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Phosphorus Efficient Phenotype of Rice

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Additional information is available at the end of the chapter

Abstract

The ideal phenotype to cope with P deficiency is suggested to be a larger root system, both in terms of length and foraging area, coupled with a high capacity for P solubilization from compounds exuded from roots. Greater soil exploration results in a large number of roots in the top soil, longer roots in general with more cortical aerenchyma, more and longer root hairs, and a shift in mycorrhizal and bacterial colonization. However, these assumptions often result from experiments in highly controlled, sterile and soil-free conditions using model plants or single ecotypes where results are then extrapolated to all genotypes and plant species. In recent years this generalization has been questioned. Here, we summarize recent rice research analyzing the natural diversity of rice root systems under P deficiency. Interestingly, while some of the high yielding genotypes do show the expected, large root system phenotype, some have a surprisingly small root system—as little as a quarter of that of the large root system varieties—but achieve similar yield and P uptake under P deficiency. This effect has recently been termed root efficiency, which we discuss in this chapter in conjunction with root foraging traits.

Keywords: phosphorus deficiency, root system, root hair, root type, xyloglucantransferase

1. Introduction

Rice (*Oryza sativa*) is the most important source of calories for millions of people [1] and, like all crops, its growth and yield is enabled by taking up water and nutrients from the soil through its root system. Yield can be constrained by many abiotic and biotic factors, including drought, nutrient deficiencies, and pathogen infections. Second, only to nitrogen (N), phosphorus (P)

is an essential major nutrient necessary for biosynthesis of nucleotides, proteins, bio mem-branes, and energy metabolism such as the provision of ATP. In contrast to most N forms, P is highly sorbed to soil particles and is therefore considered to be an immobile nutrient in soils [2]. Estimates suggest that more than half of all agricultural soils are P deficient [3] which sub-stantially affects plant growth, both below and above ground, to the extent that P is often the most yield-limiting nutrient in resource-poor farming systems in south-east Asia and Africa. In developed nations, P and N deficiencies are generally rectified by the application of mineral fertilizers to the soil, often in excess of that taken up by the crop. This leads to rapid leaching of N, more slowly also of P, into the ground water, eventually leading to eutrophication of water systems which leads to algal blooms, anaerobic water, and death of fish and other fauna [4]. In addition, mineral phosphate fertilizers are usually produced by mining rock phosphate and, although several predictions with variable numbers are present, these resources are limited and might be depleted in 50–100 years [5]. Therefore, understanding how P deficiency shapes root systems and how plants can overcome P deficiency is critical for future breeding of P effi-cient crops to ensure nutrition of the ever-growing world population in the future.

Due to the immobile nature of P in soil, increasing root foraging for P is theoretically one of the best means by which to improve the efficiency of P acquisition by plants. Research in other crops and model plants have provided indications as to what the ideal root phenotype to cope with P deficiency should look like (reviewed in [3, 6]). Overall, it is suggested that a larger root system, both in length as well as in area, is beneficial for better yield performance in low P conditions. This higher soil exploration includes a large number of roots in the top soil (soil surface until 25 cm depth), longer roots in general, with more cortical aerenchyma, smaller diameter, more and longer root hairs, more mycorrhiza, altered bacterial colonization, and higher exudation of P-solubilizing chemicals.

In this chapter, we will (1) give a general literature overview of strategies for P uptake and its optimization and (2) review the proposed ideotype for rice P efficiency and present an experi-ment for its optimization using transgenic rice.

1.1. Exudation of P-solubilizing chemicals

Mobilization of soil-bound P via the release of P-solubilizing compounds from roots is widely proposed to be a key mechanism by which many plant species enhance P acquisition in soils. These compounds include phosphatase enzymes capable of mobilizing organic P, protons that acidify the soil to solubilize calcium (Ca)-bound P and organic compounds including phytosiderophores and carboxylates that compete with P in ligand exchange reactions [7]. The release of protons and carboxylates is a strategy that is particularly important for species from the family Proteaceae, which have often evolved in extremely P deficient soils [8]. Other species that form proteiod—or cluster—roots under P deprivation such as the model legume species *Lupinus albus*, are also highly efficient at mobilizing P from aluminum (Al)- and iron (Fe)-P complexes via efflux of a targeted surge of carboxylates (predominantly citrate and malate) and protons from the cluster roots under P deficiency [9].

Beyond species that form cluster roots, however, the role of compounds exuded from roots is less clear. Experimental evidence that non-cluster root forming species can mobilize signifi-cant amounts of P through exudation of carboxylate is lacking [10] and the concentrations of

carboxylates required to mobilize P in incubation studies are much higher than those thought to occur in the rhizosphere of most plants [11]. As an example, a recent study using near-isogenic wheat (*Triticum aestivum*) lines that differed in citrate efflux failed to find evidence that higher citrate efflux led to any improvements in P uptake or crop yields [12]. The capacity for enhanced root exudation in rice to mobilize soil P is discussed in detail below (Section 2.3).

1.2. Association with mycorrhizal fungi

Arbuscular mycorrhizal (AM) fungi are recognized for enhancing nutrient availability, notably P, to most plants [13]. AM fungi are obligate biotrophs that establish mutualistic associations with the roots of over 90% of all plant species via a complex system of intraradical and extraradical hyphae, in which the external mycelium of AM fungi acts as an extension of host plant roots, thus increasing the effective surface area to absorb nutrients and water [13–15]. The transfer of nutrients from AM fungi to plant roots is typically facilitated by highly branched fungal structures within the root cortex, known as arbuscules [15]. The transfer of nutrients from AM fungi to roots occurs in exchange for sugars generated from photosynthesis and is typically facilitated by specific transporters expressed at the interface of plant root and arbuscule [15, 16].

To what extent the AM symbiosis is beneficial to crop species depends on environmental factors and varies between different crops and among varieties of the same crop. It is generally assumed that high rates of P fertilization diminish the potential benefits from AM symbiosis [17]. Further, one may assume that crops with a rather fine and dense root system would benefit less from the increase in the effective surface area provided by external hyphae compared to crops with less fine roots. Within crop species, varietal differences may be of importance and should ideally be explored in crop improvement programs. However, the multiple interactions between AM fungi, other soil microorganisms and plant roots, each potentially affected by soil properties, climatic factors, and crop management, have made a selection for a stable increase of AM-variety symbiotic efficiency a rather challenging task.

1.3. Association with beneficial bacteria and fungi other than AM

Application of free-living soil bacteria and fungi to plants can increase plant growth and nutrition status. As early as 1948 it was reported that isolated soil bacteria could enhance plant P solubilized from calcium phosphate [10]. Today efforts continue toward identifying microbiota-root interactions beyond AM that enhance crop phosphorus acquisition. These are driven by commercial and regulatory pressures to supplement mined P reserves and optimize the recycling of P from soil and biomass pools [18], and by the increasingly comprehensive genome-based methods to characterize microbiomes [19] to explore to increase agricultural fertility [20].

Beneficial microbiota includes those contributing directly to plant or soil P fertility processes, and those indirectly contributing via control of plant host diseases, soil toxicities, and weeds that compete with crop resource uptake. P fertility mechanisms include (1) promoting greater root surface area, (2) increasing inorganic P availability in the soil solution, and (3) altering organic P pools in soil (e.g. rates of turnover). The evidence is mounting that microbiota can change molecular events within the plant (e.g. induce proton release for rhizosphere acidification, gene regulation involved in ion uptake) [21].

Management of beneficial microbiota is mainly done through inoculation onto the plant (seed or shoots) of microbes that are isolated from a given environment. However, management of the naturally-associating rhizosphere microbiota through the plant (e.g. plant genetics and breeding) or soil (e.g. rotations, tillage) is also performed. Molecular characterization of the whole root and soil microbiomes will likely lead to a merging of inoculant approaches with the direct engineering of rhizosphere microbiota [22], as plant hosts are intimately connected with microbiota whose genes can be transferred with that of the host genome [23]. Although many examples report beneficial responses to bacteria or fungal strains on plants in pots in glass-houses, there are few translations to farmers' fields with formulated, scaled production [18].

1.4. Aerenchyma formation

Aerenchyma refers to plant tissue containing enlarged gas spaces, formed in roots and shoots of wetland and dryland species through cell death "lysigenous" or cell separation "schizogenous" [24, 25]. Formation of aerenchyma can be constitutive or induced by abiotic stresses such as waterlogging [26], drought, and nutrient deficiency including phosphorus, nitrogen and sulfur deficiency [27–32]. Therefore, the presence of aerenchyma can differ even within root segments when heterogeneous abiotic conditions, such as those in field soils, are present [30, 33, 34].

Several reports from maize (*Zea mays*), bean (*Phaseolus vulgaris*), and barley (*Hordeum vulgare*) results suggested that aerenchyma formation reduces the metabolic costs of soil exploration [35–37] through the decrease of root nutrient content and respiration [31]. Nutrients such as P, released during the aerenchyma formation (cell death), could be reutilized by the plant, for example, in the continued growth of apical cells. Increased soil exploration for more P resources, coupled with less P required for root functional maintenance, would be an advantageous trait under P-limitation.

1.5. Root hair production

Root hairs are unicellular extensions of epidermal cells that constitute most of the root's surface area [38]. Root hairs have been under investigation for more than a century [39], and it was shown decades ago that root hairs are involved in P uptake from soil. P uptake by root hairs was indirectly demonstrated for wheat and barley [40], and directly for rye (*Secale cereale*) root hairs [41]. Enhanced root hair development is consistently listed as one of the cheapest and most general adaptations to P deficiency [3]. And yet, how and if their presence is beneficial for P uptake remains under discussion. Within the molecular model plant *Arabidopsis thaliana*, a study investigating phenotypic reactions of >150 ecotypes to local P levels revealed unexpected results. Half of the tested genotypes did not show any root hair reaction to local low P, while one quarter responded with a production of shorter and less, and the other quarter with longer and more root hairs [42]. Another study showed that maize root hairs were more responsive to soil moisture than to soil P level [43]. While lower water content correlated with the production of more and longer root hairs, no correlation was found with soil P concentration. A recent study on barley seedlings developing normal or very short root hairs came to the conclusion that root hairs are instrumental for water uptake from drying soil when plant transpiration rates are high [44].

2. P deficiency responses found in rice

Rice roots face highly dynamic soil conditions; possibly the most complex of all cereal grains. Soils have repeated flooding, irrigation and drying events, and tilling and compacting results in very soft soils, hardpans, and furrows, with dramatic variation in aeration, pH and nutrient conditions from the surface to depth [45]. These transitions are greatest in lowland systems, but also occur in upland systems.

2.1. The rice root system

The rice root system consists of the first emerging embryonic seminal roots as well as post-embryonic crown roots emerging from shoot-borne nodes. In addition, all of these main root axiles can branch, forming lateral roots (LRs) of first and higher orders. A unique feature of rice is the formation of very distinct classes of these LRs—S- and L-type [46]. The most abundant LRs are the short and thin S-type LRs. L-type LRs are much longer, have a larger diameter and form branch roots, thus producing higher order LRs (**Figure 1**).

2.2. Root efficiency in rice

In order to analyze the natural variation of P deficiency tolerance, a large panel of diverse rice genotypes (>200 genotypes) was grown in upland fields, both in a sufficient fertilized and P deficient fields, and their root systems were analyzed and correlated to yield performance and P uptake. Interestingly, while some of the high yielding genotypes did show the estimated, large root system phenotype under P deficiency, some genotypes had a surprisingly small root system—as little as a quarter of the large root system varieties—but achieved the same yield and P uptake under P deficiency [47]. This effect has been observed in a number of field trials and recently been termed '**root efficiency**'. What could be the basis of this root efficiency? Which traits could enable a smaller root system to take up P as efficiently as or even more efficiently than a bigger one? The efficiency could be based on any or all of the aforementioned P fertility traits for plants (Section 1), and these will be elaborated on for rice in the following sections.

2.3. Exudation of P-solubilizing chemicals by rice

Rice plants exude a range of compounds from their roots, and the amount and composition of these exudates change in response to P deficiency [48–50]. A number of lines of enquiry suggest that increased efflux of carboxylates, particularly citrate, enhances the capacity of rice genotypes to acquire P. Ref. [48] reported an increase in citrate efflux under P deficiency in seven rice genotypes, and citrate efflux rates were correlated with the tolerance of these rice genotypes to P deficiency (defined as ratio of biomass grown with sparingly soluble P source compared to a soluble P source). Also found was increased citrate efflux from rice roots under P deficiency. Subsequent modeling studies indicated the importance of citrate efflux in P solubilization and uptake by rice growing under aerobic conditions [51]. However, as pointed out in a review of root traits associated with the efficient acquisition of soil P by rice [6], there is no direct evidence that increased efflux of citrate, or any other carboxylate, is responsible for higher P acquisition efficiency for any rice genotype.
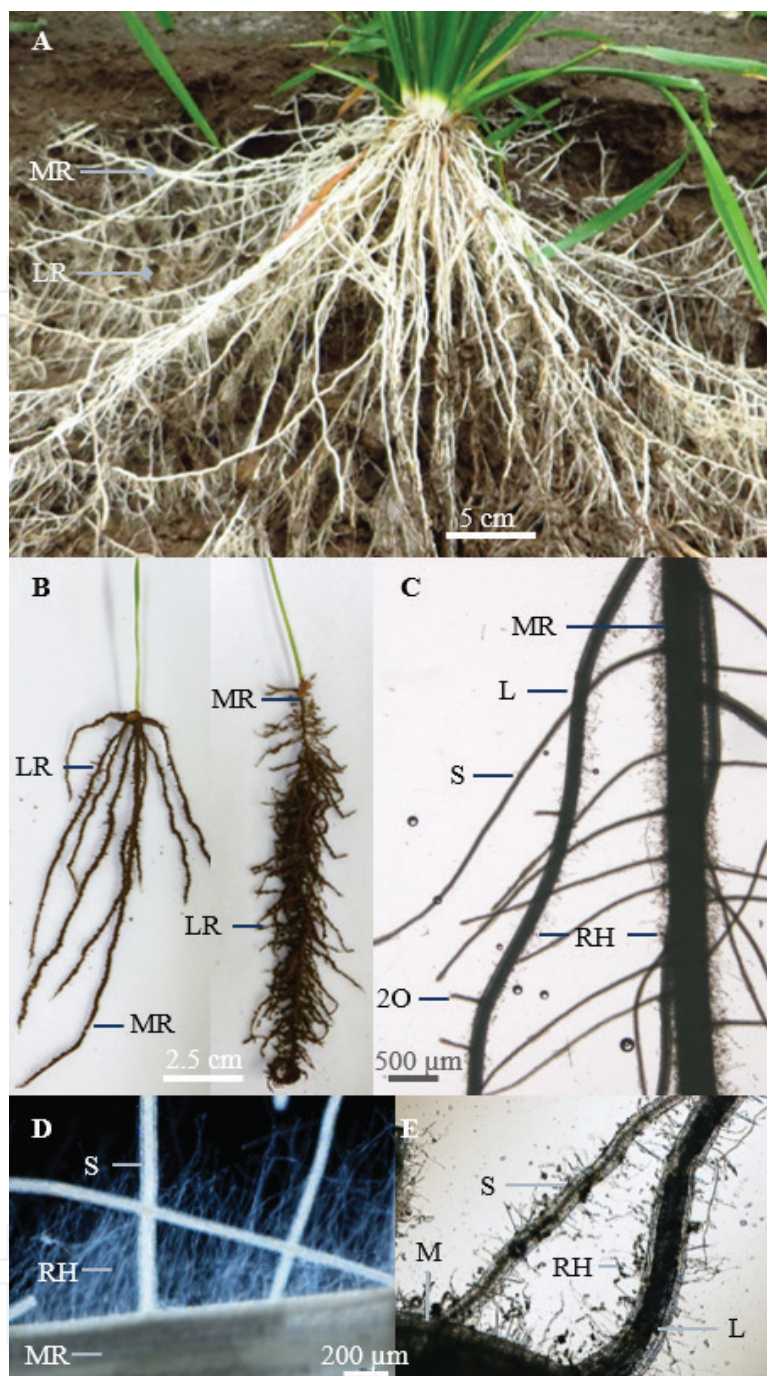
Figure 1. The rice root system structure. Top soil washed off to display the root system of a field-grown rice plant ca. 50 DAS (A). Seven DAS seedlings with many seminal roots (left) or a high proportion of lateral roots (right) (B). Stereomicroscopic image demonstrating the rice root characteristics (C). Light microscopic images of rice roots grown in nutrient solution (D) or soil (E). MR: main root (seminal or crown root), LR: lateral root, L: L-type LR, S: S-type LR, 2O: second order LR, RH: root hair.

Protons and phosphatase enzymes are also released into the rhizosphere from rice roots. The release of protons typically occurs due to an imbalance in cation/anion uptake by roots [52]. Genes encoding phosphatases are up-regulated in roots under P deficiency [53], but we are not aware of any published studies that have demonstrated that increased phosphatase efflux from the roots of rice is linked to enhanced P uptake, or that such a trait confers greater P uptake in any given rice genotype.

2.4. Rice association with mycorrhizal fungi

Rice is a host for AM fungi [15, 16] and although root colonization has been observed under irrigated lowland and upland conditions, it is generally assumed that the AM symbiosis is more important in the aerobic upland rice [54]. It has been shown that P transporters exist in rice that are specifically induced in roots colonized by AM fungi and that these P transporters (*OsPT11* and *OsPT13*) facilitate the transfer of Pi from AM fungus to plant [16]. Compared to some other crop species, however, our knowledge regarding the AM fungi community colonizing rice roots in the field remains limited, and the extent to which AM symbiosis may be exploited to benefit rice yield directly in the field is unknown. Studies comparing AM colonization in the field in a set of diverse rice genotypes indicated that considerable variation in colonization rates exists (Wissuwa, unpublished). Further, all root samples taken showed gene expression of the *OsPT11* transporter, suggesting P transfer from AM fungus to rice roots commonly occurred. Yet neither colonization rates nor gene expression levels were correlated to the large genotypic differences observed for P uptake in that set of rice genotypes (Wissuwa, unpublished), whereas P uptake correlated strongly to root size [47]. Earlier studies in one set of near-isogenic rice lines differing in root size and P uptake showed these differences remained unchanged in sterilized soil [55]. Based on the limited evidence available to date, one may tentatively conclude that the AM symbiosis contributes less to rice genotypic differences in P uptake compared to root attributes such as size, fineness or root hair length and density [56].

2.5. Rice association with beneficial bacteria and fungi other than AM

There are intense research efforts to apply rhizobacteria to rice to boost its productivity and increase environmental sustainability because of the enormous value of this crop and the resources used globally. Beneficial microbiota are tested to (1) reduce methane emissions [57]; (2) increase nitrogen uptake [58]; (3) fix atmospheric nitrogen within the root [59]; (4) reduce diseases [60], and (5) increase P nutrition, and to a lesser extent, that of Fe and other micronutrients [61]. In field experiments with farmer participation, beneficial microbiota is being combined with fertilizer applications. This mode of application and level of participation appears to be required for repeatedly validating inoculants that demonstrate high efficacy in the field [62].

The types of microorganisms previously tested for improved P uptake by rice include bacteria of the genera: *Rhizobium* [63], *Pseudomonas*, *Azotobacter*, *Azospirillum* [61, 59], and *Enterobacter* [64], as well as bacteria within a consortium of mixed genera [65]. Applied organisms may reside at the root-soil interface, or within the root (endosphere). The endosphere aerenchyma may have more stable gaseous conditions than the root-soil and outer rhizosphere zones and is a target zone for beneficial isolates, referred to as endophytes [66]. Possible functions of microbiota for P fertility are the same as those proposed for other crops (reviewed in [10, 67–69]), with the exception of much greater emphasis on endophytic diazotrophic (nitrogen fixing) bacteria, perhaps driven by the unique aerenchyma environment in rice, and/or rice-specific responsiveness to effectors associated with endophytic colonization [70]. Given the niche opportunity for endophytes within rice axile root aerenchyma, it may be beneficial to look for consortia of microorganisms or native rhizosphere (including endosphere) profiles that promote these developmental features within the roots.

2.6. Aerenchyma formation in rice

Rice roots can form lysigenous (cell death) aerenchyma in both forms: constitutive, developed from the apical parts of the roots toward the base; and inducible, promoted in all parts of the roots under anaerobic conditions such as waterlogging, drought or nutrient deficiency.

The advantage of aerenchyma formation for more soil exploration with fewer nutrients required may be greater in rice than maize, for example, due to its greater tendency for aerenchyma formation and enhanced root length under low nutrient treatments. For example, phosphorus deficiency causes a 20% increase in the percent cortical area converted to aerenchyma in rice [71]. Likewise, aerenchyma formation is enhanced by both nitrogen and oxygen-deficient conditions [72]. In addition to aerenchyma, rice possesses a barrier to radial O_2 loss (ROL) to the external environment, which promotes diffusion of O_2 toward the root apex. The O_2 increases the redox potential in the rhizosphere and causes the oxidation of Mn^{4+} and Fe^{2+} forming the plaque on the surface of rice roots [73] (which may reduce the uptake of phytotoxic elements into plant tissue).

Although the benefits of aerenchyma and ROL barrier formation are widely reported, the molecular mechanisms that regulate their formation are not completely understood. Since the rice genome has been fully sequenced and many tools are available, further insights into the molecular determinants of aerenchyma and other rice-specific tissues are expected, in order to improve rice cultivars by using modern breeding techniques.

2.7. Root hair formation in rice

An often heard assumption is that root hairs are adaptations specific to limiting conditions and are not needed for growth in optimal conditions. Consequently, elite varieties adapted to high input, optimized soil conditions should produce short and few root hairs and be less responsive to stress conditions compared to landraces.

In the next sections, we will review recent findings regarding natural variation in rice root hair formation in response to P deficiency, growth conditions, and in respect to the rice root system structure. Finally, we will also give an example of transgenic plant generation in the attempt of increasing rice P uptake under deficient conditions.

2.7.1. Root hair variation within root types

Lateral roots were recently proposed to have different, specialized functions depending on their developmental type [74]. Supporting this proposal, we showed that the thinner lateral roots of rice produced shorter and fewer root hairs [75], and found a positive linear relationship between lateral root diameter and root hair length [56]. These results were reproduced in different growth conditions, with main roots (seminal and crown axile roots) consistently producing the longest and most root hairs, followed by L-type and S-type lateral roots (**Figure 2**) (see Section 4 for experimental details). On the other hand, all root types within a genotype had the same tendencies regarding root hair length and density, and differences between genotypes were stable per root type [56]. This indicates that the phenotypic potential per genotype is first determined by its genome and then by the environment.

2.7.2. Influence of root hair longevity

Very few studies have addressed the question of root hair longevity and how long root hairs contribute to plant water and nutrient uptake. In [56] a higher proportion of living rice root hair cells was found in the low P compared to a P-fertilized field, over five tested genotypes. It can be concluded that longer living root hairs might be an adaptation to low P conditions to prolong root hair contribution to P uptake. Nevertheless, in the top soil layer fewer living root hairs were found compared to the subsoil [56]. This can most likely be attributed to the presence of younger root segments including root tips in the deeper soil layer compared to the oldest root segments in the top soil. In future experiments, more emphasis should be laid on longevity not just of root hairs, but also of lateral and main axile roots as harnessing natural variation in this trait could lead to improvements in breeding for more P deficiency tolerant varieties.

2.7.3. Root hairs impacted by experimental conditions

Our recent findings highlighted the vast influence that growth medium has on root hair formation [75]. Nutrient solution led rice seedlings to produce many, long root hairs on their main roots, while the discrepancy between root types was much smaller in soil-grown plants. We were able to reproduce these results in small plastic containers (50 ml volume) already 7 days after germination (DAG) (**Figure 2**). In addition to soil and nutrient solution, we grew rice seedlings in an artificial soil (vermiculite) and an agar solution without nutrient addition. Vermiculite led to very similar root hair growth to the soil, while roots grown in agar formed root hairs of comparable length and density as those found in nutrient solution.

One possible explanation for the observed differences among growth conditions is the absence or presence of physical restriction which can be found in soil particles, but not in a nutrient solution. To test this, another experiment was conducted to investigate the effect of growth medium strength on root hair length in rice seedlings. The experiment comprised six concentrations of agar with two replicate boxes per concentration. Root hair length after 3 weeks of growth significantly decreased with increasing agar concentration (**Figure 2**), proving that soil strength/presence of physical restrictions is one-factor influencing root hair growth. Detection of rice root hairs in undisturbed soil using a synchrotron approach also substantiated this assumption as root hairs growing in pores were found to grow to three times the length of those restricted by soil particles [75].

2.7.4. Genotype-dependent root hair formation

The commonly-stated response to P deficiency of production of longer and more root hairs may depend on genotype. For example, a wide selection of genotypes of the sup-populations indica, aus, temperate japonica, aromatic, and tropical japonica were tested in buffered, diffusion-limited solid-phase solution, and genotype-dependent variable responses to the low P condition was found [71]. We recently showed that in the soil, under controlled conditions in a greenhouse as well as in the field, some genotypes even form shorter and/or fewer root hairs in low P compared to sufficiently P-fertilized soil [56]. This highlights that increased root hair production is not a general, inevitable event upon P deficiency, but a specialized adaptation of some genotypes. Similarly, **root efficiency** (P uptake per unit root length) mentioned above
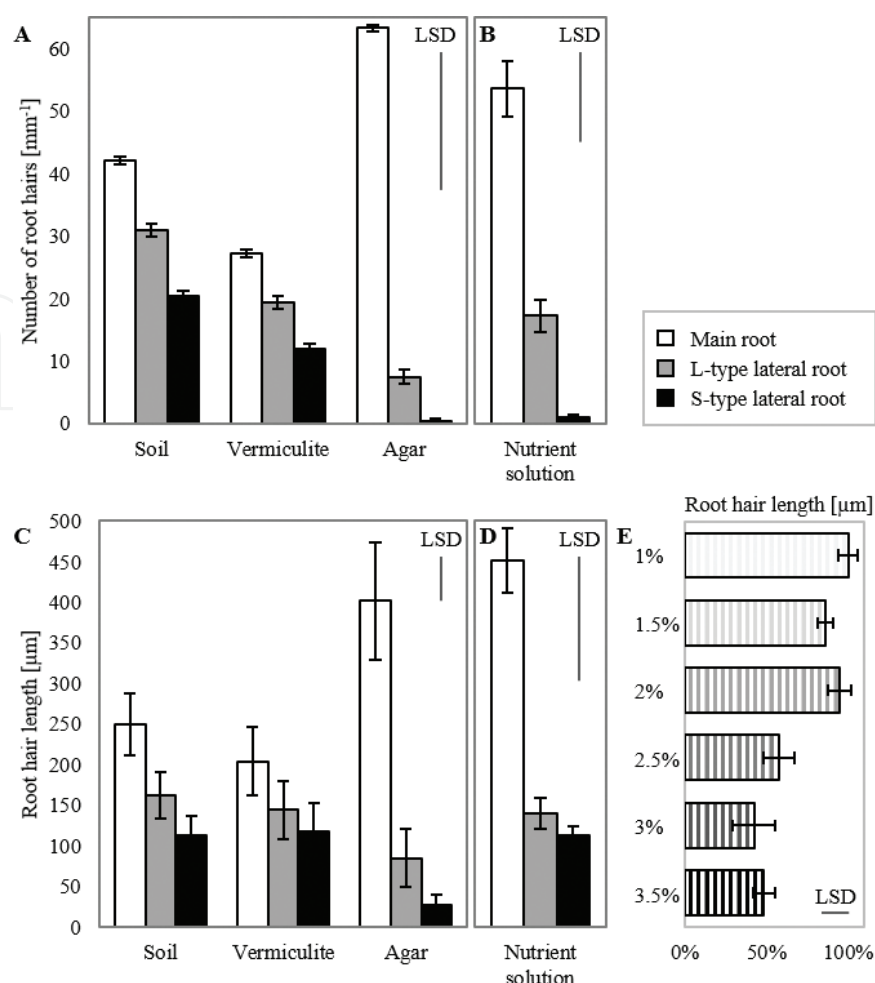
Figure 2. Dependence of root hair production on growth conditions. Determination of root hair density (A, B*) and length (C, D*) on main roots, L-type and S-type LRs. Shown are mean values over four genotypes of 4 (A, C) or 5 (B, D) replicates; +/− standard error determined at 7 DAS (A, C) or 35 DAS (B, D). Soil and nutrient solution represent low P conditions while vermiculite and agar did not receive any nutrients. Half-strength Yoshida solution with increasing amount of agar was used to measure Nipponbare root hair length (E). Least significant difference (LSD) values are indicated per graph. Please note *: data shown in (B, D) are a sub-set of data published in [56].

may not be coupled with increased root hair production, although the assumption could be that smaller root length and weight could be associated with more hairs for a given unit of P uptake. For example, one root efficient genotype had substantially increased root hair length and density upon P deficiency (DJ123). Yet another root efficient genotype (Santhi Sufaid) exhibited shorter and fewer root hairs in P deficient soil, while the inefficient genotype (Sadri Tor Misri) that took up the most P and had the greatest root weight, produced the most root hairs of all tested genotypes [56]. These results lead us to the conclusion that root hairs may respond to P deficiency for some genotypes, and may contribute to root efficiency in others, but their development is not a predictable, universal response to P.

2.8. Transgenic plants for increased P uptake: An example study

Here we present an example experiment to optimize or increase P uptake by rice roots using a transgenic approach (see Section 4 for experimental setup). Our candidate genes are based

on a previous study of transcriptomes of a P deficiency intolerant and a tolerant rice genotype. Several genes putatively associated with root cell wall loosening and root hair extension were found to have higher expression in the roots of the tolerant genotype [53]. We chose one gene to study that encodes a putative cell wall modifying enzyme, a Xyloglycantransferase (Os11g33270.1), designated XTH2, and produced transgenic plants (see Sections 4.4 and 4.5).

The ectopic overexpression of a dozen T_2 lines was tested, and three with very high, high, and moderately increased *XTH2* expression, were selected for further phenotypic characterization (**Figure 3**). Cross and longitudinal sections were prepared to compare the root anatomies of control and *XTH2* overexpressing lines and no apparent differences were found. After 4 weeks of growth either in P deficient or sufficient nutrient solution, several root parameters
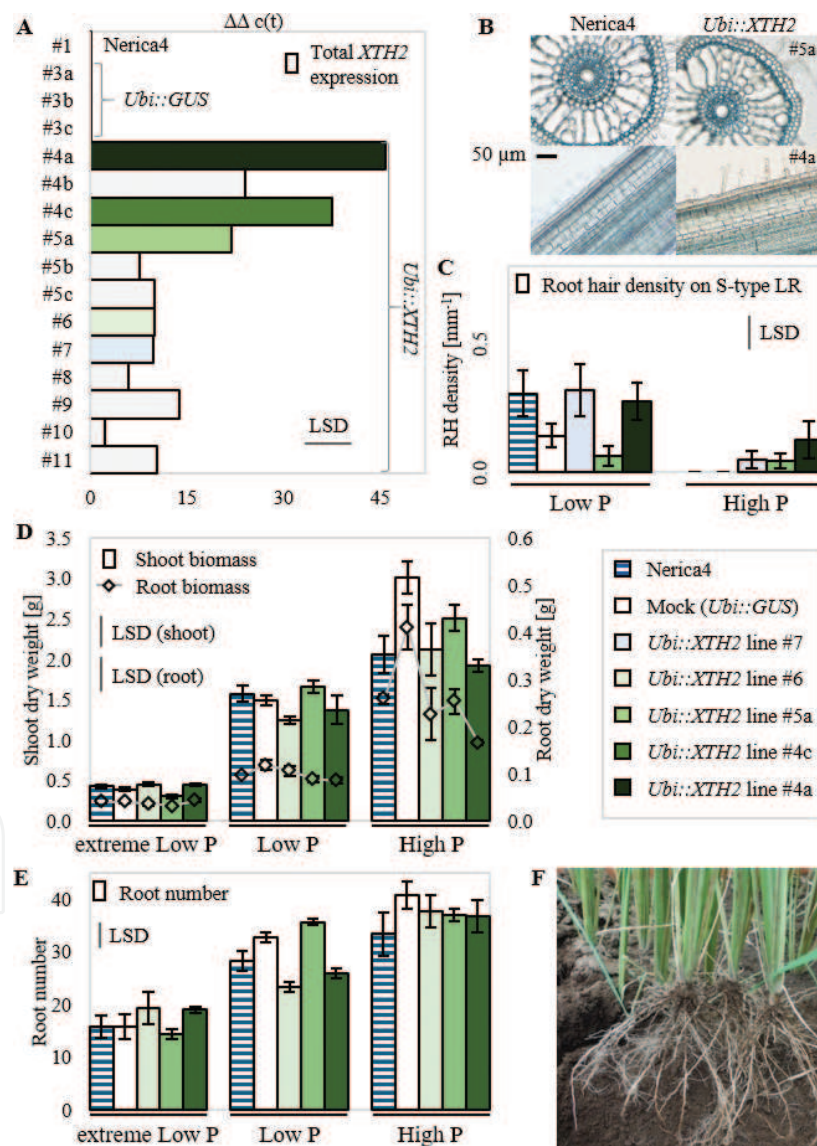

Figure 3. Phenotypic analysis of *Ubi::XTH2* lines. Total *XTH2* expression in a dozen T_2 lines was determined by qRT-PCR (A). Cross and longitudinal vibratome sections (B), and root hair density on S-type lateral roots (LRs) of selected T_2 lines grown in low or high phosphorus containing nutrient solution (C). Shoot and root dry weight (D), and root number (E) of selected T_2 lines grown in extreme low, low (example image in F) or high phosphorus soil. Least significant difference (LSD) values are indicated per graph.

were analyzed (root length and number, lateral root densities, root hair density and length on all root types); root hair density on S-type lateral roots is shown (**Figure 3**). None of the analyzed traits displayed a clear, significant difference between control and all of the *XTH2* lines, but a slightly higher number of root hairs was formed on S-type lateral roots grown in the high P nutrient solution. Also evaluated were shoot and root development after 6 weeks of growth in low or high P soil. Some of the overexpressor lines did produce more roots and a slightly increased shoot biomass in low P soil, but no consistent effect of the overexpression could be detected. In contrast, in high P soil, the overexpressor lines produced lower root biomass than the control lines (**Figure 3**).

Although so *XTH2* had been shown to have higher expression in roots of a P deficiency tolerant genotype in a previous study [53], overexpression in our experiments did not lead to better root and shoot growth or increased root hair production. In another study, tolerance to P deficiency was conferred by overexpression of *PSTOL1*, encoding a protein kinase [76], indicating that transformation can lead to tolerance to P deficiency depending on the specific gene.

3. Conclusion

Overall, it can be concluded that a number of responses to P deficiency exist in rice, yet none of these is a general mechanism found in every rice genotype. Also, often direct evidence for a beneficial effect is lacking for rice. To construct and test a rice genotype optimized for P uptake in P deficient conditions it will be necessary to harness superior traits from many sources and integrate them via marker-assisted breeding or transgenic approaches. For a future sustainable food production, it will also be necessary to overcome the dependence on mining rock phosphate as a major source of P fertilizer. This will include an increase in recycling of biomass and wastewater.

4. Experimental details

4.1. Germplasm and germination

Rice varieties Nerica4, DJ123, Taichung native, and Sadri Tor Misri were used for the phenotyping experiments (Sections 4.2 and 4.3), and Nerica4 and Nipponbare were used for transgenic plant generation (Section 4.5). Dormancy break, sterilization, and pre-germination were performed as described earlier [75].

4.2. Growth in the soil, artificial clay, and agar without the addition of nutrients

Pre-germinated seeds were subjected to different conditions in 50 ml incubation tubes. Plants were grown in low P soil (for details see [53]), vermiculite without nutrient supplementation, and water with the addition of 1% agar (Sigma Aldrich). To exclude light, all tubes were aluminum foil-wrapped and one pre-germinated seed added per tube. Five plants per genotype

and condition were grown in a growth cabinet with 16 h light (30°C) and 8 h dark (25°C) for 7 days. Root hair formation was analyzed using a light microscope as described earlier [75].

4.3. Growth in agar-nutrient solution

Half-strength Yoshida nutrient solution [77] was prepared without P. Agar (Sigma Aldrich) was added at concentrations of 1, 1.5, 2, 2.5, 3 and 3.5% to the nutrient solution. Preliminary experiments suggested that beyond 4% agar, lateral root as well as root hair growth becomes impaired (Rose, unpublished). The nutrient solution-agar mixture was poured into clear plastic boxes (200 mm high × 100 mm wide × 25 mm deep, wrapped with aluminum foil) with a 15-mm-diameter hole in the top. A duplicate set of boxes were cut open to determine the resistance of each agar concentration using a penetrometer.

Two germinated seeds per box were sown 5 mm deep in the agar and boxes transferred to a growth cabinet set to 14 h light (27°C) and 10 h dark (22°C). From week two, the boxes were watered with deionized water to weight every 3 d until harvest after 23 d.

4.4. Plasmid construction for *pBIHubi::XTH2*

To amplify the *Xyloglucantransferase2* (Os11g33270) sequence, RNA from genotype DJ123 was isolated, transcribed into cDNA and used as PCR matrix with the oligonucleotides (5′–3′) CAACCCCGGGATGGCGACGACGACGG and GATCGAGCTCTCAGGCGTCGCGGTCG, which introduced the restriction endonuclease recognition sites for *Sma*I and *Sac*I, respectively. According to manufacturer's protocols the PCR product and the target vector pBIH [78] were treated with *Sma*I and *Sac*I (Fermentas, Fast Digest enzymes), the resulting fragments purified (Promega, Wizard PCR clean-up kit), ligated (Roche, Rapid DNA ligation kit), and transformed into DH5α (Promega, library efficient DH5α). The resulting plasmid contains *XTH2* under the control of the *Ubiquitin* promoter. After *pBIHubi::XTH2* sequence confirmation (using the oligonucleotides, 5′–3′: GATGGTGGTGGCAATGTCG and CGGTCGTCGCAGTAGTTGTA) one clone (termed *Ubi::XTH2*) was selected for rice transformation.

4.5. Rice transformation and T$_2$ selection

Genetic transformation of rice varieties NERICA4 and Nipponbare were conducted by *Agrobacterium*-methods using immature embryos [79]. T$_2$ plants possessing a single copy of transgene as homozygote were selected [80] and subjected to further experiments.

4.6. Phenotyping of *Ubi::XTH2* T$_2$ lines in nutrient solution

Pre-germinated T$_2$ seedlings were grown in water supplemented with iron (12 μM) and calcium (0.1 mM) for 7 days followed by an additional 7 days in 1/3 strength Yoshida solution [77]. At 16 DAS roots were harvested and used for RNA extraction, cDNA production, and qRT-PCR analysis as described earlier [56] and for vibratome sectioning. For detection of the *XTH2* transcript, the oligonucleotides (sequences in 5′–3′) TACCACTCCTACTCCGTCCT and TGGAGTAGAGCTTCATCGGC were used. Cross and longitudinal sections of 75 μm were prepared with a vibratome (Microslicer DTK-1000, DSK) by embedding 5 mm root segments in 4% agarose followed by slicing with a frequency of 8 and cutting speed of 5–7. The

sections were then stained for 1 min with 0.05% toluidine blue, briefly washed with water and mounted with 50% glycerol for light microscope (Olympus BX50, Olympus) imaging.

Selected *Ubi::XTH2* lines with very high, high, and moderate ectopic overexpression as well as control (untransformed Nerica4 and Mock transformed) lines were grown in nutrient solution with low (1 µM) or sufficient (100 µM) P nutrition (NaH_2PO_4 $2H_2O$). The nutrient solution was changed and increased from 1/3 to 1/2 and finally to full-strength Yoshida solution [77] weekly, while pH was adjusted regularly to 5.7. At 35 DAS root hair parameters were determined as described previously [75].

4.7. Phenotyping of *Ubi::XTH2* T_2 lines in the soil

Soil containing three levels of P: extreme low P (80% low P soil mixed with 20% subsoil), low P soil, and P-replete soil (fertilized) were used. The soil was sieved and filled into boxes while softly compacted to simulate field conditions. Fertilizer was supplied in the equivalent amount to 30-0-30 or 30-30-30 kg ha^{-1} (N, P_2O_5, K_2O, respectively).

Four germinated seeds (with similar size) were sown directly in soil and thinned to two plants after 1 week of germination. Water was supplied regularly to field capacity to simulate the wet/dry cycle in upland condition. Plants were grown in a greenhouse with temperature and relative humidity varying between 25 and 32°C and 30–50%. At 40 DAS plants were harvested to evaluate plant height, number of leaves, number of tillers, main root length, root number, shoot and root dry matter.

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