Nitrogen Actively Controls the Phosphate Starvation Response in Plants

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Short title: Nitrogen controls phosphate starvation

One-sentence summary: Identification of molecular integrators shows that nitrogen actively controls the phosphate starvation response in plants.

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Abstract

Nitrogen (N) and phosphorus (P) are key macronutrients sustaining plant growth and crop yield, and ensuring food security worldwide. Understanding how plants perceive and interpret the combinatorial nature of these signals thus has important agricultural implications within the context of: i) increased food demand, ii) limited P supply, and iii) environmental pollution due to N fertilizer usage. Here we report the discovery of an active control of P Starvation Responses (PSR) by a combination of local and long-distance N signaling pathways in plants. We show that, in Arabidopsis thaliana, the nitrate transceptor CHLORINA 1/ NITRATE TRANSPORTER 1.1 (CHL1/NRT1.1) is a component of this signaling crosstalk. We also demonstrate that this crosstalk is dependent on the control of the accumulation and turnover by N of the transcription factor PHOSPHATE STARVATION RESPONSE 1/PHR1, a master regulator of P sensing and signaling. We further show an important role of PHOSPHATE 2/PHO2 as an integrator of the N availability into the PSR since the effect of N on PSR is strongly affected in pho2 mutants. We finally show that PHO2 and NRT1.1 influence each other’s transcript levels. These observations are summarized in a model representing a framework with several entry points where N signal influence PSR. Finally, we demonstrate that this phenomenon is conserved in rice (Oryza sativa) and wheat (Triticum aestivum) opening biotechnological perspectives in crop plants.
**Introduction**

Nitrogen (N) and phosphorus (P) are key macronutrients affecting plant growth and development (Heuer et al., 2017). In natural and agricultural ecosystems, plants are faced with issues of P and N availability, which are linked to mineral mobility in soil. Nitrate (NO$_3^-$), the preferred N source of plants in aerobic soils, tends to leach from the soil, while inorganic phosphate (HPO$_4^{2-}$; Pi) is relatively immobile. Increasing the supply of N and P through fertilizer use helps meet the growing demand for food; however, this also leads to increased N and P leaching into the biosphere, leading to pollution that threatens ecosystems. The estimated cost of inefficient chemical fertilizer use is several billion euros a year to the European community and likely more worldwide (Sutton et al., 2011). Furthermore, while N availability is considered virtually infinite, thanks to the Haber-Bosch process, the global P reserves are increasingly becoming scarce, and consequently a potential P crisis looms over 21st century agriculture. Thus, understanding the plant response and adaptation to availability of these two key nutrients is crucial for future reduction in fertilizer use.

Research into mineral nutrition over the past 50 years has been focused mainly on investigations of plant responses to single mineral availability. This led to an in-depth understanding of how plants perceive and adapt to N or P fluctuations. More recent investigations have started to look at interactions between two or more nutrients (Kellermeier et al., 2014a; Pal et al., 2017; Kisko et al., 2018). Recently, "omics" approaches have highlighted the strong interconnection between nutrient metabolism and signaling pathways at different integrative levels (transcriptome, proteome, metabolome and ionome) (Kellermeier et al., 2014; Li and Lan, 2015; Medici et al., 2015; Ristova et al., 2016). It is now clear that understanding the effects of crosstalk between nutritional signals, rather than single nutrient effects, is key to understanding and engineering plant adaptive responses to fluctuating nutritional environment.

The Phosphate Starvation Response (PSR) has been largely studied in Arabidopsis and rice, where several components of the signaling pathway have been identified (Puga et al., 2017). The molecular backbone of this pathway in Arabidopsis is made of SPX DOMAIN-CONTAINING PROTEIN 1/SPX1, PHOSPHATE STARVATION RESPONSE 1/PHR1 (and its homologous gene PHL1), miR399, INDUCED BY PHOSPHATE STARVATION 1/IPS1, and PHOSPHATE 2/PHO2 (Bari et al., 2006; Ham et al., 2018). In this pathway, P-dependent interaction between PHR1 and SPX1 likely perceives the levels of available Pi in cells (Puga et al., 2014; Wang et al., 2014; Qi et al., 2017; Jung et al., 2018). Upon Pi limitation, PHR1 activates the majority of Phosphate Starvation Induced (PSI) genes (i.e. miR399, IPS1, SPX1) via binding to the P1BS cis-regulatory element (Bustos et al., 2010; Puga et al., 2014). PHR1 induces miR399, which represses PHO2, an E2 ubiquitin conjugase, which triggers degradation of several phosphate transporters belonging to the PHT1 family and PHO1 (Bari et al., 2006; Liu et al., 2012; Park et al., 2014). This allows plants to increase their capacity for Pi uptake and transfer from roots to shoots.

Like phosphate, NO$_3^-$ acts as both a nutrient and a signaling molecule (Crawford and Glass, 1998; Wang et al., 2004). Interestingly, NO$_3^-$ signaling is branched, as it occurs through several interconnected signaling pathways (O’Brien et al., 2016). The first pathway is named Primary Nitrate Response (PNR) (Medici and Krouk, 2014). It corresponds to the rapid (within minutes) and NO$_3^-$-specific activation of...
sentinel genes (NITRATE REDUCTASE 1/NIA1, NITRITE REDUCTASE 1/NIR1, GLUCOSE-6-PHOSPHATE DEHYDROGENASE/G6PDH, HYPERSENSITIVITY TO LOW PI-ELICITED PRIMARY ROOT SHORTENING 1/HRS1). This pathway is the best-documented and it includes regulators such as the nitrate sensor CHL1/NITRATE TRANSPORTER 1.1/NRT1.1/NPF6.3 (Ho et al., 2009), several kinases and phosphatases (CBL-interacting protein kinase 8/CIPK8, CIPK23, calcium-dependent protein kinase/CPK10,30,32, ABA INSENSITIVE 2/ABI2) (Ho et al., 2009; Hu et al., 2009; Leran et al., 2015; Liu et al., 2017), a calcium relay (Riveras et al., 2015) and several nuclear factors (NIN-like protein/NLP6, NLP7, NITRATE REGULATORY GENE2/NRG2, squamosa promoter binding protein-like 9/SPL9) (Castaings et al., 2009; Wang et al., 2009; Krouk et al., 2010a; Marchive et al., 2013; Xu et al., 2016; Guan et al., 2017).

The second pathway can be qualified of Nitrogen Starvation Response (NSR). It is characterized by a relatively slow activation of very high affinity transporters (nitrate transporter 2.4/NRT2.4, NRT2.5) (within 24 to 48 hours) following N removal from the growth medium. Several genes have been found or have been hypothesized to be involved in plant response to NSR. These include LATERAL ORGAN BOUNDARIES DOMAIN GENE 36/LBD36,37,38 (Rubin et al., 2009) calcineurin B-like protein 7/CBL7 (Ma et al., 2015), miR169 and NFYA (Zhao et al., 2011) and HRS1 and HRS1 Homolog/HHOs (Kiba et al., 2018; Maeda et al., 2018; Safi et al., 2018).

Finally, N-related signaling pathways include long-distance signals named N-demand and N-supply (Ruffel et al., 2011; Li et al., 2014; Ruffel et al., 2015; Poitout et al., 2018). Cytokinin biosynthesis, CEP peptides and glutaredoxins have all been shown to be important in these long-distance signals (Tabata et al., 2014; Ohkubo et al., 2017). It is noteworthy that these signaling pathways are not independent from one another and their inter-connectivity is still rather elusive.

The interaction between N and P signaling has already been documented in several instances. The first hints were provided by the NITROGEN LIMITATION ADAPTION (NLA) and PHOSPHATE2 (PHO2) genes (encoding two ubiquitin conjugases) that control phosphate transporter trafficking, resulting in N-dependent Pi accumulation in shoots (Peng et al., 2007; Kant et al., 2011; Lin et al., 2013). A GARP transcription factor AtNIGT1/HRS1 was also shown to affect primary root growth according to NO$_3^-$ and PO$_4^{3-}$ signals via dual control of transcription and protein accumulation respectively (Medici et al., 2015). Recently, PHR1 was found to control AtNIGT1/HRS1 as a central regulator of the High Affinity Transport System of NO$_3^-$ (Kiba et al., 2018; Safi et al., 2018). This control is responsible for the P regulation of the NO$_3^-$ transport via NRT2.1 in particular (Maeda et al., 2018). These results represent the first mechanistic insights into the potential molecular mechanisms by which N and P signaling pathways interact.

However, the actual gene regulation following N and P combinatorial deprivation has not been reported. Therefore it is still unclear if PSR, PNR and NSR interact in a reciprocal manner, as well as what the potential molecular hubs of these interactions may be. Here we show that PSR is strongly and actively controlled by N provision. We report several observations leading to a working model describing convergent points of N signals into the PSR signaling pathway.
Results

The Phosphorus Starvation Response (PSR) strongly depends on N provision

In the course of our investigations into the N and P cross-talk mediated by HRS1, we treated Arabidopsis plants with an array of NO₃⁻ and Pi of various conditions to observe any changes in root development and gene expression (Medici et al., 2015). During these investigations, we noticed in wild type (WT) plants that PSR marker gene (IPS1, SPX1, mIR399D, PHT1-1) responses were dependent on N provision (Figure 1). Indeed, transcripts of PSI genes accumulated in P-depleted conditions only in the presence of at least 0.05 mM NO₃⁻, and these transcripts displayed an extremely low abundance at 0 mM nitrate (Figure 1).

We performed a transcriptomic analysis to evaluate the genomic impact of such cross-talk. Three independent experiments were analyzed on Affymetrix whole genome arrays (Supplemental Data 1). Data were modeled using ANOVA as previously (see Methods for details, Supplemental Data 2). We retrieved 125 non-ambiguous P-regulated genes (Figure 2A, gene lists are provided Supplemental Data 3) and 350 non-ambiguous N-regulated genes (Figure 2B, Supplemental Data 1, Supplemental Data 4) using a very stringent pvalue cutoff (<0.001, FDR < 5%). Interestingly, we observed that the vast majority (~85%) of the P-regulated genes are also significantly influenced by N (Figure 2A). The reciprocal (P effect of N response) is also true, but less dramatic (~45%, Figure 2B). This effect is statistically observable when p-values for each factor (N, P, NxP, Supplemental Data 2-4) effect are plotted against each other (Figure 2C). Thus, the cross-talk observed on maker genes (Figure 1) is a general genome-wide phenomenon (Figure 2). We then further investigated the molecular mechanisms that may be at the core of this significant cross talk.

To further investigate N/P signaling crosstalk, we first wanted to ensure that the lack of PSR under N depletion conditions was not simply due to the harsh plant growth conditions (plants were grown for 14 days on N and P varying media; under the -N/-P conditions plants were stunted (Supplemental Figure 1)), but rather due to an active loss of PSR under -N conditions. We, therefore, decided to conduct transfer experiments (Figure 3A and 3B): we grew plants in -N/-P conditions (Figure 3A) for 11 days and then transferred them for 3 days to replenishing media combining N and P provisions (Figure 3B see WT bars). In agreement with a previous observation, we noted that N provision to plants starved for P was able to reactivate the PSR, as reported by an increase in steady-state transcript levels of PSI genes (Figure 3B). However, when this experiment was performed in N and P signaling mutants for NRT1.1 (chl1-5), and PHR1/PHL1 (phr1 phl1), respectively (Ho et al., 2009; Puga et al., 2014), we noticed that the phr1 single and phr1 phl1 double mutant have a strong effect on PSR activation (Supplemental Figure 2A and Figure 3A, respectively) and upon N provision (Figure 3B). We also observed a moderate effect of chl1-5 mutation on the regulation of PSR under constant nutrient conditions experiments (Figure 3A), and only a limited effect upon plant transfer to N containing media (Figure 3B). This moderate effect of chl1 mutation can easily be explained as it has been previously shown that the effect of the chl1 mutation can be bypassed if
N starvation precedes N provision (Wang et al., 2009). As the plants have been N-starved for 11 days, it is likely that most NO$_3^-$ sensing mechanisms on N transfer conditions still rely on an unidentified sensing protein that bypasses NRT1.1 activity (fully discussed in (Wang et al., 2009; Medici and Krouk, 2014)).

Taken together, these results strongly support the idea that phosphate and nitrate signaling pathways are tightly linked and that the P-starvation signaling pathway downstream of PHR1/PHL1 activity is partly dependent on nitrate uptake and/or signaling mediated by the Nitrate sensor NRT1.1.

**phr1 phl1 double mutant still perceives NO$_3^-$**

Because the *phr1 phl1* double mutant is insensitive to N-controlled reactivation of PSR (Figure 3), we wanted to rule out the possibility that the missing activation was due to a lack of NO$_3^-$ sensing in the double *phr1 phl1* mutant. To do so, we examined whether the *phr1 phl1* double mutant was able to perceive NO$_3^-$ as the PNR is conserved in this genetic background (Supplemental Figure 2B). We confirmed that NO$_3^-$ provision was able to induce *NIR* and *NRT1.1*, and we therefore conclude that it is not possible to explain the default of PSR activation by default NO$_3^-$ sensing in the *phr1 phl1* background. This prompted us to propose that the effect of N on the PSR is likely upstream of the PHR1 sensing activity.

**Nitrogen controls PSR via local and systemic signals**

In plants, the responses to nitrate provision depend on local but also on long-distance signals, which allow their adaptation to non-homogeneous soils (Gansel et al., 2001; Ruffel et al., 2011; Li et al., 2014; Ruffel et al., 2015; Poitout et al., 2018). Two kinds of long-distance signals can be distinguished. The *N-demand signal* informs roots replete with N to compensate for roots growing in NO$_3^-$-deprived regions of the soil. The *N-supply signal* informs the nitrogen-deprived roots to adapt their physiology according to the fact that other roots are foraging for N. To determine if PSR depends on these N-related long-distance signals, we set up a split-root experiment in which we tested the response to phosphate deficiency in heterogeneous N conditions (Figure 4A). The plants were cultivated for 11 days on N and P containing media, and then subjected to double deficiency (-N and -P) for 3 days. Finally, a first batch of plants was transferred for 3 more days to media without phosphate and + or - N in homogenous (Control, C) or heterogeneous (Split, Sp) conditions. Another batch of plants was transferred to the same media plates but containing 1 mM Pi. The expression of PSI genes (Figure 4B) in the roots grown in the absence of phosphate confirmed that in homogeneous conditions the presence of nitrogen switches the PSR on, and the prolonged nitrogen starvation switches the PSR off (marked by PSI genes high expression in -P CKNO$_3$; and low expression in -P CKCl, Figure 4B). In heterogeneous conditions, the *N-supply* and *N-demand* long-distance signals (Ruffel et al., 2011) also influence PSR. Indeed, *IPS1* and *SPX1* were inhibited by the N-demand signal coming from the compartment without N (compare CNO3 and SpNO3 in Figure 4B). *N-supply* control PSR genes in different ways: for *IPS1*, the *N-supply* signal acts as an IPS1 repressor, since RNA accumulation is stronger in CKCl than in SpKCl. On the other hand, *SPX1* and *PHT1-1* show induction in roots grown under SpKCl as compared to the CKCl (Figure 4B). This demonstrates that
the *N-supply* long-distance signal indeed controls the PSR genes but that this regulation is dependent on the gene identity.

Taken together, these results strongly suggest that the PSR is under the control of N-related long-distance systemic signals (Ruffel et al., 2011). This constitutes an independent demonstration that PSR is actively shut down when N is lacking. This active repression of PSR relies at least partly on already defined local (Figures 1; 2 and 3) and long-distance (Figure 4) signaling pathways. This opens perspectives for further studies on the molecular actors involved, since several genes have already been identified in these N-related signaling pathways (Ruffel et al., 2011; Li et al., 2014; Tabata et al., 2014; Ruffel et al., 2015; Ohkubo et al., 2017; Poitout et al., 2018).

**PHO2 is transcriptionally controlled by N starvation**

PHO2 is an E2 conjugase involved in the phosphate response as it is targeted by *miR399* under P limiting conditions (Bari et al., 2006). PHO2 triggers the degradation of PHT1 transporter family members and PHO1 (Liu et al., 2012; Huang et al., 2013) under +P conditions. To investigate the existence of a link between PHO2 and N-related signals, we studied the steady state expression of *PHO2* in roots of plants grown in the presence of different NO$_3^-$ concentrations. P-provision does not strongly impact *PHO2* expression level, as previously described (Bari et al., 2006). Only a small but significant decrease in *PHO2* transcripts was recorded in -P at 0.05 mM and 0.5 mM KNO$_3$ (Figure 5A). Conversely, and more importantly, *PHO2* transcript accumulation is regulated by N provision. Indeed, *PHO2* shows mRNA accumulation in N-depleted conditions and, conversely, strong mRNA depletion by increasing concentration of NO$_3^-$ in the media (Figure 5A). This N-regulation is dependent on NRT1.1 activity, since it is partly lost in the *chl1-5* mutant (Supplemental Figure 3). Thus, PHO2 is i) repressed post transcriptionally by P starvation (as previously shown (Bari et al., 2006)) and ii) down-regulated by NO$_3^-$ provision.

**PHO2 is responsible for the N-dependent control of PSR**

Given the strong N-dependent transcriptional regulation of *PHO2* shown above (Figure 5A), we tested the hypothesis that PHO2 could integrate the N signal during the P-starvation plant response. To this end, we studied the N control of PSR in *pho2* mutants. We performed the same steady state and transfer experiments on WT and *pho2* genotypes. We observed that, indeed, in *pho2* mutants the PSR of the vast majority of PSI genes is no longer N-controlled (Figure 5B). In other words, the repressive effect of N depletion on the PSR response is strongly affected by *pho2* mutation (Figure 5B). These results further demonstrate that PHO2 functions as an integrator of the N signal into the PSR. This observation was valid for *IPS1*, *PHT1-1* and *SPX1*, as these genes appeared to be strongly de-repressed and no longer N-regulated in the *pho2* mutant (Figure 5B). Interestingly, miR399D remained under N control in the *pho2* background, revealing that the N effect on PSR contains PHO2-dependent and -independent branches (Figure 5B).
N controls PHR1 protein accumulation and half-life

It is widely accepted that PSR is controlled by the PHR1 transcription factor (Bari et al., 2006; Franco-Zorrilla et al., 2007; Liu et al., 2012; Puga et al., 2014) (Figure 2). We also hypothesized above that the N/P crosstalk likely takes place upstream of the P signaling pathway since the phr1 phl1 double mutant retains NO3− sensing capabilities (Supplemental Figure 2B). We therefore tested if PSR could be repressed in -N conditions through the regulation of PHR1 (being central to PSR (Puga et al., 2014)). First, we studied the transcriptional regulation of PHR1 under varying N and P conditions. The results of this experiment did not yield a clear conclusion concerning the transcriptional regulation of this gene either by P or by N signals (Figure 6A) as previously reported by others (Rubio et al., 2001). We, therefore, investigated if P/N signals affect PHR1 accumulation. To this end, a functional pPHR1:PHR1:GFP transgenic line was studied using antibodies directed against the GFP tag (Supplemental Figure 4). We detected a clear and reproducible decrease in PHR1 accumulation in -N deprived conditions (Figure 6B). Furthermore, we evaluated PHR1 turnover in response to N starvation (Figure 6C). We observed that the protein half-life (determined using CHX treatments) is decreased under -N conditions, explaining the potential protein accumulation observed in Figure 6B.

These results demonstrate that N deprivation modifies both PHR1 half-life and accumulation, which likely explains the repression of PSR activity by the N depletion signal (Figure 1).

PHR1 accumulation in response to N is independent of PHO2

Having shown that i) PHR1 is destabilized in response to -N and that ii) PHO2 (a ubiquitin-conjugase) is key for the downregulation of PSR by -N signal, we hypothesized that PHO2 might be responsible for the stability of PHR1 in response to -N. To this end, we also monitored the PHR1 GFP tagged protein in the pho2 background. Interestingly, we did not observe a clear and reproducible miss-regulation of the protein accumulation or stability in the pho2 background as compared to the WT (Figure 6C). This observation led us to conclude that PHR1 stability is indeed controlled by –N (Figure 6), and PHO2 is a central point of cross-talk between the N response and the PSR (Figure 5); however, this does not occur through any direct regulation of PHR1 by PHO2.

PHO2 controls the nitrate transceptor NRT1.1 and vice versa

Since PHO2 showed no influence on PHR1 accumulation, we tested the hypothesis that PHO2 could interfere with the nitrate signaling pathway instead (and affect PSR that way). To do so, we studied NRT1.1 accumulation (using specific antibodies (Medici et al., 2015)) in the WT and pho2 mutant grown on +P/+N media and then transferred (for 3 days) on +P or -P and +N media (Figure 6D). We confirmed that NRT1.1 protein accumulation is strongly repressed by P starvation conditions (as previously reported (Medici et al., 2015)). Furthermore, we demonstrated that PHO2 acts a positive regulator of NRT1.1 since in the pho2 mutant, NRT1.1 levels are lower than in the WT. This observation is consistent with previous proteomic analysis that determined that NRT1.1 is indeed strongly downregulated in the pho2 background.
This finding demonstrates once more the complexity and connectivity between P and N signals. As NRT1.1 is at the forefront of the \( \text{NO}_3^- \) sensing controlling PNR in plants, we tested the \textit{pho2} mutant response to PNR and NSR (as a control, since NRT1.1 is not known to be involved in NSR). We found that \textit{pho2} mutants are affected in the late phase of PNR (Supplemental Figure 5A) and maintain a normal NSR response (Supplemental Figure 5B). This validates that i) \textit{pho2} influences the \( \text{NO}_3^- \) response and ii) \textit{pho2} might affect the PSR partly through its control of NRT1.1.

**The dependency of the PSR on nitrogen is conserved in crop species**

Given the strong similarity between the PSR pathways in Arabidopsis and rice, we examined if the dependence of PSR on N was conserved in monocots like rice (\textit{Oryza sativa}) and wheat (\textit{Triticum aestivum}). First, we grew rice plantlets in media composed of four combinations of N and P and we tested the response of several PSI genes (Figure 7A). As shown for Arabidopsis, rice PSI genes (\textit{OsIPS1}, \textit{OsSPX1}, \textit{OsPT1}) were upregulated by P starvation only in the presence of N. Thus, in rice, N deprivation also prevents activation of PSR (Figure 7A). Interestingly, during these investigations we noted that N deprivation strongly affected shoot growth in rice. However, the effect of N deprivation seems less important for plants deprived of P (compare -N/+P vs. -N/-P conditions Figure 7B). This highlights another important interaction between N and P at the level of rice shoot growth, which will be the topic of future investigations.

We also evaluated N control of PSR genes in wheat. To do so, we grew wheat plants (cv. Chinese Spring) in -P/-N media for 11 days and then studied the PSR in roots of plants transferred for 24 hours onto media containing different combinations of P and \( \text{NO}_3^- \). The response of \textit{IPS1} and \textit{SPX1} wheat homologues (\textit{TaeIPS1}, \textit{TaeSPX1} \textit{7A}) confirmed that the PSR is repressed in -P/-N (Figure 7C). However, \textit{TaePHT1.1} does not display the prototypical N/P cross-talk response since it seems to be controlled solely by N provision.

Taken together, these investigations demonstrate that the control of PSR by N is a general mechanism that is likely conserved across a wide range of plant species. The potential conservation of the molecular mechanisms that we uncovered in Arabidopsis is discussed below.
Discussion

In the present work, we report a discovery of a conserved mechanism of PSR control by N availability in plants. Previous studies have reported such evidence for N and P interaction in the control of Acid Phosphatase 5/AtACP5, which encodes an acid phosphatase involved in P recycling (Cerutti and Delatorre, 2013); however, an advanced mechanistic explanation for these observations was lacking. Using Arabidopsis, we provide insights into the molecular actors involved in N and P signaling cross talk, leading to a model presented in Figure 7D. Briefly, N control of PSR is dependent on the NO$\text{}_3^-$ and Pi sensing related proteins, NRT1.1 and PHR1/PHL1 respectively. This demonstrates that this crosstalk is rooted in the key components of both signaling pathways, the nutrient sensors for N and a P-related sensing protein. Moreover, we show that the PHO2 gene is an essential element of the N signal transduction into the PSR as, i) the PHO2 gene is transcriptionally activated upon N deprivation and ii) pho2 mutant displays an important de-repression of the PSI genes which includes a desensitization of the PSI genes to N (Figure 5). In other words, N effect on PSR is strongly affected in the pho2 genetic background, consistent with the proposed role for PHO2 as the integrator of the two signals (Figure 5).

We also demonstrate that N affects PHR1 protein accumulation and half-life, which likely explains the effect of N on PSR (Figure 6). Interestingly, we did not found a robust and reproducible effect of PHO2 on the PHR1 protein turnover and accumulation. This led us to hypothesize that the control of the PSR by N is likely multi-pronged (Figure 7D), with an important branch of the pathway consisting of control of both PHO2 and NRT1.1 via feedback control of each other (Figure 6D, 7D, Supplemental Figure 3).

From a PSR-only perspective, our results also indirectly imply that PHO2, being downstream of the PSR ((Briat et al., 2015), Figure 7D) is also likely upstream of it. Indeed, in the pho2 mutant, we not only recorded a loss of N effect, but also a very strong up-regulation of the PSI genes (Figure 5). Thus, in the light of these results, the apparent linearity of the PSR (Briat et al., 2015; Puga et al., 2017) should be revised. A similar feedback mechanism proposed by Liu et al (2010) in rice, where OsPHO2 negatively control OsSPX1 and OsIPS1, supports our model. We believe that PHO2 can be at the same time downstream and upstream of the PSR defining a potential circular signaling pathway. We also propose that PHO2 is likely and integrator of the N signal into this pathway (as discussed above).

A suggested priority of N signal on P.

Our work provides evidence for an apparent prioritization of the plant for nitrogen. Indeed, in our experiments we demonstrated a very strong influence of N starvation on PSR, when the reciprocal (P effect of NSR) is of more modest effect in Arabidopsis (Figure 2B). Thus plants seem to bypass PSR in order to “wait” for more N favorable conditions. Indeed, in the same set of experiments (Figure 2) we observe that the core N starvation is still active regardless of P provision (Figure 2B). Explanations for such an apparent priority may reside in the fact that P foraging and retrieval is likely very dependent on plant capacity to grow, since P is very immobile in the soil. Thus, plant may invest in growth and P retrieval only if N is available potentially provided by rain, leaching and bacterial burst activity in the soil. Since P and N
responses are linked to hormonal signals on many aspects it will be interesting to investigate if this N/P cross-talk is dependent on hormones such as auxin (Krouk et al., 2010; Krouk, 2016; Ristova et al., 2016), cytokinins (Franco-Zorrilla et al., 2002; Franco-Zorrilla et al., 2005; Ruffel et al., 2011; Poitout et al., 2018; Ruffel, 2018), or Strigoloactones (Mayzlish-Gati et al., 2012) for instance.

From a molecular systems perspective, the phenomenon that we describe here resembles an electronic logic gate. Indeed, PSR genes get activated only when N AND NOT_P signals are combined. This echoes recent studies (Kellermeier et al., 2014; Ristova et al., 2016) that revealed that plant indeed perceive external and internal signals in combinations and that it is likely to be a rule rather that an exception.

In conclusion, we believe that the identification of the molecular actors (PHR1, PHO2, NRT1.1) that integrate N and P signals brings us closer to the understanding of an evolved logic gate in plant cells. This may have also important consequences in agricultural practices, or biotechnology, by opening research avenues towards the uncoupling of these signaling pathways to adapt genotypes to particular agricultural conditions.
Methods

Plant material

*Arabidopsis thaliana* (Col-0 background) mutants were previously described: *chl1-5* (Tsay et al., 1993), *phr1-3 phl1-2* (Bournier et al., 2013), *pho2-1* (Bari et al., 2006). The pPHR1:PHR1-GFP construct was prepared by PCR-amplification from wild-type plants of a genomic fragment comprising the PHR1 gene promoter region (up to 2063 bp upstream of the start site), and all exons and introns, using as primers: pPHR1HindIII.BF 5´-GGGGACAAAGTTTGTACAAAAAAGCAGGCTATAAGCTTTCAAGCAACAGAGGAAGAGGTG-3´ and pPHR1HindIII.BR 5´-GGGGACCACTTTGTACAAAAAAGCAGGCTTTGTTGGTCTGCAAGAG-3´. The PCR product was cloned using Gateway technology (Invitrogen) into the pDONR207 (Invitrogen) plasmid by the BP reaction and subsequently transferred by the LR reaction to the pGWB4 plasmid, for fusion to GFP. The resulting construct was used to transform Arabidopsis *phr1* mutant plants by the floral dip method. After T1 selection based on hygromycin resistance, the T2 segregating progenies were selected based on the complementation of *phr1* mutant phenotypes to isolate homozygous plants for the construct. pPHR1:PHR1:GFP x *pho2-1* plants were obtained by crossing the two homozygous parental lines. The generated F2 plants were verified for the presence of the GFP and the homozygous mutation of the *pho2-1* locus by sequencing the specific region (using primers listed in Supplemental Table 2). Expression of PSI genes in rice and wheat was measured respectively in *O. sativa* (cv Nipponbare) plants and on *Triticum aestivum* (cv. Chinese Spring).

Each experiment was performed at least twice (most of them 3 times). Representative results are reported in figures. Figure 1, 2, 5, and 6C represent a compilation of 3 independent experiments with several hundreds of plants pooled per sample.

Growth conditions and treatments

For Arabidopsis root transcript accumulation analysis, plants were grown on vertical plates. WT and mutant seeds were sterilized and sown on the surface of solid media consisting of nitrogen- and phosphorus-free MS basal salt medium supplemented with KNO₃ at different concentrations (0.05, 0.5, 1 and 2.5 mM), 3 mM sucrose, MES (0.5 g/l), and 0.8% (w/v) agarose. For the P-sufficient condition, 0.5 mM KH₂PO₄ was added. Different volumes of 1 M KCl solution were added to the media to keep the K⁺ concentration constant across the different conditions. Plants were grown vertically for 9 days in day/night cycles (16/8 h; 90 μmol photons m⁻² s⁻¹) at 22 °C. Light is provided by a mix of sodium-vapor and metal halide 400-W lamps (in growth chambers used for hydroponic culture) and Osram 18-W 840 Lumilux neon tubes (for in vitro plant growth).
For the transfer experiment, 9-day-old seedlings were transferred to new plates and grown for 3 more days under the same conditions. The conditions for the P or N starvation experiments in sterile hydroponic system were the same as described in (Medici et al., 2015a), with 2.5 mM KNO3. P starvation treatment was applied for 72 h. Every sample contains at least 30 plant roots from different plants. Experiments were repeated at least twice.

For rice experiments, WT Oryza sativa (cv Nipponbare) plants were grown in a hydroponic non-sterile system. After overnight soaking in deionized water in darkness, the seeds were transferred to ¼ full-strength Yoshida media for 10 days (Yoshida 1976). For treatment conditions, plants were transferred to a Yoshida modified nutrient solution (1.43 mM NH4NO3; 1.64 mM MgSO4; 0.75 mM CaCl2; 0.51 mM K2SO4; 0.33 mM NaH2PO4; 0.02 mM H2BO3; 0.01 mM MnCl2; 0.04 mM Fe-NaEDTA; 2.5 µM ZnSO4; 0.16 µM CuSO4; 0.08 µM (NH4)6Mo7O24; 2.5 µM MES buffer, pH 5.5), with P or N modified contents (see legend of the figures), that was renewed every 5 days. Plants were cultivated for 10 days in these N/P varying media in light/dark cycle (14/10 h), at 28/25°C and 80% RH. Light is provided by a mix of sodium-vapor and metal halide 400-W lamps.

For wheat experiments, seeds of Triticum aestivum (Chinese spring variety) were surface-sterilised using sodium hypochlorite (4% w/v), pre-imbibed for 3 days and transferred to a hydroponic setup where the seeds were germinated and grown for 11 days in deionised water in growth conditions of 24 °C and 16 h (light)/8h (dark). The seedlings were then transferred to four treatment levels: +P+N, -P+N, +P-N and -P-N. Modified Hoagland solution (2 mM Ca(NO3)2; 0.75 mM MgSO4; 0.7 mM K2SO4; 0.5 mM KH2PO4; 0.1 mM KCl; 100 µM Fe-EDTA; 5 µM ZnSO4; 1 µM MnSO4, 1 µM H2BO3; 0.2 µM CuSO4; 0.01 µM (NH4)6Mo7O24; pH 5.8) was used for +P+N condition. For -N treatment, Ca(NO3)2 was replaced with CaSO4 at 0.5 mM; for -P treatment, KH2PO4 was replaced with KCl at 0.5 mM; and for -P-N treatment, Ca(NO3)2 and KH2PO4 were replaced with 0.5 mM CaSO4 and 0.5 mM KCl, respectively. The plants were allowed to grow in the four treatment levels for 24 h. Then total roots were harvested, immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction. For each treatment level, three biological replicates were performed, with each sample containing root samples from four to six plants. Reverse transcription quantitative PCR (RT-qPCR) was carried out on cDNA samples using BioRad CFX 384 Real-Time System (C1000Touch) machine and whenever possible homeologue-specific primers were used. EF1a was used as the housekeeping gene.

Split root growth conditions

The split root experiment was performed in square Petri dishes (12x12 cm) based on a previously published protocol (Ruffel et al., 2011). Arabidopsis Col-0 seeds were surface sterilized and sown on a basal salt medium, with 3 mM sucrose, MES (0.5 g/l), 1% (w/v) agarose, 1 mM KH2PO4, 0.5 mM NH4-succinate, and 0.1 mM KNO3. After 12 days, the primary root was cut below the second emerging lateral root. Three days later, the two newly formed secondary roots were separated on the same media to allow proper development. Plants were the transferred on 4 different combinations of N and P. The media
respectively contained: -P/-N (KCl 3mM), +P/+N (KNO₃ 2.5mM, KH₂PO₄ 0.5mM), -P/+N (KCl 0.5mM, KNO₃ 2.5mM), +P/-N (KH₂PO₄ 0.5mM, KCl 2.5mM). KCl, KNO₃, and KH₂PO₄ solutions were spread independently on each side of the solidified media. All the root samples were collected after 3 days.

**Real Time qPCR analysis.**

Total RNA was isolated from Arabidopsis, rice and wheat roots tissues, previously harvested and stored in liquid nitrogen. DNase treatment, reverse transcription and qPCR conditions were the same as described in Medici et al, (Medici et al., 2015b). Specific primer pairs used for RT-qPCR analysis in Arabidopsis and Rice are listed in Supplemental Table 1.

**Soluble protein isolation and immunoblot analysis**

Protein isolation and immunoblot with GFP antibody were performed and quantified as described (Medici et al., 2015b). For protein loading normalization, anti–ACT antibody 1:5,000 (Agrisera, AS132640) and Goat anti-Rabbit IgG 1: 10,000 (Agrisera, AS09602) were used. CHX was applied at 100 µM in DMSO solution (used as mock control).

**Transcriptomic analyses**

Total RNA was extracted from frozen and ground root tissues using TRlzo™ reagent (ThermoFisher Scientific). RNA integrity and concentration were determined using a 2100 Bioanalyzer Instrument (Agilent) and Agilent RNA 6000 Nano kit (5067-1511, Agilent). DNA was removed by digestion with DNase I (AMPD1, SIGMA). Gene expression measurements were performed using Arabidopsis Affymetrix® Gene1.1 ST array strips. Biotin-labeled and fragmented cRNAs were obtained using GeneChip® WT PLUS Reagent kit (902280, ThermoFisher Scientific) following manufacturer's instructions. Hybridization on array strips was performed for 16 hours at 48°C. Arrays are washed, stained and scanned using a GeneAtlas HWS Kit (901667, ThermoFisher Scientific) on the GeneAtlas® Fluidics and Imaging Station.

All data manipulations were performed on R ([http://www.r-project.org/](http://www.r-project.org/)). The ANOVA was carried out using the R aov() function on GCRMA data (logged data). A probe signal has been modeled as follows: Yi = α₁.N + α₂.P + α₃.N*P + ε; where α₁ to α₃ represent the coefficient quantifying the effect of each of the factors (N, P) and their interactions (N*P), and ε represents the non-explained variance. The false discovery rate (FDR) was determined to be <5%. We determined a gene as regulated by N (Figure 2B) if the p-value associated with α₁ was <0.001. We determined a gene as regulated by P (Figure 2A) if the p-value associated with α₂ was <0.001. Raw data and whole genome ANOVA results are provided in Supplemental Dataset 1.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers CHL1/NRT1.1/NPF6.3 (At1g12110); PHO2 (At2g33770), PHR1 (At4g28610), PHL1 (AT5G29000).
Supplemental Data

**Supplemental Figure 1.** Plant phenotypes following 11 days of growth on P/N varying conditions.

**Supplemental Figure 2.** *phr1* and *phr1,phl1* mutations affect N/P cross-talk but Primary Nitrate Response (PNR) is still active in the *phr1 phl1* double mutant.

**Supplemental Figure 3.** *PHO2* mRNA level response to N is affected by the chl1-5 mutation.

**Supplemental Figure 4.** Characterisation of pPHR1:PHR1:GFP transgenic plants.

**Supplemental Figure 5.** *pho2* mutation affects PNR and not NSR consistently with its effect of NRT1.1 protein level.

**Supplemental Figure 6.** Un-cropped version of the immunoblot presented in Figure 6D.

**Supplemental Table 1.** RT-PCR primer pairs for *A. thaliana* and *O. sativa*.

**Supplemental Table 2.** primer pair for genotyping pPHR1:PHR1:GFP (*pho2-1*) plants.

**Supplemental Dataset 1.** Transcriptomic data.

**Supplemental Dataset 2.** ANOVA Tukey test results.

**Supplemental Dataset 3.** List of P regulated genes.

**Supplemental Dataset 4.** List of N regulated genes.

**Supplemental Dataset 5.** List of NxP regulated genes.

**Declaration of interests**

The authors declare no competing interests.

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Author contributions

GK, AM, WS, designed the project. AM, WS, SR, PD, AS, IMD, CS, AE, VR, HR, and GK, performed the experiments and analyzed the data. AM, WS, BL, SR, MT, HR, and GK contributed to the study design during the project course. GK, AM, MT, and HR wrote the paper.
References


Figure 1. Phosphate Starvation Response is repressed by a lack of Nitrogen.
Plants were grown on combinations of P (0 or 0.5 mM, KH₂PO₄) and N (0, 0.05, 0.5, 1, 2.5 mM KNO₃) for 14 days. Roots were harvested for measurements of PSI genes by RT-qPCR. Values are mean ± SEM (n=3). Results are from 3 independent experiments. Different letters indicate significant differences as determined by ANOVA followed by Tukey test (P<0.05). Plant phenotypes are reported in Figure S1.
Figure 2. The Phosphate Starvation Response throughout the genome is controlled by N. Plants were grown on combinations of P and N for 14 days. Roots were harvested for transcriptomic analysis using Affymetrix chips. Results are from 3 independent experiments (exp. 1,2,3). A) Cluster of genes controlled by P (ANOVA pval<0.001). B) Cluster of genes controlled by N (ANOVA pval<0.001). Heatmaps report high (yellow) and low expression (blue). C) Correlation between -log(pval) between P and NxP (left panel) and N and NxP (right panel).
Figure 3. PSR is reactivated upon nitrate provision and NRT1.1 and PHR1/PHL1 affect PSR in N varying conditions.

(A) WT, chl1-5, and phr1 phl1 genotypes were grown on combinations of P (0 or 0.5 mM, KH₂PO₄) and N (0, 2.5 mM KNO₃) for 11 days. (B) Eleven-day-old plants from -P/-N conditions are transferred towards P and/or N replenished media. Values are mean ± SEM (n=3). Asterisks indicate significant differences from WT plants (*P<0.05; ***P<0.001; Student’s t-test).
Figure 4. Local N provision and N-demand long distance signaling controls PSR.

(A) Split-root conditions on Nitrogen (schematic) were applied on P varying contexts. Roots in the same local environment are compared to evaluate the influence of distant treatments. C.KNO_3 and C.KCl indicate control conditions (homogeneous KNO_3 or KCl supply). SpKNO_3 and SpKCl indicate heterogeneous conditions with KNO_3 supply for one half of the root system and KCl supply for the other part. Differences recorded between C.KNO_3 and SpKNO_3 or between SpKCl and CKCl are due to treatments applied and sensed by the other side of the split root. (B) Measurements of PSI genes by RT-qPCR in the Nitrogen-split root context on 2 P conditions (see text for details of the experimental procedure). Values are mean ± SEM (n=3). Results are from 3 independent experiments. Different letters indicate significant ANOVA followed by Tukey test (P<0.05).
Figure 5. PHO2 is regulated by N and integrates the N signal into the PSR pathway. (A) Plants were grown on combinations of P (0 or 0.5 mM, \(\text{KH}_2\text{PO}_4\)) and N (0, 0.05, 0.5, 1, 2.5 mM \(\text{KNO}_3\)) for 14 days. Roots were harvested for measuring PHO2 by RT-qPCR measurements. Values are mean ± SEM (n=3). Results are from 3 independent experiments. (B) WT and pho2-1 genotypes were grown on combinations of P (0 or 0.5 mM, \(\text{KH}_2\text{PO}_4\)) and N (0, 2.5 mM \(\text{KNO}_3\)) for 11 days (top panel). Eleven-day-old plants from -P/-N conditions were transferred to -P and +/- N replenished media (bottom panels). Roots were harvested for measuring PSI mRNA accumulation by RT-qPCR. Values are mean ± SEM (n=3). Asterisks indicate significant differences from WT plants (*\(P<0.05\); **\(P<0.001\); Student’s t-test). Nitrogen effect (ratio between +N/-N conditions) is reported in the panels.
Figure 6. Nitrogen influences PHR1 accumulation and turnover; phosphate via PHO2 affects NRT1.1 protein accumulation.
(A) PHR1 mRNA accumulation in N and P varying conditions. Experimental conditions correspond to those described in Figure 3A. None of the responses display a significant difference. (B) PHR1-GFP immunoblot and quantification relative to ACT in three independent experiments. (C) PHR1-GFP turnover (following CHX treatment) is measured in WT and pho2 genetic backgrounds in +N or -N conditions. This is a compilation of 3 independent experiments. Comparison of linear models to zero were performed using ANCOVA [GraphPad Prism (**P<0.01; ***P<0.001)]. (D) NRT1.1 immunoblot in response to P starvation in WT and pho2-1 genetic backgrounds. NRT1.1 protein quantification and mRNA level in the same conditions. Un-cropped version of the blot showing the bands on the same membrane are provided Supplemental Figure 6.
Figure 7. N control of PSR operates in rice and wheat; proposed model of N effect on PSR (based on Arabidopsis results).

(A) PSI gene response to N/P combinations in rice (see Methods for experimental conditions). Roots were harvested for measurements of PSI genes by RT-qPCR. Values are mean ± SEM (n=3). Different letters indicate significant differences as determined by ANOVA followed by Tukey test (P<0.05). (B) Shoot phenotypes of rice grown on N/P combinations display a growth enhancement in +N/-P conditions as compared to -P/-N. (C) PSI gene response to N/P combinations in rice (see Methods for experimental conditions). Roots were harvested for measurements of PSI genes by RT-qPCR. Values are mean ± SEM (n=3). Different letters indicate significant differences as determined by ANOVA followed by Tukey test (P<0.05). (D) Model summarizing the different molecular entry points of N signals in the control of PSR. PHO2/NRT1.1 co-control is hypothesized to be central in this cross-talk.
Identification of molecular integrators shows that nitrogen actively controls the phosphate starvation response in plants

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