GY, Pup, PUE, PupE and PutE), and root growth and distribution (root length density, RLD) in 0–60 cm soil at the silking and maturation stages were evaluated in 2009 and 2010 (Table 6). As expected, L224 showed significantly higher GY and PUE than Wu312 under both NP and LP conditions over two years. Moreover, Pup and PupE, but not PutE, were significantly improved in L224 in three (09NP, 09LP and 10LP) of four

investigated trials. For RLD, it was significantly less at the maturation stage than at the silking stage mainly due to root senescence. Under LP conditions, total RLD through 0–60 cm soil was significantly higher in L224 than in Wu312 at the silking stage in 2009 and at the maturity stage in both 2009 and 2010. These results indicate that, similar to the performance in hydroponics, root growth of L224 improves in P-deficient soils which enables higher P acquisition in maize.

3. Discussion

MAS is a valuable tool for plant breeders, but it still lags behind its potential because valuable molecular markers are often unavailable. Increasing crop P efficiency is essential to maintain productivity with low costs in natural resources and environmental impacts. P-efficient rice cultivars have been successively generated by MAS and used to solve the problem of insufficient P fertilizer in Africa (Chin et al., 2010). However, this type of practice in maize breeding is limited because of a lack of well-known genomic regions for targeted genetic manipulation. In this study, we identified an important maize chromosome region *Bin3.04* controlling both RSA and PupE traits by QTL analysis of a RIL population, and subsequently developed a P-efficient inbred line by MAS of this target region from a BC₄F₃ population. Experimental evidence in both hydroponics and field trials further supports that, rather than root physiology (P acquisition ability per root length), root morphology significantly contributes to higher PUE in maize through enhanced soil exploration. Thus, this work has not only uncovered a significant genetic association between RSA and PupE, but also provided a successful application case study to genetically improve PUE in maize.

3.1 Relationship of PUE, PupE and PutE in maize

The trait of PUE consists of PupE and PutE, and can be enhanced by improving either or both of them (Manske et al., 2001; Shenoy and Kalagudi 2005; Parentoni and Lopes de Souza, 2008). It appears that the contributions of PupE and PutE to overall PUE vary among different crop species and availabilities of soil P. For soybean and wheat, under P deficiency PupE could be more relevant than PutE, while the opposite is found under P sufficiency (Manske et al., 2001; Zhao et al., 2004; Wang et al., 2010a). For maize, we found that the two parental

lines, Ye478 and Wu312, had contrasting phenotypes for PUE and PupE, but not for PutE, regardless of P levels (Table 2). This was consistent with previous studies on a set of tropical maize genotypes (Corrales et al., 2007, Parentoni and Lopes de Souza, 2008). More importantly, within our RIL population, PupE showed a higher correlation with PUE ($r = 0.48\text{--}0.54$) than PutE ($r = 0.32\text{--}0.38$) under P deficiency (Table 3). Several QTLs for PUE were also determined to coincide in the genome with those for PupE, but not PutE, under both NP and LP conditions (Fig. 2; Tables S5 and S6). In maize, consequently, PupE plays a major role in overall PUE under P deficiency, and can be considered as the central target trait for genetically developing P-efficient germplasms.

3.2. Relationship of RSA- and PupE- traits in maize

Plant roots have evolved a number of physiological, biochemical and developmental adaptations to enhance their PupE, including: 1) releasing soil P from organic and inorganic sources by secretion of some chemicals (e.g., acid phosphatases); 2) employing high-affinity phosphate transporters to effectively acquire P; 3) altering the architecture of the root system; 4) establishing symbiotic relationships (e.g., symbiosis with arbuscular mycorrhizal fungi) to increase soil exploration (Marschner, 1995; Cakmak, 2002). Among these adaptive strategies to low P availability, changes in root morphology and/or architecture are highly relevant for enhancing PupE as described in *Arabidopsis*, rice and soybean (Mashner 1995; Wissuwa 2003; Liu et al., 2008; Wang et al. 2010b; Gamuyao et al. 2012). In a recent study, comparison of LP adaptive strategies between maize and faba bean revealed that maize can respond to P deficiency by investing more biomass in its root system, while faba bean can perform rhizosphere processes (acidification, secretion of organic acids and acid phosphatases) to mobilize P (Liu et al., 2016). P-efficient maize genotypes also often display larger root systems with increased root biomass or density (Liu et al., 2004; Corrales et al., 2007; Azevedo et al., 2015). In our study, three lines of evidences further reveal the significant genetic relationship between root morphology and PupE in maize. First, phenotypic correlation analysis and PCA indicate closer positive relationships of RSA with PupE than with PutE (Fig. 1; Table S4). Negative correlations were even found between RSA and PutE, probably due to the trade-off between root growth and above-ground growth that often

influences PupE (Table S4). Second, the coincidence of QTLs for RSA and PupE was determined at the chromosomal region *Bin3.04* (Fig. 2), and all these QTLs had favorable effects from alleles coming from the parental line Ye478 that had higher PupE and larger roots (Tables S5 and S6). Noteworthy, this genomic region contains a *ZmPSTOL3.04* gene, a homolog of *OsPSTOL* that controls crown root growth and confers LP tolerance in rice (Gamuyao *et al.*, 2012). Third, in ABLs, the introgression of the genomic region *Bin3.04* into P-deficient Wu312 simultaneously enhances PupE (Tables 5 and 6) and RSA (root biomass and length) (Fig. S2; Tables 5 and 6). By contrast, root physiological processes are not altered, as represented by P acquisition per root length and *PHT* gene expression (Table 5). From a physiological perspective, root morphology is expected to play a pivotal role in effective P acquisition in maize, and our study provides solid genetic evidence establishing the relationship between them.

3.3. Breeding for P-efficient maize by MAS

Large natural variations in PUE and PupE commonly exist among maize genotypes (Gaume *et al.*, 2001; Zhu and Lynch 2004; Shenoy and Kalagudi 2005). Combined with the moderate heritability of these variations (Table S1; Chen *et al.*, 2008), it makes PUE and PupE suitable targets for maize breeding programs. To breed P-efficient genotypes by MAS, QTLs for PUE-related traits need to be determined. However, few common QTLs have so far been detected across different genetic populations, presenting an obstacle to MAS (Chen *et al.*, 2008, 2009, 2011; Li *et al.*, 2010; Mendes *et al.*, 2014; Azevedo *et al.*, 2015). Herein, we have identified an important chromosome region containing two QTL clusters (*Cl-bin3.04a* and *Cl-bin3.04b*) for P-related traits (PUE and PupE) irrespective of P levels (Fig. 2; Table S5). This genomic region was also found to harbor QTLs controlling P acquisition in two other independent maize populations (Chen *et al.* 2008; Mendes *et al.* 2014), and further determined as a consensus QTL for LP tolerance traits (*cQTL3-1*) by a meta-analysis (Zhang *et al.*, 2014). Meanwhile, several QTLs for RSA-related traits were co-localized in this genomic region, as revealed by this study (Fig. 2; Table S6) and a previous study by Azevedo *et al.* (2015). Noteworthy, a putative LP tolerant gene *ZmPSTOL3.04* is located in this region, and its expression levels vary between two maize lines with contrasting PupE (Table 5). These

findings imply that *Bin3.04* is the most promising genetic determinant for the RSA-based improvement of PupE across different P conditions and genetic backgrounds. We performed MAS to introgress two QTL clusters at *Bin3.04* into the P-inefficient recurrent line Wu312, and obtained a P-efficient line L224 that showed improved RSA and PUE, and subsequently up to 50% increases in yield in field trials across two years (Tables 5 and 6). Genetic contributions to PUE were not only detected in lines *per se* but also at the testcross level (Table 4), suggesting a great breeding value of the QTLs, since hybrid varieties of maize are often used by farmers. Because of the presence of multiple donor segments in L224, the contributions of other loci besides *Cl-bin3.04* cannot be excluded. Remarkably, a successful case was reported in rice of the development of a large-rooted near-isogenic line with enhanced PUE by transferring a single *publ* QTL (containing *OsPSTOL*) into a P-inefficient background *via* MAS (Wissuwa, 2005). Manipulation of RSA *via* selection of favorable *PSTOL* alleles opens an exciting opportunity to improve PUE in cereal crop species.

Taken together, our results revealed a close genetic relationship and a common QTL region for both RSA- and PupE- related traits, and on this basis we established a MAS approach to breed P-efficient maize. The results presented here provided not only a better understanding of the physiological adaptation and genetic components of RSA for efficient P acquisition in maize, but also a successful molecular breeding example improving root traits that are often ignored by maize breeders. In addition, the availability of high-PupE germplasm L224 for QTLs at *Bin3.04* represents a prior condition for undertaking its fine mapping and cloning, enabling further verification of the function of the *ZmPLOT3.04* allele in RSA establishment and PUE improvement in maize.

4. Materials and methods

4.1. Plant materials

An F₈ RIL population consisting of 218 lines was generated from a cross of inbred lines Ye478 (female parent) and Wu312 (male parent) as described by Li et al. (2011). Previous studies have shown that Ye478 has a large root system, whereas Wu312 has a small root system (Liu et al., 2009). A BC₄F₃ population containing 187 lines derived from Ye478 (the donor parent) and Wu312 (the recurrent parent) was further generated in a backcrossing

program as described by the previous studies (Liu et al., 2011; Cai et al., 2012).

4.2. Phenotype evaluation in the field

Maize plants were grown in a field located in Changping District, Beijing, China (40°13' N latitude, 116°12' E longitude). This field has undergone long-term P fertilization control since 1985. The chemical properties of the soil (Table S8) were evaluated before sowing for each treatment by analyzing six soil samples excavated from the 0–30 cm soil layer. Organic matter and total nitrogen (N) were determined by the $K_2Cr_2O_7$ method (Walkley, 1947) and Kjeldahl procedure (Bremner, 1996), respectively. Available K (exchangeable-K) and P (Olsen-P) were extracted using NH_4OAc and $NaHCO_3$, and determined using a flame photometer and spectrophotometer, respectively (Olsen *et al.*, 1954). The content of available P in the LP field ranged from 7.41 to 10.92 mg/kg soil, approximately 4%–13% of that in the NP field where P fertilizer was supplied (Table S8). Other chemical content in soils remained similar between the two P levels.

The experiment was arranged randomly in a split-plot design with three replicates, in which one-row plot was used for each replicate. The space between rows was 0.5 m. The row length was 2 m for 11 plants or 4 m for 13 plants, with a plant density of 100,000 or 60,000 per hectare (Table S8). Irrigation, turf machinery, and weeding were applied before sowing. N was supplied with 40% at sowing and 60% at the V9 (9-visible-leaf) stage. Other fertilizers were all supplied before sowing. Rainfall during the growing seasons ranged from 400 to 600 mm, which was sufficient to avoid water deficit stress.

Grain and straw were harvested at maturity, and weighted after oven-drying at 65°C. After calculation according to the harvest area, the grain yield and straw dry weight were obtained. The grain and straw samples were then separately ground into power, dissolved in $H_2SO_4-H_2O_2$, and analyzed for P concentrations according to the molybdate-blue colorimetric method (Murphy and Riley, 1962). The P contents of grain and straw were calculated by multiplying weight by P concentration, respectively. Total P content of plants was then obtained as the sum of that in the grain and straw. For each plot in field trials, GY, Pup, PUE, PupE and PutE were measured (see Table 1 for details).

At the silking or maturity stage, root samples were excavated from the soil. A soil volume

of 20 cm × 50 cm × 60 cm or 33 cm × 50 cm × 60 cm surrounding a plant were excavated under 100,000 or 60,000 plant density per hectare, respectively. All visible roots were picked out by hand. These roots were washed free of soil and then floated in a transparent plastic tray with water for scanning in a scanner (EPSON V700, China). Total root length was calculated based on analysis of the scanned images using WinRHIZO software (Pro 2004b, Canada). After calculation according to soil volume, the root length density (RLD) was determined (Table 1).

4.3. Phenotype evaluation in hydroponics

Maize plants were grown hydroponically in a climate chamber. In brief, after being sterilized in 10% H₂O₂, washed with distilled water and soaked in saturated CaSO₄, maize seeds were germinated on moist filter paper as a cigar roll. At the 2-visible-leaf stage, seedlings were transferred into nutrient solution and aerated with an electric pump. For NP treatment, the nutrient solution consisted of 2.0 mmol/L Ca(NO₃)₂, 0.75 mmol/L K₂SO₄, 0.65 mmol/L MgSO₄, 0.1 mmol/L KCl, 0.25 mmol/L KH₂PO₄, 1×10⁻³ mmol/L H₃BO₃, 1×10⁻³ mmol/L MnSO₄, 1×10⁻⁴ mmol/L CuSO₄, 1×10⁻³ mmol/L ZnSO₄, 5×10⁻⁶ mmol/L (NH₄)₆Mo₇O₂₄, 0.1 mmol/L Fe-EDTA. For LP treatment, the concentration of P was adjusted to 0.01 mmol/L by reducing KH₂PO₄, where K⁺ was complemented by adding KCl. The pH of the nutrient solution was adjusted to 6.0 by KOH. Nutrition was renewed every 2 days. Growth conditions were controlled at 28/22°C with a 14/10 h light/dark cycle. A photosynthetic photon-flux density of 250–300 μmol m⁻² s⁻¹ (at canopy height) was provided during the light period.

A completely randomized design for the hydroponic experiment was used with three replicates. Plants were harvested 20 days after germination (12 days after being transferred to nutrient solution) when they had 5–6 visible leaves. For each replicate, six seedlings for each line were harvested and cut at the mesocotyl to separate roots and shoots. Shoots were dried at 65°C to constant weight for weighing and P concentration analysis. Roots were stored at –20°C for measurement of root traits, and then dried to constant weight for weighing.

Maize seedling roots in the hydroponic system consisted of primary root, seminal root and crown root, and each type of roots contained its later root (Hochholdinger and Zimmermann 2008). These three root types were separated for measurement of root traits, including SRN,

CRN, PRL, SRL, CRL, TRL and LRL (see Table 1 for details).

4.4. Gene expression analysis

Gene expression was examined using quantitative RT-PCR (qRT-PCR) as described by Gu et al. (2013). Maize cDNA was reverse transcribed from total RNA extracted from hydroponic root. The PCR reactions were performed using SYBR Green dye in a 7500 Real-Time PCR System (Applied Biosystems, USA). Data were analyzed using 7500 SDS software 1.3 (Applied Biosystems). Expression of maize *Alpha tubulin4* gene (*ZmTUB4*, GenBank No. AJ420856.1) was used as an internal control. The primers for *ZmPSTOL* genes were described by Azevedo et al. (2015). The primers for *ZmPHTs* and *ZmTUB4* were listed in Table S9. Three biological replicates were performed for each sample.

4.5. Data analysis and QTL detection

Heritabilities for PUE, PupE and PutE under NP and LP conditions were calculated from an ANOVA fitting the effects of genotype (*G*), environment (*E*), and *G* × *E* interactions, as

$$h^2\% = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_{G \times E}^2 / n + \sigma_E^2 / nr} \times 100\%,$$

where h^2 is the broad sense heritability, σ_G^2 is genotypic variance, $\sigma_{G \times E}^2$ is *G* × *E* variance, σ_E^2 is error variance, *n* is the number of environments and *r* is the number of replicates (Nyquist 1991).

The genotypes of RILs and BC₄F₃ were generated in previous work (Liu et al., 2011). After updating the physical positions of SSR markers in the maize genome (www.maizegdb.org), we improved the genetic map for these two populations. Positions of markers umc1012 and phi036 were rearranged from 15th and 16th to 7th and 8th in the order of the total 24 markers on chromosome 3, respectively. The resulting map covered 2064.9 cM with an average interval of 11.2 cM, which was slightly shorter than the previous map of 2084.1 cM with an average interval of 11.3 cM (Fig. S3; Liu et al., 2011).

For each trait within a P treatment (trial), least square mean (LSMEAN) of all replicates was used for correlation analysis and QTL mapping. QTL detection was performed using the software Windows QTL Cartographer version 2.5 (Wang et al., 2012). Composite-interval

mapping (CIM) was selected to search for QTLs from each environment. Threshold limit of detection (LOD) values were determined with 1000-times permutations at the $P < 0.05$ level. The resulting LOD values were 2.5–2.9 for all trials. The LOD thresholds were set at 2.5 to retain more QTL information. Locus position was assigned as the point of maximal LOD score. Loci detected within the average interval (11.3 cM) in each trial were considered to be the same locus.

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Table 1. Summary of the traits investigated in this study and the measurements

Trait	Abbreviation	Unit	Trait measurement
PUE-related traits in field			
Grain yield	GY	kg/ha	—
Total P uptake	Pup	kg/ha	Total amount of P content in plants per hectare
P use efficiency	PUE	kg/kg	Grain yield/ total amount of P available in soil
P uptake efficiency	PupE	kg/kg	Total P uptake/ total amount of P available in soil
P utilization efficiency	PutE	kg/kg	Grain yield/ Total P uptake
PUE-related traits in hydroponics			
P acquisition per plant	—	mg plant ⁻¹	Total amount of shoot P content per plant
P acquisition per root length	—	mg/m	Total amount of shoot P content/total root length
RSA-related traits in hydroponics			
Seminal root number	SRN	number	Average number of the seminal root
Crown root number	CRN	number	Average number of the crown root
Primary root length	PRL	cm	Measured with a ruler
Seminal root length	SRL	cm	Measured with a ruler
Crown root length	CRL	cm	Measured with a ruler
Total root length	TRL	cm	Root was scanned by scanner, and TRL was determined by WinRHIZO
Lateral root length	LRL	cm	LRL=TRL – PRL – SRL – CRL
Root dry weight	RDW	g plant ⁻¹	Dried and weighted using a balance (1/1000 g)
RSA-related traits in field			
Root length density	RLD	cm/cm ³	Total root length/soil volume

646 **Table 2.** PUE-related traits of the parents and RIL population grown in field under NP and LP conditions in 2007 and 2008

Trait	Treatment	2007					2008				
		Parent		RIL population			Parent		RIL population		
		Ye478	Wu312	Mean	Range	CV (%)	Ye478	Wu312	Mean	Range	CV (%)
PUE (kg/kg)	NP	70.6	51.8*	64.3	19.3–184.9	49	76.3	62.1	56.3	6.6–199.6	56.7
	LP	389.6 [#]	249.5 ^{#*}	311.8	49.8–803.6	53.6	396.8 [#]	230.7 ^{#*}	316.0	25.4–1248.6	66.6
PupE (kg/kg)	NP	0.29	0.18*	0.25	0.11–0.46	31.9	0.30	0.21*	0.23	0.08–0.48	32.8
	LP	1.19 [#]	1.01 [#]	1.18	0.62–2.89	39.2	1.20 [#]	0.83 ^{#*}	1.40	0.53–3.70	42.1
PutE (kg/kg)	NP	265.4	262.8	244.3	94.5–411.0	24.8	258.8	302.3	219.6	58.1–412.0	28.9
	LP	346.4	318.3	318.3	119.7–547.3	23.3	358.7	341.8	267.9	45.1–514.0	35.7

647 Trait performances were calculated using the least-squares distance by SAS software. Significant difference between treatments (NP and LP) is indicated by hash ($P < 0.05$);
648 significant difference between two genotypes (Ye478 and Wu312) in each trial is indicated by asterisk ($P < 0.05$).

649 **Table 3.** Pearson's correlation coefficients between PUE and PupE or PutE under NP and LP
650 conditions in 2007 and 2008

Trait	PUE			
Treatment	NP		LP	
	2007	2008	2007	2008
PupE	0.57 ***	0.48 ***	0.48 ***	0.54 ***
PutE	0.53 ***	0.49 ***	0.32 ***	0.38 ***

651 *** represents significant correlation at $P < 0.001$

652 **Table 4.** Grain yield and PUE-related traits in testcrosses of L224 and Wu312 under NP and LP conditions in 2009 and
653 2010

Trait ^a	Treatment	2009			2010		
		L224 × 178	Wu312 × 178	<i>t</i> -test ^b	L224 × 178	Wu312 × 178	<i>t</i> -test
GY (kg/ha)	NP	7699	7215	*	5822	4831	*
	LP	6260	5747	*	2212	1407	*
Pup (kg/ha)	NP	27.21	24.54	*	31.05	23.88	*
	LP	16.40	15.11	ns	15.50	11.86	*
PUE (kg/kg)	NP	45.06	42.22	*	41.08	34.09	*
	LP	642.05	589.44	*	257.80	164.03	*
PupE (kg/kg)	NP	0.16	0.14	*	0.22	0.17	*
	LP	1.68	1.55	ns	1.81	1.38	*
PutE (kg/kg)	NP	283.05	294.50	ns	187.48	202.32	ns
	LP	381.71	380.58	ns	142.71	118.59	ns

654 ^aFor trait information, refer to Table 1. ^bSignificant difference between two genotypes (L224 × 178 and Wu312 × 178) in each trial is indicated
655 by asterisk ($P < 0.05$). ns, no significance.

Table 5. Phenotype and corresponding gene expression of L224 and Wu312 in hydroponics under NP and LP conditions

Trait	Treatment	Genotype		<i>t-test</i> ^a
		L224	Wu312	
PUE-related traits in hydroponics				
P acquisition per plant (mg plant ⁻¹)	NP	4.29	3.80	*
	LP	1.24	1.08	*
P acquisition per root length (mg/m)	NP	0.15	0.26	*
	LP	0.04	0.05	ns
Root morphology				
RDW (g plant ⁻¹)	NP	0.11	0.08	*
	LP	0.13	0.10	*
PRL (cm)	NP	405	293	*
	LP	802	350	*
SRL (cm)	NP	690	305	*
	LP	479	447	ns
LRL (cm)	NP	1644	983	*
	LP	2603	1504	*
Expression of root morphology controlling gene				
<i>ZmPSTOL3.04</i> (10 ⁶ / <i>ZmTUB4</i>) ^b	NP	1.52 ± 0.52	1.02 ± 0.02	ns
	LP	0.42 ± 0.42	0.12 ± 0.12	*
<i>ZmPSTOL3.06</i> (10 ⁴ / <i>ZmTUB4</i>)	NP	2.90 ± 0.90	2.04 ± 0.04	ns
	LP	1.47 ± 0.47	3.04 ± 0.04	ns
<i>ZmPSTOL4.05</i> (10 ⁴ / <i>ZmTUB4</i>)	NP	1.44 ± 0.44	1.67 ± 0.67	ns
	LP	1.34 ± 0.34	2.45 ± 0.45	ns
<i>ZmPSTOL8.02</i> (10 ⁶ / <i>ZmTUB4</i>)	NP	0.84 ± 0.84	0.90 ± 0.90	ns
	LP	0.63 ± 0.63	2.47 ± 0.47	*
<i>ZmPSTOL8.05.1</i> (10 ⁵ / <i>ZmTUB4</i>)	NP	1.57 ± 0.57	11.78 ± 1.78	*
	LP	0.85 ± 0.85	8.44 ± 0.44	*
<i>ZmPSTOL8.05.2</i> (10 ⁵ / <i>ZmTUB4</i>)	NP	3.22 ± 0.22	6.05 ± 0.05	*
	LP	6.77 ± 0.77	5.54 ± 0.54	ns
Root physiology				
Phosphorus acquisition per length of root (mg P/m root)	NP	0.15	0.26	*
	LP	0.04	0.05	ns
Expression of root physiology controlling gene (P uptake gene)				
<i>ZmPHT1;1</i> (10 ² / <i>ZmTUB4</i>) ²	NP	1.50 ± 0.50	1.38 ± 0.38	ns
	LP	11.79 ± 1.79	11.90 ± 1.90	ns
<i>ZmPHT1;2</i> (10 ² / <i>ZmTUB4</i>)	NP	1.36 ± 0.36	1.61 ± 0.61	ns
	LP	6.76 ± 0.76	6.19 ± 0.19	ns
<i>ZmPHT1;4</i> (10 ² / <i>ZmTUB4</i>)	NP	1.75 ± 0.75	2.01 ± 0.01	ns
	LP	6.65 ± 0.65	7.22 ± 0.22	ns

^a Significant difference between two genotypes (L224 and Wu312) in each trial is indicated by asterisk ($P < 0.05$). ns, no significance. ^b Gene expression in maize root is relative to that of control gene *Alpha tubulin4* (*ZmTUB4*).

Table 6. Phenotype of L224 and Wu312 in field under NP and LP in 2009 and 2010

Trait ^a	Treat ment	2009 L224	2009 Wu312	<i>t-test</i> ^b	2010 L224	2010 Wu312	<i>t-test</i> ^b
PUE-related traits in field							
GY (kg/ha)	NP	3091	1900	*	2803	1311	*
	LP	2921	1824	*	1297	883	*
Pup (kg/ha)	NP	17.39	11.35	*	18.97	16.48	ns
	LP	9.63	6.73	*	10.81	7.04	*
PUE (kg/kg)	NP	18.09	11.12	*	19.78	9.25	*
	LP	299.6	187.1	*	151.17	102.91	*
PupE (kg/kg)	NP	0.1	0.07	*	0.13	0.12	ns
	LP	0.99	0.69	*	1.26	0.82	*
PutE (kg/kg)	NP	177.7	167.4	ns	147.78	141.73	ns
	LP	303.3	271	ns	119.98	125.43	ns
RLD at silking stage							
Total RLD (cm/cm ³)	NP	0.15	0.15	ns	0.25	0.24	ns
	LP	0.12	0.07	*	0.15	0.18	ns
RLD_L1 (cm/cm ³) ^c	NP	0.24	0.27	ns	0.18	0.18	ns
	LP	0.15	0.13	ns	0.17	0.19	ns
RLD_L2 (cm/cm ³)	NP	0.13	0.13	ns	0.1	0.09	ns
	LP	0.16	0.06	*	0.12	0.18	ns
RLD_L3 (cm/cm ³)	NP	0.04	0.02	ns	0.05	0.04	ns
	LP	0.08	0.06	ns	0.06	0.07	ns
RLD at maturation stage							
Total RLD (cm/cm ³)	NP	0.05	0.03	*	0.07	0.06	ns
	LP	0.03	0.02	*	0.06	0.04	*
RLD_L1 (cm/cm ³)	NP	0.06	0.03	*	0.09	0.08	ns
	LP	0.03	0.03	ns	0.08	0.05	*
RLD_L2 (cm/cm ³)	NP	0.06	0.03	*	0.05	0.04	*
	LP	0.02	0.01	*	0.03	0.02	ns
RLD_L3 (cm/cm ³)	NP	0.03	0.03	ns	0.02	0.02	ns
	LP	0.01	0.01	ns	0.01	0.01	ns

^a For trait information, refer to Table 1. ^b * indicates significant difference between two genotypes within a trial at the $P < 0.05$ level. ns, no significance. ^c A soil volume of 28 cm × 35 cm around plant root and a total depth of 60 cm with three increments of 20 cm depth in each were dug out for root density measurement. These three increments were named L1 to L3 from top to bottom.

Figure legends

Fig. 1. PCA of the RIL population for PUE-related traits evaluated in the field under NP and LP conditions and RSA-related traits evaluated in hydroponics. Twenty traits are projected onto the first and second principal components. For trait information, refer to Table 1.

Fig. 2. Identified QTLs for PUE- and RSA-related traits that located in chromosome 3. The markers selected for further marker-assisted selection are labeled by red asterisk. For trait information, refer to Table 1.

Fig. 3. PUE of BC₄F₃ lines containing *Cl-bin3.04a* or *Cl-bin3.04b* or both (L224) grown under NP (**A**) and LP (**B**) conditions in 2007 and 2008. + indicates homozygous chromosome region from line Ye478.

● PUE-related traits in LP ▲ PUE-related traits in NP ▲ RSA-related traits