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Dissecting the genetic basis of root- and rhizosphere-related phosphorus use efficiency in European elite maize (*Zea mays* L.) lines and landraces

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List of Acronyms

P	Phosphorus
Pi	Phosphate
RSA	Root System Architecture
AMF	Arbuscular mycorrhizal fungi
PHT1	Phosphate transporter 1
PSR	Phosphate Starvation Response
PSI	Phosphate Starvation Induced
PDR2	Phosphate Deficiency Response 2
LRR1	Leucine-Rich Repeat 1
STOP1	Sensitive to Proton Toxicity 1
CLE14	Clavata3 / Endosperm surrounding region 14
CLV2	Clavata2
PEPR2	PEP Receptor 2
MATE	Multidrug and Toxic compound Extrusion
ALMT	Aluminum-activated Malate Transporter
PHR1	Phosphate Starvation Response 1
ROS	Reactive oxygen species
InsP / PP-InsP	inositol phosphate / pyrophosphorylated inositol phosphate
Pup1	Phosphorus uptake 1
PSTOL1	Phosphorus-starvation tolerance 1

1.Summary-Zusammenfassung

1.1 Summary

Phosphorus (P) is an essential macronutrient for the growth and development of plants, which is required as the structural component of nucleic acids, phospholipids, phospho-proteins and metabolites and plays an important role in the transport of cellular energy with ATP. However, plants can only absorb P directly as phosphate (Pi) in the soil solution, and the Pi concentration in soil solution is normally quite low, so plants often suffer from P deficiency, which affects the crop yield and quality. In agriculture, farmers massively apply P fertilizer to maintain high yield. Due to the long-term high fertilization rates and long-term organic residue accumulation, the total P pool per hectare has increased between 1900 and 2020. Since modern varieties have often been selected in high-nutrient input conditions for high yields, concerns are being raised that the beneficial traits for P uptake under a limited P supply will gradually decline in elite varieties. Regarding to maize (*Zea mays* L.), thousands of varieties have been bred since it was domesticated as a food product. It is an open question whether traits and genes related to P deficiency in European maize have changed since the Green Revolution, the start of hybrid breeding and high-intensity fertilization. This is the core research question of this dissertation. Here I present the analysis of roots in response to P deficiency using a diverse panel of European maize genotypes via several experiments.

In Chapter I, we focus on whether maize seedlings of the flint and dent heterotic pools vary in the P acquisition and utilization since the onset of hybrid breeding using 34 genotypes in mini-rhizotrons. These genotypes included 16 flint lines that were released over more than five decades ago, 7 doubled haploid lines from the flint landraces (DH_LR), 8 dent lines, and 3 hybrids. Seedling P use efficiency (PUE) and related traits were measured and compared at two P levels in a calcareous soil. Seedling PUE and P acquisition efficiency (PAE) from founder flint to representative elite flints declined over the last decades, which was associated with smaller root systems and their reduced ability to exploit external P, were paralleled by decreased rhizosphere pH and shorter root hairs in low P. Comparing flints with preselected DH_LR, old and more recently released dent elite varieties, elite dent seedlings and their hybrids revealed improved PUE and earlier start to acquire exogenous P. DH_LR were similar to modern elite flints. When evaluating early root traits associated with high P efficiency, seed P should also be considered, and it is important to stack different root traits to optimize PUE, Phosphorous utilization efficiency (PUtE) and PAE in breeding programs. The root hair length, the ability to acidify rhizosphere and root diameter in maize flint and dent pools may be utilized to further improve P use in maize agroecosystems.

In Chapter II, we compared the root exudated organic acids and mycorrhizal fungi colonization degree among 24 genotypes which have been evaluated in Chapter I. These genotypes included 16 flint lines, 6 DH_LR and 2 old dent lines. Seedling colonization with arbuscular mycorrhizal fungi (AMF) and organic

acid anion release were measured. P-uptake-related root traits were compared under P-sufficient and P-deficient conditions. Weak trends for the loss of AMF colonization or changes in organic acid anion release at low P supply were detected in modern varieties. One DH_LR was found with increased mycorrhization, whereas others were similar to modern elite lines. Overall, substantial genetic variance was encountered for these traits.

In Chapter III, using nearly isogenic maize lines, the B73 wild type and the *rth3* root hairless mutant, we quantified the effect of root hairs and AMF infection in a calcareous soil under P deficiency. Wild-type root hairs extended the rhizosphere for acid phosphatase activity by 0.5 mm compared with the *rth3* hairless mutant. Total root length of the wild type was longer than that of *rth3* under P deficiency. Higher AMF colonization and mycorrhiza-induced phosphate transporter gene expression were identified in the mutant under P deficiency, but plant growth and P acquisition were similar between mutant and the wild type. The mycorrhizal dependency of maize was 33 % higher than the root hair dependency. The results identified larger mycorrhizal dependency than root hair dependency under P deficiency in maize. Root hairs and AMF inoculation are two alternative ways to increase Pi acquisition under P deficiency, but these two strategies compete with each other.

In Chapter IV again two nearly isogenic maize lines, the B73 wild type and the *rth2* root hairless mutant, were used to address the importance of root hairs during drought and under P deficiency. The results indicate that drought and P deficiency synergistically impair maize growth; while P concentrations were little affected by the loss of root hairs, the P content was massively reduced at combined stress, showing that P deficiency is much more severe under drought.

In Chapter V, we first compared the root traits response to low P and high P of six preselected genotypes in European flint in Chapter I. We then generated RNA libraries from the roots of these lines under both low P and high P. Using an expressed genes matrix, we conducted a Weighted Genomic Coexpression Network Analysis (WGCNA), and detected general low P-induced modules and modules that were higher in founder flints. The P deficiency-responsive metabolic processes common to all six genotypes included: (1) acceleration of carbon supply for organic acid synthesis through glycolysis and TCA cycle; (2) alteration of lipid metabolism; (3) changes of activity of transmembrane transporters; (4) carotenoid metabolism. Additionally, the founder flint line EP1, F2 and doubled haploid landrace SM1 have their specific strategies and mechanism to cope with low P. Our findings well support other studies with transcriptome, proteome and metabolome experiments in maize and other species, and point to molecular events involved in the efficient alleviation of P stress in efficient maize accessions.

Altogether, this study presents informative analyses in how maize genotypes with distinct breeding history adapt to P deficiency in regard of root, rhizosphere traits and root transcription. It showed correlation between phenotypic traits and gene transcription, which is much more complex than previously reported. It also opened a novel insight into molecular regulation on Pi utilization, resulting in promotion of vegetative biomass in P deficiency. These findings will also provide precious knowledge for plant breeders and agronomists who work on P research in maize and other cereal crops.

1.2 Zusammenfassung

Phosphor (P) ist ein essentieller Makronährstoff für das Wachstum und die Entwicklung von Pflanzen, der als Strukturbestandteil von Nukleinsäuren, Phospholipiden, Phosphoproteinen und Metaboliten benötigt wird und eine wichtige Rolle beim Transport von Zellenergie mit ATP spielt. Pflanzen können P jedoch nur direkt als Phosphat (Pi) aus der Bodenlösung aufnehmen, und die Pi-Konzentration in der Bodenlösung ist normalerweise recht niedrig, sodass Pflanzen häufig unter P-Mangel leiden, was sich auf den Ernteertrag und die Qualität auswirkt. In der Landwirtschaft düngen die Landwirte massiv P-Dünger, um den Endertrag zu erhalten. Aufgrund der langfristig hohen Düngung und der langfristigen Anreicherung organischer Rückstände hat sich der Gesamt-P-Pool pro Hektar zwischen 1900 und 2020 erhöht. Da moderne Sorten häufig unter Bedingungen mit hoher Nährstoffversorung für hohe Erträge ausgewählt wurden, werden Bedenken laut, dass vorteilhafte Eigenschaften für die P-Aufnahme bei einem begrenzten P-Angebot bei Elite-Sorten allmählich abnehmen. In Bezug auf Mais (*Zea mays* L.) wurden Tausende von Sorten gezüchtet, seit er als Lebensmittel domestiziert wurde. Inwiefern sich seit der Grünen Revolution durch Hybridzüchtung und hochintensive Düngung entsprechende Merkmale und Gene des P-Mangels bei europäischem Mais verändert haben, ist ungeklärt. Dies herauszufinden ist die zentrale Forschungsfrage in dieser Dissertation. Ich untersuche dazu Wurzeln in Reaktion auf einen P-Mangel in einem Panel europäischer Maisgenotypen in mehreren Experimenten.

In Kapitel I konzentrieren wir uns darauf, ob sich die frühe Maisentwicklung von Genotypen der heterotischen Pools Flint/Dent seit Beginn der Hybridzüchtung verändert hat. Dabei kamen 34 Genotypen in Mini-Rhizotron bei zwei P-Stufen zum Einsatz. Diese Genotypen umfassten 16 Flintlinien, die zum Teil vor mehr als sechs Jahrzehnten zugelassen wurden, 7 doppelte haploide Linien, die aus den Flint-Landrassen entwickelt wurden (DH_LR), 8 Dentlinien und 3 ihrer Hybride. Die Effizienz der P-Nutzung (PUE) von jungen Pflanzen und zugehörige Merkmale wurden in einem kalkhaltigen Boden verglichen. Die Effizienz der PUE und -P-Akquisition (PAE) der ersten selektierten Flint Linien, auf denen die Hybridzüchtung beruht, bis hin zu repräsentativen neusten Elite-Flint Linien ging in den letzten Jahrzehnten zurück, was mit kleineren Wurzelsystemen und ihrer verringerten Fähigkeit zur Nutzung von externem P verbunden war. Der Vergleich von Flint mit ausgewählten DH_LR, alten und neueren Dent-Elite-Sorten und ihren

Hybriden zeigte verbesserte PUE in alten, aber nicht DH_LR Linien, welche modernen Elite-Flintlinien ähnelten. Bei der Bewertung von Wurzelmerkmalen, die mit einer hohen P-Effizienz verbunden sind, muss auch Samen-P berücksichtigt werden. Die Wurzelhaarlänge, die Fähigkeit zur Ansäuerung der Rhizosphäre und der Wurzeldurchmesser können in Mais-Flint- und Dent-Pools genutzt werden, um die P-Verwendung in Mais-Agrarökosystemen weiter zu verbessern.

In Kapitel II verglichen wir den Besiedlungsgrad der Wurzel mit Mycorrhizapilzen und die Menge und Typ der exudierten organischen Säuren anhand von 24 Genotypen, die bereits in Kapitel I untersucht wurden. Diese Genotypen umfassten 16 Flintlinien, 6 DH_LR und 2 alte Dentlinien. Die Wurzelbesiedlung mit arbuskulären Mykorrhizapilzen (AMF) und die Ausschüttung von organischen Säureanionen wurden gemessen. P-Aufnahme und Wurzelmerkmale wurden unter ausreichendem P und unter P-defizienten Bedingungen verglichen. Bei modernen Sorten wurden schwache Trends für den Verlust der AMF-Besiedlung und der geringe Verlust der Zitratausschüttung bei niedriger P-Düngung festgestellt. In einer DH_LR wurde er höchste Mycorrhizierungsgrad gefunden, während andere modernen Elite-Linien ähnlich waren. Insgesamt wurde für diese Merkmale eine erhebliche genetische Varianz festgestellt.

In Kapitel III haben wir unter Verwendung nahezu isogener Maislinien, des B73-Wildtyps und der *rth3*-wurzelhaarlosen Mutante, die Wirkung von Wurzelhaaren und von AMF-Infektionen in einem kalkhaltigen Boden unter P-Mangel quantifiziert. Wildtyp-Wurzelhaare vergrößerten die Rhizosphäre für die Aktivität der sauren Phosphatase um 0,5 mm im Vergleich zur *rth3*-Mutante ohne Haare. Die Gesamtwurzellänge des Wildtyps war länger als die von *rth3* unter P-Mangel. Eine höhere AMF-Kolonisierung und Mykorrhiza-induzierte Phosphattransporter-Genexpression wurden in der Mutante unter P-Mangel identifiziert, aber das Pflanzenwachstum und die P-Akquisition waren zwischen Mutante und Wildtyp ähnlich. Die Mykorrhiza-Abhängigkeit von Mais war 33% höher als die Wurzelhaar-Abhängigkeit. Die Ergebnisse identifizierten eine größere Mykorrhiza-Abhängigkeit als die Wurzelhaar-Abhängigkeit bei P-Mangel bei Mais. Wurzelhaare und AMF-Inokulation sind zwei alternative Möglichkeiten, um die Pi-Akquisition bei P-Mangel zu erhöhen, aber diese beiden Strategien konkurrieren miteinander.

In Kapitel IV wurden erneut zwei nahezu isogene Maislinien verwendet, der B73-Wildtyp und die *rth2*-Wurzelhaarlose Mutante, um die Bedeutung von Wurzelhaaren während Wassermangel und unter P-Mangel zu untersuchen. Die Ergebnisse zeigen, dass Trockenheit und P-Mangel das Maiswachstum synergistisch beeinträchtigen. Während die P-Konzentrationen durch den Verlust von Wurzelhaaren wenig beeinflusst wurden, war der P-Gehalt bei kombiniertem Stress massiv verringert, was zeigt, dass der P-Mangel sich unter Trockenheit viel schwerwiegender auswirkt.

In Kapitel V haben wir zuerst die Reaktion der Wurzelmerkmale auf niedrigen P und hohen P von sechs vorausgewählten Genotypen in europäischem Flint aus Kapitel I verglichen. Anschließend haben wir mRNA-Transkriptome aus den Wurzeln dieser Linien sowohl unter niedrigem P als auch unter hohem P isoliert. Mit diesen Daten führten wir anschließend eine gewichtete Koexpression-Netzwerkanalyse (WGCNA) durch und entdeckten allgemeine Module, die mit niedriger P-Versorgung höher exprimiert-wurden. Die auf P-Mangel reagierenden Stoffwechselprozesse und genetische Anpassungen, die allen sechs Genotypen gemeinsam sind, umfassten: (1) Veränderung des Kohlenstoffwechsels für die Synthese organischer Säuren durch Glykolyse und TCA-Zyklus; (2) Veränderung des Lipidstoffwechsels; (3) Aktivitätsänderungen von Transmembrantransportern; (4) Carotinoidstoffwechsel. Zusätzlich haben die effizienten Flint Linien EP1, F2 und die doppelt haploide Landrasse SM1 ihre spezifischen Strategien und Mechanismen, um mit niedrigem P Angebot fertig zu werden. Unsere Ergebnisse stimmen gut mit bestehenden Transkriptom-, Proteom- und Metabolom-Studien in Mais und anderen Arten überein. Darüber hinaus können sie helfen, die molekularen Ereignisse zu verstehen, die für P Effizienz bei Mais von Bedeutung sind.

Insgesamt präsentiert diese Arbeit Details, wie sich Maisgenotypen mit unterschiedlicher Zuchtgeschichte hinsichtlich Wurzel-, Rhizosphären-Merkmalen und Wurzeltranskription an P-Mangel anpassen. Die Korrelation zwischen phänotypischen Merkmalen und der Gentranskription, die viel komplexer ist als zuvor berichtet, bietet Hinweise darauf, welche Gennetzwerke P Effizienz ausmachen. Dies eröffnet einen Einblick in die molekulare Regulation der Pi-Nutzung, um vegetative Biomasse bei P-Mangel zu steigern. Diese Erkenntnisse werden auch Pflanzenzüchtern und Agronomen wertvolles Wissen liefern, die an der P-Forschung in Mais und anderen Getreidekulturen arbeiten.

2. Introduction

2.1 Phosphorus and adaptive responses to phosphorus deficiency

Phosphorus (P) is an essential macronutrient in plant growth and development, which is required as a structural component in nucleic acids, phospholipids, phospho-proteins and metabolites and plays a major role in transporting cellular energy with ATP. Plants can only directly absorb P as phosphate (Pi) in the soil solution and the dry weight of plants may contain up to 0.5% P (Marschner and Marschner, 2012). However, previous studies reported that over 40% of the world soils are in a P deficient level and the acid-weathered soils of tropical and subtropical regions of the world are particularly prone to P deficiency (Vance *et al.* 2003). Owing to its low availability and slow diffusion in soil, the Pi concentration in soil solution is normally quite low, ranging from 2 to 10 μM (Raghothama, 1999), which affects the crop yield and quality. Thus, it is vital to discover mechanisms and further exploit the adaptions to increase plant efficiency and manage the crop yield stability under P-limited conditions.

Under Pi limiting conditions, plants maintain cytosolic Pi levels in several ways: promoting the availability of external Pi, increasing its absorption, recycling and consumption of non-essential phosphorus-containing molecules (Pratt *et al.*, 2009). These processes mainly occur in three parts, namely shoot, root and rhizosphere (Fig. 1), but the exact order in which they function and integration remains unclear (Ajmera *et al.*, 2019). In the case of roots, these reactions occur on different biological scales, such as morphology, anatomy, physiology and biochemistry. Deferent plant species may adopt different mechanisms to gain access to soil low available Pi by altering root morphology, secreting Pi mobilizing compounds into rhizosphere and associating with mycorrhiza (Raghothama, 1999; Vance, 2001; Vance *et al.*, 2003; Lambers *et al.*, 2006).

2.1.1 Root morphology adaptation

To optimize the Pi acquisition in response to Pi deficiency, different plant species have evolved divergent adaptations to root morphology. Root System Architecture (RSA), with highly plasticity, including the shape and structure of the root system often varies among plant species (Hodge, 2004). Pi deficiency will change the RSA traits by stimulating lateral root branching, increasing the length and density of

root hairs and in some species, formation of cluster roots and inhibition primary root elongation (Carswell *et al.*, 1996; Lambers *et al.*, 2010).

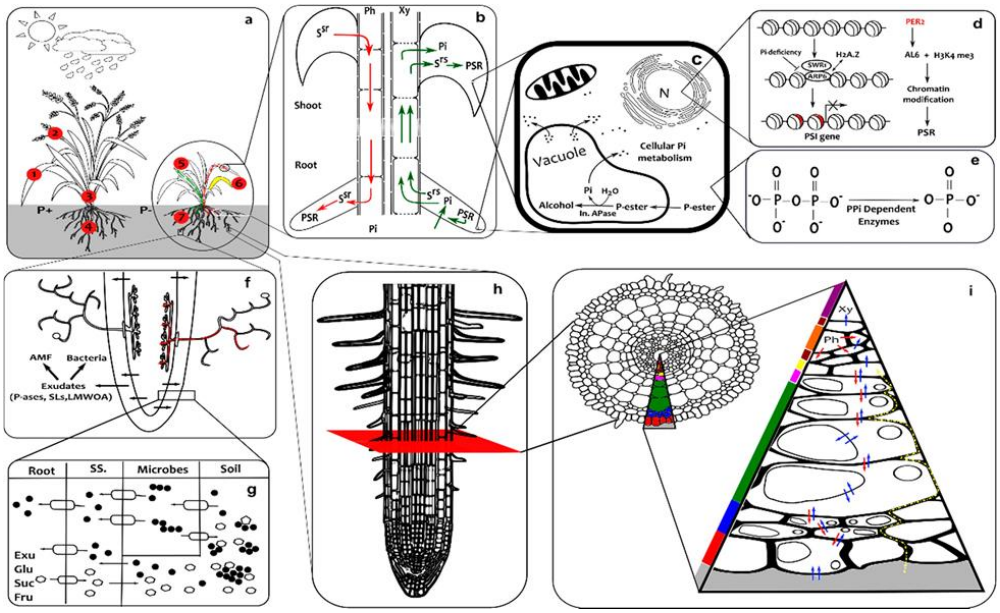

Figure 1. Integrated overview of phosphate starvation responses. The responses and signaling mechanisms operate at a range of scales and different locations which are depicted in nine connected panels: (**a**) denotes the whole plant and field scale; (**b**) denotes the whole plant scale with systemic signals; (**c**) denotes cells from any part of the plant which respond to phosphate deprivation altering the lipid content, releasing phosphate stores from the vacuole where Pi is liberated from esters by Acid Phosphatases (APase). (**d**) denotes the epigenetic effects (principally chromatin modification) that influence transcription of Phosphate Starvation Response genes. (**e**) denotes the pyrophosphate-dependent glycolytic bypass enzymes and metabolic Pi recycling system. (**f**) denotes rhizosphere activities, specifically the exudation of acid phosphatases (P-ases), Strigolactones (SLs) and Low Molecular Weight Organic Acids (LMWOA) which stimulate bacterial activity and attract Arbuscular Mycorrhizal Fungi (AMF); (**g**) denotes a close-up view of the rhizosphere boundaries between the root, soil sheath (SS), microbes and soil where exudates and sugars (Glu–glucose, Suc–sucrose and Fru–fructose) are secreted through efflux transporters respectively to solubilize Pi (**h**) denotes the alteration in meristem and elongation zone length and the formation of root hairs. (**i**) denotes a cross section through a root and the paths taken during Pi uptake: the positions of different tissues within a root, namely, epidermis, exodermis, sclerenchyma plus cortex, endodermis, pericycle, phloem, cambium and xylem are marked respectively by red, blue, green, pink, yellow, orange, pale brown and purple; and transport of shoot-to-root signal molecules, symplastic/inter-organellar Pi and apoplastic Pi are depicted respectively by red, blue and dashed yellow arrows (Ajmera *et al.*, 2019).

Lateral root branching is a key RSA trait in response to Pi deficiency. Lateral roots originate from pericyclic cells close to the xylem pole and trigger a series of asymmetric and transverse divisions (Torrey, 1950). Low Pi stress could result in a decrease of cell division rate, while the cell growth in the root elongation zone is simultaneously inhibited (Sánchez-Calderón *et al.*, 2006). Since the soil P is frequently absorbed by the soil partials, plant species with larger root system contribute more in access to available Pi in the soil for uptake (Jungk, 2001). The response of lateral root to Pi deficiency is plant species and genotypes dependent. Some Pi-efficient plant species/genotypes within a species may grow the lateral root from the basal root at an angle that allow more roots to explore the topsoil likely to contain more available Pi (Lynch and Brown, 2001) or develop an RSA that places active root area relatively rich in Pi (Smith, 2001). Some maize genotypes increase the number and length of lateral roots by distributing more roots in the sub-soil, while common beans and soybeans (Glycine *max*) with shallow roots prefer to enhance the capacity of topsoil P forage (Zhu and Lynch, 2004; Bayuelo-Jiménez *et al.*, 2011).

Root hairs - the tubular-shaped outgrowths from root epidermal cells - are one of the most important root morphologic adaptations to P deficiency (Peterson and Farquhar, 1996), because they can strongly increase the root surface area. Root hairs play an important role in acquisition of poorly mobile nutrients such as Pi by effectively extending the width of the Pi depletion zone around the root (Föhse *et al.*, 1988). Parker et al. (2000) pointed that root hairs form as much as 77% of the root surface area of field crops. Evidence from barley (Hordeum *vulgare*) suggested that genotypes with longer root hair took up more Pi, and tended to yield better when Pi was limiting crop growth (Singh Gahoonia and Nielsen, 2004). In P deficiency, maize root hairs were responsible for a >30% increase in biomass and P shoot content, but did not affect the shoot P concentration and were even more important under drought (Klamer *et al.*, 2019), which proved in the case of the limited solubility and mobility of Pi in soils, the area next to roots and the overall root surface area are most important for Pi acquisition.

2.1.2 Rhizosphere adaptation

The rhizosphere, the soil that is in close contact with the root surface is strongly influenced by exudates from the plant, is massively extended in the root hair regions compared to hairless areas in individual

plants (Ma *et al.*, 2018). Plants can alter the biochemical environment of the rhizosphere and solubilize organic and inorganic phosphate compounds through increasing the root exudates into rhizosphere (Johnson *et al.*, 1996) or formation of mycorrhizal symbionts to trap Pi for the plants in the rhizosphere (Richardson *et al.*, 2011).

Organic anions (malate, citrate and oxalate), enzymes (phosphatase, phytase), phenolic acids and proton are the main components of the root exudates involved in P deficient response (Richardson *et al.*, 2011). In general, these exudates mainly promote solubilization of insoluble phosphate compounds, by competitively binding with the cationic phosphate partners and liberating the Pi ions from organic compounds (Dakora and Phillips, 2002). The secretion of organic acids from root is highly environmental stress and plant species specific. Usually, the amounts and components varied between plant species/genotypes and even different zone of root segments (Neumann *et al.*, 1999; Liao *et al.*, 2006). Generally, dicots, particularly legumes, are more efficient in releasing organic acids to the rhizosphere for Pi mobilization than monocots (Lyu *et al.*, 2016; Wen *et al.*, 2019). Citric acid and malic acid are the predominant acids released by roots under Pi starvation conditions. For example, the roots of rape (Brassica *napus*) and white lupin excrete mainly citrate and malate for efficiently use of rock phosphates (Hoffland *et al.*, 1992; Neumann *et al.*, 1999). Root exudates of citrate increased two-fold in alfalfa that helps them solubilizing more Pi under low P conditions (Lipton *et al.*, 1987). Al induced a large amount release of malate and citrate from root tips in maize and wheat (Triticum *aestivum*) plants.

Arbuscular mycorrhizal fungi (AMF) are one of the most important beneficial microorganisms, which colonize 72% of the vascular plant species (Brundrett and Tedersoo, 2018; Bonfante, 2018). AMF play an important role in the acquisition of nutrients by their symbionts, especially P (Smith and Read, 2008). The majority of plant species, including crop species, are responsive to mycorrhizal symbiosis. AMF intimately connect with the root and transfer nutrients in cortical cell layers and can extend up to several centimeters away from the root and form a dense hyphal network (Smith *et al.*, 2011). The hyphae greatly increase the surface area and soil volumes exploited by the root and play a vital role in nutrient acquisition, especially for the sparingly soluble and poorly mobile Pi (Finlay, 2008). Previous studies have concluded that arbuscular mycorrhizal plants have two pathways for the uptake of Pi from the soil solution: the direct Pi uptake pathway via the root epidermis including root hairs, and the indirect

arbuscular mycorrhizal pathway where Pi is initially taken up by external AMF hyphae (Grace *et al.*, 2009). These different pathways are associated with distinct molecular Pi uptake transporters of the PHT1 Pi transporter family that play specific roles to the two pathways (Benedetto *et al.*, 2005; Javot *et al.*, 2007). When mycorrhizal fungi colonize plants, the mycorrhiza-specific Pi transporter gene expression (ZmPht1;6 in maize and variously named orthologs in other species) was greatly enhanced compared to the non-colonized roots (Nagy *et al.*, 2006; Liu *et al.*, 2016; Sawers *et al.*, 2017).

2.1.3 Root morphology and rhizosphere interaction

Both root morphology adaptation and microbial cooperation require plants to allocate photosynthetic carbon belowground to competing sinks, either to promote cellular hair growth or for transfer to the symbiotic partner (Lynch, 2015). The formation and maintenance of root hairs appear to be a relatively cheap process with respect to carbon and energy demand (Jungk, 2001), while AMF colonization is associated with a costly delivery of up to 15-20% photosynthetic carbon to fungi in exchange for nutrients (Wright *et al.*, 1998; Jakobsen *et al.*, 2005; Ryan *et al.*, 2012). There is compelling evidence of a general trade-off between root hairs and mycorrhizal symbiosis: plant species and genotypes with long and dense root hairs rely less on mycorrhizal fungi for P acquisition (Chen *et al.*, 2005; Brown *et al.*, 2012; Brown *et al.*, 2013). The comparison of various plant species with different root hair length and mycorrhizal dependence demonstrated that root hairs and mycorrhiza are typically inversely correlated, which the benefits of AMF were significantly less pronounced in plants with longer root hairs (Schweiger *et al.*, 1995). In maize, the importance of arbuscular mycorrhiza formation compared with root hairs under low P availability is still unclear. The AMF also influence root system architecture, most prominently, by enhancing lateral root formation (Chen *et al.*, 2017).

Meanwhile, some exudates also promote recruitment of soil microorganisms by providing a carbon source, and/or acting as a chemo-attractants (Czarnecki *et al.*, 2013). AMF colonization depends on release of such signaling compounds into the rhizosphere to germinate spores and attract hyphae for root contact; a radially extended rhizosphere was expected to be beneficial for the number of AMF-root contact sites. In response to low phosphate, exuded strigolactones, in both Lotus *japonicus* and rice, play an important role in the establishment of root and arbuscular mycorrhizal symbiosis interaction

and enhance hyphal branching and root colonization of AMF, consequently increasing the exploration for Pi (Akiyama *et al.*, 2005; Besserer *et al.*, 2006; Chagas *et al.*, 2018).

2.2 Spatial-Temporal phosphate starvation response and the molecular signaling in phosphate starvation response

All the above Phosphate Starvation Responses (PSRs) act at different temporal and spatial-physical scales, i.e., field, rhizosphere, plant, organ, tissue, cell and sub-cell (Ajmera *et al.*, 2019). Plants integrate internal and external factors to trigger such responses to Pi deficiency, which relies on both local and systemic sensing/signaling mechanisms (Fig.2). External Pi is sensed by a local system around the root-tip, particularly in the root cap (Svistoonoff *et al.*, 2007; Bonnot *et al.*, 2016). This independently slows the growth of primary-root and promotes root-hair development in Arabidopsis (Chiou and Lin, 2011). In addition to genetic regulation, the dynamics modulation of different hormones, such as auxin (AUX), ethylene (ET), gibberellin (GA) and strigolactone (SL), also plays an important role in such local responses, leading to altered RSA (Chien *et al.*, 2018). The internal Pi status is monitored by systemic signals to improve the availability, recycling, absorption and transportation of Pi (Lin *et al.*, 2014).

Lateral and cluster root growth is partially regulated at a systemic level (Zhang *et al.*, 2014). By trafficking various signals through the vasculature, systemic signaling integrates the local responses of the entire plant (Fig. 1b). This includes phloem-mediated shoot-to-root signals (microRNAs, sugars and CAX-Ca^{2+}/H^{+} transporters) and xylem-mediated root-to-shoot signals (Pi, cytokinin and strigolactones). These signals collectively trigger a cascade involving a large number of Phosphate Starvation Induced (PSI) genes (Lin and Chiou, 2008; Liu *et al.*, 2016). Most of these are described in Fig. 2 and reviewed in detail in Zhang *et al.* (2014). PSI genes are classified as early or late in expression (i.e., within a few hours or after one day of Pi starvation), and whether they are shoot-, root-or non-specific. In Arabidopsis, the early genes encode transcription factors belonging to MYB, ERF, WRKY and bHLH families, Pi transporters, protein kinases and proteins/enzymes initiating exudation, membrane remodeling and lateral root formation (whose emergence is not until later times). The late-responsive genes mainly encode the downstream regulators for Pi transport, recycling and metabolic bypass processes (Lin *et al.*, 2009;

Chiou and Lin, 2011). In roots, persisting low Pi elicits genes involved in Pi uptake, exudate synthesis and importantly, hormone regulation leading to altered RSA.

Most PSRs aim, at least in part, to increase Pi uptake and transport in the plant. Furthermore, the tissue-specific and phosphate-responsive gene expression reveals a greater level of complexity in the system. The core pathway of transcriptional regulation of Pi acquisition involves the dissociation and sequential sumoylated activation of a major transcription factor of PSRs, named PHR1 in Arabidopsis, and its orthologue PHR2 in rice (Rubio *et al.*, 2001; Miura *et al.*, 2005; Lv *et al.*, 2014; Puga *et al.*, 2014). This triggers a network of molecular responses, including gene activation, microRNA-mediated repression, a reduction in directed ubiquitination and active trafficking of Pi transporters to the plasma membrane (Liu *et al.*, 2014). PHR1/2 and its associated pathways have been extensively studied (Figure 2) and reviewed (Zhang *et al.*, 2014; Briat *et al.*, 2015; Pant *et al.*, 2015).

2.3 Breeding effect on strategic aspects in adaptation to phosphorus deficiency

Recently, increasing evidence has been obtained that root functional traits change together with evolution history (*Reinhart* et al., 2012; *Ma* et al., 2018). Because of long-term agricultural practices, especially high fertilization and long-term organic residue accumulation, the total P pool per hectare has increased between 1900 and 2010 (*Sattari* et al., 2012; *Zhang* et al., 2017). As modern varieties have often been selected under high-nutrient input conditions to provide high yields, concerns are being raised that the beneficial traits for P uptake under a limited P supply will gradually decline in elite varieties. Genes or traits related to efficient nutrient acquisition may become lost when all nutrients are directly available to plants, as plant adaptive traits to nutrient deficiency often result in additional carbon costs (*Wissuwa* et al., 2009; *Wang* et al., 2010). Indeed, in rice, a low-P tolerance QTL, Pup1 has been genetically identified for the improved P acquisition of landraces via larger roots. The *PSTOL1* gene in the QTL, Pup1, has been cloned and transferred to modern varieties and this gene enhanced early root growth, enabling more uptake when incorporated into Pi-sensitive varieties (*Gamuyao* et al., 2012; *Heuer* et al., 2017). By comparing older varieties or landraces with modern lines, the loss of root traits contributing to P uptake has also been reported in wheat cultivars, especially for traits dealing with mycorrhizal competence. High nutrient availability within selection sites is thought to reduce the benefit

of symbiotic interactions (*Hetrick* et al., 1993; *Egle* et al., 1999; *Zhu* et al., 2001). However, recent research with modern soybean cultivars selected by conventional breeding approaches for higher yield has unintentionally led to plants that are adapted better to soil P fluctuations and that acquire more P from the P-rich zones (*Zhao* et al., 2004).

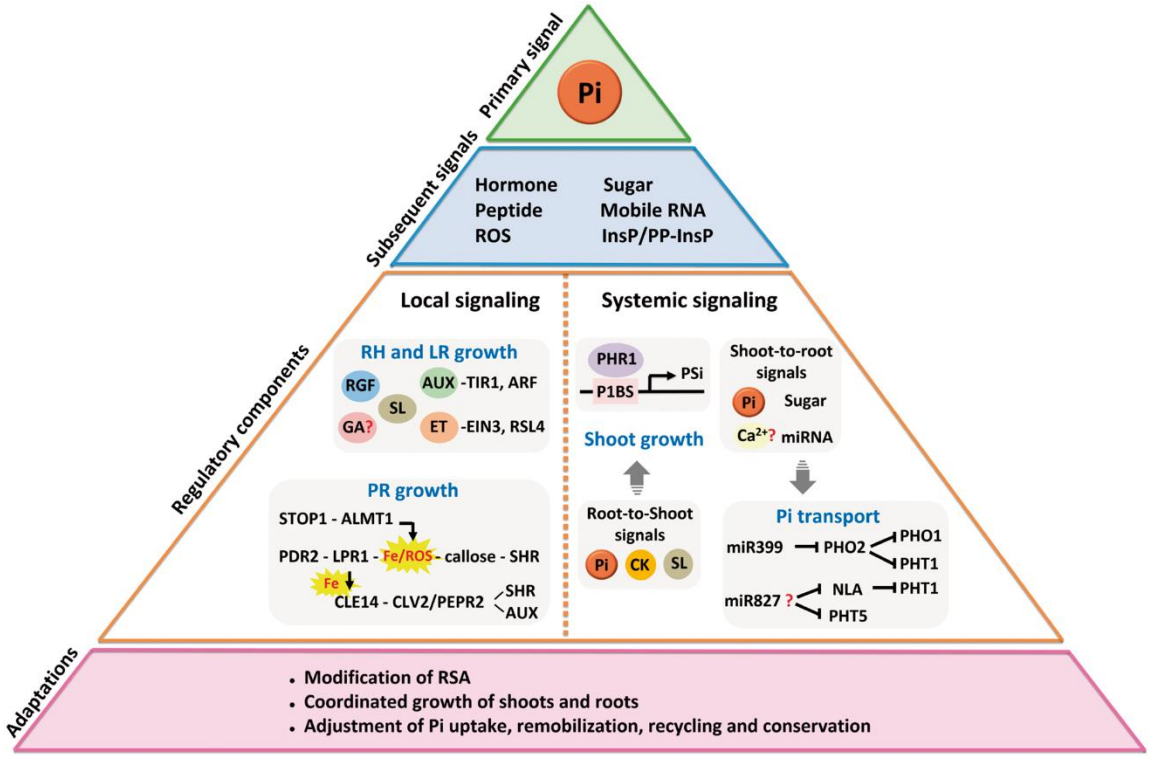

Figure 2 Hierarchical signaling pathways in response to Pi availability in plants. The pyramid represents Pi signaling pathways. From the top, Pi serves as a primary signal to trigger downstream signaling cascades. A number of molecules generated according to Pi availability act as the subsequent signals, involved in local and/or systemic signaling. Root system architecture (RSA) is altered in response to deprivation of external Pi, suggesting regulation by local signals. These local responses include suppression of primary root (PR) growth by PDR2-LPR1, STOP1-ALMT1 and CLE14-CLV2/PEPR2 modules and enhancement of root hair (RH) and lateral root (LR) growth regulated by root growth factor (RGF) peptides and hormones, including auxin (AUX), ethylene (ET), gibberellin (GA) and strigolactone (SL). On the other hand, the mobile signal molecules, such as Pi, SL and cytokinin (CK), move from roots to shoots modulating shoot growth, whereas Pi-, sugar-, miRNA- and possibly Ca^{2+}-derived signals move from shoots to roots regulating Pi uptake, remobilization and recycling. PHR1, a major transcription activator of PSRs, plays a role in regulating systemic responses. By integrating the local and systemic signaling, plants develop coordinated physiological responses to exploit the use of Pi. The question marks indicate uncertain roles of gibberellin in root growth and Ca^{2+} and miR827 in systemic signaling (Chien *et al.*, 2018).

Maize, as a major worldwide crop, have been bred thousands of varieties that can be grouped into three broad categories: landraces, open-pollinated populations, and hybrids. There are already studies implied breeding had affected on the related root traits of maize. Various maize genotypes (bred in P-rich or P-poor environments) perform differently in homogeneous and heterogeneous P soils. The genotypes bred in the P-rich environment have a competitive advantage under the heterogeneous P pattern, whereas the genotype bred in a P-poor environment has a stronger competitive ability under homogeneous P soil distribution (*Li* et al., 2019). A comparison of the mycorrhizal colonization of 141 inbred lines, 38 hybrids, and 76 landraces of maize has revealed that the percent of colonization varies greatly. Inbred lines that have been released in particular locations and years show significantly larger colonization than other lines. Modern hybrids exhibit significantly greater colonization than inbred lines and older landraces (An et al., 2010), but the year-of-release effect on colonization depends on the origin of the cultivar.

On the field scale, improvements in Phosphorus Use Efficiency (PUE) have been achieved through improved soil management (Simpson *et al.*, 2015), cultivar screening (Haling *et al.*, 2018) and selective breeding based on improved root systems (Jia *et al.*, 2018; Strock *et al.*, 2018). However, during the last decades of maize breeding, little attention has been paid on plant performance on low Pi soils, as high fertilizer loads were used and selection was performed under high available Pi conditions. Potential ways to improve Phosphate Acquisition Efficiency (PAE) include modification of RSA, rhizosphere-microbial interaction and Pi uptake. Phosphate Utilization Efficiency (PUtE) involves reducing plant phosphorus demand and/or enhancing its internal utilization/recycling. PAE and PUtE combine to give an overall PUE for a plant. Far more progress has been achieved toward understanding the mechanisms underlying PAE than PUtE, perhaps because of the greater complexity of the processes involved.

2.4 Objectives

In maize, RSA and rhizosphere traits are major components of maize PUE, especially PAE. Using a diverse panel of European maize genotypes, the individual contribution of these traits to improved PUE could be determined. Beneficial RSA (i.e., root hairs), rhizosphere traits (i.e., root exudated organic acids, mycorrhiza symbiosis) and combinations thereof may be identified.

We hypothesize that:

(1) As the consequence of breeding under high fertilizer loads, beneficial RSA and/or rhizosphere traits for high PUE may be lost in elite varieties under low Pi conditions.
(2) The contribution of AMF to plant growth and Pi acquisition is generally larger and more critical than that of root hairs, and cannot be compensated by root hairs.
(3) Genotype with different breeding history has a general molecular mechanisms of LP adaptation and its specific potential molecular mechanisms of LP adaptation.

The Chapter I and II address the first hypothesis. We determined RAS and rhizosphere traits related PUE using a panel of European maize genotypes (Chapter I 34 genotypes and Chapter II 24 genotypes) under low Pi and high Pi conditions.

The Chapter III and IV address the second hypothesis. We checked the mycorrhizal colonization, phosphatase activity and organic acids in root exudates using B73 and its root hairless mutant *rth3* under low Pi conditions, and clarified the contribution of AMF and root hairs to Pi acquisition. We also checked organic acids in root exudates and phosphatase activity using B73 and its root hairless mutant *rth2* under low Pi and water limited conditions, and clarified the contribution of root hairs to Pi acquisition.

The Chapter V address the third hypothesis. We tried to find the key genes, key pathways, and potential molecular mechanisms of LP adaptation in maize with different breeding history.

3. Publications

The present dissertation consists of five scientific articles as reflected by chapter I-V. The chapter I - IV have been published in a peer reviewed journal. The chapter V is a manuscript planned to submit to a peer reviewed journal.

Publication I

Xuelian Li*; Melissa Mang*; Hans-Peter Piepho; Albrecht Melchinger; Uwe Ludewig (2021). Decline of seedling phosphorus use efficiency in the heterotic pool of flint maize breeding lines since the onset of hybrid breeding. Journal of Agronomy and Crop Science: In press. https://doi.org/10.1111/jac.12514 **(*** shared first authorship).

Publication II

Xuelian Li; Xiuhao Quan; Melissa Mang; Günter Neumann; Albrecht Melchinger; Uwe Ludewig (2021). Flint maize root mycorrhization and organic acid exudates under phosphorus deficiency: trends in breeding lines and doubled haploid lines from landraces. Journal of Plant Nutrition and Soil Science 184: 346 - 359. https://doi.org/10.1002/jpln.202000471

Publication III

Xiaomin Ma*; **Xuelian Li***; Uwe Ludewig (2021). Arbuscular mycorrhizal colonization outcompetes root hairs in maize under low phosphorus availability. Annals of botany 127: 155–166. https://doi.org/10.1093/aob/mcaa159 **(*** shared first authorship).

Publication IV

Florian Klamer; Florian Vogel; **Xuelian Li**; Hinrich Bremer; Günter Neumann; Benjamin Neuhäuser; Uwe Ludewig. (2019): Estimating the importance of maize root hairs in low phosphorus conditions and under drought. Annals of botany 124: 961–968. https://doi.org/10.1093/aob/mcz011.

Publication V

Xuelian Li; Uwe Ludewig. Transcriptomic network analyses of the response to low phosphate supply in roots of maize with distinct breeding history (in preparation).

4. Chapter I

Decline of seedling phosphorus use efficiency in the heterotic pool of flint maize breeding lines since the onset of hybrid breeding

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Decline of seedling phosphorus use efficiency in the heterotic pool of flint maize breeding lines since the onset of hybrid breeding

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Abstract

Improved management and breeding increased maize (*Zea mays* L.) yields over the last century, but nutritional efficiency was usually not the focus. This study investigates whether old and recently released flint and dent maize seedlings vary in the phosphorus (P) acquisition and utilization. P use efficiency (PUE) and related traits were measured and compared at two P levels in a calcareous soil. PUE and P acquisition efficiency (PAE) from founder flints to elite flints declined over the last decades. This was associated with smaller root systems, reduced ability to exploit external P, decreased rhizosphere pH and shorter root hairs in low P. Comparing flints with doubled haploid landraces (DH_LR), old and elite dents and hybrids revealed that dents started to acquire exogenous P earlier and had improved PUE. Most DH_LRs had similar PUE as elite flints. When evaluating root traits associated with P efficiency, seed P was also critical, and it is important to stack different root traits to optimize PUE, P utilization efficiency (PUtE) and PAE in breeding programmes. The root hair length, the ability to acidify the rhizosphere and the root diameter in flint and dent pools may be utilized to improve P use in maize germplasm.

KEYWORDS

exudates, nutrient stress, phosphate, rhizosphere, rhizotron, root architecture

1 | INTRODUCTION

Of all essential macro-elements for plants, phosphorus (P) is typically least bioavailable in soils, despite high total P amounts within soils. Almost all P in soils is found in fully oxidized form as orthophosphate (P_i), but due to slow soil processes such as adsorption, precipitation and immobilization (often in organic molecules), the free P_i concentration even in agricultural soil solution is rarely above 10 μM and sparingly soluble P-forms are converted into insoluble forms with time (Bieleski, 1973). This low P_i concentration is more typical for micro-nutrients that are required by orders of magnitude less. If available, P is readily taken up by the roots as (di-)hydrogen phosphate ($H_2PO_4^-$ or HPO_4^{2-}) and the P_i concentrations within plant cells are approximately 5 to 20 mM (Raghothama & Karthikeyan, 2005). Although many soils have large reserves of total P, the inorganic P (P_i) and organic P (P_o)

Xuelian Li and Melissa Mang these authors contributed equally to this work.

pools of most soils are sparingly soluble and little mobile, especially at acidic and slightly basic pH (Akhtar et al., 2008).

Ensuring stable food and feed production even with dwindling productive agricultural zones with P as a finite, but an essential resource, requires resource-saving production and nutrient efficient crops that cope with these soil properties (Wang et al., 2010; Wissuwa et al., 2009). Several definitions and calculation methods are common for crop P use efficiency (PUE), an indicator of the ability of plants to produce yield or biomass under particular P conditions (Batten, 1992; Lynch, 1998; Moll et al., 1982). PUE is often separated into P acquisition efficiency (PAE) and P utilization efficiency (PUtE). PAE is the ability of plants to capture P from soils; while PUtE is the ability to produce biomass or yield using this acquired P. The P efficiency of the plant can be enhanced by improving the acquisition and/or utilization of P (Manske et al., 2001; Parentoni & Souza Júnior, 2008; Shenoy & Kalagudi, 2005).

Plants use diverse strategies to increase P acquisition and/or improve internal P utilization. The below-ground strategies to increase P acquisition can be divided into morphological traits, chemical modifications of the rhizosphere and microbial interactions. Root morphological strategies include vigorous root branching, increased frequency and length of root hairs, and shallow root systems with preferential root development in nutrient-rich topsoil, to access a large soil area (Haling et al., 2018; Postma et al., 2014; Vance et al., 2003). Additionally, plant roots can acquire P by chemical modification of rhizospheres, such as exudation of H^+, sugars, organic anions, amino acids and secondary metabolites (Hinsinger, 2001; Pang et al., 2018; Richardson & Simpson, 2011). Furthermore, P deficiency can induce the establishment of symbioses with arbuscular mycorrhizal fungi to help access P from the labile soil P pool (Irshad et al., 2012; Smith et al., 2011; van der Heijden et al., 2015).

There is substantial evidence for coordination and trade-offs among root functional traits among species and environments (Lynch, 2011; Ma et al., 2018; Reinhart et al., 2012; Wen et al., 2019). Among the major crops, maize and wheat with fibrous root systems respond to low soil P availability by altering root size and architecture. Meanwhile, legumes tend to modify the chemistry of the rhizosphere (Lyu et al., 2016). Rapeseed, barley and potato have evolved specific traits of root morphology and root exudation that enhance their P uptake capacity under low P conditions (Wang et al., 2015). The root diameter exerts a strong influence on root trait variation across plant species, growth forms and biomes (Lyu et al., 2016; Ma et al., 2018; Wen et al., 2019). Maize seedlings performed differently in different soil substrates (Liu et al., 2017). Erel et al. (2017) compared maize hybrids grown in neutral and alkaline soil and found that the soil type had a strong effect on PAE. In the neutral soil, the best genotypes were characterized by topsoil exploration, but in the alkaline soil, the 'mining strategy' was optimal.

Recently, there is also increasing evidence that root functional traits changed along with the evolution history (Ma et al., 2018; Reinhart et al., 2012). Due to long-term agricultural practices, especially high fertilization and long-term organic residue accumulation, the total P pool per hectare increased steeply between 1900 and 2010 (Sattari et al., 2012; Zhang et al., 2017). As modern varieties have often been selected under high-nutrient input conditions to obtain a high yield, there is reasonable concern that beneficial traits for uptake under limited P supply are getting lost in elite varieties or even already got lost. Genes or traits related to the efficient nutrient acquisition at low P availability may provide a trade-off when all nutrients are amply available, as plant adaptive traits to nutrient deficiency often result in additional carbon costs (Wang et al., 2010; Wissuwa et al., 2009). By comparing older varieties or landraces with modern lines, loss of root traits contributing to P uptake was already reported in wheat cultivars (Zhu et al., 2001). However, modern cultivars that were selected through conventional breeding approaches for better yield may alternatively unintentionally be better adapted to soil P fluctuations and acquire more P from P-rich zones. For example, modern soya beans have shallow root architecture and higher PAE because they can acquire more P from the soil P-rich surface zone. In contrast, wild soya bean genotypes have deep root architectures and lower PAE, and the root architecture and PAE of semi-wild soya bean are intermediate between modern cultivars and wild soya bean (Zhao, 2004). Furthermore, different maize genotypes (bred in P-rich and P-poor environments, respectively) performed differently in homogeneous and heterogeneous P soils and exhibited different root traits, as well P uptake strategies. The genotypes bred in the P-poor environments showed higher phosphatase activity as well higher mycorrhizal colonization within homogenous P soils compared with genotypes bred under P-rich environments (Li et al., 2019).

In maize, efficient P use is critical between planting and the six-leaf stage, when the young root is still too small to sustain shoot nutrient supply. P deficiency during that stage reduced grain yield (Barry & Miller, 1989). Already after the first week of seedling development, most stored P in the form of phytate was completely hydrolysed and was remobilized for P nutrition, while the uptake of exogenous P in maize was delayed and started only at around the fifth day (Nadeem et al., 2011). Maize seedling development was reported to be initially independent of exogenous P supply; within the first three weeks of growth, losses of up to 40% of seed P were reported (Nadeem et al., 2012).

In this study, we initially selected 16 representative maize genotypes from the flint heterotic pool of a public temperate maize breeding programme that spans the breeding progress since the onset of hybrid breeding from the 1950s to 2010s (Hölker et al., 2019). We hypothesized that the increase in biomass in modern elite varieties is associated with increased PUE, PAE and/or PUtE, compared to old genotypes and landraces. We additionally hypothesized that modern elite genotypes lost some beneficial root-related traits, especially under limited P supply, because of selection under ample P supply. Plants were grown in a calcareous soil-sand mixture with two P application levels and were harvested after three weeks. Moreover, rhizoboxes with observation windows enabled the collection of root physiological traits, such as exudates, and the analysis of root morphology crucial for P acquisition and P utilization. Our analysis was then expanded to doubled haploid landraces and dent

lines from different decades for a broad overview of major germplasm pools (flint and dent), as well as three hybrids (derived from crosses of studied flint and dent lines). We finally hypothesized that high PUE under high P supply is associated with trade-offs in root traits that are beneficial for P acquisition under P limitation, with the root length and root hair length exerting a major influence on other root traits across genotypes.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Thirty four maize genotypes were tested in this study, including 16 flint lines, seven doubled haploid landrace (DH_LR) lines from the flint pool, eight dent lines and three hybrids. The 16 flint lines covered a total of 67 years of hybrid breeding at the University of Hohenheim: four flint founders (FL Flint) EP1, 1,105, F2 and F7; early elite flint lines (EL Flint1, 1,102, 1,107, 1,278, 5,113), moderately old EL Flint2 (5,250, 5,267, 5,271, F012) and most recent EL Flint3 lines (F030, F047, F160, F142). The flint founder lines were registered between the late 1940s and 1974 and played a vital role during the beginning of hybrid breeding in Europe (Cartea et al., 1999; Messmer et al., 1992). The elite flint lines cover a period from the 1970s to 2016 and were among the most successful lines of the UHOH maize breeding programme in their respective time period. Seven DH_LR lines were produced from four different landraces, namely lines SM1, SM2, SM3, SF1, SF2, WA1 and CG1 from the Romanian population Satu Mare, the German population Strenzfelder, the French population Wallis and the Swiss population Campan Galade, respectively. The old dent lines B73 and Mo17 (Old Dent) are typical representatives of the BSSS ×Lancaster heterotic pair. The six elite dent inbred lines (EL Dent) were P024, P040, S072, VD03, P330 and P415. Three hybrids of the studied flint and dent lines (Hybrid) P330×F047, P330×F142, P415×F142 were crossed in 2016. The detailed background of these genotypes is given in Supplementary Table S1.

2.2 | Plant growth conditions

A long-term stored nutrient-poor subsoil (pH ($CaCl_2$) 7.6, C_{org} <0.3%, $CaCO_3$ 30%) was used, of which information is found in Supplementary Table S2. In both treatments, the soil was fertilized with 200 mg *N* (NH_4NO_3), 200 mg K (K_2SO_4), 100 mg Mg ($MgSO_4.7H_2O$), 2 mg Fe (EDTA-Fe-2Na), 2.6 mg Zn ($ZnSO_4$ 0.7 H_2O) and 1 mg Cu ($CuSO_4$ 0.5H_2O) per kg dry soil to obtain a nutrient-rich soil substrate. Then, the soil was mixed with 30% (w/w) quartz sand for improved soil texture and to facilitate root collection. Phosphorus was added to the soil as $Ca(H_2PO_4)_2{\cdot}H_2O$, with a rate of 32 mg P kg^{-1} soil for the low P treatment; the high P treatment received 132 mg P·kg^{-1}. The P availabilities in these mixtures were determined by CAL extraction (9.8 mg· kg^{-1} for LP and 61.8 mg kg^{-1} for HP, respectively) and bicarbonate extraction (Olsen-P, 6.6 mg· kg^{-1} for LP and 60.2 mg· kg^{-1} for HP, respectively). After adding the nutrient solution, the soil-sand substrate was sieved through 5 mm before potting. Soil moisture was set to 70% of the soil water holding capacity and gravimetrically adjusted every 2 days.

The experiment was conducted in a growth chamber at the University of Hohenheim, Stuttgart, Germany (48°42'44"N, 9°12'30"E). Seeds were surface-sterilized by rinsing them in 10% (v/v) H_2O_2 solution for 20 min. Afterwards, they were put in aerated 10 mM $CaSO_4$ solution at 25 °C in the dark overnight. The next day seeds were placed between filter paper soaked in a 4 mM $CaSO_4$ solution for around three days to germinate and then transferred to the soil-sand substrate gently. Flint and old dent plants were grown in half-cylinder rhizotrons (height 25 cm; diameter 10 cm), for elite dent and hybrids that developed quicker, longer rhizotrons were used (height 48 cm; diameter 10 cm). One seedling was planted per rhizotron. The open part of the half-tubes was covered with a transparent plexiglass observation window and secured with tape. The rhizotrons were arranged in an unblocked randomized design with five biological replicates for each treatment in the climate chamber. Limited by the size of the climate chamber, several growing trials ran from the summer of 2017 to the summer of 2019. The plants were harvested at three weeks after germination. The climate chamber temperature was maintained 25 °C during the day and 20°C at night, air humidity was set to 60%, and light (300–350 μmol/m^2 s^{-1}) was from 8 a.m. to 10 p.m. At harvest, the differences in shoot growth of 34 genotypes were easily observed under LP and HP conditions (Figure S4).

2.3 | Harvest and measured traits

Maize P (both shoot and root) and the root traits related to P acquisition were determined. Root traits were classified in root morphological traits and P mobilizing traits (Table S4). Firstly, root exudates were collected from 1 cm subapical root zones of roots by application of strong sorption filter paper (MN156050, Macherey-Nagel, Munich, Germany) and measured as described by Ma et al. (2021). Reverse phase-HPLC in the ion suppression mode at 40°C with isocratic elution (18 mM KH_2PO_4, pH 2.2) on a reversed-phase C-18 column (5 μm particle size, 290 × 4.6 mm, GROM-SIL 120 ODS ST) was used to determine organic acids.

After sampling the root exudates, 5 pictures were taken from the root hair zone of each rhizotron under a Stemi 2000-C microscope (Carl Zeiss, Germany). From each picture, 5 root hairs' length was measured using Axio Vision 3.1 software (Carl Zeiss, Germany). The average length of 25 root hairs was taken as one replicate.

After taking photographs of root hairs, roots were carefully lifted out of the soil and shaken to remove bulk soil. Afterwards, the whole root was soaked into 50–100 ml 0.2 mM $CaCl_2$ for a while to collect rhizosphere soil (Pearse et al., 2007). Rhizosphere soil pH was measured by a pH Meter (SevenCompact pH meter S220, Mettler Toledo), and dent rhizosphere pH was additionally

measured by soil pH meter (HANNA Instrument) directly placed next to the root in the rhizotron. After collecting rhizosphere soil, the roots were carefully washed with deionized water. Acid phosphatase activity at the root surface was assayed using p-nitrophenyl phosphate as the substrate (MP Biomedicals). Two fresh roots were carefully placed into 40 ml of acetate buffer (0.1 mmol/L, pH 5.2) containing 0.5 g·L^{-1} disodium p-nitrophenyl phosphate. The spectrophotometric assay was carried out at pH 5.2 and 25°C, as recommended by the manufacturer, for one hour. Activity was quantified by comparing the absorption at 405 nm (Hitachi U-3300, Hitachi Ltd. Corporation) to a standard curve of diluted p-nitrophenyl solution and NaOH.

After measuring the acid phosphatase activity, the root systems were stored in 70% (v/v) ethanol. After that, single roots were submerged and separated in a water film on transparent perspex trays and subsequently scanned using a flat-bed scanner at 400 dpi resolution (Epson Expression 1,000 XL). The root length and average root diameter of the scanned images were measured by using the WinRHIZO root analysis system (Reagent Instruments).

The plant materials (shoots, roots and seeds) were oven-dried at 60°C until constant weight for determining dry weight. The dried material was ground to a fine powder. 250 mg shoot dry matter was incubated with HNO_3, H_2O_2, and distilled water in a microwave (MLS Maxi 44) at a maximum of 210°C and 1,400 W for 65 min. This solution was adjusted to 20 ml and filtered through 90-μm mesh filter paper. P was measured spectrophotometrically via orthophosphate determination after the addition of molybdate–vanadate reagent (Gericke & Kurmies, 1952). Based on the P concentration, we calculated P efficiency-related indexes as detailed below.

2.4 | Definitions and abbreviations of phosphorus use efficiency

P use efficiency (PUE) here refers to the ability of plants to produce biomass under particular P conditions (Batten, 1992; Lynch, 1998; Moll et al., 1982). It was calculated by the following formula: PUE (g DW mg^{-1} P_s) = TDW/$P_{substrate}$.

where TDW is the total dry weight of the corresponding plants, $P_{substrate}$ is the P amount within the fertilized soil-sand mixture (equal to CAL-P in the soil plus added P per rhizotron), DW is short for dry weight, and P_s is short for $P_{substrate}$.

PAE refers to the ability of plants to capture P from soils, while PUtE is the ability to produce biomass using this acquired P. They were calculated by the following formulas:

PAE (mg P mg^{-1} P_s) = ($P_{content}$ − P_{seed}) / $P_{substrate}$.

with $P_{content}$ is the P amount within the plants, P_{seed} is the P amount within the corresponding seeds. For calculating PAE, we assumed that P_{seed} had been used up at harvest.

PUtE (g DW mg^{-1} P) = TDW/$P_{content}$ = 1/[P].

where [P] is the plant P concentration with the unit of mg/g DW.

We also calculated the $PUE_{w/o\ seed}$ representing only the external P acquired from the soil assuming that P_{seed} had been completely used up by the growing seedlings at harvest. It was calculated by the following formula:

$$PUE_{w/o\ seed}\ (g\ DW\ mg^{-1}\ P_s) = [(P_{content} - P_{seed}) \times PUtE] / P_{substrate}.$$

2.5 | Statistical analyses

All data were analysed by the JMP Pro 15 statistical package (SAS Institute Inc.). In general, the experiment was composed of the two key factors: P level and maize genotypes (or groups from different breeding periods).

Data validity was explored by plotting the response on the *x*-axis and the genotype or P level on the *y*-axis. Next, a mixed model with the P level, maize genotype and their interaction as fixed effects was fit, and the assumption of residual normality as well as homogeneity of within-group variances was checked by using diagnostic plots. Data showing irregular or untypical residual plots (residual by predicted plot, residual by row plot and residual quantile plot) were transformed by the logarithm of the raw data (Kozak & Piepho, 2018; Piepho, 2009). To test the time effects of the different experiment runs, random effects were added to the mixed model: Harvesting Date, Harvesting Date ×P level, Harvesting Date ×Genotype and Harvesting Date × Genotype × P level. Due to the fact that none of the investigated traits showed significant random effects, they were removed from the model. Finally, a mixed model with the P level, maize genotype and P level ×maize genotype as fixed effects was used using log-transformed data. Two-way ANOVA was based on this mixed model, followed by Student's *t* test pairwise comparisons at $p < .05$. Mean values were used when fitting linear regressions to PAE, PUtE, PUE and root traits with respect to the year of release under LP and HP separately.

3 | RESULTS

3.1 | Seedling biomass and P use efficiencies of groups of flint lines, DH_LRs, dent lines and hybrids

The average shoot dry weight of three-week-old seedlings decreased both in high P (HP) and low P (LP) conditions when grouped according to the year of their release from founder flint lines to the 2nd elite panel and stayed similar thereafter (Figure 1a). The average P concentrations in flint shoots, however, remained unchanged (Figure 1b). Recently produced DH_LRs, which represent 'old' genetics from agronomic systems prior to massive mineral P fertilization, were on average most similar to the second generation of elite flints (Figure 1a,b). The biomass of modern dent lines and hybrids, as expected, was larger than that of flints (Figure 1a). Part of the larger biomass in HP resulted from an apparently more rapid development of some genotypes/groups in HP, namely founder flint lines, dents and the hybrids (Figure 1c). However, at the time of harvest, most DH_LR and flint lines were at the six-leaf stage in HP (average 6.1 ± 0.5). Several genotypes, albeit not all, were slightly delayed in LP (average

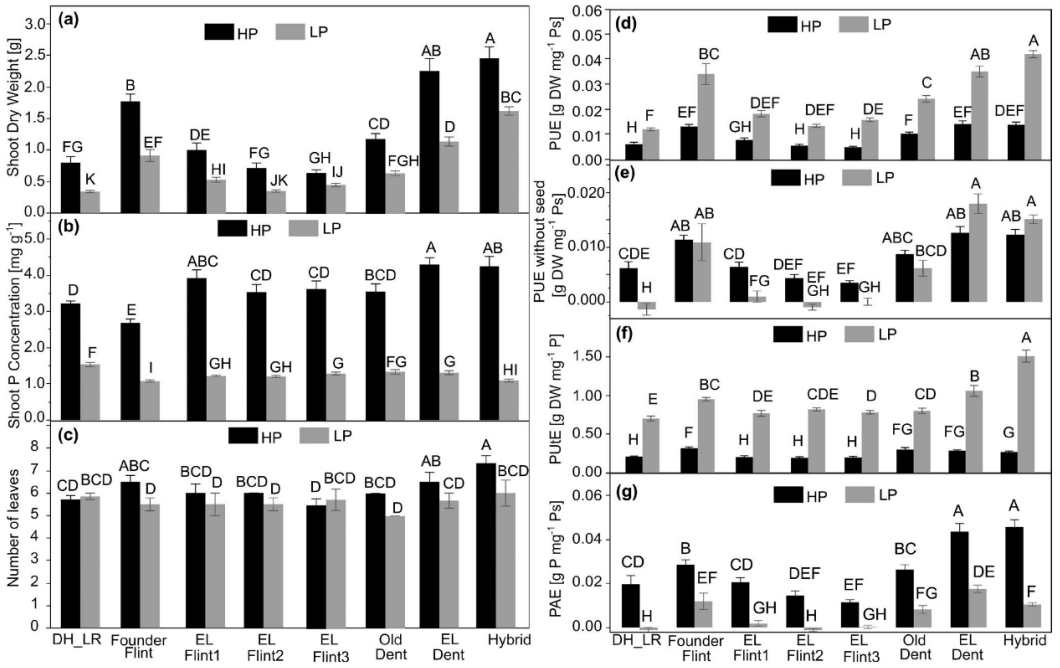

FIGURE 1 Average shoot dry weight, shoot P concentration, leaf numbers and average phosphate efficiencies of different genotype groups. Mean performance ±SE for shoot dry weight (a), shoot P concentrations (b), leaf number at harvest (c), phosphate use efficiency (d), phosphate use efficiency without seed P (e), phosphate utilization efficiency (f) and phosphate acquisition efficiency (g) were shown. Different letters represent significant differences at $p < .05$ according to the result of Student's t test all pairwise comparisons (log-transformed data were used to ensure data normality)

leaf number 5.6 ± 0.5). As expected, elite dents and hybrids developed on average more rapidly, especially in HP, with P415 and P330 × F047 having already 7–8 leaves at harvest (Figure 1c, Figure S5).

Averaged PUE, PUtE and PAE were calculated for the LP and HP treatments separately and revealed higher PUE and PUtE for each group in LP compared with HP (Figure 1d,f). PAE was always higher under HP. DH_LRs and the elite line panels 1 and 3 were the only groups where the biomass that was produced from external P (excluding seed P) divided by P in the soil (PUE without seed) exhibited significant differences between the P treatments (Figure 1e). Among the groups of flint lines that span 67 years of breeding history, the PUE and PAE declined at each P level from founder lines to the first or second group of elite lines, but did not change since then in the most modern flint elite panel (Figure 1d,g). PUtE was, on average, only higher in founder lines compared with elite lines (Figure 1f).

P efficiency measures of two classical dent lines that are often used in genetic studies (Mo17 and B73) were intermediate with respect to PUE and PAE in both LP and HP. By contrast, modern elite dent lines (and especially three hybrids that were crosses of the studied flints and dents from the same breeding programme) had larger PUtE and simultaneously higher PUE in LP, which was similar to founder flint lines (Figure 1d,f). Their high mean PUE in HP resulted from the high PAE, which was similar in hybrids (Figure 1d,g).

3.2 | Biomass, PUE, PUtE, PAE in the flint heterotic pool and comparison with DH_LRs, dent lines and hybrids

The total seedling dry weight, as well as their root and shoot dry weight, varied, but generally increased with higher P for the 34 genotypes (Figure 2). As expected, most dent seedlings and hybrids had larger biomass than flint seedlings and most of the DH_LRs. A strong growth response to HP compared with LP was found in one DH_LR line (CG1), in several older flints, and some, but not all modern dent lines. The response to P was highly variable among genotypes that were released in the same period (Figure 2). The P levels and the genotypes had significant effects on the shoot and total dry weights, but the root dry weight was little affected by P application. Meanwhile, strong genotype ×P treatment interactions were identified (Table S3).

Individual PUEs and associated efficiencies were first studied in the flint pool. Within these 16 lines, a strong decline of PUE and PAE both in LP and HP was observed, while a moderate decline of PUtE was found significant only at HP level (Figure 3a-c). The decline in PUE was paralleled by a decline in root dry weight in more modern lines, and this was more significant in HP than in LP conditions (Figure 3d). This decline in PUE was not as evident when the dent lines were included in

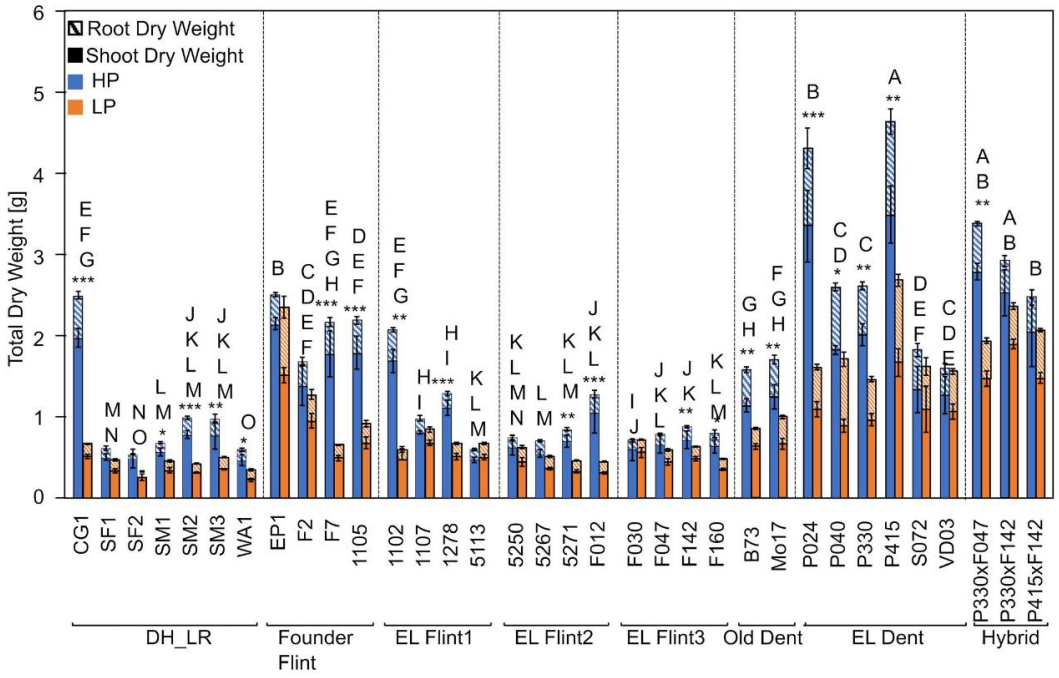

FIGURE 2 Mean performance for total dry weight (TDW) of 34 maize genotypes ($N = 5$) displayed in groups. Significant differences between P treatments and genotype tested based on Student's *t* test pairwise analysis using log-transformed data. Asterisk means differences between P treatment. *, $0.01 < p \leq .05$; **, $0.001 < p \leq .01$; ***, $p \leq .001$. Different letters mean significant difference among genotypes at $p < .05$

the graph (Figure S1). PUE and PUtE were always higher in LP than in HP (Figure 3a,b), while PAE was higher in HP (Figure 3c). The decline of the dry weight of the roots (and total biomass) of seedlings (Figure 3d) was not seen when landraces, dents and hybrids were included in the graph (Figure S1). The DH_LR lines were overall similar to recent elite flint lines concerning PUE and PAE (Figure 2, Figure S1), despite some variation between individual lines (Figure 2, Figure S1).

We furthermore measured PUE, PUtE and PAE in eight dent lines, as well as in three hybrids that were crosses of the studied flint and dent lines (Figure S1). The classical dent lines that are often used in genetic studies (Mo17 and B73) were intermediate with respect to PUE and PAE. The PUtE of old dents was similar in to elite flint lines, while modern dent lines and especially hybrids had increased PUtE in LP (Figure S1). This elevated PUtE caused higher PUEs of dent lines and hybrids in LP. These lines and hybrids also had, on average, increased PUE in HP that was caused by increased PAE (Figure S1).

3.3 | Seed P content and the origin of P in seedlings under LP and HP conditions

The P content per seed substantially varied, between 0.38 and 1.16 mg P $seed^{-1}$, but without apparent trends related to breeding history or heterotic group origin (Figure 4a). The seed P concentrations were, however, relatively stable, around 3 mg/g (Figure S2a). Due to efficient remobilization during germination, approximately 1 mg of stored P is sufficient to support seedling growth for around two weeks (Nadeem et al., 2011; White & Veneklaas, 2012). Moreover, the total seedling P content of many genotypes in LP was at seedling harvest in the same range, indicating that several genotypes did not efficiently start to acquire extra P from the soil. In HP, the majority of P in the seedlings invariably came from soil P (Figure 4b). The P from seeds contributed only ~10% of total P in HP in modern dents and hybrids, while the P fraction derived from the seed P was larger in several flint lines. It was highest in F030 (~45%) and the SF2 DH landrace (~50%) (Figure 4b). Interestingly, under LP, the seedlings of more recently released flint lines (1,278, 5,267, 5,271, F012, F142) and some DH landraces (CG1 and SF2) had even less P than was initially stored in the seeds, suggesting that these genotypes lost P during seedling establishment to the environment (Figure 4c). This loss was as large as 50% of seed P in SF2 (DH_LR) (Figure 4c). Modern dent lines, however, accumulated even in LP a large fraction of their total P (~60%) from exogenous P, while the exogenous P fraction was between 37% and 50% in the hybrids in low P. There was only a weak and non-significant trend for an increase in SDW of flints with seed P for

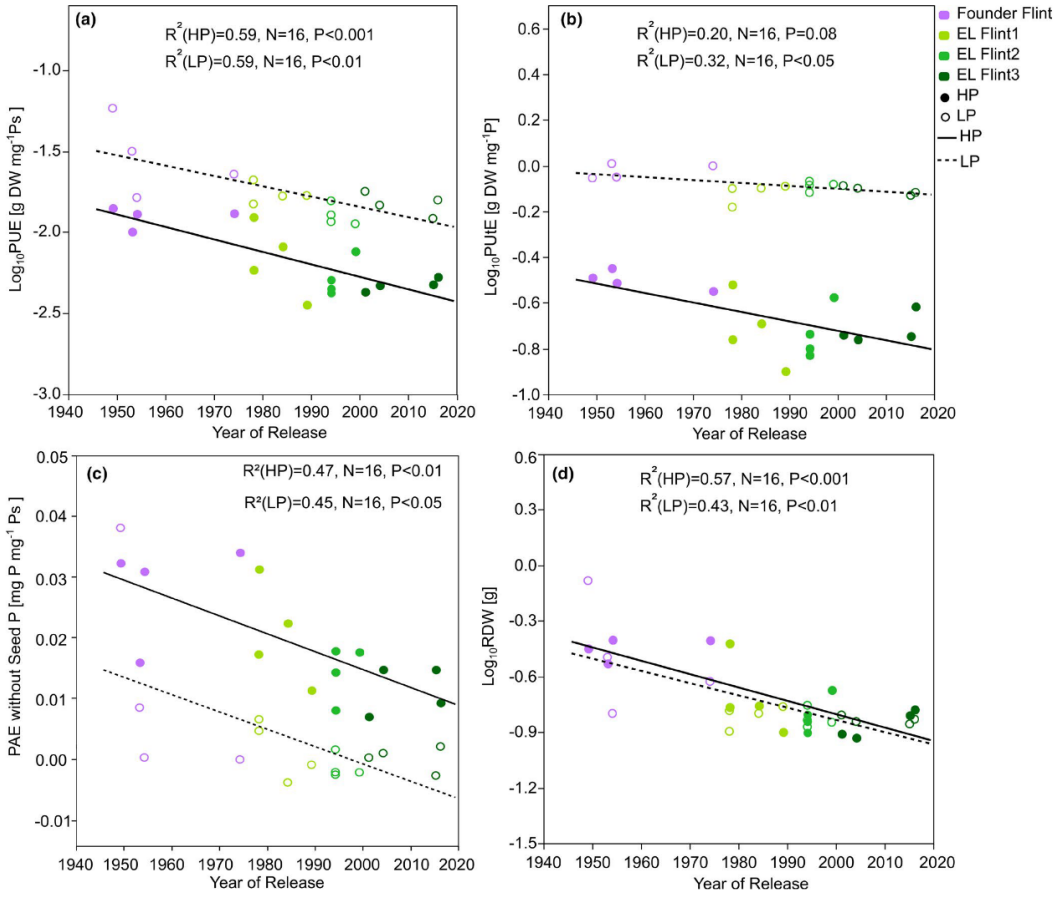

FIGURE 3 Phosphate use efficiency measures of flint material released in different decades. Mean performance for (a) phosphate use efficiency (PUE), (b) phosphate utilization efficiency (PUtE), (c) phosphate acquisition efficiency (PAE) and (d) root dry weight (RDW) of 16 flint maize genotypes plotted versus the year of release of the lines. Regression on the year of release is based on the genotypes in the LP and HP treatment separately. Different colours represent different groups. The open circle represents the mean of each genotype under LP condition; the closed circle represents the mean of each genotype under HP condition. The dashed line stands for the regression curve under LP condition; the solid line stands for the regression curve under HP condition

LP and HP, respectively (Figure 4d). Seed P did not significantly affect seedling growth at LP or HP, when analysed for all genotypes together (Figure S2b). Likewise, RDW was little affected by seed P (Figure 4e).

There was, however, a robust relationship between the logarithmic TDW of all flints with the P uptake from the soil, both at LP and at HP; this substantial correlation was still observed when DH_LRs, dents and hybrids were included (Figure S2c). Overall, the seed P content was weakly, but significantly ($p < .001$) correlated (0.29) with TDW in LP, whereas the correlation was even higher in HP (0.36, Table S7). In HP, the P concentration was not significantly affected by the seed P, but in LP the P concentration even negatively correlated (−0.43) with seed P, whereas the P content and seed P content were positively correlated mostly in HP, due to increasing TDW (Table S7).

3.4 | Root traits affected by the breeding progress

We next considered root traits in HP and LP. Root morphological traits included total root length (TRL), specific root length (SRL), average root diameter (RootDiam) and average root hair length (RootHairLen). P mobilizing traits included the amounts of organic acid anions released from root tips (Carboxylates), rhizosphere soil pH (Rhizos-pH) and acid phosphatase activity at the root surface (APase).

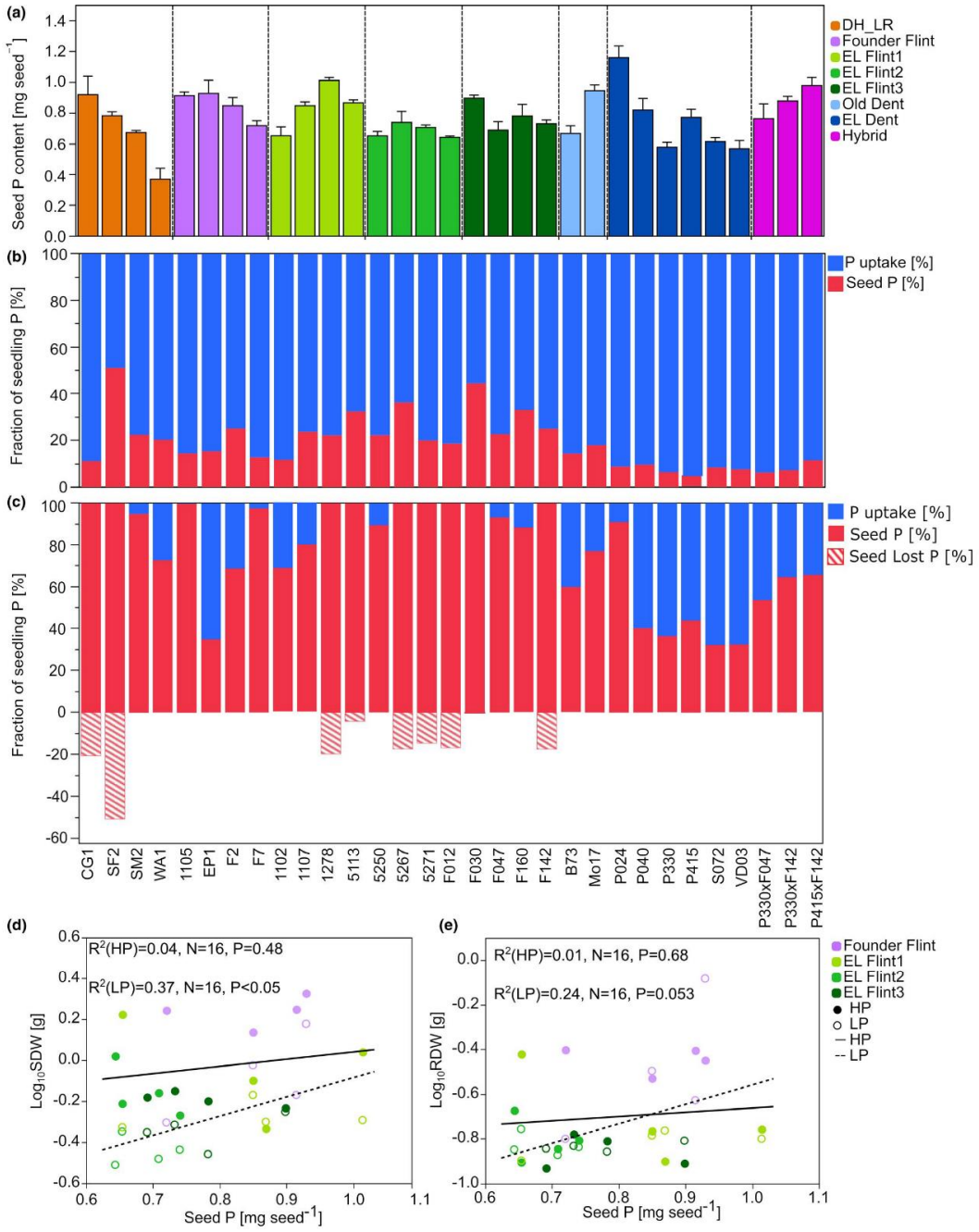

FIGURE 4 Use of seed P. (a) Seed P content in different genotypes. (b, c) The proportional origin of seedling P (red = stored seed P; blue = external P; shaded red: stored seed P lost to the soil) of different lines under high P (b) and low P conditions (c). (d) Relation between shoot dry weight (SDW) and seed P in the LP and HP treatments for 16 flints. (e) Relation between root dry weight (RDW) and seed P in the LP and HP treatments for 16 flints

There was a significant effect of genotypes on TRL and SRL, but there was no P treatment effect for these two measures (Tables S5). Genotypes, treatments and their interaction significantly influenced other root morphological traits, such as RootDiam, root-to-shoot ratio (Root/Shoot) and RootHairLen. P mobilizing traits, which include Carboxylates, Rhizos-pH and APase, were also significantly affected by the genotype, treatment and the interaction (Table S5).

When separately evaluated for the flint lines in LP, morphological traits, such as Root/Shoot ratio, TRL, SRL and RootDiam (Figure 5a-d), but also P mobilizing traits, such as APase and Carboxylates, were little affected by the breeding progress (Figure 5e-h). By contrast, Rhizo-pH and RootHairLen in LP declined in more recent released genotypes (Figure 5e-h). Root morphological and mobilizing traits were also measured in the other lines, and their measures are displayed in the same diagrams (Figure 5). Notable is the general decline of TRL with the breeding progress. This was comparable in modern dent lines and hybrids, but note the relatively thick RootDiam and increased Carboxylates in all dent lines and hybrids (Figure 5). DH_LRs were neither outstanding in morphological nor mobilizing traits and were similar to the moderately modern flints (Figure 5).

Although the internal remobilization of stored P was the crucial P source in LP at the investigated juvenile stage, all root traits except APase were significantly correlated with PAE in LP (Table 1). Overall, correlations of efficiency measures were more significant in LP than in HP. TRL and SRL were always associated with PUE and PAE, regardless of the P treatments. In LP, all root traits except APase and Root/Shoot were correlated considerably to PUE, and all root traits except APase were correlated with PAE. Furthermore, in LP, SRL, RootDiam and Rhizos-pH were correlated with PUtE, while APase was correlated with PUE and PAE in HP (Table 1). SRL was inversely correlated with all of the measured root functional traits, except TRL in LP. Most root functional traits inversely correlated with SRL in HP (Table 2). In LP, the strongest correlations for SRL were found with RootDiam ($r = -.70$), TRL ($r = .33$) and Root/Shoot ($r = -.35$), which is expected from the definitions of these traits. Within the HP treatment, correlations between SRL and TRL, Root/Shoot, RootDiam and APase were apparent. RootHairLen was significantly associated with all root traits, except SRL, Root/Shoot and APase in LP. Apart from SRL, a significantly negative correlation between two root traits under LP was only found between RootDiam and RootHairLen. In HP, TRL was negatively correlated with RootHairLen. APase showed no correlation with other root traits in LP and was therefore the least relevant trait for P use among all the eight functional traits in LP, although under HP, APase was found correlated with SRL (Table 2).

Moreover, Principal Component Analysis (PCA) with PUE, PAE and PUtE, as well as all eight root functional traits, revealed distinct patterns for founder and elite flints. The first two components together explained more than 58% of the total variation in both HP and LP treatments. Mostly in LP, founder flint genotypes were separated from the other lines (Figure 6). PUE and RootHairLen were mainly represented by PCA1, but interestingly, RootHairLen contributed oppositely in LP and HP (Figure 6).

4 | DISCUSSION

The application of mineral P fertilizers increases plant-available P in the soil and improves crop growth and yield, but its success dependents on many different factors such as type of fertilizer, application form, the timing of fertilization, weather conditions, various soil properties and plant factors (Holford, 1997; Shen et al., 2011). As P is a finite resource, breeding of major crops with improved P efficiency may have large effects on the sustainability of the agroecosystems (Ludewig et al., 2019). Efforts in breeding tropical maize for P-deficient, acid soils with high loads of toxic aluminium have already identified candidate loci for important root traits (Azevedo et al., 2015), an example of positive breeding impact on root traits.

4.1 | The decline of PUE in flint lines along with the breeding history

In this study, there was substantial variation in maize seedling growth of elite varieties under low and high P conditions. The biomass of modern flint seedlings decreased compared with founder flints, which was inconsistent with our expectations, as modern varieties are selected for higher final yields (Figures 1a and 2). In elite flints released from the 1950s to 2010s, PUE, PUtE and PAE decreased dramatically under both low P and high P (Figure 3a-c). Surprisingly, most DH_LRs performed similar to moderately modern elite flints (Figures 1a and 2). Apparently, there is no increase in P utilization accompanied with the breeding process in flints, but there are different trends in biomass and phosphorus utilization in dents and hybrids. The decline of PUE and PAE of elite flints was accompanied with smaller seedling root biomass despite the breeding progress (Figure 3), while Root/Shoot did not considerably change (Figure 5a). This may imply for flints that investment into the roots may be of less importance in well-managed soils with all nutrients amply available, as in typical breeding scenarios. The reduction in RootHairLen may explain part of the decrease in PAE under LP within the flint pool, as PAE and RootHairLen are positively correlated under LP (Table 1 and Figure 5h). In addition, elite flint lines were apparently on average not capable to acquire external P under LP conditions, in contrast to founder flint lines (Figure 4c).

In the conditions tested, most dent elite varieties performed superior than the flints. Those selected in the same public breeding programme released from the 1990s to 2010s generally had higher biomass, PUE, PUtE and PAE. These values were comparable to some flint founder lines (Figures 1d-g and 3a-c), which directly verified the hypothesis that increased biomass of modern elite varieties is associated with increased PUE, PAE and/or PUtE compared with old genotypes and landraces. PAE of all the genotypes was higher in HP than in LP, pointing to the importance of seed P under LP and probably reflecting the selection process with high P fertilization (Figure 1g). It is noteworthy that the founder flint EP1 was superior to most other flint lines in LP and similar to the modern elite dent P415. Moreover, EP1 was the only flint line that exhibited a similar P uptake as the dent lines,

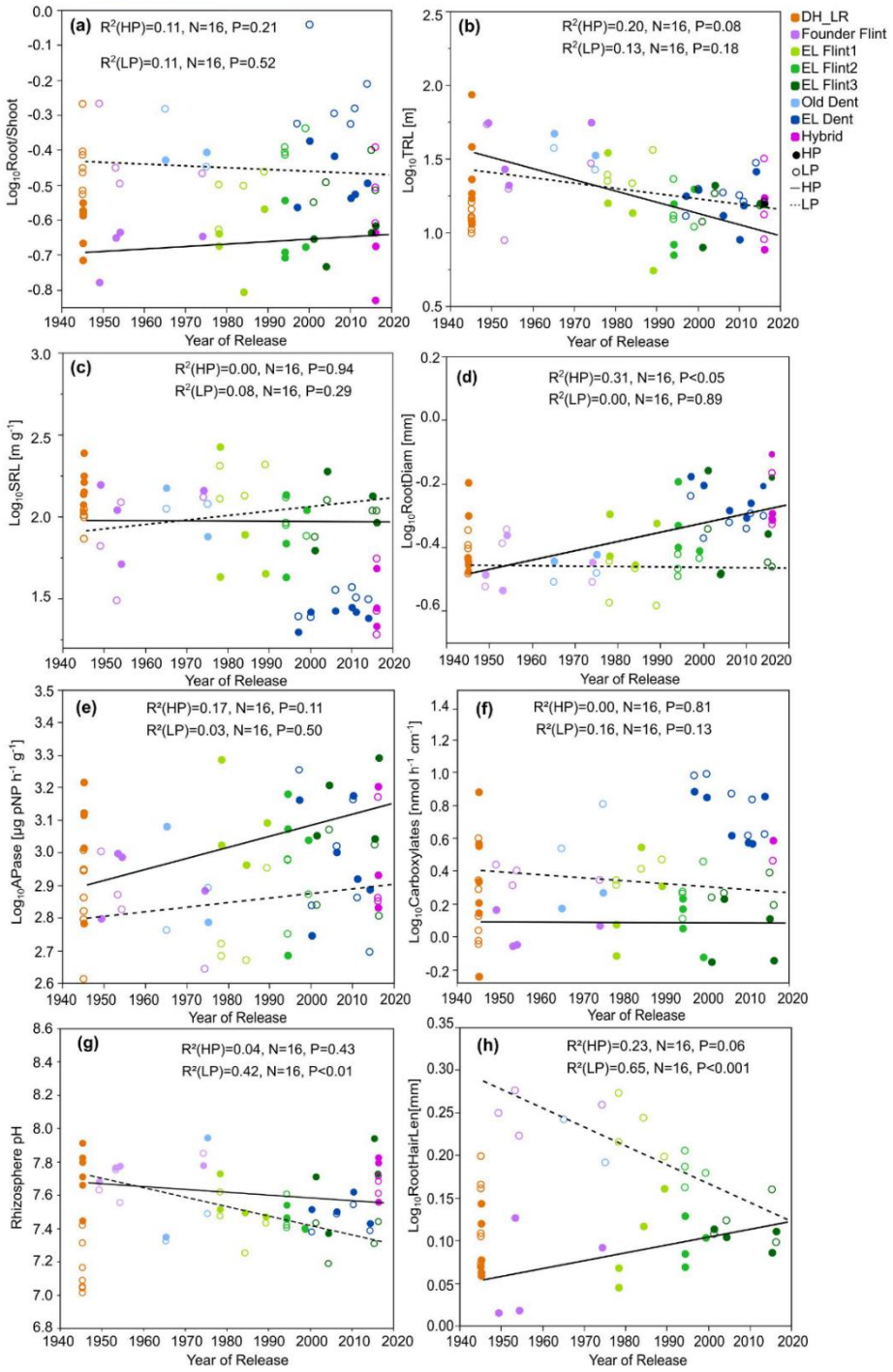
(a) R²(HP)=0.11, N=16, P=0.21
R²(LP)=0.11, N=16, P=0.52
Log10Root/Shoot
Year of Release
(b) R²(HP)=0.20, N=16, P=0.08
R²(LP)=0.13, N=16, P=0.18
Log10TRL [m]
Year of Release
DH_LR
Founder Flint
EL Flint1
EL Flint2
EL Flint3
Old Dent
EL Dent
Hybrid
HP
LP
HP
LP
(c) R²(HP)=0.00, N=16, P=0.94
R²(LP)=0.08, N=16, P=0.29
Log10SRL [m g-1]
Year of Release
(d) R²(HP)=0.31, N=16, P<0.05
R²(LP)=0.00, N=16, P=0.89
Log10RootDiam [mm]
Year of Release
(e) R²(HP)=0.17, N=16, P=0.11
R²(LP)=0.03, N=16, P=0.50
Log10APase [µg pNP h-1 g-1]
Year of Release
(f) R²(HP)=0.00, N=16, P=0.81
R²(LP)=0.16, N=16, P=0.13
Log10Carboxylates [nmol h-1 cm-1]
Year of Release
(g) R²(HP)=0.04, N=16, P=0.43
R²(LP)=0.42, N=16, P<0.01
Rhizosphere pH
Year of Release
(h) R²(HP)=0.23, N=16, P=0.06
R²(LP)=0.65, N=16, P<0.001
Log10RootHairLen[mm]
Year of Release

FIGURE 5 Root morphological and rhizosphere traits of genotypes and relation to their year of release. Mean performance of (a) root-to-shoot ratio, (b) total root length, (c) specific root length, (d) average root diameter, (e) acid phosphatase activity, (f) Carboxylates, (g) rhizosphere pH and (h) root hair length of 34 maize genotypes plotted versus the year of release of the lines and hybrids. Different colours represent different groups. The open circles represent the mean of each genotype under LP condition; the closed circles represent the mean of each genotype under HP condition. The linear regressions were separated for LP (dashed line) and HP (solid line) treatments and only included flint lines

TABLE 1 Pearson's correlation coefficients for P efficiency and root functional traits with log-transformed and non-transformed data (PAE) for 34 maize genotypes in response to high soil phosphorus availability and low soil phosphorus availability

Treatment	P efficiency	TRL	SRL	Root/Shoot	APase	RootDiam	Rhizos-pH	Carboxylates	RootHairLen
HP	PUE	**0.50****	**−0.38****	0.17	**−0.25***	0.15	−0.05	0.06	−0.19
	PAE	**0.29***	**−0.48*****	0.15	**−0.27***	**0.27***	−0.08	**0.27***	−0.22
	PUtE	**0.34*****	−0.17	−0.05	−0.1	−0.09	0.16	−0.22	−0.07
LP	PUE	**0.39*****	**−0.67*****	0.18	0.04	**0.41*****	**0.4*****	**0.45*****	**0.45*****
	PAE	**0.31****	**−0.54*****	**0.44*****	0.09	**0.20***	**0.28****	**0.42*****	**0.33*****
	PUtE	0.17	**−0.47*****	−0.03	0.03	**0.41*****	**0.26****	0.17	0.15

Note: The data for APase are without SM3 ($n = 33$); Rhizos-pH without P024 & P330 ($n = 32$); RootHairLen without SM3, dents& hybrids ($n = 22$); Carboxylates without P330 × F142 & P415 × F142 ($n = 32$). Root trait abbreviations: TRL, total root length; SRL, specific root length; Root/Shoot, root-to-shoot ratio; APase, acid phosphatase activity on the root surface; RootDiam, average diameter; Rhizos-pH, rhizosphere soil solution pH; RootHairLen, average root hair length; Carboxylates, the amounts of carboxylates in the rhizosheath. Significant correlations are in bold: *, $0.01 \le p < .05$; **, $0.001 \le p < .01$; ***, $p < .001$.

TABLE 2 Pearson's correlation coefficients for pairwise root functional traits with log-transformed data for 34 maize genotypes in response to high soil phosphorus availability (upper-right diagonal) and low soil phosphorus availability (low-left diagonal)

	TRL	SRL	Root/Shoot	APase	RootDiam	Rhizos-pH	Carboxylates	RootHairLen
TRL		**0.52*****	0.12	−0.07	−0.24	−0.01	−0.05	**−0.32****
SRL	**0.33*****		**−0.35****	**0.24***	**−0.46*****	0.07	−0.20	−0.16
Root/Shoot	**0.22***	**−0.35*****		−0.19	0.16	−0.10	0.17	−0.07
APase	−0.10	−0.18	0.16		0.02	−0.05	−0.14	0.18
RootDiam	**0.35*****	**−0.70*****	0.11	−0.07		−0.22	0.15	0.15
Rhizos-pH	0.12	**−0.31****	0.03	0.06	0.05		−0.09	−0.16
Carboxylates	0.18	**−0.43*****	**0.36*****	0.07	**0.21***	0.19		−0.08
RootHairLen	**0.37*****	−0.03	0.09	−0.20	**−0.36*****	**0.48*****	**0.22***	

Note: The data for APase are without SM3 ($n = 33$); Rhizos-pH without P024 & P330 ($n = 32$); RootHairLen without SM3, dents& hybrids ($n = 22$); Carboxylates without P330 × F142 & P415 × F142 ($n = 32$). Root trait abbreviations: TRL, total root length; SRL, specific root length; Root/Shoot, root-to-shoot ratio; APase, acid phosphatase activity on the root surface; RootDiam, average diameter; Rhizos-pH, rhizosphere soil solution pH; RootHairLen, average root hair length; Carboxylates, the amounts of carboxylates in the rhizosheath. Significant correlations are in bold: *, $0.01 \le p < .05$; **, $0.001 \le p < .01$; ***, $p < .001$.

of nearly 70% in LP. P415 was more responding to P fertilization than EP1 and doubled its biomass, while EP1 did not differ in TDW under LP and HP (Figure 2). Both lines were apparently P efficient in LP, but only P415 showed additional P responsiveness.

4.2 | Different P efficiency and root traits between flint and dent panels

Many examples have shown that P efficiency mechanisms are different from one genotype to another within a given plant species (Bayuelo-Jiménez et al., 2011; Colombi et al., 2019; Li et al., 2019; Ozturk et al., 2005; Tang et al., 2020; York et al., 2015). There are substantial variability and distinct genetic architectures in dent and flint maize diversity panels (Cartea et al., 1999; Messmer et al., 1992; Rincent et al., 2014), which is consistent with different P efficiency trends of the two panels. Due to different geographic separation and contrasting environmental conditions, phenotypic differences between these two germplasm pools are expected (Brown & Anderson, 1947; Unterseer et al., 2016). Much of the large variation in seedling biomass between these two germplasm pools was probably due to different P remobilization from seeds, and a

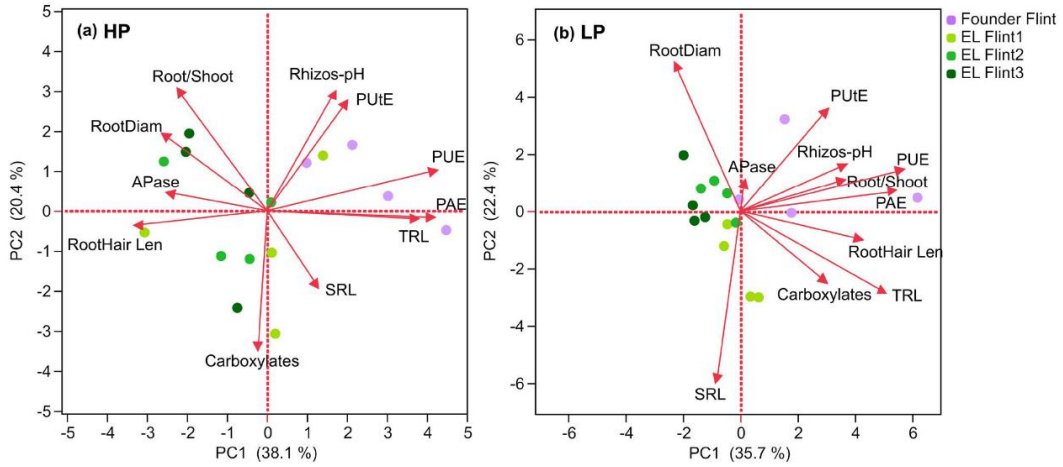

FIGURE 6 Principal component analysis (PCA) of root functional traits and phosphate efficiency for 16 genotypes in different classes with high (a) and low (b) soil phosphorus availability. Root trait abbreviations in figures: TRL, total length of the whole root system; SRL, length per dry mass of the whole root system; RootDiam, average diameter of the whole root system; Carboxylates, the total amount of organic acid anions collected by filter papers on the surface of root tips; APase, acid phosphatase activity in the rhizosheath. Rhizos-pH, the pH of the rhizosphere soil solution or pH in the rhizosheath; Root/Shoot, the ratio of root dry weight to shoot dry weight; PUE, phosphorus use efficiency; PAE, phosphorus acquisition efficiency; PUtE, phosphorus utilization efficiency

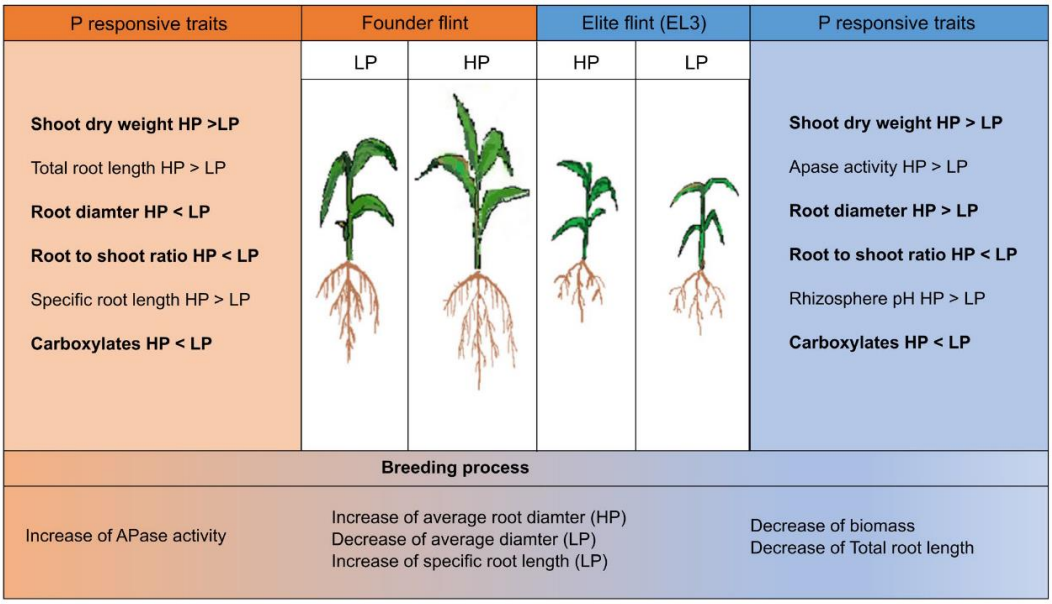

FIGURE 7 Schematic model summarizing the major observations of traits contributing to PUE, PAE and PUtE of founder flint and most modern flint EL3. Schematic plant appearance for LP and HP is shown

delayed switch to exogenous P acquisition in modern flints in LP. Especially in LP, seedlings of several genotypes (especially some in the Elite Flint2 and Elite Flint3 groups) even contained less P than originally stored in the seed (Figure 4c). This suggests that during germination these were not able to utilize the remobilized seed P and failed to import it into the seedling, resulting in a net seed P

loss. Massive losses of macronutrients and micro-nutrients during maize germination have been reported and can be prevented by silicon treatments (Moradtalab et al., 2018). In juvenile maize seedlings, re-uptake of transiently lost seed P from internal sources appears thus a major function of the root system. Previous research showed that the measurable P uptake by maize roots begins with a delay of around five days after germination, but in the variety of that study, the P uptake rate was independent of the initial seed P content and was little affected by external P (Nadeem et al., 2011; White & Veneklaas, 2012). For the different genetic backgrounds analysed here, there was a strong correlation of total dry mass with exogenous P taken up, especially in HP, but not with the seed P content (Table S7). Some dent elite lines (and hybrids) apparently switched earlier or more robustly to acquisition from exogenous P pools, so that they had already acquired substantial external P at the time of harvest (Figure 4). These consequently produced more seedling biomass.

Several root traits differed between flint and dent panels, and between different lines. On average, dent lines had increased RootDiam and released more Carboxylates, while TRL decreased in modern dents compared with old dents in HP (Figure 5). However, comparisons of the elite dents with these old dent lines must be taken with care, as their genetic origin is different, and also the sample size within these groups differed. In addition to the genetic differences, the temperature setting of the climate chamber may have been more favourable for the dent lines compared with the flints, the latter are cold tolerant and this may explain part of the differences between the flint and dent lines. While the different morphological characteristics between the flint and dent panels can be beneficial in view of hybrid breeding and the associated heterosis effect, it is important to note that beneficial traits in hybrids were more similar to dents, but were overall very little related to their flint or dent parents. The comparison of each hybrid with its parents indicated that all PUE traits were a complex additive combination of their parents that was dominated by the more efficient dent parent and was often, but not always, superior to its parents.

4.3 | Trade-offs of root traits related to PUE under LP

Several root traits were found to be associated with PUE in the breeding progress, but this was different in LP and HP. In HP, only TRL was positively correlated with PUE, implying that breeding with ample P resulted in an unintentional selection for TRL. In LP, TRL, RootDiam, Rhizos-pH, Carboxylates and RootHairLen were positively correlated with PUE, suggesting that genotypes bred under ample P remained some relationships with root traits important for limited P (Table 1). Moreover, RootHairLen and Carboxylates exhibited the highest positive contribution to PUE in LP (Table 1). Within the flint pool, several root traits were associated with the decrease in PUE in the breeding process: Rhizos-pH and RootHairLen in LP, and to a lesser extent RootDiam in HP (Figure 5). Founder flints had a weaker ability to acidify the rhizosphere and grew longer root hairs in limiting P conditions, which verified the hypothesis that modern elite genotypes lost beneficial root-related traits under limited P supply, because of selection under ample P. Interestingly, RootHairLen was the only root functional trait where Pearson's correlation of PAE significantly shifted from a negative to positive value with different P treatments (Table 1).

A wide variation and co-variation of key root and rhizosphere traits was found. This creates multiple P acquisition strategies that may be similarly efficient in an ecosystem (Brundrett & Tedersoo, 2018; Lambers et al., 2018; Zemunik et al., 2015). Previous studies showed that in controlled experimental conditions, different plant species showed different P acquisition strategies with different root and rhizosphere trait trade-offs. Maize with its fibrous root system invests relatively little into rhizosphere traits (Lyu et al., 2016; Wen et al., 2019). In our study, only TRL and SRL (morphological traits) were not significantly affected by P treatment, and all the traits (morphological traits and rhizosphere traits) showed variation among genotypes (Table S5). Meanwhile, the effect of the breeding process on TRL and SRL was not different among the P treatments (Figure 5b,c). A high co-variation of root functional traits was found in our 34 genotypes. In low P, the correlation between SRL and investigated root traits except TRL was negative (Table 2). The negative correlations between SRL and the RootDiam were already reported in other studies dealing with P responsive root traits within maize seedlings (Zhu & Lynch, 2004). This is due to its definition (SRL = TRL/RDW). Postma and Lynch (2011) claimed that with low P supply, maize roots increased cortical aerenchyma, which may lead to a decrease in root biomass and an increase in SRL, without changes in root diameter. RootHairLen was positively correlated with TRL, Carboxylates and Rhizos-pH under LP, but negatively correlated with RootDiam (Table 2), showing that in LP, genotypes with longer root hairs released more organic acids and had more fine roots. Many studies demonstrated the importance of fine roots in P uptake (Lynch, 2011). Therefore, it is assumed that the positive contribution of RootDiam to PUE is mainly based on the high correlation between RootDiam and the biomass. This may also explain the correlation between Root/Shoot and PAE in LP compared with HP.

As we have mentioned, TRL was important in both LP and HP for PUE; RootDiam, Rhizos-pH, Carboxylates and RootHairLen were more important in LP for PUE. In HP, TRL was negatively correlated with RootHairLen ($r = -.32$), and in LP, TRL was positively correlated with RootHairLen ($r = .37$) (Table 2). Moreover, the correlation between PUE and TRL decreased from 0.50 in HP to 0.39 in LP. The correlation between PUE and RootHairLen increased from −0.19 to 0.45 (Table 1). Altogether, this indicates trade-offs among the root traits, which verified our ultimate hypothesis. The variation and co-variation still existed when only considering exclusively the flint pool. In HP, there was a large variation of all these eight root functional traits; in LP, there was co-variation among TRL, RootHairLen,

Root/Shoot, Rhizos-pH and Carboxylates (Figure 6). Trade-offs between TRL and RootHairLen are found in the flint pool (Figure 6), which confirmed that breeding selection can have negative impacts under different environmental conditions.

Meanwhile, maize genotypes released in different breeding periods showed different P acquisition strategies. A schematic summary of the different strategies of old and modern elite flint lines to acquire and utilize P efficiently in LP and HP is given in Figure 7 and for dent lines in Figure S3. Dent lines exhibited the lowest SRL values, which means they produced thicker roots than flint lines. The high RootDiam within the dent lines is indicative of a different root system with long, thick seminal and crown roots (Figure 5h). On the other hand, the production of dent root biomass required less P (Table S6). Modern dent lines exhibited the highest PUE and had the highest average root diameter. The weak Root/Shoot and RootDiam correlation and that with several other traits that were different in LP and HP, potentially indicates a trade-off for acclimation to contrasting P conditions, as the increased contribution of one trait resulted in the decreased contribution of another trait (Table 2). The negative trend for PUE in juvenile flints was corroborated in another soil (Li et al., 2021), but as only young roots were considered here, field trials with the investigated genotypes are required to confirm the importance of juvenile PUE traits for adult plants and for final grain yield and grain PUE. Such experiments should also consider other traits that are important for P efficiency in adult plants, such as the presence of cortical root aerenchyma (Postma & Lynch, 2011) or the association with mycorrhiza (Li et al., 2021).

5 | CONCLUSION

When evaluating root traits associated with high P efficiency, seed P should also be considered. A decline in PUE in modern maize elite flint material was associated with smaller roots, shorter root hairs and less rhizosphere acidification, while high PUE was identified in elite dents and hybrids from the same breeding programme. Some preselected doubled haploid landraces were similar to modern elite varieties. Although the relationships of root traits and their contribution to optimal PUE, PUtE and PAE in juvenile maize flint and dent pools need to be further verified in different soils, growth conditions and in mature plants, our study may help to focus on improving P use in maize agroecosystems to contribute to more sustainable agriculture.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

We thank H. Ochott for help with nutrient analyses and Prof. Dr. Günter Neumann for discussion. X. L., M. M., A. M. and U. L. conceived the research. X. L., M. M. and U.L. designed the research. X. L. and M. M. conducted the experiments and analysed the data with help of H.-P. P. The manuscript was written by X. L. and M.M., and U.L edited the manuscript. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

Data are available in the supplement.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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5. Chapter II

Flint maize root mycorrhization and organic acid exudates under phosphorus deficiency: trends in breeding lines and doubled haploid lines from landraces

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Flint maize root mycorrhization and organic acid exudates under phosphorus deficiency: Trends in breeding lines and doubled haploid lines from landraces

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Abstract

Aims: High maize yields in modern well-managed agroecosystems depend on the use of elite varieties and hybrids. Unfortunately, because of repeated selection at high fertilizer rates, some beneficial traits, such as the interaction with arbuscular mycorrhizal fungi or the release of organic acid anions for phosphate mobilization and for attracting beneficial microorganisms, might be gradually declining in modern elite genotypes. However, old founder lines and landraces possibly carry genetic relicts that originate from pre-green revolution times that are useful for breeding elite material for low input farming systems.
Methods: Seedling colonization with arbuscular mycorrhizal fungi (AMF) and organic acid anion release were measured in flint lines that were released over more than five decades ago and in six preselected doubled haploid (DH) lines from landraces. P-uptake-related root traits were compared under P-sufficient and P-deficient conditions.
Results: Weak trends for the loss of AMF colonization or changes in organic acid anion release at low P supply were detected in modern varieties. One DH line from a landrace was found with increased mycorrhization, whereas others were similar to modern elite lines. Overall, substantial genetic variance was encountered for these traits.
Conclusions: The concern that modern elite maize varieties have lost beneficial traits for nutrient acquisition is not substantiated for the flint pool of maize, although weak trends exist. Lines associated with better P-acquisition efficiency under limited P availability should be utilized for breeding more sustainable varieties.

Supporting Information available online

Key words: AM fungi / breeding effect / maize (*Zea mays* L.) / organic anions / P-acquisition

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1 Introduction

Phosphorus (P) is globally one of the most critical plant nutrients and limits crop production in many regions all over the world. Although many soils have large reserves of total P, the inorganic P (P_i) and organic P (P_o) pools of most soils show low solubility and mobility, especially at acidic and slightly basic pH (*Akhtar* et al., 2008). In the soil, P is slowly converted chemically from sparingly soluble to insoluble forms with time, leading to relatively low concentrations of plant-available P within typical agricultural soils (< 10 μmol L^{-1}) (*Bieleski*, 1973). At the same time, further increases in agricultural output are needed to meet the global demand for food, feed and fuel (*Godfray* et al., 2010; *Davis* et al., 2016). As a result of dwindling productive agricultural zones, the cultivation of food and feed shifts to less advantageous soils. Therefore, yield increases under less favorable conditions, such as P-fixing or P-deficient soils, has become an inevitable challenge. With regard to P as a finite but essential resource, sustainable and resource-saving production is required to ensure stable food production in the future. Meeting these challenges will require improvements for nutrient efficiency traits (*Wissuwa* et al., 2009; *Wang* et al., 2010).

Arbuscular mycorrhizal fungi (AMF) play an important role in the acquisition of nutrients by their symbionts, especially phosphorus (P) (*Smith* and *Read*, 2008). The majority of plant species, including crop species, are responsive to mycorrhizal symbiosis. In maize, arbuscular mycorrhiza formation has been demonstrated to be more important than root hairs for seedling growth under low P availability (*Ma* et al., 2021). The degree to which plants respond to mycorrhizal symbiosis depends on: (1) the plant species and its root traits; (2) soil conditions, such as inherent fertility, pH, and management; and (3) AMF species. To generate agronomic benefits for maize (*Zea mays* L.) production, *Bender* et al. (2019) proposed inoculation under field conditions; however, their results show that the abundance and composition of native

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Correction added on 2 March 2021, after first online publication: Figure 3 has been corrected.

mycorrhizal communities determine the success of the establishment of the inoculant. Although conventional agricultural management can exert adverse effects on AM fungal communities (*Helgason* et al., 1998; *Oehl* et al., 2003), a high diversity of AMF can still be found in agricultural fields (*Hijri* et al., 2006; *Sasvári* and *Posta*, 2010). Thus, the use of the native AMF community to enhance mycorrhizal benefit for crops might be an alternative to the inoculation of non-native mycorrhizal strains (*Chave* et al., 2019). However, a better understanding of the effects of the native AMF community on the performance of the various crop species and crop varieties under field conditions is necessary.

Chemical modification of the rhizospheres of plant roots can also help with P_i acquisition by means such as the exudation of H^+, sugars, organic anions, amino acids, and secondary metabolites (*Hinsinger*, 2001; *Richardson* and *Simpson*, 2011; *Pang* et al., 2018). Organic acid anions, which have the potential to dissolve metals, such as iron, and phosphate from soil are typically exuded in small quantities in maize in comparison with the total root carbon costs (< 1%) (*Jones* and *Darrah*, 1995; *Jones*, 1998).

Recently, increasing evidence has been obtained that root functional traits change together with evolution history (*Reinhart* et al., 2012; *Ma* et al., 2018). Because of long-term agricultural practices, especially high fertilization and long-term organic residue accumulation, the total P pool per hectare has increased between 1900 and 2010 (*Sattari* et al., 2012; *Zhang* et al., 2017). As modern varieties have often been selected under high-nutrient input conditions to provide high yields, concerns are being raised that beneficial traits for P uptake under a limited P supply will gradually decline in elite varieties. Genes or traits related to efficient nutrient acquisition may become lost when all nutrients are directly available to plants, as plant adaptive traits to nutrient deficiency often result in additional carbon costs (*Wissuwa* et al., 2009; *Wang* et al., 2010). Indeed, in rice, a trait has been genetically identified for the improved P acquisition of landraces *via* larger roots. The *PSTOL1* gene underlying this trait has been cloned and transferred to modern varieties (*Gamuyao* et al., 2012; *Heuer* et al., 2017). By comparing older varieties or landraces with modern lines, the loss of root traits contributing to P uptake has also been reported in wheat cultivars, especially for traits dealing with mycorrhizal competence. High nutrient availability within selection sites is thought to reduce the benefit of symbiotic interactions (*Hetrick* et al., 1993; *Egle* et al., 1999; *Zhu* et al., 2001). However, recent research with modern soybean cultivars selected by conventional breeding approaches for higher yield has unintentionally led to plants that are adapted better to soil P fluctuations and that acquire more P from the P-rich zones (*Zhao* et al., 2004). Various maize genotypes (bred in P-rich or P-poor environments) perform differently in homogeneous and heterogeneous P soils. The genotypes bred in the P-rich environment have a competitive advantage under the heterogeneous P pattern, whereas the genotype bred in a P-poor environment has a stronger competitive ability under homogeneous P soil distribution (*Li* et al., 2019). Maize growth is also affected by soil microbes that are stimulated in the rhizosphere and that differ in abundance depending on nitrogen and P availabilities (*Bradáčová* et al., 2020). Improved maize growth can be obtained by optimized bacterial or fungal microbiome communities that depend on the carbon released by the roots of the plant (*Bradáčová* et al., 2019).

Maize is a major worldwide crop which in Germany is nowadays commonly grown with below-foot nutrient depots established at sowing. Thousands of varieties have been bred that can be grouped into three broad categories: landraces, open-pollinated populations, and hybrids. There is evidence that breeding has affected the extent of mycorrhizal colonization. A comparison of the mycorrhizal colonization of 141 inbred lines, 38 hybrids, and 76 landraces of maize has revealed that the percent of colonization varies greatly. Inbred lines that have been released in particular locations and years show significantly larger values than other lines. Modern hybrids exhibit significantly greater values than inbred lines and older landraces (*An* et al., 2010), but the year-of-release effect on colonization depends on the origin.

In this study, we initially selected 16 representative maize genotypes from the flint heterotic pool of a public temperate maize-breeding program that spanned the breeding progress from the onset of hybrid breeding from the 1950s to the 2010s (*Hölker* et al., 2019). We expanded these by studying doubled haploids recently produced from landraces and two commonly studied, sequenced old dent lines. Plants were grown in soil-sand mixtures with two P application levels; one of the soils was fresh and allowed mycorrhizal infection, whereas the other was essentially mycorrhiza-free (*Klamer* et al., 2019). Seedlings were grown in pots or rhizoboxes with observation windows that enabled the collection of root exudates.

We tested two hypotheses:

(1) Modern elite maize genotypes have lost the ability for beneficial mycorrhizal colonization under limited P supply, because of selection under an ample P supply.
(2) Modern elite maize genotypes release fewer organic acid anions to mobilize P under a limited P supply, but more under a high P supply.

2 Material and methods

2.1 Plant materials

Twenty-four maize genotypes were tested, including 16 flint lines, 6 doubled haploid lines (DH), and 2 dent lines (Tab. S1). The 16 flint lines covered a total of 67 years of hybrid breeding: 4 flint founder lines (EP1, DK105, F2, F7), which originated from various landraces as group FL, and 12 elite flint lines (1102, 1107, 1278, 5113, 5250, 5267, 5271, F012, F030, F047, F160, F142), which were developed by the maize breeding program of the University of Hohenheim (UHOH) and assigned to three groups, namely EL1, EL2, and EL3. The six DH lines were produced from four different landraces (group DH_LR), namely lines SM1, SM2, and SM3 from the Romanian population Satu Mare, lines SF1 and SF2 from the German population Strenzfelder, and line WA1 from the Swiss population Wallis. More details of the genetic back-

ground of these lines can be found in *Hölker* et al. (2019). The old dent lines B73 and Mo17 (as group Old Dent) are typical representatives of the BSSS × Lancaster heterotic pattern. Previous results showed that the grain P concentration of these genotypes except for WA1 (2.5 mg g^{-1} dry matter) ranged from 3 to 4 mg g^{-1} dry matter and the grain P contents were rather similar (Li et al., submitted).

2.2 Mini-rhizotron experiment

2.2.1 Plant growth

The subsoil used in the study was stored before use for more than 12 years (Tab. S2). Mycorrhization of this soil was checked with classical trypan blue staining methods and was confirmed to be nearly absent (*Neumann*, 2007; *Klamer* et al., 2019). The long-term stored subsoil was nutrient-poor, as shown in Tab. S2. The soil was collected from the calcareous C-horizon of a Luvisol (Wippenhausen near Freising, Bavaria, Germany) and so was called C-loess. Its soil water capacity was determined to be 27.4% w/w, and its soil water content was 2.9% w/w measured as the difference between the moist soil and the soil dried at 105°C, also known as the oven-dry weight.

In both treatments, the soil was fertilized with 200 mg N (NH_4NO_3), 200 mg K (K_2SO_4), 100 mg Mg ($MgSO_4 \cdot 7\ H_2O$), 2 mg Fe (EDTA-Fe-2Na), 2.6 mg Zn ($ZnSO_4 \cdot 7\ H_2O$), and 1 mg Cu ($CuSO_4 \cdot 5\ H_2O$) per kg dry soil to obtain a nutrient-rich soil substrate. The soil was then mixed with 30% (w/w) quartz sand for optimization of the soil texture. Phosphorus was added to the soil as $Ca(H_2PO_4)_2 \cdot H_2O$, with the levels of low P (LP) treatment at 32 mg P kg^{-1} soil and the high P treatment (HP) at 132 mg P kg^{-1} soil, respectively. The P availabilities in these mixtures were determined by CAL extraction, 9.8 mg kg^{-1} for LP and 61.8 mg kg^{-1} for HP, respectively. The soil–sand substrate was sieved to 5 mm before potting but after the addition of the nutrient solution. Soil moisture was set to 60% of the soil water-holding capacity and gravimetrically adjusted every two days.

The mini-rhizotron experiment was conducted in a climate chamber at the University of Hohenheim, Stuttgart Germany (48°42′44″ N, 9°12′30″ E). Seeds were surface-sterilized by rinsing them in 10% (v/v) H_2O_2 solution for 20 min. They were subsequently placed in an aerated 10 mM $CaSO_4$ solution at 25°C in the dark overnight. The next day, seeds were placed between filter paper soaked in a 4 mM $CaSO_4$ solution for around 3 d to germinate, and were then gently transferred to the soil-sand substrate. Plants were grown in half-cylinder rhizotrons (height 25 cm; diameter 10 cm). The open part of the half-tubes was covered with a transparent plexiglass observation window and secured with tape. The rhizotrons were arranged in an unblocked randomized design with 5 biological replicates for each treatment in the climate chamber. Because of the limited size of the climate chamber, several runs of growth were carried out from the summer of 2017 to the summer of 2018; experiment runs were therefore taken as a random effect when the results were interpreted. Photosynthetic Photon Flux Density was set to 300–350 μmol m^{-2} s^{-1} by adjusting the table height. Plants were harvested at 3 weeks after germination. The climate chamber temperature was maintained 25°C during the day and 20°C at night, air humidity was set to 60%, and day time was from 8 am to 10 pm.

2.2.2 Measured root traits

Eight root traits were determined at harvest; which included root to shoot ratio (Root/Shoot), total root length (TRL), specific root length (SRL), average root diameter (RootDiam), root hair length (RootHairLen), the amount of organic acid anions released from root tips (Carboxylates), rhizosphere soil pH (Rhizos-pH), and acid phosphatase activity at the root surface (APase).

Root exudates were collected from 1-cm subapical root zones of roots by application of sorption filter papers according to the method described by *Neumann* et al. (2014). For each mini-rhizotron, sampling was conducted with five replicates, and subsequently, the sorption filter papers were pooled. The pooled samples were re-extracted in 1 mL 80% (v/v) methanol and centrifuged at 12,000 rpm for 5 min. Aliquots of the supernatants (approximately 900 μL) were evaporated to dryness at 30°C using a Speed Vac Concentrator (Savant, Farmington, USA) and re-dissolved in isocratic elution buffer (18 mM KH_2PO_4, pH 2.2 adjusted with H_3PO_4). Organic acids were determined by RP-HPLC in the ion suppression mode according to the method described by (*Haase* et al., 2007) with isocratic elution buffer on a reversed-phase C-18 column (GROM-SIL 120 ODS ST, 5 μm particle size, 290 × 4.6 mm) at 40°C with direct UV detection at 210 nm. Identification and quantitative determination were conducted by comparison with known standards. Rhizosphere traits were measured as discussed previously (Li et al, submitted).

2.2.3 P and Mn status in shoots

The maize shoots were oven-dried at 60°C until constant weight for determining shoot dry weight. The dried material was ground to a fine powder. Shoot dry matter (around 250 mg per sample) was incubated with 5 mL HNO_3 (65%) and 3 mL H_2O_2 (30%) in a microwave (MLS Maxi 44, Germany) at a maximum of 210°C and 1400 W for 65 min. Then the solution was adjusted to 20 mL and filtered over activated charcoal and through 90-μm mesh filter paper. Shoot P was measured spectrophotometrically *via* orthophosphate determination after the addition of molybdate–vanadate reagent (*Gericke* and *Kurmies*, 1952). Shoot Mn was determined *via* Atomic Absorption Spectrophotometer (iCE 3000, Thermo Fisher Scientific, USA).

2.2.4 Calculation of P use efficiency (PUE)

Some measures of PUE were calculated by a modification of methods in *Hammond* et al. (2009).

$$\Delta\ Apparent\ agronomic\ P\ efficiency(\Delta APE,\ g\ DM\ g^{-1}P_s) = \left[(TDW_{high}) - (TDW_{low})\right] / \Delta P_{substrate}, \quad (1)$$

$$\Delta P\ uptake\ efficiency(\Delta PUpE,\ mg\ P\ mg^{-1}P_s) = [(P_{content\ high}) - (P_{content\ low})]/\Delta P_{substrate}, \quad (2)$$

$$\Delta P\ utilization\ efficiency(\Delta PUtE,\ g\ DM\ mg^{-1}P) = [(TDW_{high}) - (TDW_{low})]/[(P_{content\ high}) - (P_{content\ low})], \quad (3)$$

where TDW = Total dry weight, TDW_{high} = Total dry weight on a high P fertilized soil, TDW_{low} = Total dry weight on a low P fertilized soil, $P_{substrate}$ = P amount within the fertilized soil–sand mixture, $\Delta P_{substrate}$ = difference in amount P between high and low P treatment, $P_{content}$ = P content per plant, $P_{content\ high}$ = P content of a high fertilized plant, $P_{content\ low}$ = P content of a low fertilized plant.

2.3 Pot experiments

2.3.1 Plant growth

Two soils (C-loess and fresh soil) mixed with sand were used as substrates. Each pot contained 3 kg fresh soil, 1.5 kg C-loess, and 1.5 kg sand, giving 6 kg mixed soil–sand substrate in each pot. Fresh soils were collected from the grassland topsoil of Hohenheim Botanical Garden (Stuttgart, Baden-Wurttemberg, Germany) on 3rd October 2018, the chemical properties of which are shown in Tab. S2. Soil water capacity was determined to be 31% w/w, and soil water content was 10.7% w/w.

The pot experiment was conducted in the greenhouse of the Institute for Nutritional Crop Physiology (340h), Universität Hohenheim (48°42'44" N 9°12'30" E) in autumn, daytime was set to 14 hours. Seed germination, water moisture, and fertilizer application (except P) were the same as for the mini-rhizotron experiment. Phosphorus was added to the soil as $Ca(H_2PO_4)_2 \cdot H_2O$, but only for the HP treatment at 95 mg P kg^{-1} soil. At 5 weeks after germination, 50 mL of a 0.1 mol L^{-1} NH_4NO_3 solution was added per pot to avoid nitrogen deficiency. Plants were harvested at 8 weeks after germination. At harvest, differences in the shoot growth of the 24 genotypes could be observed under LP and HP conditions. Plants were at approximately the same developmental stage as the plants grown in rhizotrons.

2.3.2 P and Mn status of shoots

The plant shoots and roots were separated, and the shoot part was then oven-dried at 60°C until constant weight. Shoot P and Mn measurement were the same as those described in the previous section.

2.3.3 Root mycorrhizal colonization

After shoot excision, the roots were washed with deionized water in order to remove all soil from the root surface. Root samples were stored in 70% ethanol until further analysis. The roots were then stained according to *Brundrett* et al. (1994) with slight modifications. Briefly, the roots were cut into 1-cm length segments, cleared with 10% KOH at 90°C for 90 min (two runs, 45 min per run), rinsed three times with tap water, and acidified in 2 M HCl for 2 min. Finally, the samples were stained with 5% ink-acetic acid for 30 min at 60°C. Root pieces were then soaked in tap water acidified by drops of acetic acid overnight to remove excess ink (*Koske* and *Gemma*, 1989; *Vierheilig* et al., 1998). At least 30 randomly selected root pieces (around 1 cm long) from each replicate were fixed on microscope slides (10 per slide), and their AMF colonization was determined under a bright-field light microscope (Axioskop2, Zeiss, Germany). Mycorrhizal colonization was estimated for each root fragment dependent on the percentage of the root length colonized by AMF (*Trouvelot* et al., 1986). In brief, the mycorrhization degree of the root system (M) was calculated as follows:

$$M = \begin{pmatrix} 0.95\times N_5 + 0.7\times N_4 + 0.3\times N_3 + 0.05\times N_2 \\ +0.01\times N_1 \end{pmatrix} / N_{total} \times 100\%, \quad (4)$$

with N_x = number of root fragments in five intensity classes, namely 0.95, 0.70, 0.30, 0.05, and 0.01 as the relative coefficients of each intensity class, and N_{total} = total number of root fragments examined under the microscope. The mycorrhization intensity of mycorrhized root fragments (m) was determined as:

$$m = M\times N_{total}/N_m, \quad (5)$$

with N_m = number of mycorrhized root fragments. The arbuscule abundance of mycorrhized root fragments (a) was calculated as:

$$a = mA_3 + 0.5\times mA_2 + 0.1\times mA_1, \quad (6)$$

$$\text{with } mA_x = \begin{pmatrix} 0.95\times A_x^5 + 0.70\times A_x^4 + 0.30\times A_x^3 \\ +0.05\times A_x^2 + 0.01\times Ax_3^1 \end{pmatrix} / N_m \times m. \quad (7)$$

A_x^y represents, within the AMF colonization intensity class y, the number of root fragments with arbuscule abundance level A_x. The arbuscule abundance (A) of the root system was calculated as:

$$A = a\times M. \quad (8)$$

2.4 Statistical analyses

Before further processing, data validity was explored in histogram plots. A mixed model with the P level, maize genotype, and their interaction as fixed effects (other parameters were set random) was used for data analysis, and the normality of residuals and homogeneity of within-group variances was checked by using diagnostic plots. Data showing irregular or untypical residual plots (residual by predicted plot, residual by row plot, and residual quantile plot) were log-transformed. The transformed data were used to conduct the following analysis as its residual plots were more acceptable (*Piepho*, 2009; *Kozak* and *Piepho*, 2018).

In the mini-rhizotron experiment, the mixed model contained three fixed factors, namely P level, genotype, and their interaction, and four random effects, namely experiment run,

experiment run × P level, experiment run × genotype, and experiment run × genotype × P level. No significant contribution of random effects was found in the model, and so the random effects were removed from the mixed model. Finally, the mixed model with the P level, maize genotype, and their interaction as fixed effects was applied using transformed data for both experiments. The employed two-way ANOVA was based on the mixed model, followed by Student's t-test of all pairwise comparisons taking $p < 0.05$ as being significant. Mean values were used for fitting the generalized linear regression and Pearson's correlation analysis for each P treatment. Based on the P-efficiency and the P-responsiveness, 22 genotypes were then divided into four different efficiency and responsive classes, and the root trait contribution to the P-efficiency and the P-responsiveness was checked by a mixed model with the P-efficiency, the P-responsiveness, and their interaction as fixed effects. Subsequently, a Principal component analysis was performed to determine the multivariate ordination of nine root traits for 22 genotypes within the classes in each P treatment. All data were analyzed by the JMP Pro 15 statistical package (SAS Institute Inc., Cary, NC, USA).

3 Results

3.1 Seedling growth under two different conditions depends on P level and genotype release date

Flint genotypes from different breeding periods (founder lines, elite 1–3) were pooled into four groups according to their release date, and their seedling growth was analyzed at a similar developmental stage (5–7 leaves). As shown previously for slightly alkaline soil (Tab. S2; Li et al., submitted), mean shoot dry weights (SDW) of seedlings and the P concentrations of all groups were larger under high P (HP) than under low P (LP) conditions. The growth conditions in the two experiments varied in several ways: different soils (Tab. S2), different growth environments (climate chamber *versus* greenhouse), different growth temperatures, and pots *versus* rhizoboxes were used. Seedling SDW was highest in the founder lines in both experiments, whereas P concentrations were lower in founder lines (Fig. 1a, b). The P content tended to decline from the founder line group to more modern elite flints under HP, but not under LP conditions. Two old dent lines (B73, Mo17) performed similarly to the flint founder lines with respect to SDW and were used as controls. The group of DH from landraces performed most closely to the EL2 group. A significant correlation was found between genotype SDW performance in the two soil mixtures, and this was tighter under HP than under LP conditions (Fig. 1d). In agreement, the shoot P accumulations in both experimental soils were highly correlated (Fig. 1f), and the P concentrations in genotypes grown at LP always had lower shoot P concentrations than those grown at HP (Fig. 1e).

3.2 Breeding influence on mycorrhizal infection of roots

The mycorrhization of all genotypes was examined in the upper, middle, and lower parts of the roots in the soil mix with

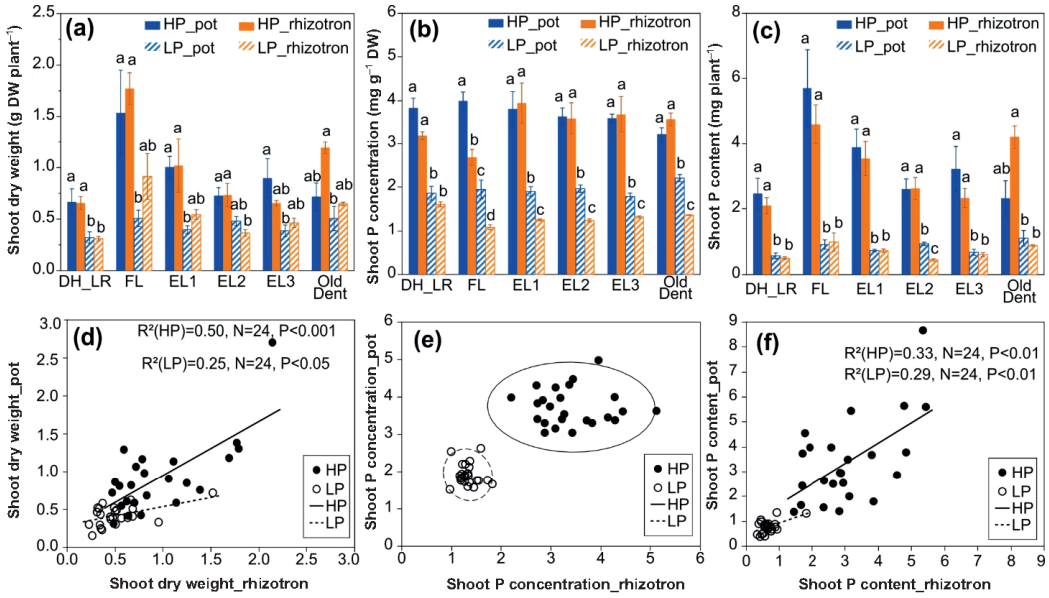

Figure 1: Mean performance for (a–c) shoot dry weight, shoot P concentration and shoot P content in the minirhizotron and the pot experiments. (d– f) Correlations between the two experiments (climate chamber/greenhouse, rhizotron/pot, alkaline/neutral soil). Different letters stand for significance at $p < 0.05$ within in each group (DH_LR, doubled-haploid lines from landraces: FL, founder lines; EL1–3, elite lines1–3) according to the result of Student's-t all pairwise comparisons.

fresh soil (Tab. S2) supplemented with mycorrhiza starter cultures and revealed various levels of mycorrhization (Fig. 2a, b). The employed scoring system had five categories, from 1 (low) to 5 (high), for the mycorrhization degree of the root system (M), the mycorrhization intensity of the infected fragments (m), the arbuscular abundance in the root system (A), and the arbuscular abundance in the mycorrhized root fragments (a) (see methods; *Trouvelot* et al., 1986). The P level had a strong effect on each of these values, whereas no genotype effect was observed on the arbuscular abundance in the mycorrhized root fragments (a) (Tab. S3). The mycorrhization parameters M, m, and A were affected by the genotype, but no genotype × P level interaction effects were found. Irrespective of the genotype, mycorrhization was always higher under LP than under HP conditions (Fig. 2c–f).

Broad variation for mycorrhization was encountered among the genotypes, but no significant trend for gain or loss of mycorrhization was apparent for the breeding lines when these were plotted against their release date (Fig. 2c–f). Most DH

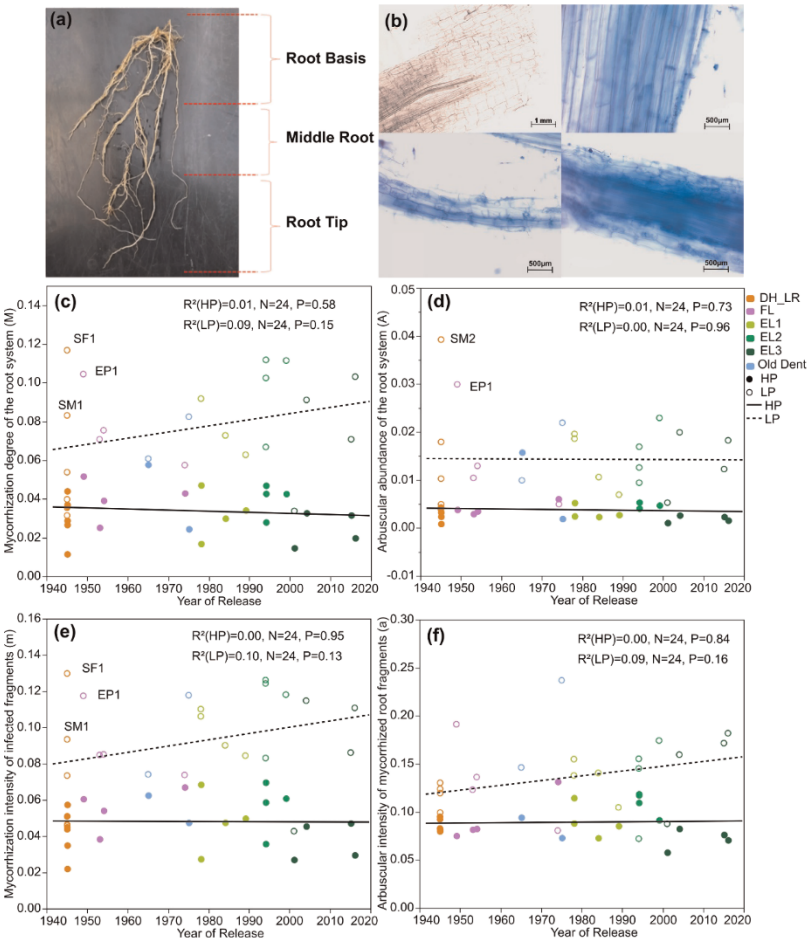

Figure 2: (a) Image of root sampling part and (b) Image of different levels of mycorrhized root fragment in the pot experiment. Scaling bar on the bottom right in figure (b). Mean performance for (c) mycorrhization degree of the root system (M), (d) arbuscular abundance of the root system (A), (e) mycorrhization intensity of infected fragments (m) and (f) arbuscular abundance of mycorrhized root fragments (a) of 24 flint maize genotypes plotted *versus* the year of release of the lines. Different colors represent groups from different breeding time periods. Open circles represent mean values under LP condition; closed circles represent mean values under HP condition. The regression line under LP and HP condition is dashed and solid, respectively. Note that the doubled-haploid lines from landraces were positioned before all breeding lines, although they were produced only recently.

lines from landraces were similar in mycorrhization compared with the breeding lines, but interestingly, the genotype SF1 from the German landrace Strenzfelder turned out to be the genotype with the highest mycorrhization degree, followed by the founder line EP1 (Fig. 2c). Furthermore, the arbuscular abundance of the root system was highest in line SM2 from the Romanian landrace Santu Mare, followed by EP1, but was low in other DH lines from this landrace (Fig. 2d).

Under LP but not under HP conditions, the mycorrhization degrees of root fragments (m) and of the entire root (M) were weakly correlated with the P concentrations and the P contents, but not with the Mn concentrations or Mn contents (Tab. S4). Arbuscular abundance in the root system (A) weakly correlated with the P content at LP and HP, but with SDW only at LP (Tab. S4).

The mycorrhization degree (M) and the arbuscular abundance (A) of the root system were weakly correlated with shoot P content only in the basal parts of the root ($R^2 = 0.29$, $p < 0.01$ and $R^2 = 0.37$, $p < 0.01$, respectively) under low P, and not in the middle or tip regions (Fig. S1). These correlations essentially vanished at HP (Fig. S2).

3.3 Breeding influence on organic acid anion release from roots

The major organic acid anions released from maize roots were malate, acetate, succinate, and citrate (Fig. 3a), of which only malate and citrate differed between LP and HP conditions. Whereas malate release decreased at LP, citrate release from the roots increased on average. *Trans*-aconitate, which was found in other studies as being highly exuded from maize roots, was unchanged between the two P levels and was of relatively low abundance (Fig. 3a). The influence of the genotype was generally weak on the exudation of individual organic acid anions, but a strong effect of the P level was encountered on the citrate, acetate, and succinate efflux (Tab. S5). Under HP conditions, the release of individual organic acid anions was not correlated with nutritional or yield components (Tab. S6), but citric, succinic, and *trans*-aconitic acid were correlated with SDW and the P content under LP conditions (Tab. S6). No correlation was found between the detected organic acids and shoot Mn concentration or shoot Mn content under either P treatment. No striking characteristics were exhibited in the exudates from the DH lines from the six landraces, although an interesting time trend was found for the release of citrate in the elite lines released during the different decades. Citric acid, which has the largest potential of the identified organic acids for P solubilization from soils, was most significantly correlated with SDW ($R^2 = 0.54$, $p < 0.01$; Tab. S6) and was found to decrease exudates in more recently released elite lines under LP conditions (Fig. 3b–e). Furthermore, weak declines of succinic acid at HP and of *trans*-aconitic acid at LP were encountered, and a slight increase of citric acid at HP was also identified associated with the history of the breeding lines (Fig. 3b–e).

3.4 Differences in P-responsiveness and P use efficiency among lines released within different decades

The relationship of TDW under LP with P-responsiveness, defined as the P content increase attributable to P application, and with other definitions of the apparent agronomic P use efficiencies is shown (Fig. 4a–c) based on data from the mini-rhizotron experiment. This procedure separated the genotypes into four classes: efficient and responsive genotypes (ER), non-efficient and responsive genotypes (NER), efficient but non-responsive genotypes (ENR), and non-efficient and non-responsive genotypes (NENR). Two genotypes (5113 and F030) even exhibited a slightly higher TDW under LP than under HP. Despite this interesting detail, these lines could thus not be assigned to any of the four groups.

Based on this classification, the DH lines from landraces and the most modern of the elite flint lines, EL Flint3, were consistently grouped into non-efficient classes, in agreement with their poor P utilization efficiency (Li et al., submitted). Founder flint lines were classified as efficient, with the most efficient EP1 line being classified as ENR (Fig. 4a). Its P uptake response (Fig. 4b) was classified as ER, whereas the P utilization efficiency was characterized as ENR (Fig. 4c).

Only a few root functional traits contributed highly significantly to the P-responsiveness (acid phosphatase activity; $p < 0.0001$) and P-efficiency (total root length; $p < 0.0001$) under HP conditions (Tab. S7). Under LP, the P-responsiveness was most highly correlated with total root length ($p = 0.0004$), whereas the contributions of total root length, root hair length , and root to shoot ratio to P-efficiency were most significant (Tab. S8). Interaction effects between P-efficiency and P-responsiveness were exclusively found under LP (Tab. S8).

3.5 Different strategies used to cope with low P in different classes

Four genotypes of each efficiency and response class were then selected to represent the impact of root traits on the P-efficiency and the P-response (Fig. 4d–h). These four genotypes were founder flint EP1 from the ENR class, founder flint DK105 from the ER class, F047 from EL3 in the NENR class, and F012 from the EL2 in NER class. The shoot/root ratio, total root length, average root diameter, and specific root length had characteristic differences between the efficiency classes (Fig. 4d–h). The efficient non-responsive EP1 line was characterized by a large increase in shoot root ratio and average root diameter under LP and lower specific root length with maintained total root length (Fig. 4d–g). The root diameter class between 0.2 and 0.4 mm (fine roots) was favored under LP in all genotypes (Fig. 4h). In comparison with the non-responsive genotypes, the TRLs of DK105 and F012 were significantly decreased under LP (Fig. 4e).

Moreover, differences between these representative lines of each efficiency class were encountered for rhizosphere traits, with the observation that efficient lines were typically characterized by longer root hairs and altered rhizosphere acidifica-

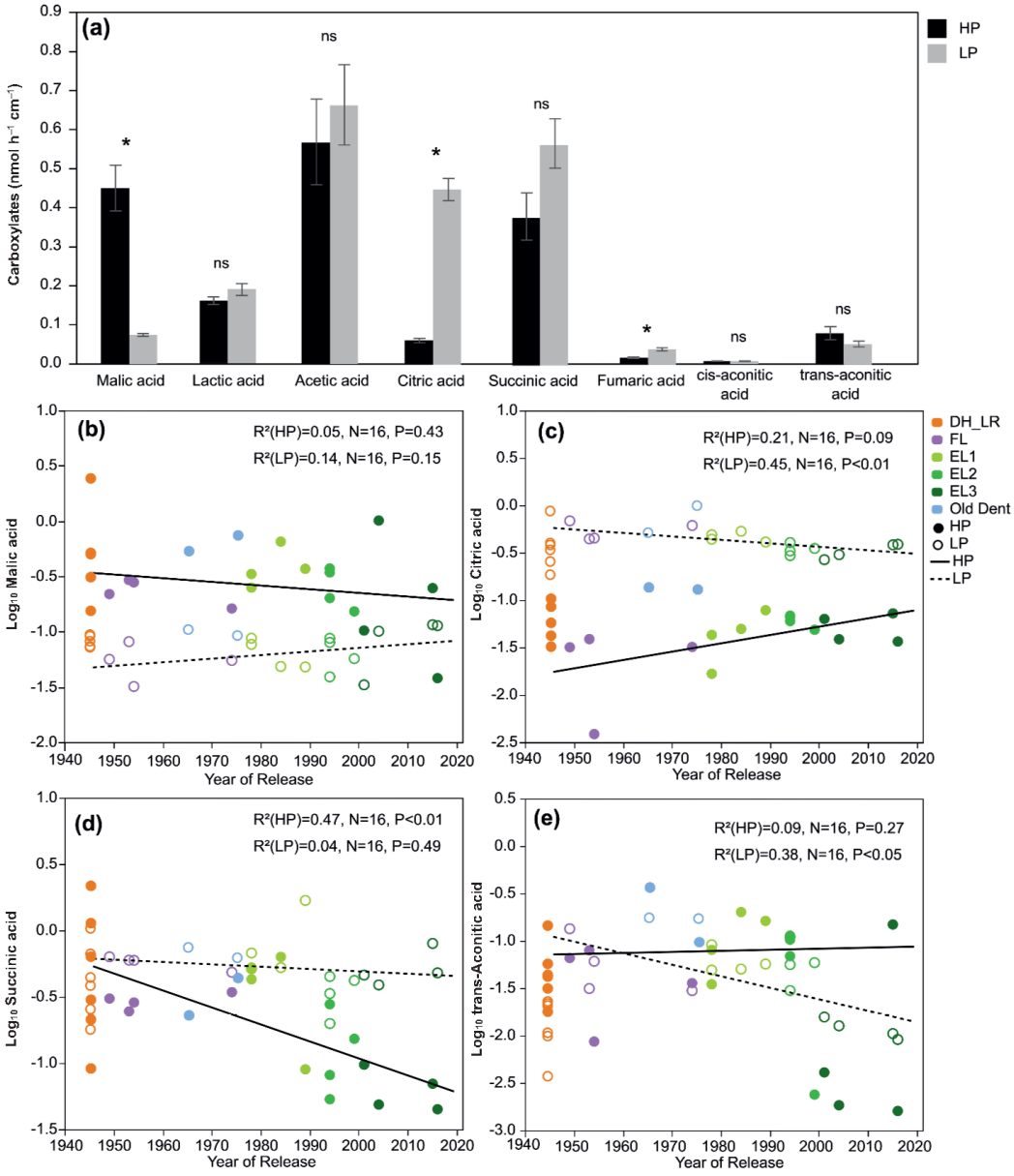

Figure 3: (a) Release of individual organic acids averaged over all genotypes under low P and high P conditions. Asterisks stand for significance at $p < 0.05$ according to the result of Student's-t all pairwise comparisons using transformed data; ns stands for no significant difference. Abundance of (b) malic acid, (c) citric acid, (d) succinic acid, and (e) trans-aconitic acid in exudates of all genotypes plotted *versus* the year of release using log transformed data. Different colors represent groups from different breeding time periods. Open and closed circles represent mean values under LP and HP conditions, respectively. The regression line under LP and HP condition is dashed and solid, respectively. Note that the doubled-haploid lines from landraces were positioned before all breeding lines, although they were produced only recently. And note that the regression lines were based on only 16 flint maize genotypes.

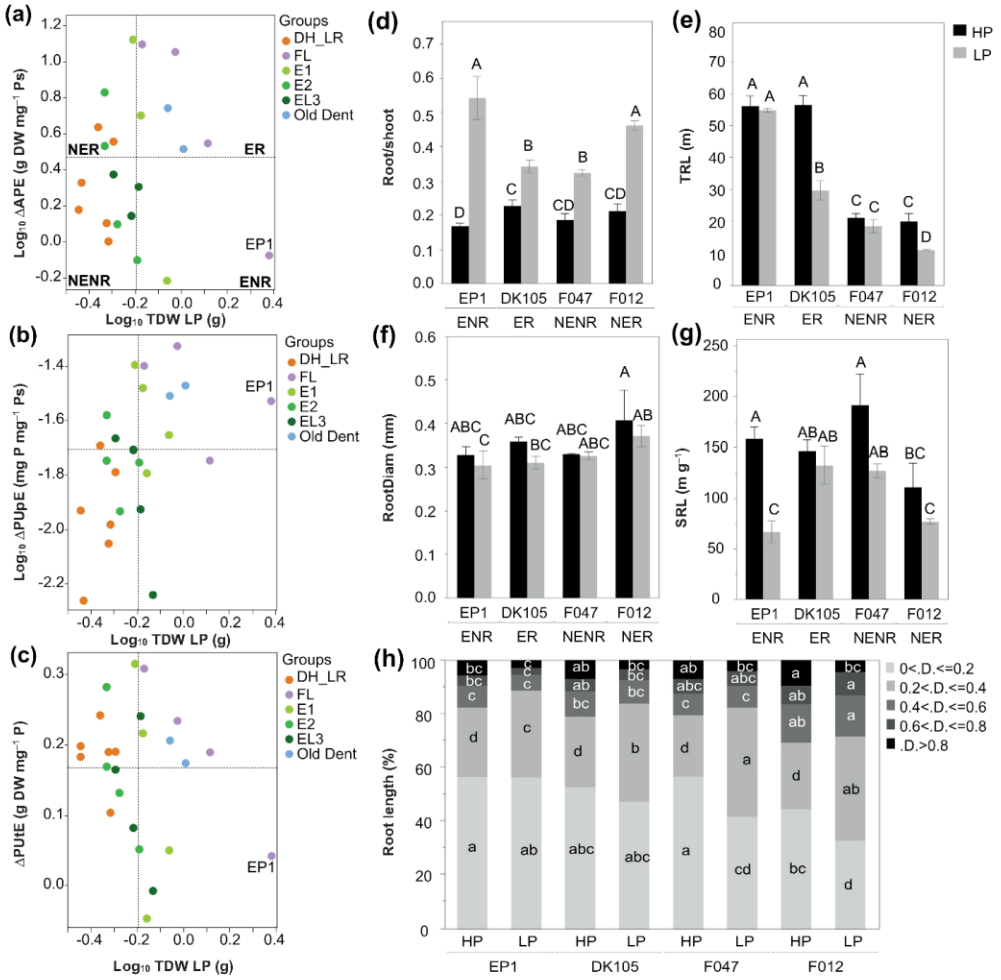

Figure 4: Relationship between total dry weight under low P (TDW) and responsiveness to P measured as (a) Δ apparent agronomic P use efficiency (ΔAPE), (b) Δ P uptake efficiency (ΔPUpE), and (c) Δ P utilization efficiency (ΔPUtE). Each circle represents the mean of each genotype with five replicates. Dashed lines represent the mean value of the axis. NER = non-efficient and responsive, ER = efficient and responsive, NENR = non-efficient and non-responsive, ENR = efficient and non-responsive. Data without genotypes 5113 and F030 (n = 22). The genotype with highest TDW in LP was EP1. Mean performance ± SE for (d) root to shoot ratio, (e) total root length, (f) average root diameter, (g) specific root length, and (h) root diameter classes of four typical genotypes grouped in four different classes based on P efficiency and responsiveness. Different letters stand for significance at $p < 0.05$ according to the result of Student's-t all pairwise comparisons using log transformed data.

tion (Fig. 5b, d). In contrast, responsive genotypes significantly differed with respect to their release of carboxylates (Fig. 5c). The moderate differences between the different components of mycorrhization were non-significant (Fig. S3).

3.6 Multivariate ordination of efficient and responsive genotypes

Principal component analysis (PCA) was conducted to address relationships among root traits and to determine the major trait components that explained the variation in the original data. The genotypes grown under HP (Fig. 6a) and LP (Fig. 6b) were associated with nine root functional traits. At HP, the principal component 1 (PC1) was dominated by TRL, SRL, and mycorrhization degree, and RootDiam. PC1 explained 22.7% of the variance, whereas the second principal component (PC2) explained 19.7% of the variance.

Under LP, PC1 (dominated by RootHairLen, TRL, Rhizo-pH, and RootDiam) explained 28.7% of the variance, whereas

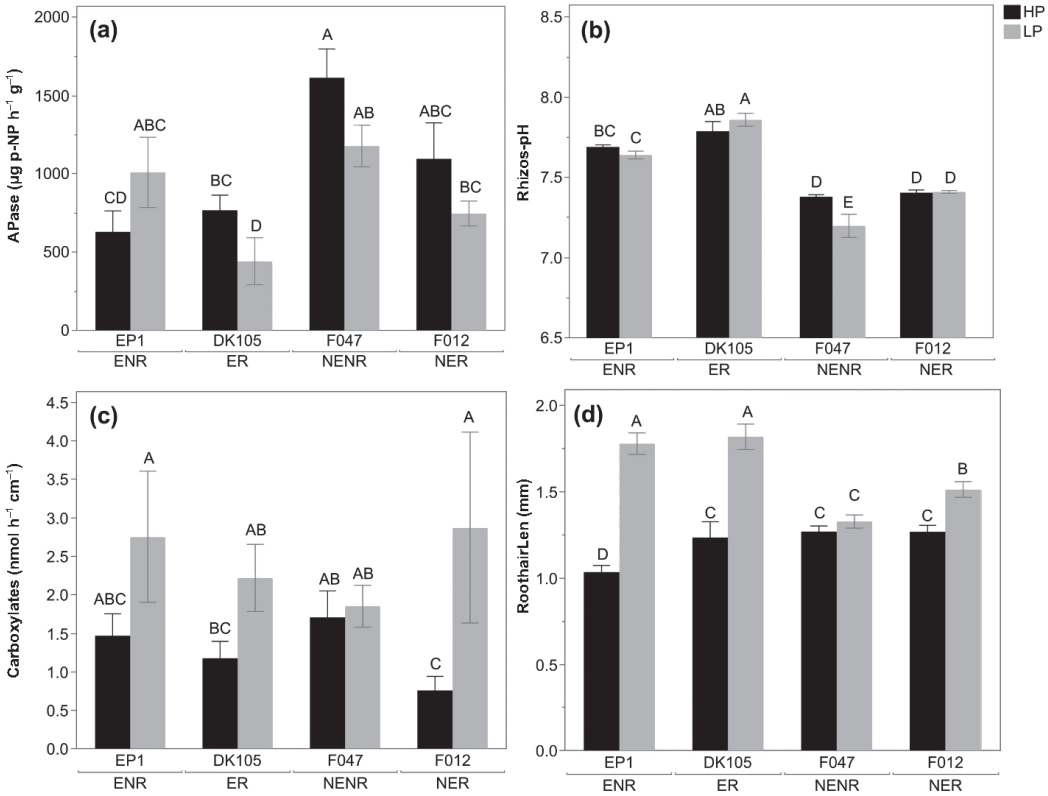

Figure 5: Mean performance ± SE for (a) Acid Phosphatase activity, (b) Rhizosphere pH, (c) Carboxylates, and (d) Root hair length of typical four genotypes grouped in four different classes based on P efficiency and responsiveness. ENR = efficient and non-responsive, ER = efficient and responsive, NENR = non-efficient and non-responsive, NER = non-efficient and responsive. Different letters stand for significance at $p < 0.05$ according to the result of Student's-t all pairwise comparisons using log transformed data.

PC2 explained 20.4% (dominated by Root/Shoot, APase, SRL). This separated both the different response classes and the genotype groups from the different decades (Fig. 6b). P-efficiency under LP were mostly separated by PC1 and efficient genotypes clustered at the right side of the diagram, irrespective of being P-responsive or not. Whereas P-responsiveness was poorly explained by each PC component under LP. By contrast, P-responsiveness was more clearly explained by PC2 under HP (Fig. 6a, b).

4 Discussion

4.1 AM colonization changed little under limited P supply during maize breeding process

We grew European flint maize genotypes with different breeding histories in two soil mixes under different conditions and observed that seedling biomass was more substantially determined genetically at HP compared with LP (Fig. 1), whereas shoot content was similarly correlated between both experiments at both LP and HP (Fig. 1). The group of founder lines had higher seedling biomass compared with that of modern lines, whereas recently developed DH from landraces were, on average, similar to moderate recent elite lines (Fig. 1). AMF colonization, a typical response to coping with P deficiency in native environments and crops (*Smith* et al., 2011), modulates the relationship between root growth and nutrient acquisition in maize (*Ramírez-Flores* et al., 2019). In our conditions, AMF formation was restricted to one soil mix where it was analyzed in more detail. All genotypes in our study showed increased colonization under low P availability (Fig. 2), and only under this condition was the mycorrhizal colonization correlated with shoot P content, although arbuscular frequency was also correlated to some extent to the shoot concentration and the shoot dry mass (Tab. S4). Interestingly, we found that only the mycorrhizal colonization of root in the top soil was significantly correlated with the shoot P accumulation, whereas the middle and bottom regions of pots were not (Fig. S1). This is consistent with previous findings showing that species with thicker roots, such as young maize, tend to have a higher colonization by AMF (*Li* et al., 2017;

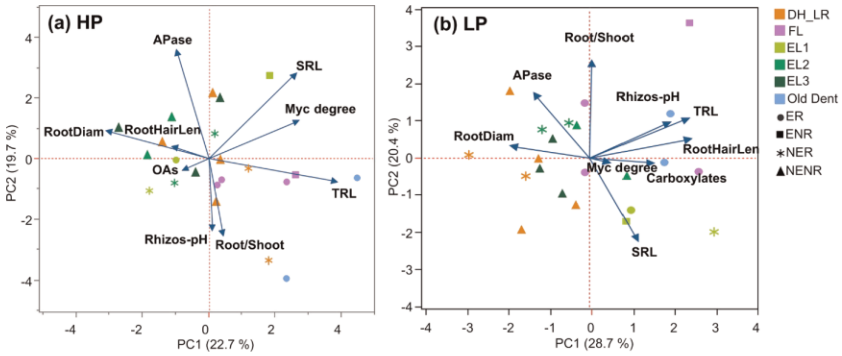

Figure 6: Principal component analysis (PCA) of nine root functional traits for 22 genotypes in different class with high (a) and low (b) soil phosphorus availability. Root trait abbreviations in figures: TRL, total length of the whole root system; SRL, length per dry mass of the whole root system; RootDiam, average diameter of the whole root system; Carboxylates, the total amount of organic acid anions collected by filter papers on the surface of root tips; APase, acid phosphatase activity in the rhizosheath. Rhizos-pH, the pH of the rhizosphere soil solution or pH in the rhizosheath; Root/Shoot, the ratio of root dry weight to shoot dry weight; Myc degree, mycorrhizal colonization degree of the root

Wen et al., 2019), but contrasts with data from chickpea genotypes in which thinner roots had a relatively higher colonization by AMF (*Wen* et al., 2020). These differences indicate that alternative AMF interaction strategies are adopted by different species and possibly by different genotypes within a species. Concern has been expressed that, for crops such as maize, mycorrhizal colonization and responsiveness have decreased, even during the relatively short period of breeding selection from the 1950s to 2000s, although only a very limited number of lines has been evaluated for this trait (*Chu* et al., 2013, 2020). On evolutionary time scales, plants have clearly reduced their dependence on symbiotic mycorrhizal fungi since they first emerged in land ecosystems (*Ma* et al., 2018). Indeed, a comparison of a landrace with hybrid maize varieties has revealed a better response to AMF mycorrhization (*Londoño* et al., 2019), whereas mycorrhizal infection was even more pronounced in a modern European elite line compared with an African line (*Wright* et al., 2005). Here, no obvious trend for a loss of mycorrhization during the breeding process for European flint genotypes was observed, although the founder line EP1 showed particularly high mycorrhization (Fig. 2). Furthermore, the DH line SF1 had the highest mycorrhization degree/intensity and SM2 the highest arbuscular abundance of the root system (Fig. 2), but interestingly, several other DH lines from the same landrace were even less colonized than the modern flints. SF1 or SM2 may therefore be good candidates for breeding for superior AMF mycorrhization or can be used as parents for quantitative trait loci (QTL) studies to identify genetic components of mycorrhization. However, although such material might contribute interesting genetics to modern elite lines, DH lines from landraces are not generally superior with regard to these traits than modern elite lines.

4.2 Modern elite flints release less citrate/succinate than founder flints under LP to mobilize P

Despite limited relevance for larger quantities of dissolution of soil P, root organic acid anion exudation (*Pearse* et al., 2006, 2007; *Oburger* et al., 2011; *Lyu* et al., 2016; *Wang* et al., 2016) for the mobilization of adsorbed P from soil particles can increase the absorption of phosphorus by plants (*Gerke* et al., 2000). The amount of carboxylates exuded from roots varied in the 24 maize genotypes under both LP and HP conditions, but on average, citrate was the only major carboxylate that was more highly released under LP (Fig. 3a). Citric acid was correlated with shoot dry weight and shoot P accumulation, together with succinic acid and *trans*-aconitic acid (Tab. S5). Importantly, the mobilization efficiency of P_i by the organic acid anions in many soils is citrate > oxalate > malate > trans-aconitate > succinate > acetate (*Jones*, 1998; *Gaume*, 2000), and interestingly, the amount of citric acid and *trans*-aconitic acid in exudates has declined during the breeding process under LP conditions (Fig. 3b–e). This suggests that modern elite genotypes release less beneficial organic anions to mobilize P under limited P supply than old genotypes. Other functions of root exudation, such as attracting other beneficial microbes, may be related to the trends to release more malate under HP conditions or other changes in root organic acid anion exudates. The release of malate and citrate by Aluminum-Activated Malate Transporters (ALMT) and Multidrug and Toxic Compound Extrusion (MATE) families are among the well-understood transporter candidates involved in exudation (*Sasse* et al., 2018). Previous findings also showed that AM induces malic acid accumulation in the roots of water-stressed maize plants (*Hu* and *Chen*, 2020), but the organic acid in exudates do not increase.

4.3 Patterns of efficient and responsive flints

The lines were grouped into four categories, according to the definitions of apparent agronomic use efficiency and their responsiveness to P, with the founder line EP1 being exceptionally efficient (Fig. 4a–c). The increase in the Root/Shoot ratio is a well-known strategy of plants for coping with P-deficiency and was consistently found in our experiments. However, the P-efficiency was not significantly affected by the ratio. In terms of P-efficiency and P-responsiveness, root traits associated with physiology and architecture are more important than the root biomass itself (Tab. S8).

Root traits associated with efficient P use (such as in EP1) were characterized by a large investment into not only long laterals, but also thick shoot-borne roots. Roots thicker than 0.2 and thinner than 0.4 mm are lateral roots (*Tai* et al., 2016), and these were promoted most strongly under low P, irrespective of genotype and class (Fig. 4d–h). Because of the different root types contributing to P-efficiency in maize, the specific root length, a typically valuable measure of the high P use efficiency of plants, was studied and shown to be low in the most P-efficient genotypes (Fig. 4d–h). Rhizosphere pH and root length were associated with P-efficiency (Fig. 5) and contributed similarly to the prediction of the P-efficient genotypes under LP conditions, *e.g.*, TRL and RootDiam (Fig. 6). The latter two criteria contributed in an opposite way to PCA1 under HP conditions, revealing the dilemma that beneficial traits under LP conditions may be different from those under HP and underpinning the importance of the environment in which crops are selected during the breeding process.

5 Conclusions

Mycorrhizal colonization is apparently little affected by the period of intensive breeding in maize during the past five decades, but trends for loss of citrate release have been found in the European elite flint maize breeding lines. Two of the studied DH lines from landraces, but no other examined DH lines produced from this germplasm pool, showed the highest mycorrhization colonization/arbuscule numbers under limited P conditions. Lack of effects on the mycorrhization and other traits during intensive breeding may indicate that (1) the selection pressure on these traits was low, (2) that these traits have low heritability, or (3) the traits are little correlated with the selected features. The latter would argue that one could select for mycorrhization without to change the agronomically important characteristics of maize in a negative way.

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Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

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6. Chapter III

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

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

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• **Background and Aims** An increase in root hair length and density and the development of arbuscular mycorrhiza symbiosis are two alternative strategies of most plants to increase the root–soil surface area under phosphorus (P) deficiency. Across many plant species, root hair length and mycorrhization density are inversely correlated. Root architecture, rooting density and physiology also differ between species. This study aims to understand the relationship among root hairs, arbuscular mycorrhizal fungi (AMF) colonization, plant growth, P acquisition and mycorrhizal-specific P_i transporter gene expression in maize.
• **Methods** Using nearly isogenic maize lines, the B73 wild type and the *rth3* root hairless mutant, we quantified the effect of root hairs and AMF infection in a calcareous soil under P deficiency through a combined analysis of morphological, physiological and molecular factors.
• **Key Results** Wild-type root hairs extended the rhizosphere for acid phosphatase activity by 0.5 mm compared with the *rth*3 hairless mutant, as measured by *in situ* zymography. Total root length of the wild type was longer than that of *rth3* under P deficiency. Higher AMF colonization and mycorrhiza-induced phosphate transporter gene expression were identified in the mutant under P deficiency, but plant growth and P acquisition were similar between mutant and the wild type. The mycorrhizal dependency of maize was 33 % higher than the root hair dependency.
• **Conclusions** The results identified larger mycorrhizal dependency than root hair dependency under P deficiency in maize. Root hairs and AMF inoculation are two alternative ways to increase P_i acquisition under P deficiency, but these two strategies compete with each other.

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

INTRODUCTION

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

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

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

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

Maize (*Zea mays*) is one of the most widely cultivated crops for production of both food and fodder in tropical and temperate soils worldwide (Gore *et al.*, 2009). Two maize genotypes with contrasting root hair morphology, the root hairless mutant 3 (*rth*3) (Wen and Schnable, 1994) and its corresponding wild type (B73), were used in this study. The wild-type and *rth3* roots had similar diameter but contrasting root hair morphology: the wild type's roots were covered by dense root hairs around 1 mm long, while the root epidermis of *rth*3 had only very short bumps in the root hair zone and appeared completely bald (Fig. 1). Previous studies showed that there was no growth difference between the wild type and *rth3* in terms of shoot biomass, root biomass, mycorrhizal colonization and shoot P content under P-sufficient conditions (Kumar *et al.*, 2019), and *rth3* can grow vigorously under standard conditions with sufficient nutrition (Wen and Schnable, 1994; Hochholdinger *et al.*, 2008). In this study we aimed to understand the effects of the presence of root hairs, AM colonization and its interaction with root hairs for P acquisition and plant growth under P deficiency. We hypothesized that (1) the wild type is more adaptive to P deficiency than *rth*3, because of the presence of root hairs; (2) the contribution of AMF to plant growth and P_i acquisition is generally larger (especially large in *rth3*) and more critical than that of root hairs, and cannot be compensated by root hairs.

MATERIALS AND METHODS

Plant material and cultivation

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

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

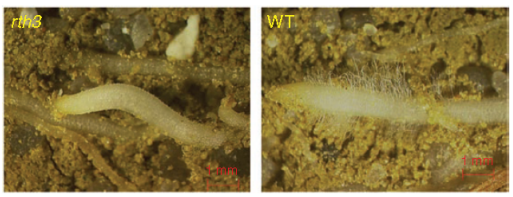

FIG. 1. Microscopic images of maize root hairless mutant *rth3* (left) and wild type (WT, right) grown in soil in rhizoboxes.

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

Root hair length and organic acids in the root exudates

At harvest, pictures from the root hair zone were taken with a Stemi 2000-C video microscope equipped with Axio Vision 3.1 software (Zeiss, Oberkochen, Germany). From these pictures, root hair length was determined by taking the average length of ten root hairs per plant (Weber *et al.*, 2018). Afterwards, according to the method described by Neumann *et al.* (2014), root exudates were collected from a 1-cm subapical root area, visible on the observation window, by applying special sorption filter papers (MN156050, Macherey-Nagel, Munich, Germany). Sampling was conducted with five replicates for each minirhizotron, and after 2 h of collection the sorption filter papers were collected. The collected samples were re-extracted with 1 mL of 80 % (v/v) methanol and centrifuged at 18 000 *g* for 5 min. The supernatants (around 900 µL) were evaporated using a SpeedVac Concentrator (Savant, Farmington, USA) until dryness at 30 °C and re-dissolved in HPLC elution buffer (18 mM KH_2PO_4, pH 2.2 adjusted with H_3PO_4). The organic acids were determined by reversed-phase HPLC in the ion suppression mode with direct UV detection at 210 nm according to the method described by Haase *et al.* (2007). Isocratic elution was performed with 18 mM KH_2PO_4, pH 2.2, on a reversed-phase C-18 column (290 × 4.6 mm, GROM-SIL 120 ODS ST, 5 µm particle size), which was equipped with a 20 × 4.6 mm guard column with the same stationary phase (Dr Maisch, Ammerbuch, Germany). Identification and quantitative determination were conducted by comparison with known standards.

Rhizosphere acid phosphatase activities determined by zymography and image analysis

To visualize the acid phosphatase activity in the rhizosphere, direct zymography was used after the collection of the root exudates (Sanaullah *et al.*, 2016). Methylumbelliferyl (MUF) phosphate substrate was dissolved in MES buffer (pH 6.5) to obtain a working solution with a final concentration of 1 mM. Thin polyamide membrane filters (Tao Yuan, China) with a pore size of 0.45 mm were saturated with substrate by soaking in the working solution. The membrane saturated with the substrate was applied directly to the rooted soil surface and incubated for 1 h. Fluorescent substrates in the membranes diffused into the soil and were hydrolysed by enzymes to release fluorescent MUF, which diffused back and was visualized under UV light (Spohn and Kuzyakov, 2014). After incubation the membranes were carefully lifted off the soil surface and all attached soil particles were gently removed using a soft brush. The membranes were placed under UV light (excitation, 365 nm) in a Geneflash Documentation System (Syngene, Cambridge, UK) in a dark room and photographs of the membranes were taken. The imaging equipment, the camera (DSLR-A300, Sony, Japan) settings, exposure time (5 s) and positions of the camera and the membrane were fixed and constant for all samples. A calibration line was set up using membrane squares (4 cm^2) that were soaked in solutions of increasing MUF concentration (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mM). The amount of MUF per unit area was calculated by dividing the volume of the solution absorbed by the membrane by the size of the membrane (Ma *et al.*, 2019). The membranes used for the calibration line were imaged in the same way as the samples.

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

Shoot dry weight and P analysis

The maize shoots were oven-dried at 60 °C for 3 d to determine shoot dry weight. Dried shoot material was ground to a fine powder. Two hundred and fifty milligrams of shoot dry matter was incubated with 65 % HNO_3, 10 mM H_2O_2 and distilled water in a microwave (MLS Maxi 44, Germany) at a maximum of 210 °C and 1400 W for 65 min. This solution was adjusted to 20 mL and filtered over activated charcoal and through 90-µm mesh filter paper. Phosphorus was measured spectrophotometrically by orthophosphate determination after the addition of molybdate-vanadate reagent (Gericke and Kurmies, 1952) using a spectrophotometer (U-3300, Hitachi, Tokyo, Japan).

Root analyses and mycorrhizal colonization

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

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

The roots were stained according to Brundrett *et al.* (1994) with slight modifications. Briefly, the roots were cut into segments 1 cm long, cleared with 10 % KOH at 90 °C for 1 h, rinsed three times with tap water, acidified in 2 M HCl for 2 min and stained with 5 % ink–acetic acid for 30 min at 60 °C. Root pieces were then soaked in tap water acidified with drops of acetic acid overnight to get rid of excess ink (Koske and Gemma, 1989; Vierheilig *et al.*, 1998). Finally, 30 randomly selected root pieces (1 cm long) from each replicate were placed on microscope slides (ten per slide) and observed for AMF colonization under a bright-field light microscope (Axioskop 2, Zeiss, Germany). As in the method described by Trouvelot *et al.* (1986), the mycorrhizal intensity of colonization (MIC) was determined using colonization classes, which were estimated for each root piece depending on the percentage of the root length colonized by AMF (#0 = 0 %, #1 ≤ 1 %, #2 = 2–10 %, #3 = 11–50 %, #4 = 51–90 %, #5 > 90 %). The MIC was calculated as an average for 30 root pieces using the formula MIC = (95n5 + 70n4 + 30n3 + 5n2 + n1)/*N* × 100 %, where n5, n4, n3, n2, n1 are the numbers of root pieces scaled as 5, 4, 3, 2 and 1, respectively, and *N* is the total number of fragments observed. The total colonized root length was calculated as total root length multiplied by MIC.

Mycorrhizal and root hair dependency

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

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

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

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

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

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

Gene expression analysis

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

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

Statistical analyses

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

RESULTS

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

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

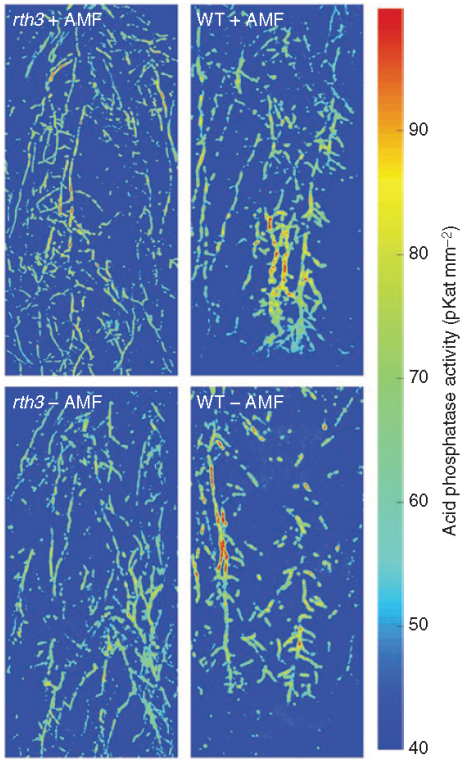

FIG. 2. Spatial distribution of acid phosphatase activity in the rhizosphere of maize root hairless mutant *rth3* (left) and wild type (WT, right). Representative examples are shown with AMF (top panels) and without inoculation (lower panels). The colour scale on the right shows linear enzyme activities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

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

Shoot and root dry weight, total root length and specific root length differed between the two genotypes when grown under limited P_i supply (Fig. 4). The nutritional P status (measured as shoot P concentration, which was around 0.1 %) indicated severely P-deficient conditions (Fig. 1). The non-inoculated *rth*3 had the lowest shoot and root biomass, shortest total root length and lowest P content. Values for these traits in the wild type were 1.9- to 3.6-fold of those of the root hairless mutant *rth3*

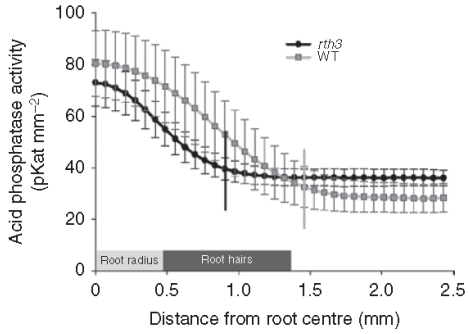

FIG. 3. Profiles of acid phosphatase activity as a function of distance from the maize root centre in the root-hairless mutant *rth3* and the wild type (WT). The root radius (0.45 mm) is shown as a light grey rectangle. The root hair length of wild type (0.9 mm) is shown as a dark grey rectangle. Vertical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

(Fig. 4A–C, F and Supplementary Data Fig. S1). The specific root length of the wild type was ~25 % higher than that of *rth*3 (Fig. 4D). The P concentration in the shoot was very low, at around 0.1 %, indicating severe P deficiency, but it was not different between the two genotypes (Fig. 4E and Supplementary Data Fig. S1).

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

In AMF-inoculated plants, fine extraradical hyphae were visible across the entire root, but these were absent in non-inoculated conditions (Supplementary Data Fig. S2). Inoculation with AMF significantly improved plant growth and P_i assimilation, and the enhancement was greater than that of root hairs (Table 1, Fig. 4A–C, E, F). The shoot and root biomass of AMF inoculated plants, as well as P content, were 4.0- to 7.4-fold greater those of non-inoculated plants in *rth*3, while only 1.8- to 4.6-fold greater in wild type (Fig. 4A–C, F). Stimulation of plant growth and P_i acquisition in *rth*3 by AMF was 1.5- to 3.9-fold greater than in wild type (Fig. 4A–C, E, F), but both AMF-colonized genotypes ultimately had almost the same biomass. The total root length of AMF-inoculated wild-type plants was double that of non-inoculated wild-type plants and was 5-fold increased in *rth3*, while specific root length was almost not affected (Fig. 4D, Table 1). AMF colonization strongly improved the nutritional status of the plants and increased the internal P concentration ~2-fold to ~0.2 %, but these plants still experienced P deficiency, as the critical sufficiency level for maize is commonly around 0.3 %. Inoculation of AMF in the presence and absence of root hairs did not affect organic acid anion release from roots (Supplementary Data Table S2).

There was significant interaction between mycorrhizal colonization and the presence of root hairs (P = 0.002) (Table 1). The average mycorrhizal colonization of non-inoculated roots was <2 %, but AMF inoculation increased colonization to 46 % for the wild type and to 80 % for *rth*3 (Fig. 5A, B). However,

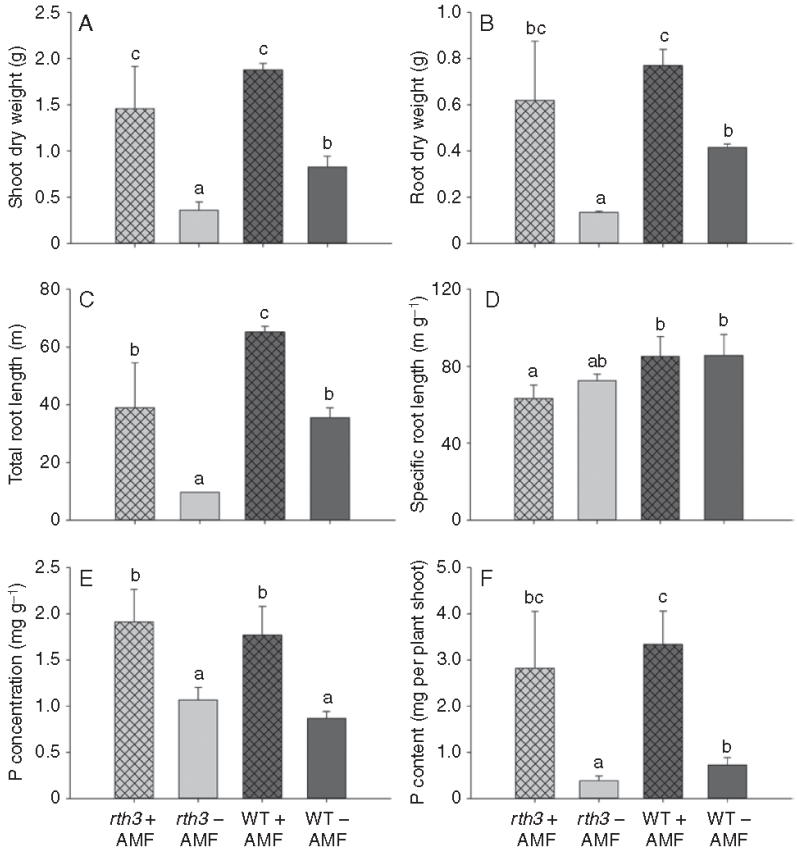

FIG. 4. The growth defect of the root hairless mutant *rth3* on P-deficient soil is compensated by AMF inoculation. Shoot dry weight (A), root dry weight (B), total root length (C), specific root length (D), P concentration (E) and P content (F) of maize *rth3* and wild type (WT) inoculated with AMF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

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

ZmPht1;6, the primary mycorrhiza-specific P_i transporter gene, is exclusively found in AMF-colonized roots and is expressed at a high level. In agreement with the greater infection of *rth3* roots, *ZmPht1;6 gene* expression was higher in the hairless roots. The less expressed, but also mycorrhiza-induced P_i transporter gene *ZmPht1;11* and the AMF colonization marker gene *Am*3 were also induced by mycorrhization, both in AMF-colonized roots of *rth*3 and in the wild type (Fig. 6).

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

Mycorrhizal colonization was positively correlated with *ZmPht1:6* and *Am*3 gene expression, shoot and root biomass, as well as with P concentration and P content (Table 2). The expression of *ZmPht*1:6 was positively correlated with shoot dry weight, P concentration and P content, indicating the importance of this gene in P_i uptake in maize (Table 2). Both shoot P concentration and shoot P content were also positively correlated with total root length, root and shoot biomass, as well as with each other.

DISCUSSION

Maize root hairs improved growth and P_i acquisition

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

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

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

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

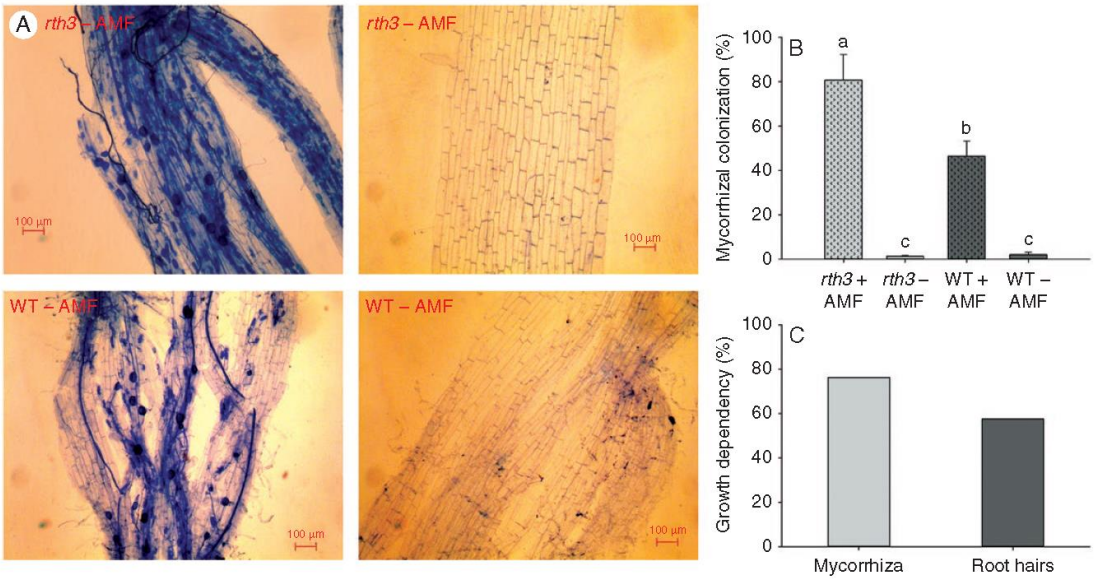

FIG. 5. Root hairs impair AMF infection. (A) Visualization of AMF colonization in maize roots of *rth3* and wild type (WT) inoculated with AMF (left panels) or not inoculated with AMF (right panels). (B) Mycorrhizal colonization and (C) growth dependence on mycorrhiza and root hairs.

Pang *et al.*, 2018). This was confirmed by up to 0.5 mm broader rhizosphere extent of acid phosphatase activity in the wild type compared with the hairless mutant (Figs 2 and 3), irrespective of AMF inoculation. This is in line with previous studies; root hairs greatly improved and extended rhizosphere acid phosphatase activity (Giles *et al.*, 2018) and may contribute 70–90 % of the total surface area relevant for nutrient absorption (Bates and Lynch, 1996; López-Arredondo *et al.*, 2014). Our study further verified that the root hair dependency of maize growth was 57 %. By contrast, the root hair dependency of barley was, according to results shown in Chen *et al.* (2005) and Jakobsen *et al.* (2005), around 75 %, which is notably higher than our results in maize, indicating that root hairs play more important roles in barley than in maize. Furthermore, the root hairs enhanced the P content by 44 %, but this increase was not significant and the P concentration was similar or tended to be even slightly decreased in plants with root hairs (Fig. 4E, F). This is in line with previous research (Weber *et al.*, 2018; Klamer *et al.*, 2019; Ludewig *et al.*, 2019). However, root-hairless plants were severely depressed in biomass formation compared with the wild type, meaning that any additionally acquired P_i was immediately invested in producing more shoot and root biomass, leading to bigger shoots and longer roots (Fig. 4). Specific root length is also an important root morphology measure that can affect nutrient acquisition (Lambers *et al.*, 2006). Maize wild type had a higher specific root length than *rth*3, which indicated that the wild type has longer root length per unit invested in dry mass. This further allowed a greater soil volume to be explored per unit C invested and thus enhanced P uptake efficiency (Laliberté *et al.*, 2015). Therefore, maize root hairs not only improved P acquisition efficiency but also indirectly improved root morphology, with longer total root length and higher specific root length that further improved plant growth compared with the *rth*3 hairless mutant.

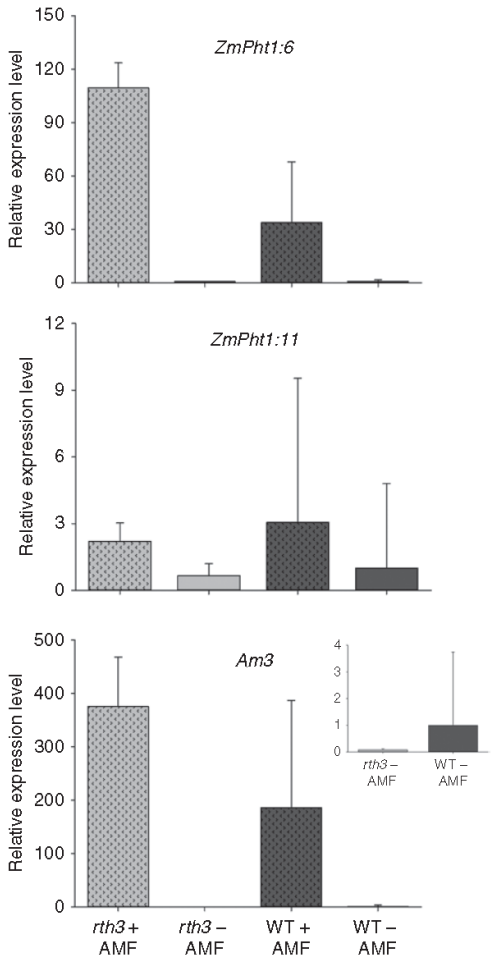

FIG. 6. Induction of mycorrhiza-induced *Pht* genes for phosphate transfer to the plant. Relative expression levels of *ZmPht1;6*, *ZmPht1;11* and *Am3* genes in *rth3* and wild-type (WT) maize root inoculated or not inoculated with AMF under P deficiency. Data points are means ± s.e.m. (n = 3 biological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

Inoculation of AMF improved plant growth and P_i acquisition more effectively in rth*3 than in wild type*

Inoculation of AMF increased plant growth and P_i acquisition of both the wild type and the *rth*3 root hairless mutant 1.8- to 7.4-fold (Fig. 4). The P nutritional status, i.e. the shoot P concentration, was massively improved by AMF, facilitating maize growth and further P_i acquisition by AMF and by roots. Phosphorus uptake by the root and root hairs generates a radial depletion zone of up to 2 mm around the roots (Joner *et al.*, 1995) due to the low mobility of P_i (Fontes and Weed, 1996). The hyphal network of AM fungi, by contrast, can extend up to 25 cm from the root, which is far beyond the depletion zone (Li *et al.*, 1991; Jansa *et al.*, 2003). Furthermore, root P_i assimilation is mainly in the root hair zone (Smith *et al.*, 2011), while the AMF also colonizes more mature regions of the root and supplies these regions with assimilated P_i (Richardson *et al.*, 2011). Therefore, hyphae can greatly increase exploration of the soil volume for phosphate. Moreover, the diameter of extracellular hyphae is about one order of magnitude smaller than that of fine roots and often even thinner than that of root hairs; thus, hyphae can enter small soil pores that are inaccessible to thick roots (Drew *et al.*, 2003; Smith and Read, 2008). Even when roots and hyphae have a similar volume, the surface area of hyphae in contact with soil is higher than that of the root due to its small diameter, which can greatly increase P_i assimilation (Jakobsen *et al.*, 2005). All these factors probably contribute to the much larger biomass and greater P_i uptake of AMF-inoculated *rth*3 compared with wild type under the non-inoculated condition (Fig. 4).

The root-hairless mutant *rth*3 particularly profited more from the AMF inoculation than the wild type, which is in line with previous studies where root hair length negatively correlated with mycorrhizal dependency of various species, because root hair length contributed to plant P_i uptake (Tawaraya, 2003). Thus, root hairs and AMF provide alternative, inversely correlated pathways for P_i foraging. Although root hairs and AMF provide alternative mechanisms to increase contact with the soil, the 33 % higher mycorrhizal growth dependency than root hair dependency strongly argues that AMF provide a more efficient way to acquire P_i even in young maize (Fig. 5C). Furthermore, the total colonized root length was similar in the two genotypes under the AMF inoculation condition and was accompanied by almost the same final biomass, indicating that AMF played a more important role than root hairs for P_i acquisition in maize. This is in line with previous studies showing that AMF play a critical role in nutrient acquisition, while root hairs are dispensable in maize (Wen and Schnable, 1994; Cozzolino *et al.*, 2013). However, similar studies in barley showed that the root hairs play more important roles than AMF (Jakobsen *et al.*, 2005; Chen *et al.*, 2005; Li *et al.*, 2014). This is probably due to the fact that the root hair length of barley wild type was almost doubled under the P-deficient condition compared with that under the higher P condition (Brown *et al.*, 2012), while the root hair length in maize B73 wild type was not significantly different between P-deficient (this study, 0.90 mm) and P-sufficient conditions (0.83 mm) (Weber *et al.*, 2018). This explanation is supported by observations in *Plantago lanceolata*, whose root hair length was not responsive to P availability but was highly dependent on AMF for P_i acquisition under P deficiency (Brown *et al.*, 2013*a*). Other root morphological traits also influence plant dependency on AMF symbiosis; for example, species with thin roots rely less on AMF symbiosis for nutrient uptake than species with thick roots (Kong *et al.*, 2014; Z. Ma *et al.*, 2018). Similar phenomena exist among different root architectures, where the mycorrhizal colonization was lower in the thin first-order root than in the thick second-order roots (Eissenstat *et al.*, 2015). This further indicated that root morphological properties (e.g. root hairs and root diameter) and mycorrhizal colonization act as alternative strategies in P_i uptake. It is also noteworthy that root hairs are already endogenously found

TABLE 2. *Pearson correlations among mycorrhizal colonization,* ZmPht*1;6,* ZmPht*1;11 and* Am*3 expression, shoot dry weight, root dry weight total root length, P concentration and P content*

	Mycorrhizal colonization	*ZmPht*1;6	*ZmPht1*;11	*Am*3	Shoot dry weight	Root dry weight	Total root length	Specific root length	Shoot P concentration
*ZmPht*1;6	0.945**								
ZmPht1;11	0.326	0.117							
Am3	0.887**	0.911**	0.065						
Shoot dry weight	0.732*	0.590*	0.564	0.682*					
Root dry weight	0.677*	0.530	0.637*	0.567	0.969**				
Total root length	0.520	0.352	0.547	0.511	0.941**	0.943**			
Specific root length	–0.375	–0.464	–0.158	–0.154	0.089	0.052	0.366		
Shoot P concentration	0.822**	0.692*	0.412	0.786*	0.955**	0.893**	0.851**	0.015	
Shoot P content	0.917**	0.824**	0.241	0.889**	0.766**	0.666*	0.591*	–0.166	0.903**

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

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

Mycorrhizal colonization and mycorrhiza-specific P_i transporter *ZmPht*1:6 expression were positively correlated with each other ($P < 0.01$) and both also positively correlated with plant growth, P status and P acquisition (Table 2). This indicated that the colonization rate and transporter gene expression are positively correlated with symbiotic function and activity. Mycorrhizal colonization promoted maize growth, P status, P_i acquisition and related P_i transporter gene expression in a plant genotype-dependent manner, in which the *rth*3 hairless mutant benefited more than the wild type. As a consequence, plant biomass and P_i uptake were induced 1.6- to 2.5-fold by AMF colonization in *rth*3 compared with the wild type (Fig. 4A, B, F). The mycorrhizal colonization of *rth*3 was 1.7-fold that of the wild type (Fig. 5A, B), leading to higher AM-induced P_i transporter *ZmPht*1;6 expression in *rth*3 compared with the wild type (Fig. 6A). The following two mechanisms can explain the lower dependency of the wild type than the hairless mutant on AMF. Morphologically, the wild type, with long and dense root hairs, has a larger root surface area than the root-hairless mutant; thus, dependency on mycorrhiza for P_i acquisition was decreased. Physiologically, root hair growth and AM hypha establishment are both carbon- and energy-consuming and compete for resources (Kuzyakov and Domanski, 2000; Ryan *et al.*, 2012). Hence, the abundance of AM colonization was not only induced by limited soil-available P, but also controlled by phytohormones and molecular mechanisms (Akiyama *et al.*, 2005; Kobae *et al.*, 2018). The phytohormone strigolactone plays an important role in the interaction of root establishment with AM symbiosis interaction (Akiyama *et al.*, 2005; Besserer *et al.*, 2006; Chagas *et al.*, 2018). Colonization by AMF depends on the release of such signalling compounds into the rhizosphere to germinate spores and attract hyphae for root contact; a radially extended rhizosphere was expected to be beneficial for the number of AMF–root contact sites. However, root hairs expand the soil volume into which strigolactone is released, but had only a minor role in maize, as AMF colonization was higher in the hairless mutant than in the wild type (Fig. 5). Molecular signals such as CLE (CLAVATA3/Embryo Surrounding Region-Related) peptides also play a key role in AMF colonization of various plants (Handa *et al.*, 2015; Karlo *et al.*, 2020). Future investigations of genetic and molecular pathways such as the relationship between the release of strigolactones and CLE peptides with AMF colonization are needed to reveal the contrast in mycorrhizal colonization between wild type and *rth3* in maize.

Conclusions

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

SUPPLEMENTARY DATA

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

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7. Chapter IV

Estimating the importance of maize root hairs in low phosphorus conditions and under drought

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SPECIAL ISSUE ON ROOT TRAITS BENEFITTING CROP PRODUCTION IN ENVIRONMENTS WITH LIMITED WATER AND NUTRIENT AVAILABILITY

Estimating the importance of maize root hairs in low phosphorus conditions and under drought

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• **Background and Aims** Root hairs are single-cell extensions of the epidermis that face into the soil and increase the root–soil contact surface. Root hairs enlarge the rhizosphere radially and are very important for taking up water and sparingly soluble nutrients, such as the poorly soil-mobile phosphate. In order to quantify the importance of root hairs for maize, a mutant and the corresponding wild type were compared.
• **Methods** The *rth2* maize mutant with very short root hairs was assayed for growth and phosphorus (P) acquisition in a slightly alkaline soil with low P and limited water supply in the absence of mycorrhization and with ample P supply.
• **Key Results** Root and shoot growth was additively impaired under P deficiency and drought. Internal P concentrations declined with reduced water and P supply, whereas micronutrients (iron, zinc) were little affected. The very short root hairs in *rth2* did not affect internal P concentrations, but the P content of juvenile plants was halved under combined stress. The *rth2* plants had more fine roots and increased specific root length, but P mobilization traits (root organic carbon and phosphatase exudation) differed little.
• **Conclusions** The results confirm the importance of root hairs for maize P uptake and content, but not for internal P concentrations. Furthermore, the performance of root hair mutants may be biased by secondary effects, such as altered root growth.

Key words: Macronutrients, micronutrients, phosphate, fine roots, water, rhizosphere.

INTRODUCTION

In low-phosphate soils and under dry conditions, root hairs comprise a cheap, 'cost'-effective strategy to increase plant uptake of the sparingly soluble phosphate and water from soils (Jungk, 2001; Lynch and Ho, 2005; Brown *et al.*, 2013). Water enters roots primarily in the root hair zone and is directly taken up via root hairs (Cailloux, 1972). The water (and nutrient) taken up via root hairs may, however, make only a limited contribution to transpiration during the day (Segal *et al.*, 2008).

The contribution of long and dense root hairs to depleting Olsen P from soil was initially estimated with diverse wheat and barley genotypes (Gahoonia *et al.*, 1997). A spontaneous barley mutant without root hairs (*brb*) depleted less P from the soil and was less competent on low-P soil compared with the wild type (Gahoonia *et al.*, 2001). When P-limited barley was exposed to drought, deficiency symptoms and growth retardation were most severe in plants lacking root hairs, confirming the synergism in nutrient and water uptake in this species (Brown *et al.*, 2012). Interestingly, at low P supply, root-hair-deficient barley mutants tended to have elevated internal P concentration, although this effect was not significant (Brown *et al.*, 2012). Furthermore, the *brb* barley root-hair-deficient mutant was impaired in water uptake from drying soil, at least at high evaporative demand (Carminati *et al.*, 2017) but less at low evaporative demand (Dodd and Diatloff, 2016).

In addition to root hairs, symbiotic vesicular arbuscular mycorrhiza help to acquire P and water via extension of the below-ground uptake surface. Mycorrhiza may compensate for root hairs, if successfully established. Mycorrhization reduced root hair density and length in wild-type maize by ~40 % (Kothari *et al.*, 1990). Furthermore, seedling root hairs are thought to help mechanical root anchorage during establishment and root tip penetration into compacted soil (Bengough *et al.*, 2011). Finally, root hairs are crucial for the interaction with rhizobacteria and may serve as entry points for endophytes (Prieto *et al.*, 2011).

The patterning of root hairs in the rhizodermis and root-hair-specific gene expression are best characterized in *Arabidopsis* (Lan *et al.*, 2013) and experiments with this species and mutants have suggested that root hairs provide a competitive advantage in mixed cultures in low-P soil (Bates and Lynch, 2001). As P in soil is hardly mobile and typically only enriched in the upper soil layers, root hairs are especially effective in soil fractions close to the surface. Thus, their functional contribution cannot be viewed independently of other root architectural and morphological features (Lynch, 2013).

The maize crop is especially sensitive to P deficiency in juvenile phases, after internal stores have been used up

(Nadeem *et al.*, 2011). The internal P stores are large enough to provide sufficient P for several days, but a proteomic study revealed that phosphate uptake transporters were already more abundant in root hairs from just germinated seedlings exposed to a nutrient solution lacking P (Li *et al.*, 2015).

We hypothesized that the loss of root hairs increases the severity of P deficiency in maize and strongly represses plant growth under drought, especially when combined with low P availability. The root-hair-defective maize mutant *rth2* (Wen and Schnable, 1994) was used to estimate the importance of maize root hairs under low P availability and drought. Importantly, a soil mix was used in which mycorrhization was essentially absent (Neumann, 2007), as mycorrhization might compensate for the loss of root hairs (Kothari *et al.*, 1990). We observed that juvenile shoot P concentrations were strongly decreased by the stresses, but were unaffected by the very short root hairs in *rth2*, irrespective of water limitation or P level. However, plant growth and plant P content (the product of concentration and biomass) were severely compromised by very short root hairs under combined drought and low P. Interestingly, *rth2* roots also had more fine roots.

MATERIALS AND METHODS

Plant material

We used the maize inbred line B73 as wild type and the *rth2* mutant, which was backcrossed to B73 more than seven times and is therefore nearly isogenic (Wen and Schnable, 1994). The seeds were surface-sterilized by rinsing them for 2 min in a 10 % H_2O_2 solution and were then placed in a 10 mM $CaSO_4$ solution for 24 h. Seeds were put between foam sheets soaked in a 3 mM $CaSO_4$ solution for 4 d to germinate and were then transferred gently to soil or nutrient solutions.

Growth experiments in soil

Plants were grown in 5-L ceramic, cylindrical Mitscherlich pots (diameter 20 cm, height 18 cm) in the greenhouse (48°42′41.04″ N, 9°12′34.20″ E) in a warm spring and were harvested after 44 d. For the first experiments, a long-term-stored nutrient-poor subsoil (pH 7.6, C_{org} <0.3.%, $CaCO_3$ 30 %) with the following mineral concentrations was used (mg kg^{-1}): 7.9 total P; 59.9 Ca; 11.3 Mg; 15 Mn; 7.8 Fe; 0.6 Zn; 0.2 B; and 0.7 Cu (VDLUFA, 1997). This subsoil was mixed with quartz sand (17 % w/w) and fertilized with (mg kg^{-1} soil): 200 NH_4NO_3; 200 K_2SO_4; 100 $MgSO_4$; 1.9 Fe-Sequestrene; 2.6 $ZnSO_4$; 1 $CuSO_4$; and 37.5 (–P) or 150 (+P) $Ca(H_2PO_4)_2$. The rationale for using this carbonate-rich subsoil mix was its very low basal nutrient content and especially low P availability. Carbonate-rich soils may limit plant P availability and growth even when the soil pH is adjusted by liming to an optimal pH of 6–7 (Rothwell *et al.*, 2015).

Plants were additionally fertilized at day 17 with 100 mg nitrogen kg^{-1} substrate and 2.6 mg Zn kg^{-1} at day 27. In this subsoil mix, mycorrhization was previously checked in hundreds of samples over several years and was essentially absent across a wide array of plant species, including maize (Neumann, 2007). The absence of mycorrhiza was confirmed in our samples by using trypan blue stain (Neumann, 2007).

Each pot contained one plant and 6 kg of substrate. For the well-watered control (W+), the 15 % initial soil water content (total water-holding capacity 30 %) was raised at day 11 to 20 % water. For the drought treatments, the plants were gradually exposed to less water to allow adaptation to the dry conditions. These plants were treated like the controls until day 19, when water was reduced to 15 % and at day 27 further to 12 %. The soil moisture level was gravimetrically measured and adjusted on a daily basis.

For later experiments a nutrient-rich peat soil (Einheitserde type T, Einheitserde- und Humuswerke, Sinntal-Jossa, Germany) was mixed with 15 % of the loamy loess subsoil and 10 % sand in 5-L Mitscherlich pots, to obtain a substrate with high P availability. Soil P was 130 mg kg^{-1}, pH 6, and plants on this substrate were additionally fertilized twice with 100 mg kg^{-1} P (as $Ca(H_2PO_4)_2$) in the last two growing weeks.

Growth experiments in nutrient solution

Hydroponic plants were grown for 6 weeks in a climate chamber at 24 °C, 60 % humidity and photosynthetically active photon flux density of 400 µmol m^{-2} s^{-1} for 14 h. Hydroponics started with six seedlings per pot in pots containing 2.8 L of a diluted maize nutrient solution, containing 0.1 mM K_2SO_4, 0.12 mM $MgCl_2$, 0.5 mM $Ca(NO_3)_2$, 200 µM KH_2PO_4, 0.2 µM H_3BO_3, 0.1 µM $MnSO_4$, 0.1 µM $ZnSO_4$, 0.04 µM $CuSO_4$ and 2 nM $(NH_4)_6Mo_7O_{24}$. Three days later the seedlings were separated into two seedlings per pot and the macronutrient concentrations were increased to 0.5 mM K_2SO_4, 0.6 mM $MgCl_2$, 2.5 mM $Ca(NO_3)_2$ and 0.1 mM KH_2PO_4. The KH_2PO_4 concentration was progressively raised to 0.2 mM and finally to 0.5 mM in week 4, while micronutrients were 1 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$ and 100 µM Fe-Sequestrene, which was raised to 200 µM at the first nutrient solution change and to its final amount of 300 µM at the second solution change. The first nutrient solution change was done after 1 week and from then on every 3 d until harvest.

Elemental analysis

Element concentrations of P, Zn and Fe were determined from oven-dried (60 °C) shoot material that was ground to fine powder. Half a gram of shoot dry matter was incubated with 5 mL of HNO_3, 4 mL of H_2O_2 and 2 mL of distilled water in a microwave (MLS Maxi 44, Germany) at a maximum of 210 °C and 1400 W for 65 min. This solution was adjusted to 20 mL and filtered over activated charcoal and through 90-µm mesh filter paper. Concentrations of Zn and Fe were determined by atomic absorption spectroscopy (AAS, ATI Unicam Solaar 939; Thermo Electron, USA). Before measuring Fe concentrations, caesium chloride and lanthanum chloride buffer (Merck, No. 116755) was added at 1:50 ratio to eliminate spectral interferences. Phosphorus was measured spectrophotometrically via orthophosphate determination after addition of molybdate–vanadate reagent (Gericke and Kurmies, 1952).

Root analyses

Root dry biomass, specific root length (total root length per unit dry mass) and root diameter fractions were measured after washing the soil from the roots. To determine root traits, the roots were scanned and digitized at a resolution of 600 dpi and analysed with WinRHIZO (Regent Instruments Inc., Canada) software. Mycorrhization of root samples in the carbonate-rich soil–sand mix was checked with classical trypan blue staining methods and confirmed to be essentially absent (Neumann, 2007).

Exudate analyses and phosphatase activity

Exudates were collected from 5-week-old hydroponically grown plants that were P-starved for 2 d before sampling. Collection was for 1 h in 1-L pots containing sufficient 1 mM $CaSO_4$ solution that all roots were completely covered by the buffer solution. The total carbon in exudates was quantified with a total organic carbon analyser (Elementar). Phosphatase activities were photometrically quantified in 1250-µL extracts shaken for 1 h at 27 °C at pH 5.3 using *p*-nitrophenyl phosphate (Tabatabai and Bremner, 1969).

Statistical analyses

A three-way ANOVA and pairwise Tukey tests were used to investigate genotype ζ environment interactions between wild type, *rth2* mutant and different treatments. Data are given as mean ± s.d.

RESULTS

Maize biomass under drought, low P and combined stress

Maize plants were grown with two different water and P supply levels under greenhouse conditions for 6 weeks. Representative maize plants are shown in Fig. 1A–D and indicate that mutant shoots grew similarly under optimal conditions, but were smaller with increasing stress compared with the wild type. The unstressed control plants were largest, while drought (–W) reduced the wild-type and *rth2* mutant shoot biomass by 30 and 38 %, respectively. The root dry biomass in –W was 39 and 38 % lower than in controls, respectively.

The low-P (–P) treatment severely decreased wild-type shoot biomass by 75 % and that of the mutant by 83 %, while root dry mass was reduced in the wild type and mutant by 72 and 76 %,

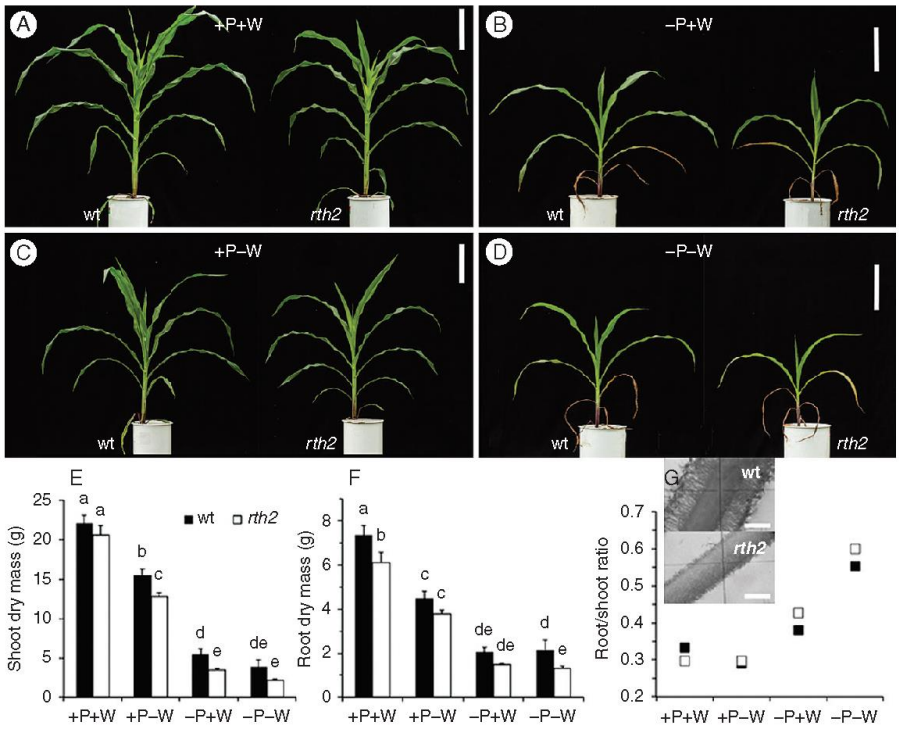

FIG. 1. Phenotype and biomass of 5-week-old maize plants. (A–D) Representative shoots (wt, wild type, left; *rth2*, *root hair defective2*, right) with (A) optimal P nutrition and water (+P+W), (B) low P but water (–P+W), (C) optimal P but low water (+P–W) and (D) low P and low water supply (–P–W). Scale bars = 20 cm. (E) Dry shoot, (F) root biomass and (G) root/shoot ratio under different P and water levels. The inset in G shows primary roots of both genotypes with root hairs at day 7 on filter paper. Significant differences ($P < 0.01$) are indicated by different letters above columns.

respectively. Shoot size and dry shoot and root biomass of the *rth2* mutant tended to be always smaller than in the wild type, although this was not always statistically significant in all treatments (Fig. 1E, F). The combined stress did not further reduce shoot and root biomass. The root/shoot ratio was little affected by drought as the only stress, but this ratio was higher in –P, especially when plants were additionally exposed to drought (Fig. 1G). The root/shoot ratio was higher in the mutant than in the wild type in –P, potentially indicating more investment in root growth in the mutant (Fig. 1G).

The *rth2* mutant was not completely devoid of root hairs, but short root hairs with similar density were detected, although their length was reduced by >80 % in the primary root just after germination (inset in Fig. 1G). This decrease in total root hair length was somewhat less at later growth stages and in seminal roots, but very short root hairs were consistently observed on all *rth2* root types. While wild-type root hairs at harvest in soil were on average 0.84 mm in length, the root hairs of the *rth2* mutant were only ~0.19 mm long (Weber *et al.*, 2018).

Effect of water supply, P level and root hairs on shoot water content and nutrients

Both genotypes contained ~90 % water in their fresh shoot biomass under control conditions (Fig. 2A), but both the wild type and the mutant had marginally reduced water content, at about 88–89 %, in the fresh shoot biomass under drought. The same lower water content was measured in –P, while the water content was further reduced in –P–W to ~86 % in both genotypes (Fig. 2A).

Shoot P content per plant (product of P concentration and dry biomass) followed the same trend as shoot biomass (Figs 1E and 2B). Except for the well-supplied condition, the mutant had a lower shoot P content, which is commonly taken as evidence for the importance of root hairs in taking up P (Zhu *et al.*, 2010; Brown *et al.*, 2012). This increased P content in the wild type relative to the mutant was only due to higher biomass of these plants, as the wild-type and mutant shoot P concentrations were indistinguishable in each condition (Fig. 2C). By contrast, the shoot P concentration of both genotypes in –W was below the

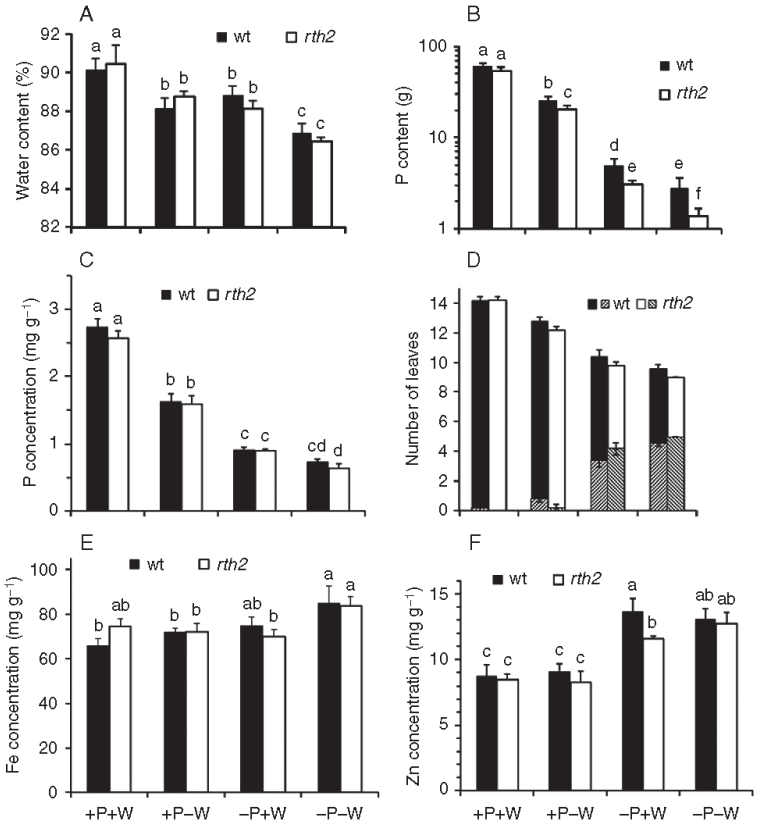

FIG. 2. Water and P contents, nutrient concentrations and visible deficiency symptoms with different P and water supply. (A) Water content (%) at harvest. (B) Shoot P content (note logarithmic scale) and (C) shoot P concentrations of wild type (wt) and *rth2*. (D) Total numbers of leaves and (hatching) number of leaves that were >50 % chlorotic or necrotic. (E) Shoot Fe and (F) shoot Zn concentrations. Significant differences ($P < 0.01$) are indicated by different letters.

sufficiency threshold (around 2.5 mg g^{-1}). The P concentration was further drastically lowered in –P and was lowest in the –P–W condition. Wild-type and *rth2* plants were progressively delayed in development under increasing stress, as both genotypes had fewer leaves. In –P, these plants also showed necrotic old leaves, the typical visible signs of P starvation (Fig. 2D). The severity of this symptom was greater in the mutant, suggesting that although shoot P concentration was identical to that in the wild type, the mutant suffered more under the –P condition.

Since root hairs are also considered important for the uptake of sparingly soluble micronutrients, such as Fe and Zn, the concentrations of these elements were also measured. While the Fe concentrations were increased in the most severe stress condition, the Zn concentration was higher in –P. However, except for the Zn concentration in –P with sufficient water supply, the mutant and wild-type element concentrations matched and were independent of root hairs (Fig. 2E, F).

The relationship and interaction of individual factors (P supply, water supply and genotype) with plant biomass, root/shoot ratio and nutrient concentrations are summarized in Table 1. The P supply significantly affected root and shoot dry biomass, root/shoot ratio and P, Zn and Fe concentrations. Drought also affected all these parameters, except for Zn concentration. Genotype, i.e. whether long root hairs were present or not, significantly affected plant biomass but not internal nutrient concentrations (Table 1). Most interestingly, interactions between factors were only significant for the combination of limited water and P supply, which affected root and shoot biomass, root/shoot ratio and P concentrations. Phenotypic plasticity for these factors, also called genotype × environment interaction, however, was entirely absent (Table 1).

Very short root hairs are associated with more fine roots

We then quantified root length in different diameter classes, to test whether the mutant had altered root morphology, in addition to root hair phenotype. Most of the roots were fine roots of diameter <0.2 mm, which represent lateral roots (Tai *et al.*, 2016). Despite substantial variation amongst individual plants, *rth2* clearly tended to have more lateral roots, independent of the P supply (Figs 2B and 3A). We did not detect differences between the tested conditions, probably because the variance among the samples was relatively large. Therefore only the common trend for all roots per genotype is shown. Thinner root fractions in *rth2* were observed despite the overall reduced root dry biomass (Fig. 1F), suggesting a morphological switch to the production of thinner roots and an increase in specific root length (length per unit root dry mass).

In a second experiment the growth of wild-type and *rth2* plants was compared in another, slightly acidic, but nutrient-rich soil substrate with contrasting texture and sufficient moisture, as well as in hydroponics, since root hairs may represent an extra metabolic cost to the plant in P-rich, moist conditions. In the nutrient-rich substrate, we failed to identify significant genotypic differences between the shoot and root biomass, as well as in the shoot P concentration after 5 weeks, in agreement with the +P+W conditions in the first experiment. Furthermore, in hydroponics, where both genotypes had the same, unrestricted access to water and otherwise immobile nutrients, the *rth2* mutant accumulated slightly, but significantly, higher shoot and root biomass (Fig. 3B, C).

Furthermore, root hairs participate in releasing protons, sugars and organic anions from roots, to feed the soil microflora and mobilize P from sparingly soluble soil fractions (Holz *et al.*, 2018). Short-term carbon release from the roots into the nutrient solution, which accounts for the sum of all organic solutes released, was therefore quantified in hydroponically grown plants after 2 d of P starvation. However, the released total C_{org} was similar in the plants without root hairs (Fig. 3D). Likewise, the (low) phosphatase activity of P-starved roots was also similar in the two genotypes (Fig. 3E), suggesting minor effects of the very short root hairs on rhizosphere P mobilization processes, at least when plants were grown in hydroponics. High shoot P concentrations occurred after 5 weeks of growth under luxury P supply in hydroponics, as expected, with no differences between mutant and the wild type (Fig. 3F).

Table 1. *Factorial analysis (three-way ANOVA)*

	DM shoot	DM root	R/S ratio	P ($g\,g^{-1}$)	Zn ($g\,g^{-1}$)	Fe ($g\,g^{-1}$)
P	*	*	*	*	*	*
W	*	*	*	*	ns	*
G	*	*	ns	ns	ns	ns
P × W	*	*	*	*	ns	ns
P × G	ns	ns	ns	ns	ns	ns
W × G	ns	ns	ns	ns	ns	ns
P × W × G	ns	ns	ns	ns	ns	ns

Main effects and significant interactions ($P < 0.05$) are indicated with an asterisk. ns, interaction not significant.

P, phosphate supply; W, water supply; G, genotype; DM, dry mass; R/S, root shoot ratio.

DISCUSSION

This study confirms the importance of root hairs for water and P uptake in dry conditions and when P bioavailability is low in maize, similar to results in other species (Jungk, 2001; Lynch and Ho, 2005; Brown *et al.*, 2013). The bald root barley (*brb*) line, a root-hairless mutant (Gahoonia *et al.*, 2001), has frequently been used to analyse the function of crop root hairs (Zuchi *et al.*, 2011; Holz *et al.*, 2018). However, the genetic cause of the *brb* phenotype is unknown and it was recently reported that in this mutant other plant properties are also affected, besides the formation of root hairs. Indeed, *brb* shows increased root growth in dry soil, which compensated for the surface loss of root hairs (Dodd and Diatloff, 2016). This was apparently P-independent and young (non-tillering) plants of this mutant had shoot growth similar to that of the wild type, in both low- and high-P soil (Dodd and Diatloff, 2016). A somewhat different genetic compensation of root growth was found for the maize *rth2* mutant. The fine root fraction was rather constitutively increased, independently of the stress, but the *rth2* mutant had less total root biomass (Fig. 1). Although we cannot rule out that the genetic defect in *rth2* also affects other processes in addition to root hairs, our observation that P

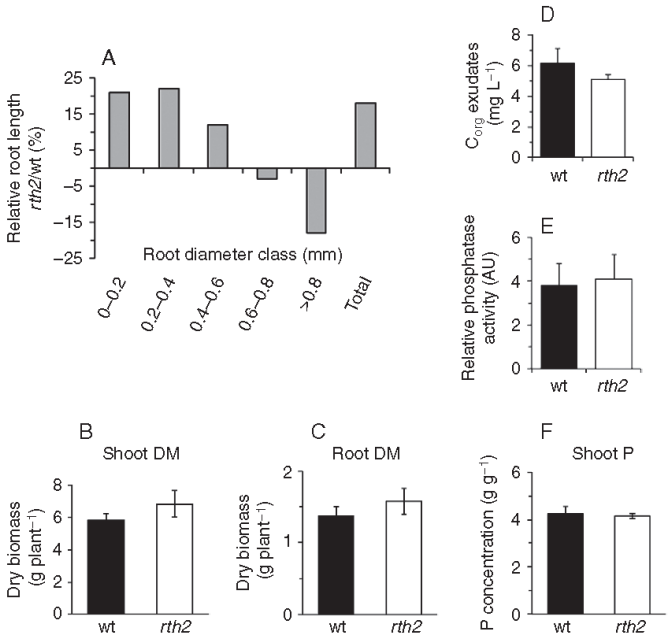

FIG. 3. Compensation for root hair loss by fine roots. (A) Relative amounts of roots of different diameters in mutant (*rth2*) and wild type (wt), averaged across all treatments in soil. (B) Shoot and (C) root dry biomass (DM) in full-nutrient hydroponics. (D) C_{org} released and (E) phosphatase activity for hydroponic, P-starved roots. AU, arbitrary units. (F) Shoot P concentrations in hydroponics. Black bars, wild type; white bars, *rth2*. Mean ± s.d. is shown for all values.

concentrations in the mutant are not affected suggests that the metabolism may be little different in the mutant. Importantly, mycorrhization was absent in our set-up, excluding the possibility that fungal symbioses compensated or aggravated the lack of root hairs (Zhu *et al.*, 2010; Brown *et al.*, 2012).

Under P limitation, as well as under drought stress in the carbonate-rich soil, the *rth2* mutant performed worse than the wild type. Thus, growth of *rth2* was most drastically impaired under combined stress with low P and drought (Fig. 1), in agreement with the substantial importance of root hairs for P and water uptake in maize under low availability. Indeed, the longer root hairs and root hairs that were induced by low P conferred a yield advantage on maize plants grown in low-P environments (Zhu *et al.*, 2010). The limited P and water availability in our experiments strongly reduced internal shoot P concentrations below the critical value of 2.5 mg g^{-1} and induced P deficiency symptoms in the plants, such as darker coloration of young leaves and necrotic old leaves (Jones *et al.*, 1991; Bergmann, 1993). The P content per plant (the product of concentration and dry biomass per plant) was higher for the wild type compared with the mutant in each condition, indicating how much the root hairs contributed to P uptake: +14 % in +P+W, +24 % in +P-W, +61 % in −P+W and +103 % in −P−W. The shoot P concentration, typically used by farmers, researchers and the plant itself to estimate crop nutritional status, did not indicate that *rth2* was more deficient than the wild type. Indeed, the inverse of the P concentration is proportional to P utilization in plant tissue, i.e. how much biomass is generated at a given tissue P supply. This was apparently unchanged in the mutant, indicating that at least this aspect of metabolism was similar in *rth2* and the wild type, at least when the wild type and mutant were exposed to the same external P concentration. The P content, by contrast, a measure of how much total P was acquired at a given external supply, indicated the massive growth benefit conferred by the root hairs (Fig. 2), especially under combined stress. Non-significant differences in internal P concentrations among barley root hair mutants and wild type (higher P concentrations in mutants) were also reported in barley, but these did not drop below 2 mg g^{-1} (Brown *et al.*, 2012).

At higher soil P levels, a large collection of modern maize hybrids differed substantially in their juvenile P concentrations, but this poorly predicted ($r_E = 0.04$) their final yield (Melchinger *et al.*, 2016). Here, the nutrient concentrations indicated that the plants were apparently already P-deficient under drought conditions (although water was probably the growth-limiting factor), in agreement with the idea that deficiencies of soil-immobile nutrients are a rapid consequence of low soil moisture. The poorly soluble micronutrient Zn was also low (deficiency threshold ~10–20 ppm), although substantial Zn fertilizer was added to the alkaline soil. Zinc was increased under the −P condition, which is not uncommon, as Zn is frequently co-mobilized by rhizosphere processes induced by −P (Neumann and Römheld, 2002). Under −P with sufficient water, such rhizosphere processes might be less efficient in the root hair mutant (as the rhizosphere did not extend as deep into the soil as in the wild type), so in low P the Zn concentration was higher in the wild type than in the mutant (Fig. 2). Iron is taken up in maize by an independent phytosiderophore complexation mechanism

(Marschner and Römheld, 1984), potentially explaining why root hairs did not affect internal Fe concentrations across environments (Fig. 2).

Plant strategies to adapt to low soil phosphorus bioavailability generally can be divided into 'foraging' (by adapting root architecture and morphology) and 'mobilizing' (by solubilization of fixed P via rhizosphere processes) strategies (Hinsinger, 2001; Lynch, 2011). Maize is a crop that mainly responds to insufficient P with root architectural changes (Lyu *et al.*, 2016), while this species is thought to invest relatively little in rhizosphere processes, such as proton and organic acid release, secretion of phosphohydrolyses, interaction with rhizosphere microbes, etc. However, substantial organic metabolites are released in hydroponics in a nutrient deficiency-specific way; these comprise amino acids, sugars and organic anions (Carvalhais *et al.*, 2011). The very short root hairs in *rth2* were apparently not associated with major differences in some rhizosphere-related processes, such as the sum of organic molecules released and phosphatase activity (Fig. 3).

The mutant apparently had, in addition to the root hair phenotype, altered root architecture. Based on previous analyses of the maize root system, thick roots of diameter >0.8 mm can be classified as primary and shoot-borne roots, while the category of 0.6–0.8 mm comprises the seminal roots and the 0.4- to 0.6-mm class contains a mixture of seminal and lateral roots. Roots thinner than 0.4 mm are lateral roots (Tai *et al.*, 2016), indicating an increased amount of laterals in *rth2*. Interestingly, genotype × environment interaction was apparently low or absent, suggesting that the effects of the very short root hairs were similar in each environment. Thus, the root architecture changes were seemingly not a response to short root hairs under P-limiting conditions. Importantly, the altered root architecture in *rth2* and *brb* in barley (Dodd and Diatloff, 2016) may also have influenced P uptake, so mechanistic conclusions on root hair function from these mutants must be drawn with care.

The importance of root hairs has been questioned in some rice varieties recently, where root hairs improved P efficiency only in some genotypes (Nestler and Wissuwa, 2016). In a population of native *Arabidopsis*, root hair density and length responded surprisingly heterogeneously to differential P supply and some genotypes reduced hair length or density when locally lacking P in agar plates (Stetter *et al.*, 2015). However, predictions of root hair behaviour in real soils from root hair density and length on agar plates must be made with great care, as enormous variability in root hair traits was found in single genotypes between synthetic growth substrates and real soils (Nestler *et al.*, 2016). Surprisingly, the mutant performed better than the wild type in well-supplied hydroponics, which may reflect a substantial energetic cost of building root hairs in conditions where they are not required.

Conclusions

Long root hairs were substantially important for growth and P content of maize under drought and low P conditions, but they were dispensable for internal P concentrations and even slightly detrimental in well-supplied conditions. Because the reduction in root hair length in *rth2* was associated with secondary effects on root architecture, future experiments on the relevance of root hairs should involve several independent mutants (especially those with complete absence of root hairs). The collection of root hair mutants in maize is growing and it will be interesting to see whether other maize root hair mutants are associated with secondary root phenotypes (Hochholdinger *et al.*, 2018).

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8. Chapter V

Transcriptomic network analyses of the response to low phosphate supply in roots of maize with distinct breeding history

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Abstract

Phosphorus (P) deficiency is a global problem in maize production. Although macro/microarray technologies have significantly increased our general knowledge of maize responses to P deficiency, an integrative and deeper understanding of the diversity of responses in maize genotypes is still needed.

In this study, we first compared the root traits response to low P (LP) and high P (HP) of six preselected genotypes in European flint in a mini-rhizotron experiment for three weeks. We then generated RNA libraries from the roots of these lines under both LP and HP. Using the expressed gene matrix, we subsequently conducted Weighted Genomic Coexpression Network Analysis (WGCNA). The P deficiency-responsive metabolic processes common to all six genotypes included: (1) acceleration of carbon supply for organic acid synthesis through glycolysis and TCA cycle; (2) alteration of lipid metabolism; (3) changes of activity of transmembrane transporters; (4) carotenoid metabolism. Additionally, the founder flint line EP1, F2 and doubled haploid landrace SM1 have their specific strategies and mechanism to cope with LP. Our findings could help to understand of the molecular events involved in the diversity and efficiencies of P stress responses among maize accessions.

Keywords: maize (Zea Mays. L), Phosphorus, RNAseq, WGCNA

1. Introduction

Phosphorus (P) is essential for the normal growth and development of plants because it is vital for regulating energy metabolism, enzymatic reactions and signal transduction processes (Raghothama 1999). P is readily taken up by the roots as (di-)hydrogen phosphate ($H_2PO_4^-$ or HPO_4^{2-}), and almost all P in soils is found in fully oxidized form as orthophosphate (P_i). P is typically least bioavailable in soils of all essential macro-elements for plants, despite high total P amounts within soils. Because it usually forms insoluble complexes, especially with aluminum and iron under acidic conditions and calcium under alkaline conditions (Péret et al. 2011), the free P_i concentration even in agricultural soil solution is rarely above ten µM, and sparingly soluble P-forms are converted into insoluble forms with time (Bieleski 1973). So, plants always suffer from P deficient stress under natural conditions.

Plants have evolved diverse strategies to adapted to P deficiency, including morphological modification to access a large soil area (Postma et al. 2014; Haling et al. 2018); chemical modifications of the rhizosphere (Hinsinger 2001; Pang et al. 2018); microbial interactions with arbuscular mycorrhizal fungi to help access P from the labile soil P pool (Smith et al. 2011; van der Heijden et al. 2015); and bypassing the metabolic steps that require ATP (Ganie et al. 2015). These adaptations to variable P availability depend at least in part on changes in gene expression. Some key regulators of P homeostasis, mainly been characterized by Arabidopsis (*Arabidopsis thaliana* L.) and rice (*Oryza sativa* L.), include the MYB transcription factor PHOSPHATE STARVATION RESPONSE REGULATOR 1 (PHR1), which acts as a central regulator by binding to the P1BS cis-element (GNATATNC) in the target genes (Chiou and Lin 2011); members of WRKY (Devaiah et al. 2007; Chen et al. 2009; Dai et al. 2016; Wang et al. 2014) and PHO families (Hamburger et al. 2002; Bari et al. 2006); the miRNAs miRNA399 and miRNA827 (Fujii et al. 2005; Kant et al. 2011); E3 ligase NLA and SIZ1 (Kant et al. 2011; Miura et al. 2005); and IPS1/At4 (Franco-Zorrilla et al. 2007; Hou et al. 2005). In contrast, only the bHLH transcription factor ZmPTF1 has been shown to increase the low P tolerance of maize (Zea *mays*. L); it does so by regulating carbon metabolism and root growth (Li et al. 2011). Many phytohormone were found to be related to plant P deficient responses. Wang et al. (2013) found that heterologous expression of ZmPHR1 in Arabidopsis increased the shoot biomass and inorganic Pi content. Huang et al. (2018) found that auxin response factors ARF7 and ARF19 function upstream of PHR1 to modulate its transcription in Arabidopsis roots. Pi loading into the xylem or root-to-shoot translocation were also important in low P tolerant plants, which was mediated by PHO1 (Poirier et al. 1991). PHO2, a gene in the same family as PHO1, was found to be regulated by PHR1 and miRNA399 at the transcription level, and could degrade PHO1 and Pi transporters to down-scale Pi absorption and translocation (Bari et al. 2006). PHOSPHATE TRANSPORTER 1 (PHT1) family plays crucial roles in both Pi acquisition and Pi translocation (Nussaume et al. 2011). In Arabidopsis, the knockout of AtPHT1;1

significantly reduced Pi uptake, and AtPHT1;5 regulated Pi translocation to maintain Pi homeostasis (Shin et al. 2004; Nagarajan et al. 2011).

Maize is not only an important food and feed crop in the worldwide, but also an important raw material in energy production and many other industries (McLaren 2005). In maize, efficient P use is critical between planting and the six-leaf stage, when the young root is still too small to sustain shoot nutrient supply. P-deficiency during that stage reduced grain yield (Barry & Miller, 1989). Our previous study has found different strategies to cope with P deficiency between old European landraces and modern flint maize seedlings, which may also differ in changes in gene expression.

In recent decades, the development of molecular biological methods has promoted the potential genotypic response mechanisms of physiological metabolic responses of plants under biotic and abiotic stresses. In these molecular biological methods, RNA sequencing (RNA-seq) has showed advantages in quickly and comprehensively obtaining the gene expression to abiotic stresses in different developmental stages, tissues, and organs (Martin et al. 2013). RNA-seq results could provide insights into the discovery of new genes, including annotation genes and differentially expressed genes (DEGs), and molecular markers (Zhang et al. 2018). Compared with traditional sequencing methods, RNA-seq can provide high-throughput sequencing results with lower cost but high sensitivity, and can detect low abundance expressed genes. A large quantity of DEGs associated with P deficient response have been reported in various species by RNA-seq, such as wheat (Wang et al. 2019), maize (O'Rourke et al. 2013; Du et al. 2016; Sun et al. 2016; Yu et al. 2018), rice (Deng et al. 2018), and soybean (Zeng et al. 2016). Another method, Weighted Gene Co-expression Network Analysis (WGCNA) has also been used to analyze large gene data sets like micro-array and RNA-seq data sets. In WGCNA, a matrix of all the genes or filtered genes is built first and soft threshold of the matrix is performed, and then the scale-free network is established through the soft threshold (Langfelder and Horvath 2008). These genes are clustered into different modules in scale-free networks, and the genes in the same module have the same expression pattern. After correlating these modules with the external phenotypic traits of the sample, modules with a high correlation with the sample traits are selected. Finally, vital genes with high connectivity in the module could be identified. These genes play key roles in the concerning traits and development.

In this study, we selected 6 representative maize genotypes from the flint heterotic pool of a public temperate maize breeding program that spans the breeding progress since the onset of hybrid breeding from the 1950s to 2010s (Li et al., submitted). Through an oligonucleotide microarray platform, a total 1179 P-deficient responsive genes (high P vs. low P) in the roots of a low P-tolerant genotype were detected in maize by Calderon-Vazquez et al. (2008); among the genes, at least 33 % lack an orthologue in the Arabidopsis genome, implying that some P-deficiency responsive pathways are unique in maize (Calderón-

Vázquez et al. 2011). Therefore, the purpose of the present study was to find the key genes, key pathways, and potential molecular mechanisms of efficient P adaptation in maize.

2.Materials and Methods

2.1 Plant Materials

Six maize genotypes, of which seeds obtained from Institute of Plant Breeding of University of Hohenheim (UHOH), had been selected from previous study in the climate chamber. These six genotypes were: two flint founder lines (EP1, F2) originating from landraces, two elite flint lines (F160, F142) developed by the maize breeding program of UHOH, and two doubled haploid lines from landraces (SF1 produced from the German population Strenzfelder, SM1 produced from the Romanian population Satu Mare).

2.2 Plant growth conditions

The experiment was conducted in a growth chamber at the University of Hohenheim, Stuttgart Germany (48°42'44"N, 9°12'30"E). Seeds were surface-sterilized by rinsing them in 10%(v/v) H_2O_2 solution for 20 minutes afterwards they were put in the aerated 10 mM $CaSO_4$ solution at 25 ºC in the dark overnight. The next day seeds were placed between filter paper soaked in a 4mM $CaSO_4$ solution for around three days to germinate and then transferred one seedling to soil-sand substrate gently as described (Li et al, submitted). Plants were grown in half-cylinder rhizotrons (height 25 cm; diameter 10 cm) and the rhizotrons were arranged in an unblocked randomized design with five biological replicates for each treatment in the climate chamber. The climate chamber temperature was maintained 25 ºC during the day and 20 ºC at night, air humidity was set to 60%, day time was from 8 a.m. to 10 p.m. P content, root and rhizosphere traits were measured as discussed previously (Li et al, 2021).

2.3 RNAseq Analysis

The roots of maize plants were washed and harvested in five replicates, one plant per replicate, at 3 weeks after transferring into the rhizostron. Total RNA was extracted from ground root tissues with an innuPREP Plant RNA Kit (Analytik Jena AG, Jena, Germany) following the manufacturer instructions. The RNA concentration was determined by Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, US). Three of the replicates were sent to Novogene Co., Ltd where the RNAseq libraries were constructed using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) and the Novaseq 6000 instrument was used for sequencing. Reads were aligned to B73 genome RefGen_v4 with HISAT2 and counted using HTSeq.

2.4 Differential Expression Analysis

Differentially expressed genes were analyzed with DESeq2 package in R (Love et al. 2014; McCarthy et al. 2012). Genes with readcount fewer than two counts per million reads were filtered out, and analysis was carried out under False Discovery Rate < 0.05 as the significant measure.

2.5 Weighted Coexpression Network Analysis

WGCNA package was necessary to construct the co-expression network (Langfelder and Horvath, 2008). The correlation between genes was performed by Tukey's Biweight correlation (Horvath 2011), and the correlation results were used to calculate the distance matrix. The calculations were done using WGCNA package in R (Langfelder and Horvath 2008). The distance matrix was later used for the dynamic hierarchical clustering and to build the edges (connections) between nodes (genes) in the network.

2.5.1 Co-expression Network Construction

First, unqualified genes of samples were excluded from subsequent studies. Then, an appropriate soft-thresholding power (sft, in our study sft was 7) based on a scale-free topology criterion was chosen according to the function pickSoftThreshold and the weighted adjacency matrix was built using the sft. The correlation between one gene and all other ones was incorporated into an adjacency matrix, and the adjacency matrix was later transformed into the topological matrix (TOM) (Yip and Horvath 2007). These genes demonstrated hierarchical clustering according to the TOM-based dissimilarity (1-TOM) measure. After hierarchical clustering, highly correlated genes were assigned to the same module (Ravasz et al. 2002).

2.5.2 Identification of Significant Modules and Functional Annotation

After the samples' trait information imported into the network, the module eigengene (ME), module membership (MM), and gene significance (GS) were calculated. Eigengenes were representative gene of the principal component 1 in a module, ME representing the expression pattern of eigengenes in the module. MM was the degree of correlation between eigengenes and module. If MM is close to 1, the eigengene is highly correlated with the module. GS could be considered as the association of individual genes with samples' trait information. The module could be a candidate key module if it has a high ME valve with the samples' trait information (Langfelder and Horvath 2008).

2.5.3 Identification and visualization of Hub gene

Hub genes are defined as genes with a high correlation in candidate modules. The hub gene was filtered to meet the absolute value of the geneModuleMembership >0.80 and geneTraitSignificance >0.60. After identifying hub genes highly associated with P utilization related traits, the annotating the function of relevant

modules was preformed to the potential mechanisms for the effects of corresponding trait. Gene ontology (GO) functional annotation and the Kyoto encyclopedia of genes and genomes (KEGG) were analyzed by gProfiler2 (Kolberg et al. 2020) and visualized the results by clusterProfiler, enrichplot, DOSE (Yu 2020) packages in R. Meanwhile, search tool for the retrieval of interacting genes (STRING) (Szklarczyk et al. 2015) database was used to construct a Protein-protein-interaction (PPI) prediction analysis and Cytoscape (Shannon et al. 2003) visualize the PPI network. If a gene has high degrees in a PPI network, it will be defined as playing a critical role in the module.

3. Results

3.1 Transcriptomic analyses of P response in the seedling maize root

Previous analyses demonstrated of P response related traits of 24 European flint maize released from the onset of hybrid breeding. Under Pi limitation, founder flint EP1 and F2 showed higher Pi uptake and biomass accumulation compared with the other four genotypes (Fig. 1 a-d). In general, root hair length and root to shoot ratio increase under low P compared to high P condition were larger in founder flint lines (Fig. S1); and doubled haploid landrace SM1 has relatively higher mycorrhizal colonization (Li et al, 2021). To capture the transcriptional changes coinciding with these physiological and morphological changes in maize roots, plants roots harvested in the same experiment were used for RNAseq analysis.

Principal component analysis (PCA) clarified two principal components (PCs), which altogether explain 59% of the total sample variance in the transcriptomic data. Specifically, the first PC corresponded to the P treatments and explained 39% of the total sample variation, whereas the second PC (PC2; 20% of sample variance) delineated six genotypes analyzed (Fig. 1e). These results showed that in addition to a response to P treatments, the gene expression of samples also exhibited a genotypic difference.

The gene expression levels were analyzed and normalized using the VST method in DEseq2; $|\log2(\text{Fold-Change})| \geq 1$ and adjusted p-value < 0.05 were set as the threshold for significant differential expression. We found with a range of 3293 to 5863 genes which showed differential expression between LP and HP in the six genotypes. SF1 had the most DEGs and F160 had the least DEGs among these six genotypes. EP1 and F2 had similar numbers of DEGs, which a total of 2332 and 2575 DEGs were upregulated, whereas 2215 and 2499 DEGs were downregulated in EP1 and F2, respectively. F142, SF1 and SM1 had similar numbers of DEGs (Fig. 2a).

To identify common genes in different genotypes, the overlaps of all genotypes and unique genes were shown in a Venn diagram (Figs. 2b and c). A total of 144 downregulated genes (Fig. 2b) and 594 upregulated genes (Fig. 2c) overlapped with those of six genotypes, respectively. The unique downregulated genes

were more than the shared DEGs in every genotype, suggesting different plant strategies after P deficiency stress (Fig. 2b). The unique upregulated genes varied in every genotype, which SM1 had the most unique upregulated DEGs and SF1 had the least unique upregulated DEGs (Fig. 2c).

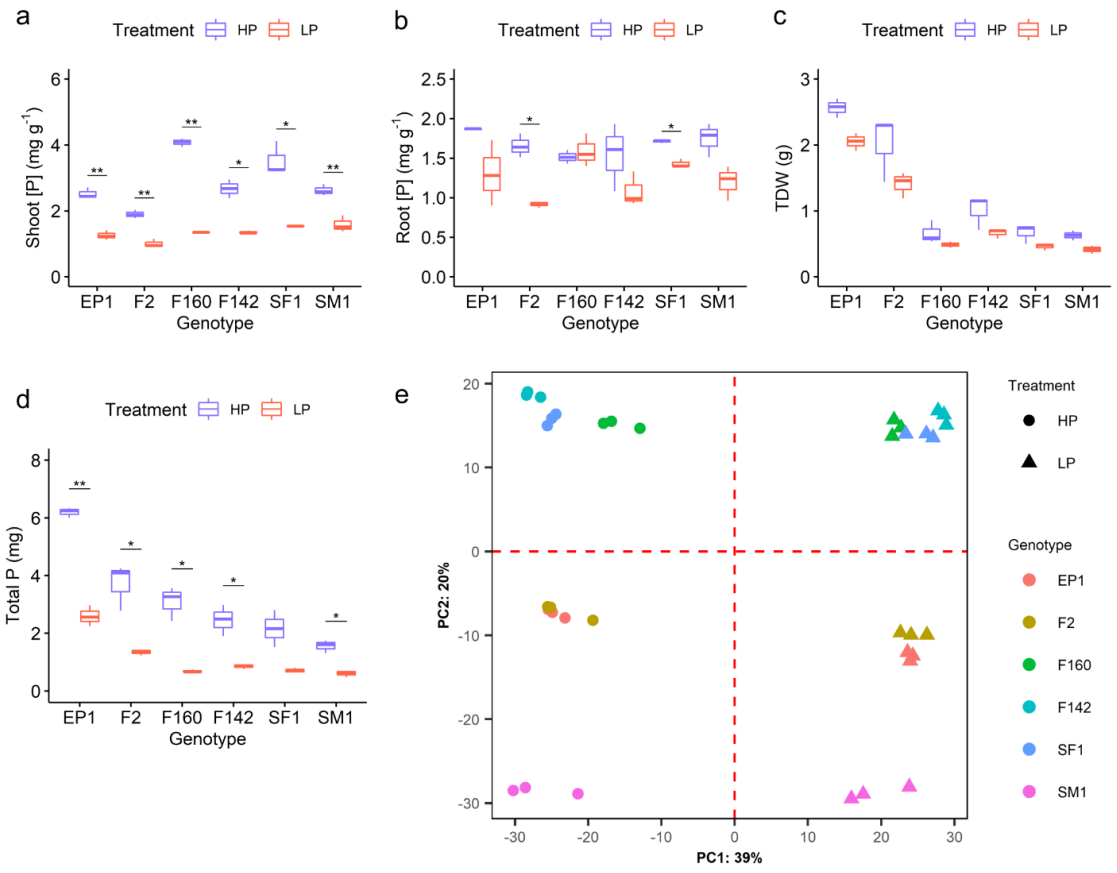


Figure 1. Biomass and P content of six genotypes (a-d). (e)PCA identified two PCs corresponding to the P treatments (PC1) and genotypes (PC2) in our RNA-seq analysis. Each point corresponds to one RNAseq sample. Dots correspond to high P treatment, triangle points correspond to low P treatment; Each color corresponds to a specific genotype.

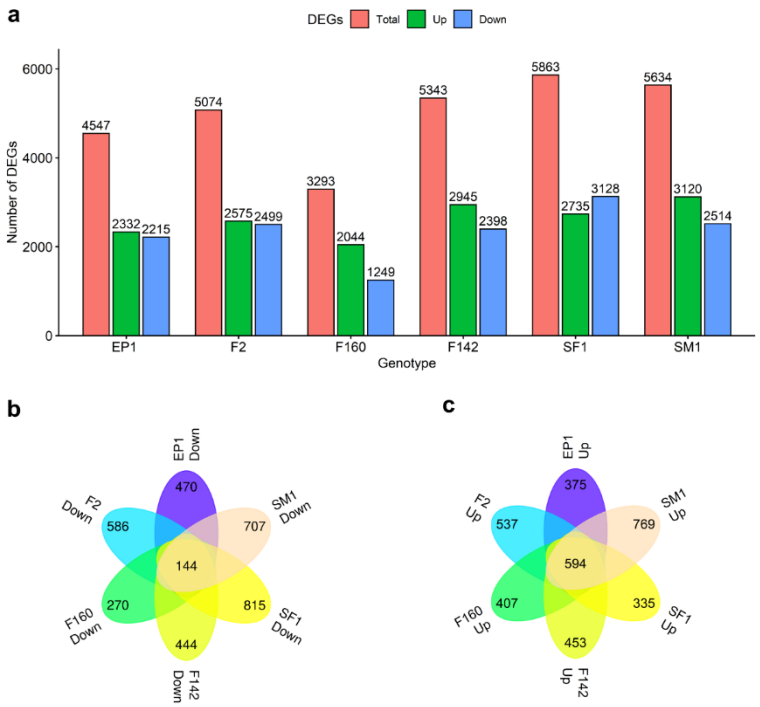


Figure 2. Up- and downregulated DEGs and venn diagrams across six genotypes. **(a)** Numbers of Differently Expressed Gene (DEG) under low P condition (LP) relative to high P condition (HP) of genotypes in our RNAseq analysis. **(b-c)** Venn plots showing numbers of unique up- or down-regulated genes in each genotype (in the petals), and shared up- or down-regulated gene number of all genotypes (in the center).

3.2 A Weighted Coexpression Network Analysis (WGCNA) identifies candidate regulatory genes for P response

Genes differentially expressed were queried for relationships to known P starvation response genes identified previously in Arabidopsis (Table S1). In general, maize presented two or more duplicate loci with high homology to individual Arabidopsis P starvation response genes. Detailed summaries of the pathways for plant P starvation response and in-depth transcript accumulation patterns of predicted maize candidate genes expressed during P deficiency are summarized according to previous reviews (Calderón-Vázquez et al. 2011; Ajmera et al. 2019) and provided in Table S1. We next used a gene coexpression network analysis (Langfelder and Horvath 2008, 2012) to identify additional candidate genes involved in regulating the P utilization of the maize. In a WGCNA, each edge (correlation between gene expression levels) was calculated to indicate the strength of its co-expression relationship with every other node in the network. In this way, a WGCNA was constructed based upon the expression-level correlations of all 22764 transcribed genes identified in our RNAseq analysis.

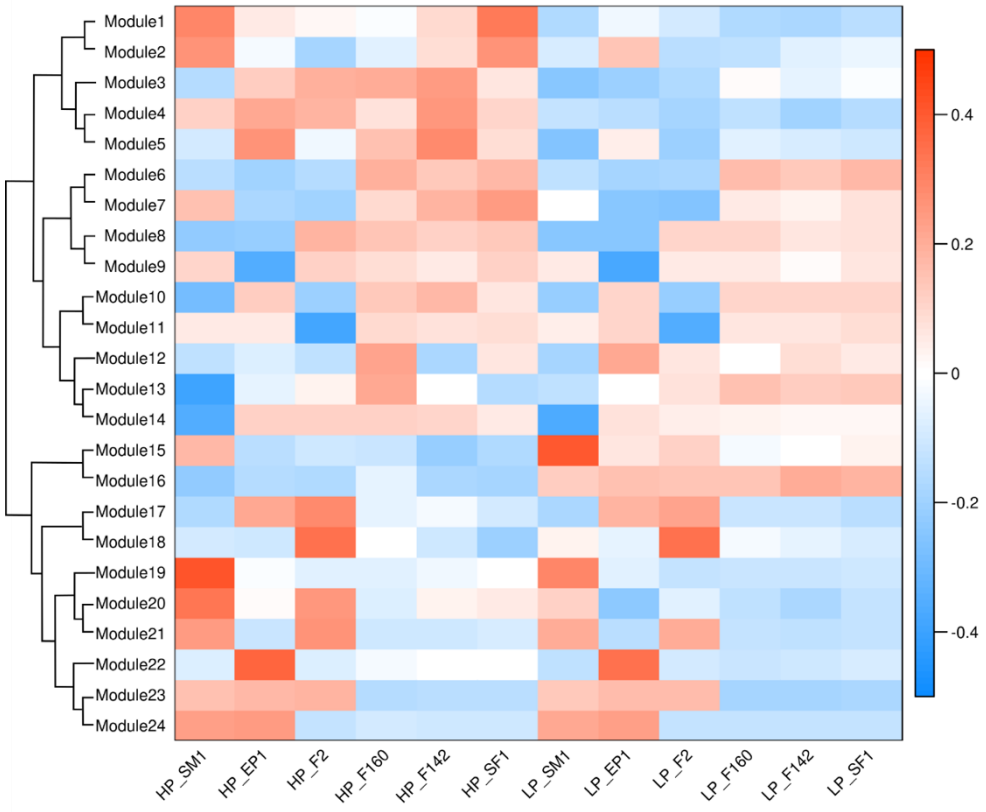


Figure 3. The expression levels of eigengenes within the 24 modules identified in our WGCN analysis at each of the six genotypes analyzed under LP and HP.

Our WGCNA partitioned the transcriptome of six genotypes into 24 coexpression modules. Fig. 3 illustrates the expression levels of eigengenes (idealized representative genes) within these 24 modules at each of the six genotypes analyzed under LP and HP conditions. Expression levels of genes within modules 15 and 16 are associated with the P treatments (Fig. 3). Thus, comparisons of transcript accumulation levels between P treatments at each genotype (Fig. 2) reveal interesting correlations, clearly identifying a set of "suppressed" by P deficiency (1 to 5), "induced" by P deficiency (15 to 16) and "genotype-specific" modules (6 to 14 and 17 to 24). Modules which are significantly correlated with specific root traits contain genes from known P starvation response and regulatory genes were collected. Within module 16, for example, the PHT1;1 homologs Zm00001d004305 showed correlation coefficients of 0.76 with Root/Shoot and 0.63 with Root hair length, whereas the PHR1 homolog Zm00001d029020, found in module 14, has a correlation coefficient 0.51 with rhizosphere pH. Most known P starvation response and regulatory genes (Table S1) could be found in Module 16. Moreover, Module 16 was highly corelated to PUtE, PUE, root/shoot, root hair

length and root exudated organic acids (Fig. 4), so it was selected to investigate the molecular mechanism of P adaptation under P deficiency.

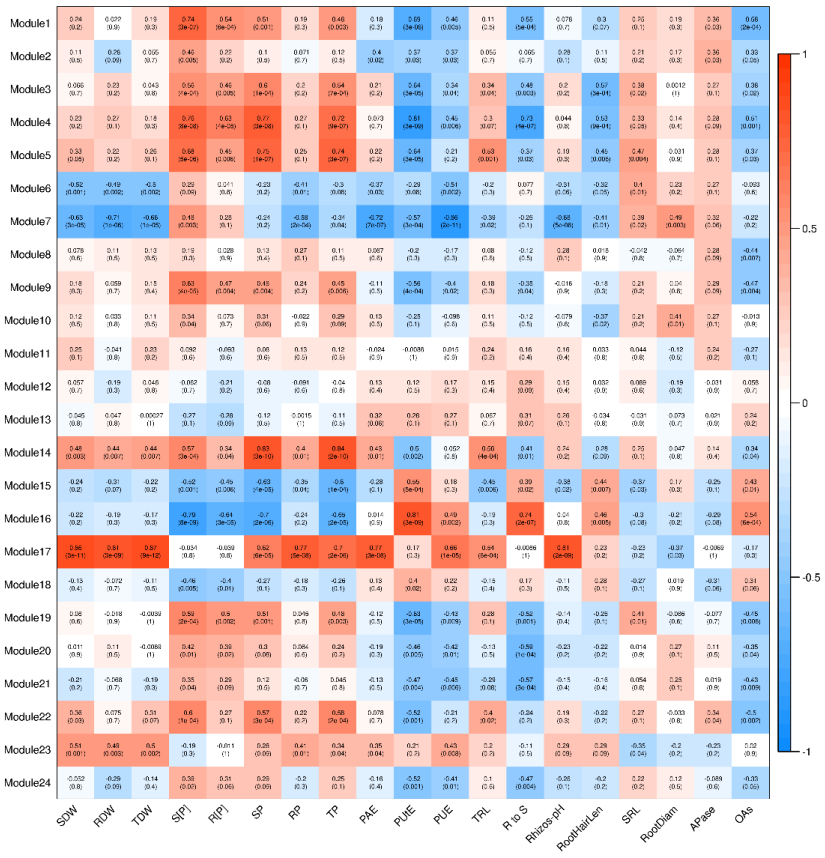


Figure 4. Heat map depicting the correlation of each trait (x axis) to the 24 coexpression modules (y axis) identified in transcriptomic analyses of the six maize genotypes. Colors (red to blue) correspond to the values of the Pearson's pairwise correlations, where red (+1) is positively correlated and blue (−1) is negatively correlated.

3.3 Functional analysis of module 16

Module 16 is one of the modules "induced" by P deficiency. GO term analysis of the eigengenes in coexpression module 16 showed significant enrichment for oxidation-reduction process, transmembrane transport and cellular response to phosphate starvation (Fig. S1). KEGG term analysis of the eigengenes in coexpression module 16 showed significant enrichment for biosynthesis of secondary metabolism and carbon related metabolism (Fig. S1). Hub genes related to PUtE of Module 16 were selected to meet the absolute value of the geneModuleMembership >0.80 and geneTraitSignificance for PUtE >0.60 (Fig. 5a). GO term analysis of hub genes related to PUtE in coexpression module 16 showed significant enrichment for

cellular response to phosphate starvation, including 89 genes (Fig. 5b). KEGG term analysis of hub genes related to PUtE in coexpression module 16 showed significant enrichment for Glycerolipid metabolism, Glycerophospholipid metabolism, Carotenoid biosynthesis and ABC transporters (Fig. 5c). The PPI network found top 10 key genes related to PUtE, which is Zm00001d005925 (1_Glucose-6-phosphate isomerase), Zm00001d041243 (2_Malate dehydrogenase), Zm00001d010038 (3_CASP-like protein 5), Zm00001d026156 (4_Putative glycerol-3-phosphate transporter 1), Zm00001d031428 (5_Monogalactosyldiacylglycerol synthase 2 chloroplastic), Zm00001d022496 (6_ Anther-specific proline-rich protein APG), Zm00001d027936 (7_15-cis-phytoene desaturase chloroplastic/chromoplastic), Zm00001d043442 (8_Carotenoid cleavage dioxygenases8), Zm00001d050428 (9_NADP-dependent glyceraldehyde-3-phosphate dehydrogenase) and Zm00001d048835 (10_ Phosphatidate phosphatase PAH2) (Fig. 5d).

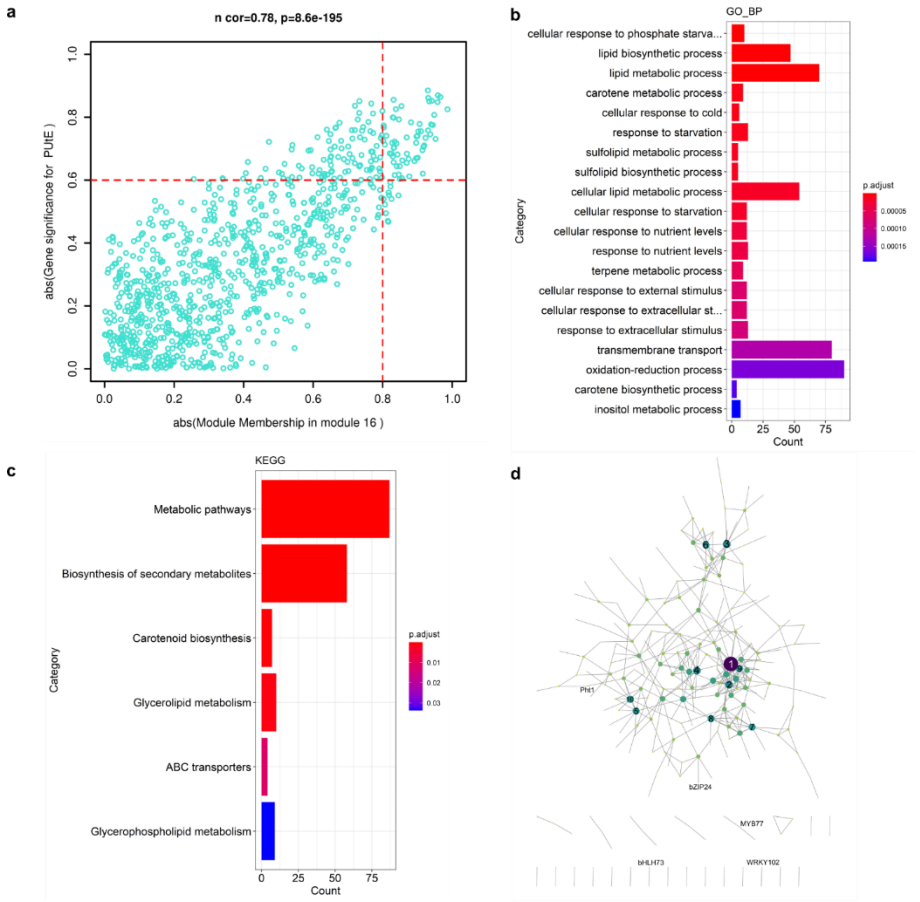
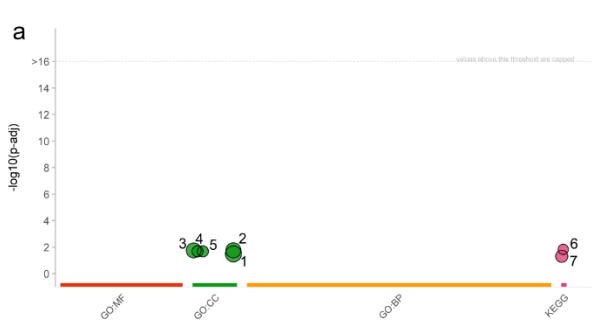


Figure 5. Net-work analysis the hub genes in Module 16 which had the highest correlation coefficient (0.81) with PUtE. (a) Hub genes of the Module 16. Hub genes were genes having a gene significance over 0.6 and a module membership over 0.8.; (b) Top 20 GO categories of the hub genes involved in Module 16; (c) KEGG categories of hub genes involved in Module 16; (d) Protein-protein-interact (PPI) prediction analysis of the hub genes in Module 16. Node size means the degree of genes.

3.4 Functional analysis of module 17

Module 17 is one of the modules which specifically expressed higher in founder flint. GO term analysis of the eigengenes in coexpression module 17 showed significant enrichment for peptidase complex, endopeptidase complex and proteasome complex (Fig.6a). KEGG term analysis of the eigengenes in coexpression module 17 showed significant enrichment for proteasome and Glutathione metabolism (Fig.6a). The PPI network found top 10 key genes related to SDW, which is Zm00001d035136 (1_Succinate-CoA ligase), Zm00001d008245 (2_ribosomal protein L30), Zm00001d027514 (3_Ubiquitin domain-containing protein DSK2b), Zm00001d022573 (4_26S proteasome regulatory subunit 4 homolog A), Zm00001d034667 (5_Fes1A), Zm00001d005248 (6_ Dolichyl-diphospho-oligosaccharide--protein glycosyltransferase 48 kDa subunit), Zm00001d020506 (7_ 26S proteasome non-ATPase regulatory subunit 9), Zm00001d015788 (8_proteasome component4), Zm00001d021020 (9_ 60S ribosomal protein L32) and Zm00001d037700 (10_Heat shock protein 4) (Fig. 6b).



id	source	term_id	term_name	term_size	p_value
1	GO:CC	GO:1905368	peptidase complex	176	3.2e-02
2	GO:CC	GO:1905369	endopeptidase complex	137	1.7e-02
3	GO:CC	GO:0000502	proteasome complex	130	1.7e-02
4	GO:CC	GO:0005838	proteasome regulatory particle	57	2.1e-02
5	GO:CC	GO:0022624	proteasome accessory complex	57	2.1e-02
6	KEGG	KEGG:03050	Proteasome	50	1.4e-02
7	KEGG	KEGG:00480	Glutathione metabolism	71	4.9e-02

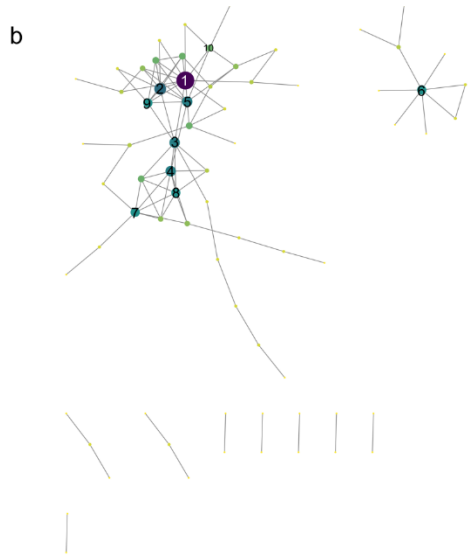


Figure 6. Network analysis the genes in Module 17 which had the highest correlation coefficient (0.86) with SDW. (a) GO categories and KEGG categories involved in Module 17; (b) Protein-protein-interact (PPI) prediction analysis in Module 17. Node size means the degree of genes.

4. Discussion

4.1 General molecular mechanism to LP response in the six maize genotypes

The adaptations to variable P availability depend at least in part on changes in gene expression, including morphological modification to access a large soil area (Postma et al. 2014; Haling et al. 2018); chemical modifications of the rhizosphere (Hinsinger 2001; Pang et al. 2018); the establishment of symbiotic relations with AMF to help access P from the labile soil P pool (Smith et al. 2011; van der Heijden et al. 2015); recycling and mobilization of internal Pi (Cruz-Ramírez et al. 2006); and bypassing the metabolic steps that require ATP (Ganie et al. 2015). Previous research in maize reported changes in some specific proteins' response to Pi starvation by altering the balance of carbohydrate, protein, nucleotide, and secondary metabolites (Li et al. 2007). Pi deficiency's effect on the expression of genes encoding proteins that mediate these pathways in maize roots remains to be determined.

4.1.1 Pi deficiency integrates P and C metabolism in maize roots

The protein modification in the alteration of the balance of carbohydrate mainly happened in the pathway of glycolysis and tricarboxylic acid (TCA) cycle (Li et al. 2007), which is consistent with gene expression pattern (Calderon-Vazquez et al. 2008) and our functional enrichment in module 16. Identifying changes in the expression of several sugar-related genes, including genes involved in sugar synthase, photosynthate product distribution and glycolysis in Pi-deficient maize roots, proved that there is direct crosstalk between sugar metabolism and Pi deficiency in plants (Hammond and White 2011). Tesfaye et al. (2007) noted a dark/light directed expression of several carbohydrate and metabolism genes in response to Pi stress in white lupin roots. Glycolysis is modified through bypassing reactions that require ATP, as reflected by the cluster of genes encoding phosphoglycerate mutase (PGM), phosphoenolpyruvate carboxylase (PEPCase), and PEPcase kinase (PEPK) in module 16. However, increased gene expression in the transcription levels were observed only PEPK in all genotypes, probably to provide the carbon skeletons necessary for the next intermediary reactions in C metabolism. In addition, the synthesis and excretion of organic acids have been documented in maize as a response to Pi deficiency (Gaume et al. 2001). Significant induction of several genes encoding Aluminum-activated malate transporter 10 (ALMT10) and malate dehydrogenase 2 (MDH2) in all genotypes were observed, whose activity are necessary for the synthesis of malate and citrate, indicating that in maize roots ALMT10 or MDH2 is a limiting step for the exudation of citrate/malate.

4.1.2 P recycling under Pi deficiency

P recycling changes in these maize roots in the gene expression level was related to the lipid metabolism, cell wall organization and transmembrane transporter. Internal Pi recycling involves phospholipid

degradation and sulfolipids/galactolipid synthesis (Essigmann et al. 1998; Hammond et al. 2004). The expression increased phospholipid degradation related genes included phospholipase A2 (PLA2), phospholipase C (PLC), phospholipase D (PLD), phosphatidate phosphatase 2 (PAH2) and glycerophosphodiester phosphodiesterase (GPPDs), in which PLC and PLD were also found to mediate phospholipid degradation in Arabidopsis (Cruz-Ramírez et al. 2006) and PLA2 and PAH2 was stronger induced in maize (Calderon-Vazquez et al. 2008). The expression increased sulfolipids/galactolipid synthesis included UDP-sulfoquinovose synthase, Sulfoquinovosyl transferase SQD2 and Monogalactosyldiacylglycerol synthase 2 (MGD2). Similarly, transport systems were strongly affected under Pi deficiency (Supplementary Database S1). The alterations in the transcript level of phosphate, sulphate, Fe, and ABC transporters as well as sugars and oligo-peptides encoding genes was identified. In addition, the genes related to establishment of the Casparian strip membrane domain (CSD) and the subsequent formation of Casparian strips was found in module 16, which in rice also determined similar genes under Pi deficiency (Wasaki et al., 2003).

4.1.3 Carotenoid metabolism under Pi deficiency

Carotenoid metabolism related genes were found to be induced under Pi deficiency in module 16. Carotenoid cleavage dioxygenases (CCDs) drive carotenoid catabolism to produce various apocarotenoids and immediate derivatives with particular developmental, ecological, and agricultural importance. Carotenoids can be sequentially cleaved by plastid CCD7 and CCD8, generating carlactone as the strigolactone precursor (Alder et al. 2012), which were highly induced under low P in all the six genotypes. Strigolactones coordinate shoot and root development, promote germination of parasitic seeds, and trigger AMF branching (Umehara et al. 2008). Loss-of-function mutation of CCD7 or CCD8 leads to dramatically more axillary branches in Arabidopsis. OsCCD8b also regulates rice (Oryza *sativa*) tillering, whereas ZmCCD8 plays essential roles in root and shoot development, with smaller roots, shorter internodes, and longer tassels in Zmccd8 mutant plants (Arite et al. 2007; Guan et al. 2012). According to our WGCNA analysis, ZmCCD8 is one key genes in coexpression Module 16, which is typically a set of genes highly expressed under LP and significantly correlated with P utilization of all the six preselected maize genotypes (Fig. 6d). A particularly interesting feature of a number of CCD genes is their active involvement in P-deficiency responses. Upon Pi limitation, enhanced CCD8 expression boosts strigolactone production, reducing tiller proliferation and stimulating AMF-mediated Pi acquisition (Umehara et al. 2008; Czarnecki et al. 2013).

4.2 Specific molecular mechanism to LP response in founder flint

In founder flint EP1 and F2 showed higher biomass and Pi content in LP and can be considered efficient. The results in our study suggested that the enhanced biomass accumulation maybe related to the ability of integrating C and N metabolism. The ability of these two maize roots to preserve N- and C containing

metabolites under Pi starvation is notable. An extensive repression of genes related to proteasome and peptidase involved in protein degradation was found in module 17, the up-regulation of protein degradation in a low Pi tolerance maize was also found under LP by microarray (Lin et al. 2013). Possible reasons could be the low Pi tolerance maize uses amino acids as an alternative C source to maintain biomass (Calderon-Vazquez et al.,2008). It was also found that genes involved in glutathione transferase were induced under Pi deficiency. Glutathione was widely studied and found to be involved in stress management like drought, oxidative stress, chilling, high temperature (Kocsy et al. 2001; Štolfa et al. 2016; Pardo-Hernández et al. 2020). A previous study linked low P availability in the soil with photo-oxidative stress in plants (Hernández and Munné-Bosch 2015), which implies that glutathione transferase accumulation played a role as antioxidant regulator in founder flints.

5. Conclusions

In maize, as in other crops, the general mechanism of regulating P utilization under LP is related to balance of carbohydrate through glycolysis and TCA cycle, alteration of lipid metabolism, changes of gene expression of transmembrane transporters and carotenoid biosynthesis. Additionally, the P use efficient founder flint lines EP1, F2 and doubled haploid landrace SM1 have their own specific strategies and mechanism to cope with LP. This may help to identify network constraints for efficient P use. Gene networks identified in this study and their proposed role in Pi adaptation are supported by the analysis of the data available from existing transcriptome, proteome and metabolome experiments conducted in maize and the other species. These genes provide an opportunity to identify different alleles involved in the adaptive response to Pi deficiency; they also provide a basis for identifying candidate genes and processes that may improve the tolerance to Pi deficiency in maize and other cereal crops. This work also provides a framework for the production of Pi-specific maize arrays to study global gene expression changes between Pi high-efficiency and low-efficiency maize genotypes.

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Authors' Contributions

X. L. and U. L. conceived the research. X. L. and U. L. designed the research. X. L. conducted the experiment and analyzed the data. X. L wrote the manuscript. All authors read and approved the manuscript. The authors declare that they have no conflict of interest.

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Supplemental materials

Table S1. Phosphate Starvation-Induced genes in Arabidopsis and their respective orthologs in maize. (n.d. means not detected.)

Gene	Arabidopsis	Maize	Module	Gene name	Description
ARP6	At3g33520	Zm00001d024059	1		uncharacterized
IPS1	At3g09922	Zm00001d022669	16	lncRNA819, pilncr1 - pi-deficiency-induced long non-coding RNA1	inhibits ZmmiR399-guided cleavage of ZmPHO2
bHLH32	At3g25710	Zm00001d047878	3	bhlh143 - bHLH-transcription factor 143	Locus designated and assigned to a transcription factor family by the GRASSIUS project (Yilmaz et al 2009) , which also provided the mappings to the B73_Reference Genome sequence v2 gene models.
		Zm00001d039764	13	bhlh104	Locus designated and assigned to a transcription factor family by the GRASSIUS project (Yilmaz et al 2009), which also provided the mappings to the B73_Reference Genome sequence v2 gene models.
CAX1	At1g08960	Zm00001d023377	4		Cation/calcium exchanger 5
CAX3	At3g51860	Zm00001d044533	13	cax3 - calcium exchanger3	Expression affected by ABP4, auxin, and Ca++. Homolog of Arabidopsis CAX1 involved in auxin transduction pathway.
IPK1	At5g42810	Zm00001d001974	6		Uncharacterized Protein: inositol-pentakisphosphate 2-kinase 1
LPR1	At1g23010	Zm00001d040035	21	mco1 - multicopper oxidase1	LiPocalin-Related (LPR), LOW PHOSPHATE ROOT (LPR), LPR1b (per Zhang, XR), (LPR1) Cupredoxin superfamily protein (per Zhang, XR), LPR2 (per NCBI), multicopper oxidase LPR1 homolog 1 (per NCBI)
		Zm00001d040034	10	pza02427	candidate gene for root system architecture and nitrogen use efficiency
MYB62	At1g68320	Zm00001d008528	16	myb22 - MYB-transcription factor 22	Locus designated and assigned to a transcription factor family by the GRASSIUS project (Yilmaz et al 2009), which also provided the mappings to the B73_Reference Genome sequence v2 gene models. similar to myb domain protein 116 of A. thaliana

		Zm00001d040019	23	myb137 - MYB-transcription factor 137	Locus designated and assigned to a transcription factor family by the GRASSIUS project (Yilmaz et al 2009), which also provided the mappings to the B73_Reference Genome sequence v2 gene models. similar to myb domain protein 62 of A. thaliana
PHF1	At3g52190	Zm00001d019048	16	pco144169	pco144169(539), SEC12-like protein 1 (per NCBI)
PHO1	At3g23430	Zm00001d051945	16	phos2 - phosphate transporter2 PHO1-like phosphate transporter	phosphate transporter PHO1-2-like (per NCBI), ZmPHO1, ZmPho1;2a (per Sawers, RJH)
PHO2	At2g33770	Zm00001d038972	16	uce10 - ubiquitin conjugating enzyme10	similar to Arabidopsis Ubiquitin-conjugating enzyme family protein AY109797 (per Old Canonical Name), CL10211_1, phosphate2 (per Du, QG), putative ubiquitin-conjugating enzyme E2 24 (per NCBI), ZmPHO2 (per Du, QG)
PHR1	At4g28610	Zm00001d029020	14	glk17 - G2-like-transcription factor 17	Locus designated and assigned to a transcription factor family by the GRASSIUS project (Yilmaz et al 2009), which also provided the mappings to the B73_Reference Genome sequence v2 gene models. GLK17 putative MYB DNA-binding domain superfamily protein (per NCBI), PHR1 (per NCBI), ZmMYB-CC1 (per Bai, JR), ZmPHR1 (per Xu, YJ)
		Zm00001d019536	10	glk15 - G2-like-transcription factor 15	Locus designated and assigned to a transcription factor family by the GRASSIUS project (Yilmaz et al 2009), which also provided the mappings to the B73_Reference Genome sequence v2 gene models. CDPK substrate protein 1 (per NCBI), pco106271(668) Protein PHOSPHATE STARVATION RESPONSE 1 (per NCBI), ZmMYB-CC9 (per Bai, JR), ZmPHR15 (per Xu, YJ)
PHT1;1	At5g43350	Zm00001d004305	16	pht13 - phosphate transporter protein13	**Annotation**: can completely or partly complement the yeast Pi-uptake mutant

					Expression: induced under low Pi conditions **Expression**: up-regulated by arbuscular mycorrhizal fungi (AMF) **Map Note**: Genetic coordinate based on bp coordinates in B73 v4 (EHC Aug 2019) Pht1;2 (per NCBI), ZmPHT1;4 (per Liu, F), Zmpt4 (per Liu, F)
PHT1;4	At2g38940	Zm00001d004301	n.d.	pht3 - phosphate transporter protein3	similar to Arabidosis inorganic phosphate transporter 1-4, PT3 (per Wright, DP), ZmPHT1;8 (per Liu, F), Zmpt8 (per Liu, F) **Map Note**: Genetic 2 coordinate based on bp coordinates in B73 v4 (EHC Nov 2017) **Annotation**: can completely or partly complement the yeast Pi-uptake mutant **Expression**: induced under low Pi conditions
		Zm00001d032850	16	pht2 - phosphate transporter protein2	Pht1;1 (per Nagy, R), pt2 (per Wright, DP), PT2 (per Wright, DP), ZmPHT1;9 (per Liu, F), Zmpt9 (per Liu, F) **History**: Mapped in silico by inference from BAC sequence match (EH Coe Jul 2008) **Annotation**: can completely or partly complement the yeast Pi-uptake mutant **Expression**: up-regulated by arbuscular mycorrhizal fungi (AMF) **Expression**: induced under low Pi conditions **Map Note**: Genetic 1 coordinate based on bp coordinates in B73 v4 (EHC Sep 2017)
PHT2;1	At3g26570	Zm00001d017069	n.d.		Inorganic phosphate transporter 2-1 chloroplastic
PLD1	At3g16785	Zm00001d037946	1	pld13 - phospholipase D13	phospholipase D p1 (per NCBI), phospholipase D zeta 1 (per NCBI), ZmPLDζ (per Chen, L), ZmPLDZeta
PTF1	At5g58010	Zm00001d045046	n.d.	ptf1 - Pi starvation-induced transcription factor1	single copy, may be involved in regulating carbohydrate metabolism bhlh171 (per Grassius), bHLH transcription factor PTF1 (per NCBI), putative transcription factor (ptf) (per Hawkins, JS), umc2362, ZmbHLH194 (per Gramene) **Annotation**: bhlh171 locus designated and assigned to a transcription factor family by

					the GRASSIUS project (Yilmaz et al 2009) , which also provided the mappings to the B73_Reference Genome sequence v2 gene models. **Map Note**: Genetic coordinate based on re-combination mapping and bp coordinates in B73 v4 (EHC May 2019) **Expression**: overexpression allows improved root system; increased ABA content; and acti-vated ABA-, CBF4-, ATAF2-and NAC30-mediated stress responses; increased drought tolerance (Li et al., 2019)
SPX1	At5g20150	Zm00001d053626	16	cl49602_1	cl49602_1(357), IDS4-like protein (per NCBI), SPX domain-containing protein 1 (per Benfey, PN)
SPX3	At2g45130	Zm00001d044541	16		SPX domain-containing protein 3
		Zm00001d029460	16		SPX domain-containing protein 3
		Zm00001d033047	16		SPX domain-containing protein 3
SQD2	At5g01220	Zm00001d018595	16	GRMZM2G100652	ortholog associated with root-to-shoot ratio in A. thaliana pco094720a (per NCBI), Sulfoquinovosyl transferase SQD2 (per NCBI)
		Zm00001d028539	9	IDP329	sulfoquinovosyl transferase SQD2 (per NCBI)
ZAT6	At5g04340	Zm00001d029586	4		zinc finger protein 36

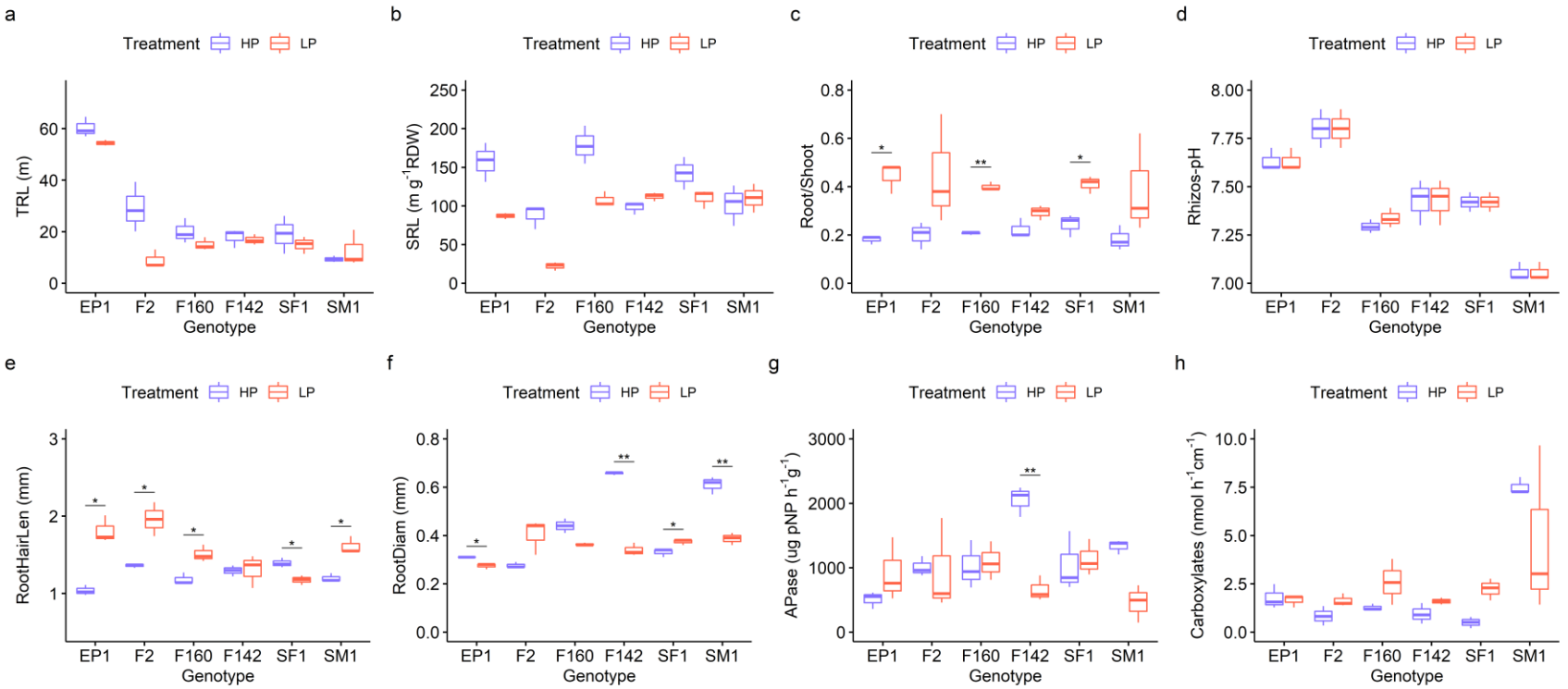

Figure S1. Root traits of six genotypes between low P and high P. (a) total root length; (b) specific root length; (c) root to shoot ratio; (d) rhizosphere soil solution pH; (e) root hair length; (f) average root diameter; (g) root secreted acid phosphatase activity; (h) root exudated oganic anions.

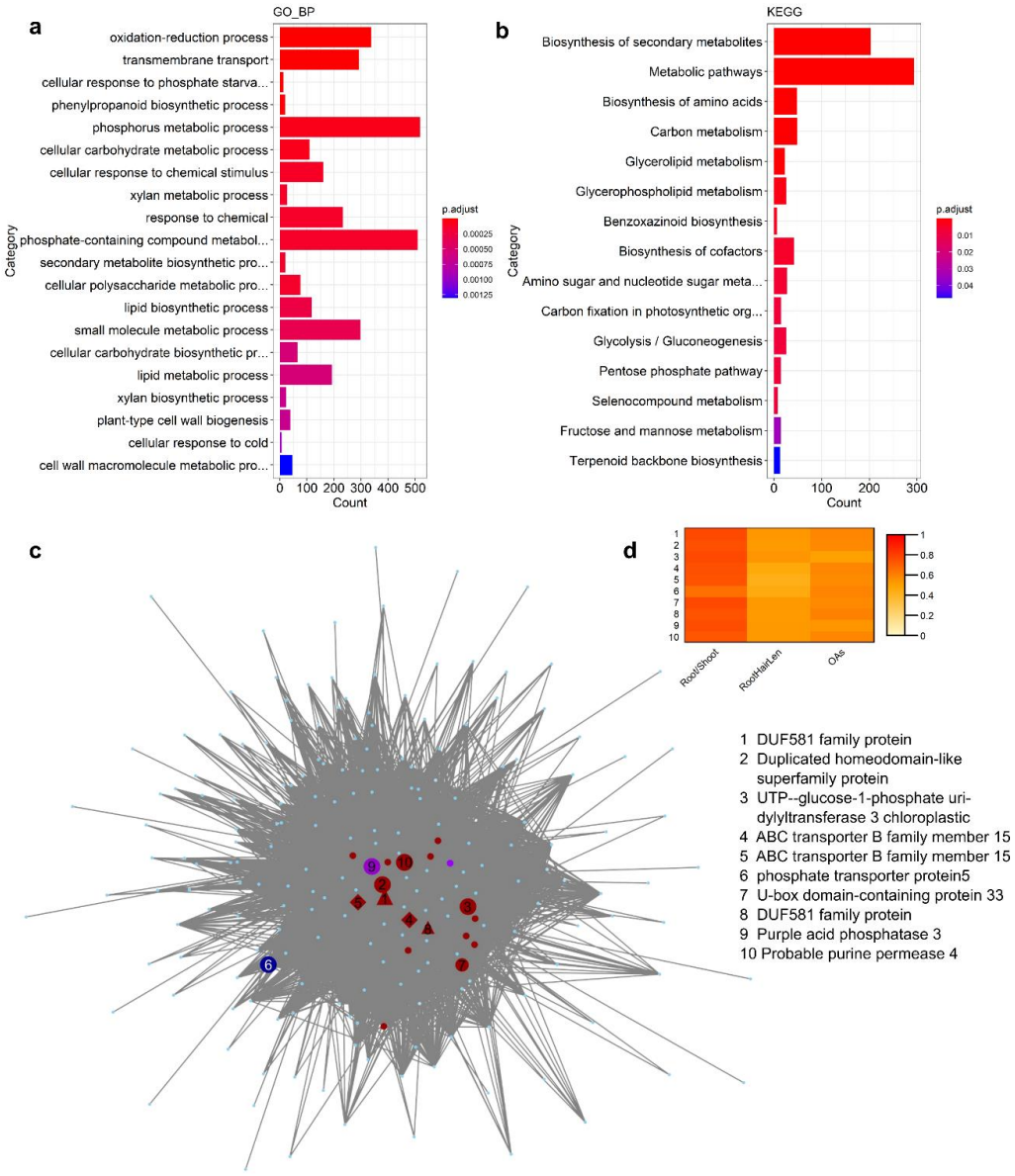

Figure S2. Net-work analysis the eigengenes in Module 16 which had the highest correlation coefficient (0.81) with PUtE. (a) Top 20 GO categories of the eigengenes involved in Module 16; (b) Top 20 KEGG categories of eigengenes involved in Module 16; (c) Net-work analysis of the eigengenes in Module 16. Node size means the degree of genes; (d) Correlation between representative root traits and key eigengenes.

9. General discussion

The application of mineral P fertilizers increases plant-available P in the soil and improves crop growth and yield, but its success dependents on many different factors such as type of fertilizer, application form, the timing of fertilization, weather conditions, various soil properties and plant factors (Holford, 1997; Shen *et al.*, 2011). As P is a finite resource, breeding of major crops with improved P efficiency may have large effects on the sustainability of the agroecosystems (Ludewig *et al.*, 2019). Efforts in breeding tropical maize for P-deficient, acid soils with high loads of toxic Aluminum have identified candidate loci for important root traits (Azevedo *et al.*, 2015), which gave a good example that breeding indeed has an impact on root traits. But it is still ambiguous how the breeding affected maize root traits for P-deficient, neutral or alkaline soils, and the molecular mechanism of LP tolerance was lack of an integrative understanding. In this dissertation, we will discuss the breeding effect on European maize root traits and the trade-off of different root traits under LP first, then try to summarize an integrative understanding of maize Pi utilization molecular mechanism.

9.1 Root and rhizosphere related responses of European maize flint lines to P deficiency

There are substantial variability and distinct genetic architectures in dent and flint maize diversity panels (Messmer *et al.*, 1992; Cartea *et al.*, 1999; Rincent *et al.*, 2014), which is consistent with different P efficiency trends of the two panels showed in Chapter I. Due to different geographic separation and contrasting environmental conditions, phenotypic differences between these two germplasm pools are expected (Brown and Anderson, 1947; Unterseer *et al.*, 2016). In Chapter I, the PUE, PUtE and PAE of elite flints released from the 1950s to 2010s decreased dramatically under both low P and high P. Most DH-LRs, surprisingly, preformed similar to moderately modern elite flints. However, most dent elite varieties in the study performed superior. Those selected in the same public breeding program released from the 1990s to 2010s generally had higher biomass, PUE, PUtE and PAE, comparable to some flint founder lines.

Much of the large variation in seedling biomass differences between these two germplasm pools was probably due to different P mobilization from seeds and a delayed switch to exogenous P acquisition. Especially under LP, seedlings of several genotypes (especially some in the Elite Flint2 and Elite Flint3 groups) even contained less P than originally stored P in the seed. It suggested that during germination they were not able to utilize the seed P and translocate it into the seedling, resulting in a seed P loss. Massive losses of macronutrients and micronutrients during germination have been reported and can be prevented by silicon treatments (Moradtalab *et al.*, 2018). In those juvenile plants have a seed P loss, re-uptake of transiently lost P from internal sources could appear as a major function of the root system. Indeed, previous research showed the measurable P uptake by maize roots begins with a delay of around five days after germination, but the P uptake rate was shown to be independent of the initial seed P content (Nadeem *et al.*, 2011; White and

Veneklaas, 2012). For the different genetic backgrounds analyzed here, there was a strong correlation of total dry mass with exogenous P taken up, especially in HP, but not with the seed P content. Some dent elite lines (and hybrids) apparently switched earlier or more robustly to acquisition from exogenous P pools so that they had already acquired substantial external P at the time of harvest, consequently produced more biomass.

Although the internal remobilization of stored P was dominant in LP at the investigated juvenile stage, all root traits except APase were significantly correlated to PAE in LP. When only focusing on the flint pool, the decline of PUE and PAE of flint were accompanied with smaller seedling root biomass in elite flint varieties despite the breeding progress, while Root/Shoot had no considerable change. This implies for flint, investment into the roots may be less important when all nutrients are amply available, as in typical breeding scenarios. Several root traits of flints were associated with the decrease of PUE in the breeding process, Rhizos-pH and RootHairLen in LP and to a lesser extent RootDiam in HP were accompanied with the decline of PUE of flint, which means founder flints have a weaker ability to acidify the rhizosphere and grow longer root hairs under limited P conditions.

In Chapter II, we grew European flint maize genotypes with distinct breeding histories in two soil mixtures under two P levels and observed that seedling biomass was more substantially determined genetically at HP compared with LP, whereas shoot P content was similarly correlated between both experiments at both LP and HP. The group of founder flints had higher seedling biomass compared with that of elite flints, whereas recently developed DH from landraces were, on average, similar to Elite Flint2 and Elite Flint3. Despite limited relevance for larger quantities of dissolution of soil P, root organic acid anion exudation (Pearse *et al.*, 2006; Oburger *et al.*, 2011; Wang *et al.*, 2016; Lyu *et al.*, 2016) for the mobilization of adsorbed P from soil particles can increase the absorption of phosphorus by plants (Gerke *et al.*, 2000). The volume of carboxylates secreted from root tips varied in the 24 maize genotypes under both LP and HP conditions, but on average, citrate was the only major carboxylate that was more released under LP. Citrate was correlated with shoot dry weight and shoot P accumulation, together with succinic acid and *trans*-aconitic acid. Importantly, the mobilization efficiency of P_i by the organic acid anions in many soils is citrate > oxalate > malate > trans-aconitate > succinate > acetate (Jones, 1998; Gaume, 2000), and interestingly, the amount of citric acid and *trans*-aconitic acid in exudates has declined during the breeding process under LP conditions. This suggests that modern elite genotypes release less beneficial organic anions to mobilize P under limited P supply than old genotypes. Other functions of root exudation, such as attracting other beneficial microbes may be related to the trends to release more malate under HP conditions or other changes in root organic acid anion exudates. The release of malate and citrate by Aluminum-Activated Malate Transporters (ALMT) and Multidrug and Toxic Compound Extrusion (MATE) families are among

the well-understood transporter candidates involved in exudation (Sasse *et al.*, 2018). Previous findings also showed that AM induces malic acid accumulation in the roots of water-stressed maize plants (Hu and Chen, 2020), but the organic acid in exudates do not increase.

AMF colonization, a typical response to coping with P deficiency in native environments and crops (Smith *et al.*, 2011), modulates the relationship between root growth and nutrient acquisition in maize (Ramírez-Flores *et al.*, 2019). In Chapter II, all genotypes in our study showed increased colonization under LP, and only under this condition was the mycorrhizal colonization correlated with shoot P content. Concern has been expressed that, on evolutionary time scales, plants have clearly reduced their dependence on symbiotic mycorrhizal fungi since they first emerged in land ecosystems (Ma *et al.*, 2018). Indeed, a comparison of a landrace with hybrid maize varieties has revealed a better response to AMF mycorrhization (Londoño *et al.*, 2019), whereas mycorrhizal infection was even more pronounced in a modern European elite line compared with an African line (Wright *et al.*, 2005). Even during the relatively short period of breeding selection from the 1950s to 2000s, mycorrhizal colonization and responsiveness have decreased, although only a very limited number of lines has been evaluated for this trait (Chu *et al.*, 2013; Chu *et al.*, 2020). Here, no obvious trend for a loss of mycorrhization during the breeding process for European flint genotypes was observed, although the founder line EP1 showed particularly high mycorrhization. Furthermore, the DH line SF1 had the highest mycorrhization degree/intensity and SM2 the highest arbuscular abundance of the root system, but interestingly, several other DH lines from the same landrace were even less colonized than the modern flints. SF1 or SM2 may therefore be good candidates for breeding for superior AMF mycorrhization or can be used as parents for quantitative trait loci (QTL) studies to identify genetic components of mycorrhization. However, although such material might contribute interesting genetics to modern elite lines, DH lines from landraces are not generally superior with regard to AMF colonization than modern elite lines.

9.2 Tradeoffs of root traits related to PUE under low P

A wide variation and co-variation of key root and rhizosphere traits have been found to create multiple P acquisition strategies that may be similarly efficient in an ecosystem (Zemunik *et al.*, 2015; Brundrett and Tedersoo, 2018; Lambers *et al.*, 2018). Previous studies showed in controlled experimental conditions, different plant species showed different P acquisition strategies with different root and rhizosphere trait tradeoffs, while maize with its fibrous root system investing relatively little into rhizosphere traits (Lyu *et al*., 2016; Wen *et al*., 2019). In our study in Chapter I, only TRL and SRL (morphological traits) are not significantly affected by P treatment, and all the traits (morphological traits and rhizosphere traits) showed variation among genotypes. TRL was important under both LP and HP to PUE; RootDiam, Rhizos-pH, Carboxylates and RootHairLen were more important under LP to PUE. While under HP, TRL was negatively correlated to RootHairLen ($r = -0.32$); and under LP, TRL was positively correlated to RootHairLen

(r = 0.37). Moreover, the correlation between PUE and TRL decreased from 0.50 under HP to 0.39 under LP, meanwhile, the correlation between PUE and RootHairLen increased from -0.19 to 0.45. This can be taken as one kind of trade-off among the root traits, which verified our third hypothesis. The variation and co-variation still exist when only considering the flint pool. Under HP, there is a large variation of all these eight root functional traits; under LP, there is a visible co-variation among TRL, RootHairLen, Root/Shoot, Rhizos-pH and Carboxylates. The trade-off between TRL and RootHairLen is also still found in the flint pool. As only young roots were considered here, field trials with the investigated genotypes are required to confirm the importance of juvenile PUE traits for adult plants and final grain yield. Such experiments should also consider other traits that are important for P efficiency in adult plants, such as the presence of cortical root aerenchyma (Postma and Lynch, 2011).

The genotypes in Chapter II were grouped into four categories, according to the definitions of agronomic use efficiency and their responsiveness to P, with the founder line EP1 being exceptionally efficient. The increase in the Root/Shoot ratio is a well-known strategy of plants for coping with P-deficiency and was consistently found in our experiments. However, the P-efficiency was not significantly affected by the ratio. In terms of P-efficiency and P-responsiveness, root traits associated with physiology and architecture are more important than the root biomass itself. Root traits associated with efficient P use (such as in EP1) were characterized by a large investment into not only long laterals, but also thick shoot-borne roots. Roots thicker than 0.2 and thinner than 0.4 mm are thought to be lateral roots (Tai *et al.*, 2016), and these were promoted most strongly under low P, irrespective of genotype and class. Because of the different root types contributing to P-efficiency in maize, the SRL, a typically valuable measure of the high P use efficiency of plants, was studied and shown to be low in the most P-efficient genotypes. Rhizosphere pH and root length were associated with P-efficiency and contributed similarly to the prediction of the P-efficient genotypes under LP conditions, e.g., TRL and RootDiam. The latter two criteria contributed in an opposite under HP conditions, revealing the dilemma that beneficial traits under LP conditions may be different from those under HP and underpinning the importance of the environment in which crops are selected during the breeding process.

In Chapter IV, the *rth2* maize mutant apparently had, in addition to the root hair phenotype, altered root architecture. Roots thinner than 0.4 mm are lateral roots (Tai *et al.*, 2016), indicating an increased number of laterals in *rth2*. However, the genotype × environment effect on the root dry weight was apparently low or absent, suggesting the root architecture changes were seemingly not a response to short root hairs under P-limiting conditions. However, changes in the root architecture of *rth2* and the barley mutant *brb* (Dodd and Diatloff, 2016) may also affect P uptake. Therefore, these mutants must be used cautiously to draw conclusions on root hair function. Surprisingly, the mutant *rth2* performed better than the wild type in well-

supplied hydroponics, which may reflect a substantial energy cost of building root hairs in conditions where they are not required.

9.3 Maize root hair and mycorrhizal interaction under P limiting conditions

As root hairs play an important role in Pi acquisition (Jungk, 2001; Lambers *et al.*, 2006; Lynch, 2015), it was expected that the hairless mutant performed much worse than the wild type under P-deficient conditions in Chapter III. The primary role of root hairs is to extend the root surface area and to increase the radial rhizosphere diameter to explore a larger soil volume (Ma *et al.*, 2001; Pang *et al.*, 2018). This was confirmed by up to 0.5 mm broader rhizosphere extent of acid phosphatase activity in the wild type compared to that of the hairless mutant, irrespective of AMF inoculation. Our study further verified that the root hair dependency of maize growth was 57%. The root hair dependency of barley, by contrast, was according to results shown in Chen *et al*., (2005) and Jakobsen *et al*., (2005) around 75%, which is notably higher than our results in maize, indicating root hairs play more important roles in barley than in maize. Furthermore, the root hairs enhanced the P content by 44%, but this increase was not significant and the P concentration was similar or tended to be even slightly decreased in plants with root hairs, which is in line with previous research (Weber *et al.*, 2018; Klamer *et al.*, 2019; Ludewig *et al.*, 2019). However, root hairless plants were severely depressed in biomass formation compared with the wild type, meaning that any additionally acquired Pi was immediately invested in producing more shoot and root biomass, leading to bigger shoots and longer roots. Maize wild type had a higher SRL than *rth3*, which indicated that the wild type has longer root length for a per-unit invested dry-mass. This further allowed a greater soil volume to be explored per unit C invested and thus enhanced P uptake efficiency (Laliberté *et al.*, 2015). Therefore, maize root hairs not only improved PAE but also indirectly improved root morphology, with longer total root length and higher SRL that further improved plant growth compared to the *rth3* hairless mutant.

AMF inoculation increased the plant growth and Pi acquisition of both wild type and *rth3* root hairless mutant 1.8-7.4 times. The P nutritional status, i.e., the shoot P concentration, was massively improved by AMF, facilitating maize growth and further Pi acquisition by AMF and by roots. The root hairless mutant *rth3* particularly profited more from the AMF inoculation than the wild type, which is consistent with previous studies where root hair length negatively correlated with mycorrhizal dependency of various species, because root hair length contributed to plant Pi uptake (Tawaraya, 2003). Thus, root hairs and AMF provide alternative, inversely correlated pathways for Pi foraging. Although root hairs and AMF provide alternative mechanisms to increase the contact with soil, the 33% higher mycorrhizal growth dependency than root hair dependency strongly argues that AMF provide a more efficient way to acquire Pi even in young maize. Furthermore, the total colonized root length was similar in the two genotypes under AMF

inoculation condition and was accompanied with almost the same final biomass, indicating that AMF played a more important role than root hairs for Pi acquisition in maize (Wen and Schnable, 1994; Cozzolino *et al.*, 2013). However, similar studies in barley showed that the root hairs play more important roles than AMF (Jakobsen *et al.*, 2005; Chen *et al.*, 2005; Li *et al.*, 2014). This is probably due to the fact that the root hair length of barley wild type was almost doubled under the P-deficient condition compared to that under higher P condition (Brown *et al*., 2012), while the root hair length in maize B73 wild type was not significantly different between under P deficient (this study 0.90 mm) and sufficient condition (0.83 mm) (Weber *et al*., 2018). This explanation is supported by observations in Plantago lanceolata L., whose root hair length was not responsive to P availability, but was highly dependent on AMF for Pi acquisition under P deficiency (Brown *et al*., 2013). It is also noteworthy that root hairs are already endogenously found in young seedlings from the first days of growth, while AMF establishment requires several weeks to be functionally established (Smith and Read, 2008). Ultimately, AMF could compensate the loss of root hairs and even played a more critical role than root hairs for Pi acquisition under P-deficiency even for juvenile maize. As a consequence, the plant biomass and Pi uptake were induced by AMF colonisation in *rth3* by 1.6-2.5 times compared to that in the wild type. The phytohormone strigolactone plays an important role in the establishment of the root and AM symbiosis interaction (Akiyama *et al*., 2005; Besserer *et al*., 2006; Chagas *et al*., 2018). AMF colonisation depends on the release of such signaling compounds into the rhizosphere to germinate spores and attract hyphae for root contact; a radially extended rhizosphere was expected to be beneficial for the number of AMF-root contact sites. However, root hairs expand the soil volume into which strigolactone is released, but had only a minor role in maize, as the AMF colonisation was higher in the hairless mutant than in the wild type. Molecular signals such as CLE peptides also play a key role in AMF colonisation of various plants (Handa *et al.*, 2015; Karlo *et al.*, 2020). Future investigations about genetic and molecular pathways such as the relationship between the release of strigolactones and CLE peptides with AMF colonization are needed to reveal the contrasting mycorrhizal colonisation between wild type and *rth3* in maize.

9.4 Different molecular mechanism patterns of flint maize roots under low P

In Chapter IV, the adaptations to low P availability in the expression in roots of six preselected genotypes in European maize flint depended mainly on bypassing the metabolic steps that require ATP (Ganie *et al.*, 2015); and recycling and mobilization of internal Pi (Cruz-Ramírez *et al.*, 2006). Glycolysis is modified through bypassing reactions that require ATP, as reflected by the cluster of genes encoding phosphoglycerate mutase (PGM), phosphoenolpyruvate carboxylase (PEPCase), and PEPcase kinase (PEPK) in a module induced by P deficiency in WGCNA result. P recycling changes in these maize roots in the gene expression level was related to the lipid metabolism, cell wall organization and transmembrane transporter. Internal Pi recycling involves phospholipid degradation and sulfolipids/galactolipid synthesis (Essigmann *et al.*, 1998;

Hammond *et al.*, 2004). The expression increased phospholipid degradation related genes included phospholipase A2 (PLA2), phospholipase C (PLC), phospholipase D (PLD), phosphatidate phosphatase 2 (PAH2) and glycerophosphodiester phosphodiesterase (GPPDs), in which PLC and PLD were also found to mediate phospholipid degradation in Arabidopsis (Cruz-Ramírez *et al*. 2006) and PLA2 and PAH2 was stronger induced in maize (Calderon-Vazquez *et al.*, 2008). The expression increased sulfolipids/galactolipid synthesis included UDP-sulfoquinovose synthase, Sulfoquinovosyl transferase SQD2 and Monogalactosyldiacylglycerol synthase 2 (MGD2). Similarly, transport systems were strongly affected under Pi deficiency. The alterations in the transcript level of phosphate, sulphate, Fe, and ABC transporters as well as sugars and oligo-peptides encoding genes was identified. In addition, the genes related to establishment of the Casparian strip membrane domain (CSD) and the subsequent formation of Casparian strips was found in module 16, which in rice also determined similar genes under Pi deficiency (Wasaki *et al.*, 2003).

Founder flint EP1 and F2 showed specific molecular mechanism to LP response. The higher biomass of these two genotypes maybe related to the ability of integrating C and N metabolism. A relatively higher expression of genes related to proteasome and peptidase involved in protein degradation was found in these two genotypes under LP. It was also found that genes involved in glutathione transferase were induced under Pi deficiency. Glutathione was widely studied and found to be involved in stress management like drought, oxidative stress, chilling, high temperature (Kocsy *et al*., 2001; Štolfa *et al*., 2016; Pardo-Hernández *et al.*, 2020). Previous study linked low P availability in the soil with photo-oxidative stress in plants (Hernández and Munné-Bosch, 2015), which imply that glutathione transferase accumulation played a role as antioxidation under low Pi conditions in founder flints.

Maize general mechanism of regulating P utilization under LP is related to balance of carbohydrate through glycolysis and TCA cycle, alteration of lipid metabolism, changes of gene expression of transmembrane transporters and carotenoid biosynthesis. Additionally, the founder flint line EP1, F2 and doubled haploid landrace SM1 have their own specific strategies and mechanism to cope with LP. The genes identified in this study and their proposed role in Pi adaptation are supported by the analysis of the data available from existing transcriptome, proteome and metabolome experiments conducted in maize and the other species. These genes provide an opportunity to identify different alleles involved in the adaptive response to Pi deficiency; they also provide a basis for identifying candidate genes and processes that may improve the tolerance to Pi deficiency in maize and other cereal crops. This work also provides a framework for the production of Pi-specific maize arrays to study global gene expression changes between Pi high-efficiency and low-efficiency maize genotypes.

9.5 Future perspectives

Improving PUE is vital to increase the nutritional value of grains, improve farm economies and reduce environmental burden. However, due to insufficient understanding of the molecular mechanisms underpinning traits and their interactions, current breeding strategies have hardly been successful (Veneklaas *et al.*, 2012). Therefore, it's important to clarify the components of (and their relative contributions to) traits of interest from molecular to field scales, and provide a starting point for integrative research to improve PUE.

From the outset, we should keep in mind that different growth conditions (light regimes, growth matrices, etc.) influence the plant response to the same external Pi treatment. So, it would be good to collaborate with other groups, modellers and statisticians to capture the related processes in different growth conditions or scales. The comparison of these processes within relevant genotypes (particularly, those performing well in low-P landscapes) or multiple conditions, for example, a certain range of constant external Pi levels, will also be both informative and closer to field conditions. Using novel drugs (such as Phostin and Phosphatin) and Pi analogs (such as phosphite and methylphosphonate (Arnaud *et al.*, 2014; Jost *et al.*, 2015)) may help to further decipher plant responses to Pi starvation. Regarding to the phosphate-starvation responses, many core gene regulatory networks involving PHR1/2 have been identified in Arabidopsis and rice, and there are some genes seem to be conserved in a variety of plant species (Fang *et al.*, 2009; Ajmera *et al.*, 2019), which provides a set of candidate genes/molecular components for maize research for improving PUE.

Furthermore, we have used transcriptomic technique to identify the components of the system concerned but note that at the molecular scale, individual omics techniques may give only one-side of which players are important. This could be resolved by using multiple omics techniques on the same samples (Wilson *et al.*, 2015). This will help to refine and prioritize the regulatory pathways and provide the interactive topology on which dynamic models can be developed. The initial dynamic models should focus on a smaller part of the entire network, these models can be integrated later to discover the different aspects of the PSR affect each other.

Taking all together, to get deeper understanding of phosphate research, interdisciplinary team working is a good choice. Interdisciplinary team working on the different aspects of phosphate research (especially of different scales) must come together and share terminology, skills and concepts in order to be able to build models that link genotypes to desirable traits or genes. In addition, interdisciplinary cooperation is likely to accelerate output, strengthen understanding, and promote the transformation of purely scientific endeavors into practical applications.

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Affidavit

Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

Dissecting the genetic basis of root- and rhizosphere-related phosphorus use efficiency in European elite maize (*Zea mays* L.) lines and landraces

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

16.02.2021, Stuttgart — Xuelian Li

Place, Date — Signature

Curriculum Vitae

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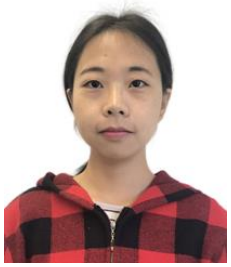

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Li, Xuelian; Quan, Xiuhao; Mang, Melissa; Neumann, Günter; Melchinger, Albrecht; Ludewig, Uwe (2021): Flint maize root mycorrhization and organic acid exudates under phosphorus deficiency: trends in breeding lines and doubled haploid lines from landraces. Journal of Plant Nutrition and Soil Science 184: 346-359. DOI: 10.1002/jpln.202000471.

Ma, Xiaomin*; **Li, Xuelian***; Ludewig, Uwe (2021): Arbuscular mycorrhizal colonization outcompetes root hairs in maize under low phosphorus availability. Annals of botany 127: 155–166. DOI: 10.1093/aob/mcaa159.

Li, Xuelian; Ludewig, Uwe: Transcriptomic network analyses of the response to low phosphate supply in roots of maize with distinct breeding history. (in preparation)

He, Mingjie*; **Li, Xuelian***; Mang, Melissa; Ludewig, Uwe; Schulze, Waltraud X.: A systems-biology approach to identify regulatory modules in response to low phosphate supply in maize-lines of different breeding history. (submitted to The Plant Journal)

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Liu, Ying; Jia, Zhongtao; **Li, Xuelian**; Wang, Zhangkui; Chen, Fanjun; Mi, Guohua et al. (2020): Involvement of a truncated MADS-box transcription factor ZmTMM1 in root nitrate foraging. In Journal of experimental botany 71 (15), pp. 4547–4561. DOI: 10.1093/jxb/eraa116.

Wang, Liyang; **Li, Xuelian**; Mang, Melissa; Ludewig, Uwe; Shen, Jianbo: Root hairs and fine roots: complementary functional root traits in response to heterogenous phosphorus. (submitted to Annals of Botany)

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