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Nitrogen and Phosphorus interactions in plants: From Agronomic to Physiological and Molecular Insights.

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Abstract
Nitrogen (N) and Phosphorus (P) are the two most essential nutrients ensuring food production and security. The ever growing population demands more N and P based fertilizers. Even though the N provision to the agricultural system is virtually infinite (Haber and Bosch process) it triggers pollution when it is not used by the plant and leaks into the environment. On the other hand, P is predicted to be a limited source worldwide. P use is also responsible for water eutrophication. Thus understanding plant response to combinations of N and P has clear implications for sustainable human development. Recent works have shed new light on how N and P closely interact to control plant responses. Several molecular actors have been revealed controlling the molecular interaction between these two essential elements drafting a working model of N and P interactions. We summarize here these new findings as well as several previous lines of evidence in agronomy and physiology studies preceding this new trend of investigation in the molecular world.

Introduction

Terrestrial and marine ecosystems are accepted to be nutrient limited [1]. This explains life blooms following diverse fertilizations [2]. Nitrogen (N) and Phosphorus (P) are among the most essential elements that sustain yield in agricultural systems since they alleviate such limitations. N and P are ensuring food security worldwide, and fertilizations have been considered as a key factor of the human population growth and is part of the so called *green revolution*. Since the 60's, anthropic activities have dramatically modified the cycle of these elements in the biosphere threatening natural ecosystems [3]. Thus a basic knowledge of such phenomenon is essential for a sustainable human development.

To date, the effect of N and P have been largely considered in isolation but many literatures report that these elements are interacting at several levels of integration. The present report is meant to summarize the different levels of P and N interactions, starting from an ecological and an agronomic perspective to physiological and more recent molecular insights.

Historical observation about N/P interactions.

Liebig's law of the minimum, proposed in the nineteenth century, states that plants' growth is constrained by a single limiting nutrient. Since then, the law has been applied as a basic principle in various ecological and agronomic studies on N and P [4,5]. However, a number of studies have suggested that there are interactions between them [6–8]. For instance, Elser et al. (2007) analyzed the nature of their interaction in a meta-analysis of N- and P-amendment experiments in aquatic and terrestrial ecosystems [3]. They concluded that N and P co-limitation is widespread in ecosystems and there is a synergistic interaction between them because addition of both nutrients together produces a much higher response than adding either one alone. In agronomic studies, a synergistic effect of N and P co-fertilization on yield are well documented in many crops, including wheat, rice, corn, and cotton (reviewed in [9–11]). Physiological observations that N act positively on P uptake [12,13] and P starvation negatively on nitrate uptake and assimilation [14–16] have suggested that there is a mutual interaction between N and P. Together, it is now clear that the interaction exists. Such interaction must be a strategy for plants to coordinate N and P acquisition and usage under fluctuating nutrient conditions for growth optimization, however, our understanding of the molecular basis of the interaction is still in its early stage.

Control of Phosphate molecular responses by Nitrogen

Description of N and P related simple pathways

P and N-related signaling pathways have been studied for decades. They can be sorted into 2 classes. Pathways triggered when the nutrient is removed or when mineral is provided.

For P, the most studied phenomenon is named Phosphate Starvation Response (or PSR; Figure 1) that is characterized by the slow (within days) activation of Phosphate Starvation Induced (PSI) genes (*IPS1, At4, miR399s, PHTs*) when P is removed from the media [17,18]. P sensing mechanism upstream of PSR is performed by the inositol polyphosphate (insP) triggered protein-protein interaction between the transcription factor PHOSPHATE STARVATION RESPONSE (PHR) and SPX-domain-containing (SPX) proteins [19–22]. When P is sensed it prevents the PSR activation. PHR1 thus induces *miR399* (and *IPS1*, target mimicry that dampers *miR399* effect) that represses PHOSPHATE 2 (PHO2), an ubiquitin conjugase that works with NITROGEN LIMITATION ADAPTATION (NLA) to repress phosphate transporters (PHOSPHATE TRANSPORTER 1.1, PHOSPHATE 1) [23,24].

N related signaling pathways are more diverse. The most studied N related signaling pathway is named the Primary Nitrate Response (or PNR, Figure 1) being rapidly (within minutes) triggered when plants deprived of NO_3^- are provided with this nutriment [25,26]. Transcriptional markers of the PNR are for instance *NIA1*, *NIR*, HYPERSENSITIVITY TO LOW PI-ELICITED PRIMARY ROOT SHORTENING 1 (*HRS1*) [27]. One of the identified sensors in PNR is the NO_3^- transceptor CHLORINA1/NITRATE TRANSPORTER 1.1 (CHL1/NRT1.1) [28]. CHL1/NRT1.1 triggers calcium response likely decoded by CALCIUM-DEPENDENT PROTEIN KINASEs (CPK) that phosphorylate NIN-LIKE PROTEINs (NLP) [29–31]. These latter are central transcription factors controlling PNR marker genes [32,33]. This constitutes the backbone of PNR but many other genes, including kinases, phosphatases, and other transcription factors have been shown to belong to this pathway (for detailed review see [34]).

Nitrogen Starvation Response (NSR) is manifested when N is removed from the media. It is characterized by a slow (days) activation of marker genes *NRT2.4, NRT2.5*, *GDH3 [35]*. Recently genes belonging to this pathway have been identified. They include NITRATE-INDUCIBLE, GARP-TYPE TRANSCRIPTIONAL REPRESSOR 1/HRS1/HRS1 HOMOLOGs (NIGT1/HHOs) [36–38], LBD36,37,38 [39], NFYA [40], CBL7[41]. But the interplay between these molecular elements is still under investigation.

Finally it is worth mentioning that PNR and NSR pathways are not independent. For instance NIGT1/HHOs being marker genes of PNR are key regulators of the NSR (Figure 1, [36–38]). These pathways can also be studied in the context of their role in distant organs as they trigger long distance signaling adding a layer of complexity to their interactions (for review see [42]).

Control of Development by N x P

Variations in N and P are known to control plant development adaptation. For instance P starvation triggers primary root shortening and lateral root outgrowth [43] via the action of several molecular elements including LPR1/2, STOP1, ALMT [44,45]. This affects plant architecture to explore shallow soil horizons supposed to contain more abundant P. Root responses to N fluctuation are quite diverse and was recently reviewed in an excellent paper [46]. In short, nitrate provision triggers lateral root initiation and elongation, when N starvation tends to repress LR development and favor primary root elongation to seek for N (supposed to leak in the form of NO_3^- towards deeper soil horizons). Several dozens of genes are involved in these responses [46], including some genes also controlling PNR and NSR, as well as long distance components. Concerning root development, several studies pointed to N and P interactions [47] but only a few so far identified molecular actors.

The study of direct targets of the HRS1 (NIGT/HHO family) transcription factor, being an excellent marker of the PNR [27], showed that P-related genes are enriched in this target list [48]. This brought Medici et al, [48] to study the interaction of P and N signals and the role of this transcription factor in the N/P interaction. It has been shown that *hrs1,hho1* double mutant is resistant to P limitation primary root response only when limitation is applied in presence of NO_3^-. This interaction is supposed to be the result of the strong transcriptional control of HRS1 and a post-translational control of the protein level by P [48]. Interestingly, recent work also demonstrated that NPF7.3/NRT1.5 (nitrate transporter) mutants display a very strong root phenotype on -P conditions [49].

Regulation of Phosphate Starvation Response (PSR) by Nitrogen Availability

A number of physiological evidences have shown that N availability affects PSR. Under P starvation N supplement activates PSR, while N starvation strongly represses it [12,50–54], indicating that plants have a regulatory system to prioritize N over P. Considerable progress has been made recently in understanding the underlying mechanism of the system and three major PSR signaling factors at N-P interface have been identified. They are SPXs, PHRs and PHO2. In *Arabidopsis*, the expression of *SPX1*, *SPX2*, and *SPX4* is repressed in response to N supplement by NIGT1/HHOs [36,55]. In rice, OsSPX4 is degraded by the 26S proteasome pathway in a nitrate-stimulatory manner. It was shown that the interaction between OsSPX4 and OsNRT1.1B (a homologue of CHL1/NRT1.1 in rice), which is facilitated by nitrate, triggers degradation of OsSPX4 by recruiting NRT1.1B INTERACTING PROTEIN 1 (OsNBIP1), an E3 ligase [53]. PHRs are positively regulated by N at transcriptional and

post-transcriptional levels. The expression of *PHR1-LIKE 1* and *OsPHR3* is activated by N supplement [56,57] and PHR1 protein stability is decreased by N-starvation [52], though the N-related factor involved in these regulations is unknown. PHO2 is recognized as an important interface because *pho2* mutants display severe impairment in N starvation repression of PSR [52,58]. *PHO2* expression is upregulated by N starvation, and NIGT1/HHOs and CHL1/NRT1.1 are implicated in the regulation [36,52]. Furthermore, the mutant of *NLA* shows *pho2*-like phenotype in N-dependent PSR regulation and the translation of NLA is repressed by N starvation [58,59], suggesting that PHO2 and NLA act together in N-dependent PSR regulation. In addition to these, several potential factors involved in N-dependent PSR regulation have been reported in *Arabidopsis*, including miR399 [60] and NPF7.3/NRT1.5 [49]. However, their exact roles in N-dependent PSR regulation remain to be explored.

Control of Nitrogen molecular responses by Phosphorus

The effect of P availability on N response has been almost exclusively studied from the aspect of P starvation. Typical symptoms of P starvation on N response is reduction of uptake, translocation, and assimilation [14–16]. Transcriptome analysis in *Arabidopsis* revealed that many PNR genes, including *NR1*, *NIR*, and CHL1/NRT1.1, are repressed as early as 24 h after the onset of P deprivation treatment [17,23], suggesting that PSR and PNR pathways are closely connected. Two N signaling factors have been identified to be regulated by PSR pathway. Firstly, AtCHL1/NRT1.1 is negatively regulated by P starvation in transcript accumulation and protein stability [48]. PHO2 and LEAF TIP NECROSIS 1 (a homologue of PHO2 in rice) are implicated as positive regulators of the transceptor expression in *Arabidopsis* and rice because the mutants of both species have reduced transceptor expression and are affected in PNR [52,61,62]. Secondly, NIGT1/HHOs are directly activated by PHR1 and PHL1 under P starvation [36,38]. Recently it was reported that OsNLP3 action is hindered by OsSPX4 in rice. OsSPX4 interacts with OsNLP3 and abrogates nitrate-induced cytoplasmic-nuclear shuttling of OsNLP3 [53]. The amount of OsSPX4 available for interaction is controlled by inositol phosphates-facilitated OsSPX4-OsPHR complex formation [53], and degradation via SPX4 DEGRADATION E3 LIGASEs (OsSDELs) under P starvation [63] and via OsNBIP1 in response to nitrate [53]. Hu et al. proposed that OsSPX4 action on OsNLP3 is intricately regulated at the protein level and interacting affinity to yield optimal N response under different N and P conditions. However, further experimental evidence would be required to validate this proposal and to know whether such regulation is universal in plants.

Since PSR is strongly attenuated under N starvation [52,53,55], it is reasonable to assume that P starvation has little effect on NSRHowever, there are a few studies against this assumption. For example, N starvation induced expression of *PtNRT1.1*

and *PtNRT2.1* was shown to be down-regulated by N and P co-starvation in poplar [64]. P starvation might negatively regulate NSR in certain phases of N starvation (e.g. early phase). Given that NIGT1/HHOs have roles in controlling genes for both NSR and PSR [36], it is tempting to speculate that NIGT1/HHOs are involved in the regulation.

Conclusions: Towards a more complex picture and perspective to unleash plant nutrition

We believe that the important control of P responses by N and *vice versa* are likely the tip of the iceberg. Indeed, it is now quite obvious that plants monitor signal combinations (nutritional or not) rather than any simple signals to adapt their physiological and molecular responses [47,65,66]. So we expect investigations to be extended to Light, C, N, P, K, S, Fe, ... in the near future.

For instance, light through the HY5 transcription factor action could be a relevant nexus since it represses PHR1 expression [67], while it activates *NRT2.1* by traveling from shoot to roots [68]. This shows that many more connections from other signaling pathways are forking on N and P signalings, and probably the other way around.

To conclude and open perspectives, we would like to emphasize two particular striking examples of signal interactions taking place at the level of single sensor proteins. The first one is going beyond simple nutritional control. Indeed the presence of $insP_6$ has been reported as a potential co-factor in the auxin sensing system made of TIR1 and IAA17 [69]. Even if the function of $insP_6$ in auxin sensing still needs to be fully investigated, this demonstrates that signals of different kinds can interact very closely and that nature have evolved proteins being able to intimately mediate crosstalk. The second very elegant example is the control of IRT1 (iron/metal transporter) by metals [70]. A cytoplasmic loop of IRT1 is able to bind metals (Mn2+, Zn2+) and recruit the sequential activation of CIPK23 and ubiquitination that remove the transporter from the membrane, preventing the plant from poisoning itself with heavy metals [70]. This again shows that nature evolved highly responsive sensing proteins having several tasks in the cell that necessitate a tight control of their function by a combination of signaling elements.

Finally, N and P interconnections are complex traits that plants evolved in natural environments and they may not be optimal in agricultural practices. For instance, we observe that plants seem to wait for N to trigger PSR [52]. Being able to uncouple N and P signal by *pho2* mutation [52] may provide an advantage to plants in conditions where P and N are lacking. This would allow plants to react to P starvation with or without N. In the same line, NIGTs/HHOs manage a P connection towards NSR (Figure 1). We observe that NIGTs/HHOs mutations derepress nitrate transport activity which can raise up to 2.5 fold increase of HATS [36,37]. This may be the results of uncoupling N and P signals. We thus think that a fundamental knowledge of signal interactions may be an

interesting path to enhance N and P Use Efficiency (NUE and PUE) by giving the plant a new degrees of freedom in reaction to the fluctuating environment.

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Conflict of interest statement
Nothing declared

Recommended reading
* Of special interest
** Of outstanding interest

**Kiba et al (2018)
The authors demonstrate cross talk between NSR and PSR, in which NIGT1/HHOs act as a hub in *Arabidopsis*. They show that NIGT1/HHOs directly repress the expression of *NSR* genes and negative regulators of PSR (SPXs and PHO2), resulting in suppression of NSR and activation PSR.

**Maeda et al (2018)
This paper shows that NIGT1/HHOs act as integrators of PNR and PSR signaling in *Arabidopsis*. The authors demonstrated that NIGT1/HHOs are directly activated by NLPs and PHR1 at the transcription level and act as negative regulators of *PNR* genes.

*Medici et al (2019)
This study proposes an interesting model describing convergent points of N signals into the PSR signaling pathway in *Arabidopsis*. Observations indicating that NPF6.3/NRT1.1, PHR1 and PHO2 are the molecular actors playing roles in the process are presented. Furthermore, authors provide evidence that control of PSR by N signals is a general mechanism across a wide range of plant species.

**Hu et al (2019)
The authors revealed a mechanism for coordinated utilization of N and P in rice. They identified OsSPX4 as a factor acting at the N-P interface. OsSPX4 is degraded by 26S proteasome pathway in a nitrate-stimulatory manner through the action of OsNRT1.1B and OsNBIP1 and inhibits the action of OsPHR2 and OsNLP3 through protein-protein interaction, thus coordinates PSR and PNR in response to nitrate.

*Ueda et al (2019)
In this work, authors provide evidence for the involvement of NIGT1-SPX-PHR1 module in nitrate-responsive regulation of PSR in *Arabidopsis*. They show that repression of *SPXs* by nitrate-inducible NIGT1/HHOs and subsequent modulation of PHR1 activity is the main pathway for the regulation.

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Figure legend

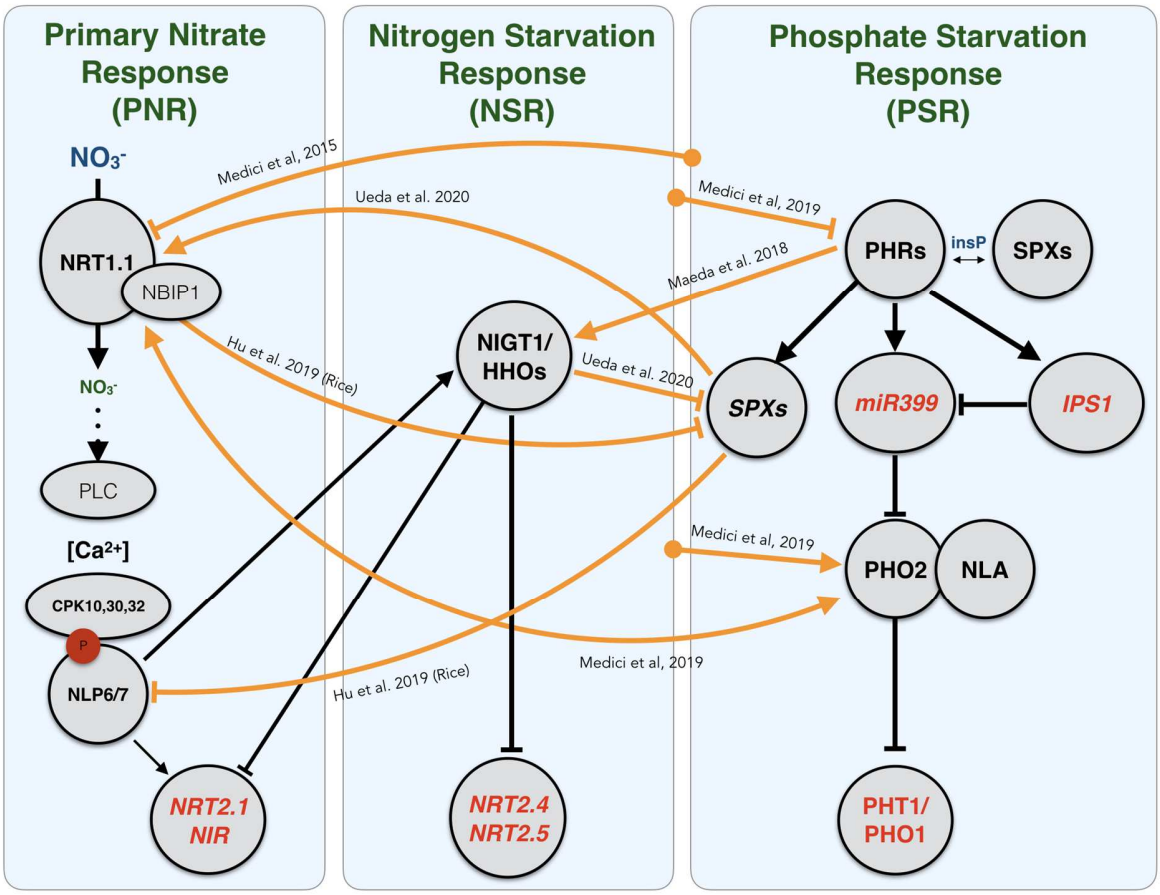
Figure 1. Inter-relationships between Primary Nitrate Response (PNR), Nitrogen Starvation Response (NSR) and Phosphate Starvation Response (PNR) explaining N/P interactions identified so far.

Molecular elements belonging to PNR, NSR and PSR (see text for their thorough definition) are organized vertically from sensors to marker genes (red font). Cross talks between pathways are represented using orange arrows. Some arrows originate from defined molecular actors, other arrow origins are undefined yet (orange ball). PHRs are the master transcriptional regulators of Phosphate Starvation Response that is

characterized by the activation of Phosphate Starvation Induced (PSI) genes and activation of phosphate uptake and translocation by phosphate transporters (PHT1s and PHO1). PHRs are negatively regulated by SPXs through inositol polyphosphate (insP)-triggered protein-protein interaction. Under P starvation, PHRs upregulate the expression of *PSI* genes, including miR399 and IPS1. miR399 represses PHO2, an E2 ubiquitin conjugase that acts in concert with an E3 ligase NLA to target phosphate transporters (PHT1s and PHO1) for degradation. OsSPX4 is degraded by the 26S proteasome pathway in response to N supplement (+N) through the action of OsNRT1.1B and an E3 ligase OsNBIP1 (OsNRT1.1-OsNBIP). The transcription of *SPXs* is directly repressed in response to +N by NIGT1/HHOs (NIGT1s). PHRs are transcriptionally activated in +N and post-translationally repressed by N-starvation (-N) through yet unidentified mechanisms. *PHO2* expression is down-regulated in response to +N by NIGT1/HHOs and CHL1/NRT1.1 (NRT1.1). Together, PSR is attenuated under N-starvation because the level of negative regulators, SPXs and PHO2, is increased, while that of the positive regulator PHRs is decreased.

NLPs are the master transcriptional regulators of PNR. Nitrate (NO_3^-) triggers calcium waves ($[Ca_2^+]$) through NRT1.1. Then the calcium signal is decoded by CPKs that phosphorylate NLPs. Upon phosphorylation, NLPs activate the expression of PNR-related genes (*PNR* genes), including the transcriptional repressor NIGT1s. NIGT1s directly repress NSR marker genes (*NRT2.4*, *NRT2.5*). NIGT1s also directly repress genes involved in Nitrogen Starvation Response (*NSR* genes). NRT1.1 is negatively regulated by P starvation in transcript accumulation and protein stability. PHO2 is implicated in NRT1.1 regulation. NIGT1s are directly activated by PHRs under P starvation.

Figure 1


Primary Nitrate Response (PNR)
Nitrogen Starvation Response (NSR)
Phosphate Starvation Response (PSR)
NO3-
NRT1.1
NBIP1
NO3-
PLC
[Ca2+]
CPK10,30,32
P
NLP6/7
NRT2.1
NIR
NIGT1/ HHOs
NRT2.4
NRT2.5
PHRs
insP
SPXs
SPXs
miR399
IPS1
PHO2
NLA
PHT1/ PHO1
Medici et al, 2015
Ueda et al. 2020
Medici et al, 2019
Maeda et al. 2018
Hu et al. 2019 (Rice)
Ueda et al. 2020
Medici et al, 2019
Hu et al. 2019 (Rice)
Medici et al, 2019