Invited Review

Modulation of plant root traits by nitrogen and phosphate: transporters, long-distance signaling proteins and peptides, and potential artificial traps

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As sessile organisms, plants rely on their roots for anchorage and uptake of water and nutrients. Plant root is an organ showing extensive morphological and metabolic plasticity in response to diverse environmental stimuli including nitrogen (N) and phosphorus (P) nutrition/stresses. N and P are two essential macronutrients serving as not only cell structural components but also local and systemic signals triggering root acclimatory responses. Here, we mainly focused on the current advances on root responses to N and P nutrition/stresses regarding transporters as well as long-distance mobile proteins and peptides, which largely represent local and systemic regulators, respectively. Moreover, we exemplified some of the potential pitfalls in experimental design, which has been routinely adopted for decades. These commonly accepted methods may help researchers gain fundamental mechanistic insights into plant intrinsic responses, yet the output might lack strong relevance to the real situation in the context of natural and agricultural ecosystems. On this basis, we further discuss the established—and yet to be validated—improvements in experimental design, aiming at interpreting the data obtained under laboratory conditions in a more practical view.

Key Words: root system architecture, root morphology, nitrogen, phosphate, transporter, peptide, signaling.

Introduction

Nitrogen (N) and phosphorus (P) are two vital mineral nutrients for plant growth and development as well as for human health (Huang et al. 2020). Nitrate/ammonium and inorganic orthophosphate (Pi) are the major forms of N and P accessible to plant root, which is the predominant organ for plant uptake of these nutrients and water (Giehl and Wirén 2014, Gutiérrez-Alanís et al. 2018). Root morphology is determined by several parameters regarding root system architecture (RSA) and other morphological traits (e.g. root hair [RH] development). RSA refers to the spatial configuration of a plant’s entire root, including primary root (PR)/crown root (CR) elongation, lateral root (LR) branching, and root growth angle (Giehl and Wirén 2014, Uga et al. 2013). All these root traits together with root physiology, which are regulated by both external and internal stimuli, collectively contribute to the root phenotypic plasticity. The highly plastic plant root can actively drive a series of adaptive responses to environmental stimuli, including N/Pi availabilities.

Plant adaptations to N/Pi availabilities are active cellular processes and can be grouped into local and systemic (long-distance) responses. Local responses are largely regulated by external Pi level in environments, whereas systemic responses tend to be controlled by internal Pi concentration (Chien et al. 2018). It has long been recognized that nitrate, ammonium and Pi can all act as a local signals triggering LR development (Drew 1975). The contact of root tip with the medium lacking Pi or that supplied with ammonium leads to an inhibition of PR growth (Li et al. 2010, Svistoonoff et al. 2007), suggesting the important role of root tip in sensing local nutrient availabilities. In addition, nitrate and ammonium play complementary roles in LR development, whereas Pi starvation is usually considered to stimulate the proliferation of LRs and RHs (Pérez-Torres et al. 2008). Nevertheless, it has been demonstrated recently in Arabidopsis that at least some of the root responses fluctuate with the external nutrient concentration. Low external Pi stimulates first-order LR density, whereas Pi deficiency suppresses this trait. Likewise, a middle level of external N enhance total root length and LR length, whereas this enhancement is not observed when an even lower level of N is supplied (Gruber et al. 2013).

All the root responses to N/Pi availabilities are fine-tuned by elaborate molecular signaling networks, in which genes of diverse functional categories are implicated.
Recently, several reviews have well summarized the progresses in deciphering these networks (Chien et al. 2018, Gutiérrez-Alanis et al. 2018, Liu et al. 2020, Liu and Wirén 2017). Furthermore, in spite of these accumulating progresses, it seems that there is still a yawning gap between the established knowledge and practical application even in the long run. One potential cause responsible for this gap could be the artificial experimental conditions conventionally adopted which might not reflect the real situation in the context of natural and agricultural ecosystems (Nestler et al. 2016, Zheng et al. 2019). In this article, we focus on recent progress made in two classes of regulators for root responses to N/Pi, namely transporters and long-distance mobile proteins/peptides, which largely mediate root responses to local and systemic nutrient signals, respectively. Furthermore, we also discuss how to update the existing methodologies for further approaching the truth.

1. Plasma membrane-localized N and Pi transporters are potent regulators of RSA

An increasing number of N/Pi transporters, especially those localized to PM, are demonstrated to affect different aspects of RSA/root morphology; however, their involvement in root responses to N/Pi nutrition/stresses and the underlying molecular mechanisms are not fully explored (Table 1). Nevertheless, some transporters have been demonstrated/inferred to act as transceptors linking extracellular N/Pi availabilities and intracellular signals for root development.

1.1 Nitrate transporter

It seems to be a conserved phenomenon between dicots and monocots that post-embryonic LR rather than embryonic PR/CR is more sensitive to external nitrate (Forde 2014, Steffens and Rasmussen 2016, Tian et al. 2014), although limited studies provide evidence supporting that nitrate can exert an inhibitory effect on PR growth (Patterson et al. 2015, Yan et al. 2014). Several nitrate transporters crucial for nitrate uptake under different nitrate regimes have been recognized as potent regulators of LR development, uncoupled from their activity for mediating nitrate transport (Fig. 1, Supplemental Fig. 1).

**CHLORATE-RESISTANT 1 (CHL1) / NITRATE TRANSPORTER 1.1 (AtNRT1.1) / NRT1-PEPTIDE TRANSPORTER FAMILY 6.3 (AtNPF6.3)** is defined as a transceptor (transporter and receptor) because it is not only a dual-affinity nitrate transporter in *Arabidopsis thaliana* (Arabidopsis), but also a sensor of external nitrate availability. Rather than the trans-membrane transport of nitrate, the binding of nitrate to CHL1 leads to its conformational change which activates the nitrate signaling characterized by transcriptional changes known as primary nitrate response (Ho et al. 2009, Liu et al. 1999, Liu and Tsay 2003). Under low nitrate conditions, nitrate bind to the high-affinity site of CHL1. This binding induces Threonine 101 (T101) phosphorylation and triggers low levels of primary nitrate response. Under high external nitrate conditions, the low-affinity site of CHL1 is occupied by nitrate which inhibits T101 phosphorylation, leading to high levels of primary nitrate response (Ho et al. 2009).

By using a classic split-root system in which one side was supplied with high nitrate whereas the other side was provided with low nitrate, Remans et al. (2006) has shown that mutation of CHL1 suppresses LR growth in the nitrate-rich patch. In a system in which nutrients are supplied homogeneously, *chl1* mutants show increased LR branching in the absence or at low concentration of nitrate, and this phenotype cannot be rescued by external glutamine (Gln). These findings suggest that CHL1 can sense external nitrate levels, and acts as a positive regulator of LR elongation in response to localized high nitrate and also as a negative regulator of LR branching at homogeneous low nitrate availability (Fig. 1A; Krouk et al. 2010, Remans et al. 2006). Nutrient-dependent alterations in RSA are thought to be closely related to hormones, yet the underlying mechanisms are widely unknown (Giehl and Wirén 2014). Interestingly, unlike its counterpart AtNRT1.2, CHL1 is demonstrated to facilitate auxin influx which is negatively regulated by high nitrate concentration. Mutation of CHL1 results in enhanced auxin accumulation in LR primordia and newly emerged LRs in response to severely low external nitrate concentration. Similar to that found in the case of LR branching, external Gln does not counteract the accumulation of auxin in *chl1* mutants (Krouk et al. 2010), indicating that nitrate itself acts as a signal regulating LR development/emergence but not initiation via CHL1-mediated sensing of external nitrate and transport of auxin.

Very recently, CHL1 has been reported to be responsible for auxin biosynthesis as well, reinforcing the notion that CHL1 acts as a linker of nitrate nutrition, auxin homeostasis and root development (Maghiaoui et al. 2020).

The functional homolog of CHL1 in rice, OsNRT1.1B, has been reported to integrate nitrate and Pi signaling, transducing the signal from plasma membrane to nucleus (Hu et al. 2019). However, its contribution in regulating RSA is unknown. Interestingly, two more homologs, OsNRT1.1A and OsNRT1.1C, are present in rice genome. OsNRT1.1A is a tonoplast-localized nitrate transporter regulating N utilization and flowering (Wang et al. 2018). It would be of interest and significance to investigate the contribution of rice NRT1.1s in root development, especially considering the developmental difference between the root systems of dicots (tap root system; e.g. Arabidopsis) and monocots (fibrous root system; e.g. rice).

Arabidopsis LR development is coordinately regulated by CHL1 (dual-affinity nitrate transporter) as well as the high-affinity transporters of the NRT2 family. AtNRT2.1 is probably the most important high-affinity nitrate transporter in Arabidopsis, since it together with AtNRT2.2 are responsible for over 70% of the high-affinity transport activity (Filleur et al. 2001). AtNRT2.1 functions as a
### Table 1. Summary of the plasma membrane-localized N/Pi transporters involved in root responses to external N/Pi. *At*: *Arabidopsis thaliana*; *Os*: *Oryza sativa*

| Gene name/Gene ID | Response to N/Pi | Tissue localization in root | Alteration in RSA in mutants/Ox lines | Regulatory mechanism |
|-------------------|------------------|-----------------------------|--------------------------------------|---------------------|
| **Nitrate transporter** | | | | |
| *AtNRT1.1 / AtCHL1 / AtNPF6.3*<br>At1g12110 | induced by nitrate. | Epidermis, cortex and endodermis | repress lateral root growth at low nitrate availability | As a nitrate sensor; As a negative regulator of the TAR2 auxinbiosynthetic gene<br>Okamoto *et al.* 2003<br>Ho *et al.* 2009<br>Krouk *et al.* 2010<br>Maghiaoui *et al.* 2020<br>Muñoz *et al.* 2004<br>Little *et al.* 2005<br>Wei *et al.* 2018 |
| *AtNRT2.1*<br>At1g08090 | induced by nitrate | The epidermal, cortical and endodermal cell layers | the repression of LR initiation can be relieved<br>decreased lateral root number and length | As a nitrate sensor and signal transducer<br>Muñoz *et al.* 2004<br>Little *et al.* 2005 |
| *OsNRT2.4*<br>LOC_Os01g36720 | induced by NO$_3^-$, suppressed by NH$_4^+$ | The base of the lateral root primordia | N.A. | |
| **Ammonium transporter** | | | | |
| *AtAMT1;1*<br>At4g13510 | induced by –N | root tips, epidermis, cortex, pericycle and root hairs | N.A. | Transceptor sensing external ammonium<br>Mayer and Ludewig 2006<br>Loqué *et al.* 2006<br>Lima *et al.* 2010 |
| *AtAMT1;3*<br>At3g24300 | induced by –N | LR cap and epidermal cells of LR tips; cortex and rhizodermis of root zones where LR primordia emerge | restore third-order lateral root formation; decrease lateral root branching | Potentially as a sensor<br>Loqué *et al.* 2006<br>Lima *et al.* 2010 |
| **Organic N transporter** | | | | |
| *OsLHT1*<br>LOC_Os08g03350 | NA | RH, epidermis, cortex and stele | repression of PR/CR elongation by external aspartate is relieved | N.A. | Guo *et al.* 2020 |
| **Pi transporter** | | | | |
| *AtPHT1;1*<br>At5g43350 | induced by –Pi | epidermis of roots, cortical cells of the root hair zone; in columella and lateral root cap of roots | Double mutation of *AtPHT1;1* and *AtPHT1;4* leads to enhanced length of RH (–Pi condition) and LR (in the high-Pi patch of a horizontal split-root system) | N.A. | Mudge *et al.* 2002<br>Misson *et al.* 2004<br>Shin *et al.* 2004 |
| *AtPHT1;4*<br>At2g38940 | induced by –Pi | epidermis, cortex of the roots, and the root cap; at the lateral root branch of the primary root and in the stele of lateral roots | N.A. | |
| *AtPHT1;5*<br>At2g32830 | induced by –Pi | weak expression in stele of some roots | Ox: increased root hair formation and reduced primary root growth | N.A. | Mudge *et al.* 2002<br>Nagarajan *et al.* 2011<br>Sun *et al.* 2012 |
| *OsPHT1;1*<br>LOC_Os03g05620 | constitutively expressed | all cell types | Ox: augmented RH development | N.A. | |
| *OsPHT1;3*<br>LOC_Os10g30770 | induced by –Pi | all cell types | Ox: an increase in primary/crown root length in response to Pi starvation stress | N.A. | Chang *et al.* 2019 |
| *OsPHT1;8*<br>LOC_Os10g30790 | induced by –Pi | all cell types | Ox: a constitutive induction of RH and LR number | Up-regulating auxin biosynthetic and transport genes<br>Jia *et al.* 2011<br>Jia *et al.* 2017 |
negative regulator of LR initiation in response to a combination of high sucrose and low nitrate (Little et al. 2005). The repression of LR initiation can be relieved either by increased level of external nitrate or upon AtNRT2.1 mutation. Given that AtNRT2.1 is a component of the high-affinity nitrate uptake system, it has been proposed to act as nitrate sensor or a signal transducer downstream of the real sensor, independent of its role in nitrate uptake (Little et al. 2005). Interestingly, AtNRT2.1 expression in root is induced by carbohydrate photosynthate and a bZIP transcription factor (HY5, Elongated Hypocotyl 5), both of which are translocated from shoot to root (Chen et al. 2016; discussed below). In rice, OsNRT2.1 and OsNRT2.2 expressed in all cell types of root with stronger activity in epidermis, and OsNRT2.3a expression is detectable in root stele and emerged LR (Feng et al. 2011, Tang et al. 2012); however, their roles in root development have not been documented. The expression OsNRT2.4 is restricted in the basal region of LR primordia and, to a lesser extent, in the companion cells of crown root. Consistent with the cellular localization of OsNRT2.4, the osnrt2.4 mutants show a defect in LR length and number under both high- and low-nitrate supplies (Wei et al. 2018).

1.2 Ammonium transporter

Ammonium is the other major form of N for plant uptake. It lays a suppressive effect on the elongation of both PR and LR in Arabidopsis. Interestingly, Li et al. (2010) showed that the contact of primary root tip with ammonium is necessary and sufficient to drive the growth arrest of PR under ammonium nutrition. The authors also provided evidence that the elongation of the cells in root tip is the principal target of ammonium-induced inhibition of root growth, while meristematic cell division and/or meristem cell size are a minor but significant cause (Li et al. 2010). Liu et al. (2013b) further demonstrated that cell division and expansion contribute at least equally to ammonium-induced inhibition of PR growth. The slight difference in the conclusions between the two groups might be attributed to different experimental conditions. To our knowledge, no ammonium transporter has been linked with the ammonium-induced PR inhibition (Fig. 1, Supplemental Fig. 2). Furthermore, complementary to nitrate which stimulates LR elongation (in the high-nitrate patch of the split-root system), localized ammonium has a positive effect on LR initiation and higher-order LR branching (Lima et al. 2010).

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A yeast ammonium transporter Mep2 has been demonstrated to act as a transceptor as evidenced by the uncoupling of its transport activity from ammonium signaling (Marini et al. 2006, Rutherford et al. 2008). The AMMONIUM TRANSPORTER (AMT)-type ammonium transporters from Arabidopsis cannot complement for Mep2 function in yeast (Van Nuland et al. 2006). However, the role of plant ammonium transporters as transceptors has been inferred. The threonine residue T460 in the C-terminus of AtAMT1;1 is phosphorylated upon high external ammonium, which inhibit the forming of AMT1 trimer and ammonium transport activity. Neither increased intracellular ammonium generated by using methionine sulfoximine (MSX, an inhibitor of Gln synthetase), nor Gln derived from ammonium assimilation can lead to T460 phosphorylation. These indicate that AtAMT1;1 acts as a sensor of extracellular ammonium and monitors ammonium uptake via phosphorylation-mediated inactivation of AMT trimer (Lanquar et al. 2009).

Despite of the involvement of AtAMT1;1 in external ammonium sensing, it does not affect higher-order LR branching upon supply of this nutrient. By contrast, one of AtAMT1;1 counterparts, AtAMT1;3, stimulates higher-order LR branching in response to localized ammonium supplied to the high-N patch of the split-root system (Table 1; Lima et al. 2010, Loqué et al. 2006), suggesting that distinct signaling pathways are triggered by their sensing of localized external ammonium. Noteworthy, AtAMT1;1 and AtAMT1;3 are both mainly expressed in the epidermis of first- and higher-order LRs, where the sensing of external ammonium takes place. Thus, these two AMT proteins are proposed to act in concert to balance ammonium acquisition and foraging (Lima et al. 2010). On the other hand, to date, very limiting information on the roles of rice ammonium transporters in root development is available, although some of the homologs have been functionally characterized regarding their contribution to ammonium uptake and improving N use efficiency (Lee et al. 2020). Given that ammonium is the major and preferential N source for rice plants growing in paddy field, it is of significance and interest to investigate rice ammonium transporters further.

1.3 Organic N transporter

Inorganic N absorbed by plants undergoes reductive assimilation, giving rise to amino acids which is a less important but non-negligible source of N for plant uptake (Xu et al. 2012). The amino acid transporters represent a large family comprised of more than 10 subfamilies (Rentisch et al. 2007, Zhao et al. 2017). They have been reported to be responsible for the uptake of organic N and distribution of N to both vegetative and reproductive organs (Peng et al. 2014, Tabuchi et al. 2007, Xu et al. 2012). Nonetheless, their roles in plant N nutrition and RSA modulation remain obscure. Very recently, an amino acid transporter of the LHT subfamily in rice, OsLHT1, has been functionally characterized (Guo et al. 2020, Wang et al. 2019a). OsLHT1 expression is detected in all cell types in almost all root segments including root cap, and OsLHT1 can mediate the influx of a wide range of amino acids. The repression of PR/CR elongation by external aspartate (one of the major dissolved organic N source in soil) is relieved upon mutation of OsLHT1 (Guo et al. 2020). Given the root cap localization of OsLHT1, it is tempting to speculate that OsLHT1 might act as a sensor of external amino acid(s) and could mediate root responses to organic N in environment. Nevertheless, the information regarding the functions of amino acid transporters and their potentials as transceptors is lacking (Dinkeloo et al. 2018). Thus, intensive work is needed in future to gain further insights into this field.

1.4 Pi transporters of the PHT1 family

The elucidation of the SPX-inositol polyphosphate (InsP)-PHR module pins SPX to the Pi signaling network as an intracellular P sensor (Dong et al. 2019, Wild et al. 2016). However, the PHR TFs are largely involved in systemic Pi starvation signaling (Thibaud et al. 2010), and the knowledge on the sensor(s) of extracellular Pi is widely unestablished. In addition to the cell wall-localized ferredoxidase LOW PHOSPHATE ROOT1 (LPR1) (discussed below), limited evidence suggests that PHT1 (probably the only influx transporter for Pi uptake) may also act as sensors of extracellular Pi, defining them as transceptors (Ayadi et al. 2015; Supplemental Fig. 3). Several transgenic lines with altered expression of PHT1 genes show drastic alterations in several root traits. In rice, OsPHT1;3 overexpression lines show an increase in primary/crown root length in response to Pi starvation stress (Fig. 1B; Chang et al. 2019), phenocopying that of OsPHR2 overexpressors and ospho2 mutants (Hu and Chu 2011, Zhou et al. 2008). OsPHR2 transcription factor acts as a positive regulator of several PHT1 genes via direct binding to their promoters, whereas OsPHO2 functions downstream of OsPHR2 and negatively regulates PHT1 protein abundance via the 26S proteasome pathway (Fig. 1C; Chang et al. 2019, Hu and Chu 2011, Liu et al. 2010, Wang et al. 2009). Therefore, it is pertinent to assume that the regulation of OsPHR2 and OsPHO2 on rice root elongation is at least in part attributed to PHT1s. Interestingly, a similar PR response (enhanced PR/CR elongation upon low-Pi) has been reported for Arabidopsis AtPHT1;9 overexpression lines (Fig. 1A; Remy et al. 2012), but not for the overexpression plants of OsPHT1;8 (Jia et al. 2011, 2017), a homolog of OsPHT1;3. Given that the intracellular Pi levels are dramatically increased upon overexpression of either OsPHT1;3/OsPHT1;8 or AtPHT1;9 (Chang et al. 2019, Jia et al. 2011), we speculate that the PR/CR elongation depends on the presence of PHT1 proteins rather than on the quantity of Pi taken up. This is reminiscent of the case of ammonium-triggered LR development in Arabidopsis, namely AtAMT1;3 but not AtAMT1;1 stimulates higher-order LR proliferation though these two
ammonium transporters display similar transport properties and localization (Lima et al. 2010). The differential effects of these PHT1 members on root elongation suggest that some PHT1 proteins (e.g. OsPHT1;3) may serve as sensors of extracellular Pi and even they can facilitate hormone transport. If the latter is true, it would be of significance to know whether PHT1 proteins directly facilitates the hormone transport as CHL1 does (Krouk et al. 2010) and/or indirectly regulates the transcription of genes for hormone biosynthesis and transport through a yet to be identified cascade (Jia et al. 2017).

In addition to the elongation of primary/crown root, the development RH and LR are also important traits for the uptake of mineral elements including Pi (Liu et al. 2013a, Ma et al. 2001). In rice, OsPHT1;8 overexpressors show a constitutive induction of RH and LR number when high level of Pi is supplied, which could be attributed to enhanced auxin biosynthesis and transport under that condition (Jia et al. 2017). Overexpression of OsPHT1;1 also results in augmented RH development in response to a sufficient level of external Pi; however, unlike that found in OsPHT1;8 overexpressors, the RH number and length of OsPHT1;1 overexpression lines under +Pi condition are even higher than that under –Pi condition (Sun et al. 2012). In Arabidopsis, overexpression of AtPHT1;5 leads to decreased PR length and enhanced RH number and length irrespective of the Pi regimes (Nagarajan et al. 2011). It seems that OsPHT1;1, OsPHT1;8 and AtPHT1;5 all play a positive role in PR elongation and/or LR/RH development. By contrast, double mutation of AtPHT1;1 and AtPHT1;4 leads to enhanced length of RH (~Pi condition) and LR (in the high-Pi patch of a horizontal split-root system; Misson et al. 2004, Shin et al. 2004). Two possibilities could be the causes of the seemingly contrasting effect of these PHT1 proteins on root development: i) the root traits have not been examined in the mutant lines of OsPHT1;1, OsPHT1;8 and AtPHT1;5, which might show similar response to external Pi as atpht1;1 atpht1;4 mutant; ii) the alteration of root traits observed in atpht1;1 atpht1;4 mutant could be an indirect effect of impaired Pi nutrition. Noteworthy, the expression of OsPHT1;1, AtPHT1;1 and AtPHT1;4 is detected, although not restricted, in the root cap of PR/CR, which is an important tissue for both Pi uptake and local Pi sensing (Kanno et al. 2016, Sun et al. 2012). These findings suggest that these PHT1 proteins may have differential sensitivities to external Pi and thus elicit distinct signaling pathways involving auxin biosynthesis and transport, the underlying molecular basis of which would be of interest to be investigated in future work.

1.5 Other plasma membrane-localized transporter involved in the root responses to Pi

Aluminum Activated Malate Transporter 1 (ALMT1) is a plasma membrane-localized transporter mediating root malate efflux. ALMT1 and a transcription factor activating ALMT1 expression, Sensitive To Proton Rhizotoxicity (STOP1), have been found to be implicated in aluminum and proton tolerance (Hoekenga et al. 2006, Iuchi et al. 2007). Interestingly, STOP1 is regulated post-translationally and its accumulation in nucleus is enhanced upon –Pi, whereas ALMT1 is a downstream target of STOP1 and transcriptionally induced by –Pi (Fig. 2; Balzerque et al. 2017, Mora-Macías et al. 2017, Wang et al. 2019b). Similar to that found in lpr1 single mutant and lpr1 lpr2 double mutant, the PR growth of the single

![Fig. 2.](image-url) A complex network regarding primary root growth in response to external phosphate (Pi) and iron (Fe), which is subjected to the influence of artificial experimental condition (i.e. exposure of root to blue light). The arrowhead pointing at ‘Mobilization of Fe accumulation’ is shown as a dotted line because that the Fe accumulation in stem cell niche, elongation zone or maturation zone is not the cause but likely the consequence of the sensitivity of PR growth to Pi deficiency; instead, the mobilization of Fe accumulation might be the cause, which needs to be further validated. The arrowhead pointing at ‘Calllose deposition’ is also present as a dotted line because whether ‐OH leads to calllose deposition in this case is unknown, although ROS‐mediated callose deposition has been reported. The arrowhead pointing at ‘LPR1’ is also shown as a dotted line because LPR1 expression seems not to respond to –Pi, yet the distribution of its transcript is extended to proximal regions of the root tip. RAM, root apical meristem; PR, primary root. Red line: transcriptional regulation; blue line: post-transcriptional/post-translational regulation; green line: transcriptional or post-translational regulation.
mutants of either STOP1 or ALMT1 is insensitive to \(-\)Pi. Mutation of ALMT1 results in decreased Fe accumulation and relieved PR growth inhibition upon \(-\)Pi (Balzergue et al. 2017, Wang et al. 2019b). However, this is due to an indirect effect of the chelating of extracellular Fe\(^{3+}\) by exported malate, which facilitates a Fe redox cycling within cell wall. The byproduct of this Fe redox cycling, hydroxyl radical, is the real cause of PR growth arrest. ALMT1 functions downstream of ALS3/STAR1 (Wang et al. 2019b). The STOP1-ALMT1 and PDR2-LPR1 modules control PR growth under Pi deficiency in an interdependent manner involving the promotion of malate-dependent Fe accumulation in roots (Balzergue et al. 2017, Dong et al. 2017, Liu and Wirén 2017, Mora-Macías et al. 2017, Müller et al. 2015, Ticconi et al. 2004, 2009, Ward et al. 2008). However, the role of Fe in PR growth inhibition is controversial (Balzergue et al. 2017, Mora-Macías et al. 2017, Müller et al. 2015). More recently, Wang et al. (2019b) provided further evidence demonstrating that it could be the mobilization but not the level of Fe accumulation in roots determining the PR growth under \(-\)Pi. Despite of these interesting findings regarding the STOP1-ALMT1 and PDR2-LPR1 modules, these established understandings of their roles in root responses to external Pi availability has been reported to be affected by experimental design (Zheng et al. 2019; discussed below).

2. Long-distance mobile protein and peptides

As discussed above, the systemic signaling is equally important for plant root adaptions to N/Pi signals. The vascular system serves as a highway for the long-distance mobile molecules mediating intercellular nutrient stress signals. Small RNAs, mRNAs and proteins/peptides have all been detected in phloem sap of diverse plants and are thus recognized as such mediators of systemic signaling (Buhtz et al. 2008, Notaguchi et al. 2015, Paulitre et al. 2016, Zhang et al. 2016). However, the studies focusing on the involvement of long-distance mobile proteins/peptides in controlling root responses to N and Pi availabilities as well as the underlying mechanisms are just emerging.

2.1 Long-distance mobile protein

The coordination of shoot photosynthetic carbon fixation with root nutrient uptake (e.g. nitrate) is vital for optimizing plant growth. A bZIP transcription factor in Arabidopsis HY5 has turned out to be a shoot-to-root mobile signal mediating the light-promoted root growth and nitrate uptake (Chen et al. 2016, Oyama et al. 1997). In shoot receiving illumination, HY5 stimulates both carbon assimilation (via activating photosynthesis- and carbon metabolism-related genes) and translocation (through promoting sucrose efflux transporter genes). Meanwhile, shoot-derived HY5 is translocated downward to root to activate AtNRT2.1 expression and nitrate uptake (Chen et al. 2016, Kobayashi et al. 2012, Toledo-Ortiz et al. 2014). AtNRT2.1 is a negative regulator of LR initiation in response to a combination of high external sucrose and low nitrate (Little et al. 2005). Recently, it has been reported that HY5 suppresses total LR length under N-replete condition regardless if the root is illuminated or not (Zhang et al. 2019a). In addition, the activation of the expression of several PHT1 genes and thus Pi uptake require the phytochrome B-HY5 module, and HY5 has been demonstrated to directly bind to the promoter of AtPHT1;1 (Sakuraba et al. 2018). Two open questions awaits to be answered: i) whether the alteration in total LR length upon HY5 mutation is at least in part due to impaired LR initiation (LR number); ii) does this regulation of LR development by HY5 depend on AtNRT2.1/AtPHT1;1 and occur under low-N or low-P conditions as well? Furthermore, Chen et al. (2016) provided evidence that, in addition to HY5, AtNRT2.1 is also required for the elongation of PR of seedlings grown in middle or relatively high light fluence rates, which is not observed in the work by Little et al. (2005). The contrasting results are probably attributed to the exposure or shielding of root to light, and need to be further validated.

2.2 Peptides

Emerging evidence has shown that some small peptides of the C-TERMINALLY ENCODED PEPTIDE (CEP) and CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE) families are responsive to N/Pi signals and are involved in modulation of RSA (Liu et al. 2020, Taleski et al. 2018). Upon induction, they are perceived and bound by their respective receptors, CEP receptor (CEPR) and CLAVATA (CLV), to relay the signals to downstream components.

It has been reported that the expression of seven out of the 15 CEP/CEP-like genes in Arabidopsis (AtCEP1, 3, 5, 6, 7, 8, and 9) is rapidly up-regulated in N-starved roots. Overexpression of these seven CEP genes as well as AtCEP2, AtCEP4 and AtCEP11 leads to enhanced expression of the high-affinity nitrate transporter gene AtNRT2.1, although the latter three are not positively responsive to N starvation (Table 2; Roberts et al. 2013, Tabata et al. 2014). CEP peptides induced in the roots colonized in low-N patch are translocated upward through xylem to shoot where they are perceived by two CEP receptors (leucine-rich repeat-receptor kinases, AtCEPR1 and AtCEPR2; Tabata et al. 2014). Subsequently, secondary signaling molecules in shoot, CEP DOWNSTREAM 1 (AtCEPD1) and AtCEPD2, move back through phloem to roots where they activate the expression of AtNRT2.1 (Ohkubo et al. 2017, Tabata et al. 2014). Very recently, Arabidopsis CEPD-like 2 (AtCEPDL2) has been reported to work coordinately with AtCEPD1/2 to promote high-affinity uptake and root-to-shoot transport of nitrate (Ota et al. 2020). AtNRT2.1 senses local nitrate signal and represses LR initiation upon a combination of high external sucrose and low external nitrate (discussed above; Little et al. 2005). A cepr1 cepr2
double mutant shows enhanced LR elongation (Tabata et al. 2014). Nonetheless, it is elusive whether the CEP-CEPR-CEPD module regulates LR development via monitoring AtNRT2.1 expression or through an unknown pathway independent of AtNRT2.1. Notably, the ascending CEPs are transported through xylem, whereas the descending CEPs are delivered via phloem where AtCEPRI is expressed. Thus, the signal transduction is probably achieved by the diffuse of the AtCEP1 peptide from xylem to phloem (Ohkubo et al. 2017). These findings demonstrate that the systemic regulation of N acquisition and probably RSA requires a signal switch event between different domains of the vascular system. It would be interesting to know whether this mode of long-distance signaling is universal or is applicable to systemic N signaling only.

Four out of the 32 CLE genes in Arabidopsis, namely AtCLE1, 3, 4, and 7, are transcriptionally induced by –N in root pericycle cells where the initiation of LR primordia occurs (Table 2; Gutiérrez-Alanís et al. 2017), suggesting their critical roles under N-deprived conditions. Constitutive overexpression of any one of AtCLE1, 3, 4, 5, and 7 results in suppressed LR emergence as evidenced by decreased number and length of emerged LRs but normal development of LR primordia. In contrast, mutation of a CLE receptor gene encoding a leucine-rich repeat receptor-like kinase, CLAVATA1 (CLV1), leads to exaggerated LR length under N-deficient conditions, and this phenotypic defect can be rescued by introducing AtCLV3 driven by its native promoter (Araya et al. 2014). A transgenic line harboring the AtCLV1 promoter-driven AtCLV1-GFP construct shows fluorescence signal in phloem companion cells (Araya et al. 2014). The inconsistent cellular localization of the CLEs (pericycle cells) and their receptor (companion cells) indicates that CLE peptides can also undergo intercellular transport as CEPs and trigger non-cell autonomous events via binding with their receptor AtCLV1.

Unlike the aforementioned small peptides (AtCEP1, AtCEPD1/2 and AtCLE1/3/4/7), some small peptides may not be mobile in the vasculature; instead, they tend to be translated and localized in the same site where their mRNAs are transcribed (e.g. AtCLE14). Another group of CLE genes in Arabidopsis (AtCLE14, AtCLE22 and AtCLE26) have been found to be transcriptionally up-regulated in the root tip of Arabidopsis by –Pi in the presence of Fe (Table 2; Gutiérrez-Alanís et al. 2017). AtCLE14 expression domain in root tip (+P: lateral root cap and columella; –Pi: extended to cortex, endodermis, stele and the cortex/endodermis initial daughter cell) in part coincide with that of AtLPR1 (+P: SCN, endodermis and provascular tissues; –Pi: extended into maturation zone). The induction of AtCLE14 expression upon –Pi is dependent on LPR1/LPR2 function and –Pi-induced Fe mobilization in RAM, indicating that AtCLE14 functions downstream of LPR1/LPR2 and the effect of Fe on root growth (Fig. 2; Gutiérrez-Alanís et al. 2017, Müller et al. 2015). Moreover, AtCLE14 receptor CLAVATA2 (CLV2) and PEP1 RECEPTOR 2 (PEPR2; a receptor-like kinase) function redundantly in transducing AtCEP14 signal upon –Pi (Gutiérrez-Alanís et al. 2017). A downstream event of the signaling cascade mediated by the AtCLE14-AtCLV2/AtPEPR2 module is the suppression of the transcript and/or protein levels of SHR/SCR and several auxin exporter genes (Fig. 2; AtPIN1, 2, and 3). Furthermore, several other CLE genes including the two homologs induced by –Pi (AtCLE22 and 26) are up-regulated upon AtCLE14 mutation (Gutiérrez-Alanís et al. 2017). The implications of other –Pi-induced CLE genes in plant adaptations to this stress as well as the potential interactions and genetic complementation among them remain to be investigated.

3. Concerns over the experimental design

Given the complexity of natural and agricultural environments, all the researchers know that the data obtained in laboratory conditions may not always reflect the real situation. However, we may never quit from labs even we know there are insufficiencies in laboratory experiments which may result in artifacts and prevent us from approaching the truth (Hanlon et al. 2018, Zheng et al. 2019). It is just like when we are trying to work out a physics problem, the frictional force and air resistance are often assumed to be negligible. Nonetheless, there is still more we need to rethink about updating the existent experimental design.

In a considerable proportion of excellent work focusing on root responses to various stimuli, the transparent Petri dish system is utilized. Therefore, plant roots are constantly exposed to light, which may barely happen in natural environments. Very recently, Zheng et al. (2019) reported that one of the typical Arabidopsis root responses to –Pi, namely PR growth arrest, results from the illumination of roots with blue light. Specifically, blue light can trigger malate-mediated photo-Fenton reaction followed by a canonical Fenton reaction, leading to an Fe redox cycle in root apoplast and a by-product hydroxyl radical. The hydroxyl radicals, which will not be produced in light-shielded –Pi roots, are responsible for the inhibited growth of illuminated –Pi roots (Fig. 2; Zheng et al. 2019). Development of new methodologies by which plant roots are shielded from light is of significance in future work. In addition to the aluminum foil method utilized in several studies (Chen et al. 2016, Zhang et al. 2019a, Zheng et al. 2019), some other platforms enabling light-shielding and/or in situ imaging of roots (e.g. GLO-Roots) have been demonstrated to be equally feasible (Rellán-Alvarez et al. 2015). It is also important to examine the potential effect of light on the research focusing on other stimuli.

As for the research in rice and many other model plants, hydroponic systems are often the first priority for investigators, owing to its advantages such as easy manipulation and monitoring. It has long been realized that rice plants subjected to –Pi nutrient solution produce more and longer RH (Guo et al. 2015, Nestler et al. 2016). However, in rice
Table 2. Summary of AtHY5 and the long-distance mobile and/or locally expressed peptides involved in root responses to N/Pi. *At: Arabidopsis thaliana; Os: Oryza sativa*

| Gene/Annotation/ID | Response to N/Pi | Tissue localization/Mobility | Alteration in RSA in mutants/Ox lines | Regulatory mechanism | Reference |
|--------------------|------------------|-----------------------------|--------------------------------------|----------------------|-----------|
| **Protein**        |                  |                             |                                      |                      |           |
| *AtHY5* bZIP TF    | activates NRT2.1 | meristem and transition zone of mature LR | shoot-illumination promotion of root growth | shoot-root mobile HY5 activates root NRT2.1 via a mechanism that is amplified by auto-activation of root HY5 | Chen et al. 2016 |
| At5g11260          | Expression and NO\(^3\) Uptake |                             |                                      |                      |           |
| **Peptide**        |                  |                             |                                      |                      |           |
| *AtCEP1 At1g47485* | induced by –N    | apical meristem; leaf margin; inner layer; LR primordium; vasculature | NA | translocated upward through xylem to shoot in low-N; interact with CEPR1; up-regulate *AtNRT2.1* in high-N patch | Roberts et al. 2013 Tabata et al. 2014 Ohkubo et al. 2017 |
| *AtCEP3 At2g23440* | induced by –N    | the tip of the cotyledons; roots base | NA | interact with CEPR1; up-regulate *AtNRT2.1* | Roberts et al. 2013 Tabata et al. 2014 |
| *AtCEP5 At5g66815* | induced by –N    | phloem pole pericycle and adjacent phloem; petioles; roots base lateral root primordia and lateral roots excluding the meristem region; aerial tissues | Ox: shorter PR decrease lateral root density | interact with CEPR1; up-regulate *AtNRT2.1* | Roberts et al. 2013 Tabata et al. 2014 |
| *AtCEP6 At5g66815* | induced by –N    | NA | NA | up-regulate *AtNRT2.1* | Roberts et al. 2013 Tabata et al. 2014 |
| *AtCEP8 Between At5g66817-At5g66820*; AtCEP9 At3g50610 | induced by –N | NA | NA | up-regulate *AtNRT2.1* | Roberts et al. 2013 |
| *AtCEP7 Between At5g66817-At5g66820* | induced by –N | NA | NA | interact with CEPR1; up-regulate *AtNRT2.1* | Roberts et al. 2013 Tabata et al. 2014 Ohkubo et al. 2017 |
| *AtCEPDJ At1g60830* | induced by –N    | Phloem; Signals move back through phloem to roots | Double mutant plants produced longer LRs mutant; reduction in shoot nitrate content and plant biomass | cooperatively with CEPD1 &CEPD2; a shoot-derived descending signal and root-to-shoot transport of nitrate | Ota et al. 2020 |
| *AtCEPD2 At2g47880* | induced by –N    | Vascular translocation from root to shoot | | | |
| *CEPDL2 At3g62960*  | induced by –N    | | | | |
| *AtCLE1 At1g73165* | induced by –N    | root pericycle cells | Ox: suppress LR emergence feedback regulated by CLV1 (except CLE1) | | Araya et al. 2014 Goad et al. 2017 |
| *AtCLE3 At1g60225* | induced by –N    | | | | |
| *AtCLE4 At2g31081* | induced by –N    | | | | |
| *AtCLE7 At2g31082* | induced by –N    | | | | |
| *AtCLE14 At1g63245* | induced by –Pi   | +P: lateral root cap and columella; –Pi: extended to cortex, endodermis, stele and the cortex/endodermis initial daughter cell | inhibition of root elongation trigger full root meristem differentiation through CLV2/PEPR2 receptors; acts downstream of LPR1/LPR2 | | Gutiérrez-Alanís et al. 2017 |
plants grown in upland fields and rhizoboxes, the length of RHs is generally lower than that in hydroponic cultured plants and is not very responsive to –Pi, and the RH density is higher in high-Pi conditions (Nestler et al. 2016). These observations again push us to reevaluate the relevance of previous results to plants grown in soil and potential measures to optimize the experimental design.

In addition, other characters conferred by soil affect plant RSA/nutrient uptake and are not readily to be recreated in regular laboratory conditions. These characters include the highly inhogeneity of nutrients and water, the changing soil strength (Colombi et al. 2017), and the interaction of roots with beneficial (e.g. rhizobia and arbuscular mycorrhiza fungi) and adverse microorganisms (Castrillo et al. 2017, Fabiańska et al. 2019, Zhang et al. 2019b). On the other hand, the fine manipulations during the treatment and sampling processes represent an additional level of artificial effect, since plants are sensitive to mechanical force and can record the mechanical signals and trigger adaptive responses accordingly (Jensen et al. 2017, Lange and Lange 2015). Actually, such influence as well as other environmental stimuli (i.e. other abiotic and biotic stresses) are inevitable in natural and agricultural ecosystems and are universal to all research fields (Braam and Davis 1990, Swanson et al. 2015). One advantage of laboratory conditions is that the researchers can choose to eliminate these stresses or to impose them homogeneously.

4. Conclusions and prospects

Three major strategies have been proposed for breeding of nutrient-efficient crops: 1) conventional and marker assisted selection breeding; 2) transgenic modification; 3) better P fertilization and cultivation management. Among these strategies, molecular breeding via transgenic approaches require a fully understanding of the gene regulatory networks (Tian et al. 2012). While the number of the genes in these networks is boosting (Liu et al. 2020, Liu and Wirén 2017), several recognized issues and emerging areas deserve more attention.

4.1 Interactions among different nutrients and the effect of other stimuli in soil

Extensive interactions among different mineral nutrients occur in soil. The physiological and molecular mechanisms underlying the crosstalk between different macronutrients (e.g. N and P) and that between macronutrients and micronutrients (e.g. P and Zn) have been studied (Bouain et al. 2014, Briat et al. 2015, Hu et al. 2019, Takehisa and Sato 2019). In future work, in addition to the potential interactions among different nutrients, other factors rendering by the nature of soil could be integratively considered in experimental design: i) soil chemistry (e.g. soil pH, buffering capacity and adsorption; Hanlon et al. 2018, Liang et al. 2013); ii) a much more complicated heterogeneity of nutrients in soil; iii) intraspecific and interspecific competitions for nutrients which is largely determined by spatial constraint, planting density and root exudates (Fang et al. 2013, Joseph et al. 2015, Takehisa and Sato 2019); iv) water availability in soil which affects nutrient availability and RSA (Araus et al. 2020, Plett et al. 2020); v) rhizosphere microbiota associated N/Pi nutrition (Castrillo et al. 2017, Fabiańska et al. 2019, Zhang et al. 2019b).

4.2 Other components of RSA

Compared with the RSA parameters most intensively used such as the length of PR/LR/RH and the density of LR/RH, some other components contributing to RSA is less studied. Root growth angle is such a root traits significant for foraging of both nutrient and water (Miguel et al. 2015, Uga et al. 2011). Mobile nitrate and immobile Pi are more abundant or available in the deep and top soil layers, respectively (Giehl and Wirén 2014). Therefore, deep rooting to lower soil layer may facilitate plants with the exploration of water and nitrate, whereas a shallow root system may enhance the scavenging of Pi (Giehl and Wirén 2014, Uga et al. 2013). Developing smart plants having a root system with balanced distribution of roots to different vertical soil layers could be an important strategy for breeding nutrient- and water-efficient crops.

4.3 Phytohormones: the cause or the consequence?

Most of the chemicals currently defined as phytohormones have been connected with root responses to N and/or Pi (Gu et al. 2017, Hammond and White 2008, Jiang et al. 2007, Maghiaoui et al. 2020, Mayzlish-Gati et al. 2012, Song et al. 2016, Tong et al. 2014). However, it is still unclear at least for some of the hormones that whether they and the genes for their biosynthetic and transport are the causes or the consequences of the altered RSA in response to the changing N/Pi availabilities.

It is a long way to the fully understanding of the gene regulatory network controlling RSA and then to genetically modified agriculture. In future fundamental research, it is pertinent and necessary to put much more emphasis on studies dealing with roots under laboratory conditions better mimicking the natural environments, or ideally, directly in natural and agricultural ecosystems. These will require the development and expansion of novel experimental platforms, like those facilitating in situ assessment of root traits.

Author Contribution Statement

MG and GHX envisaged the concept. XH and TTW collected recent information on transporters and long-distance mobile proteins/peptides involved in root responses to nitrogen/phosphate. MG wrote the manuscript. MG, XH, TTW, and GHX approved the final manuscript.
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